DEVELOPMENT

Running head: Arabidopsis seed aging

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Proteome-wide characterization of seed aging in
Arabidopsis. A comparison between artificial and
natural aging protocols[w]

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**Abbreviations** - 2D, 2-dimensional; CDT, controlled deterioration treatment; DTT, dithiothreitol; IEF, isoelectrofocusing

**Keywords** - seed longevity, seed germination, proteomics, aging, Arabidopsis thaliana
ABSTRACT

A variety of mechanisms has been proposed to account for the extension of life span in seeds (seed longevity). In the present work, we have used 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 seven days. 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, the 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 unveiled essential mechanisms for seed vigor as translational capacity, mobilization of seed storage reserves and detoxification efficiency. Finally, the present work shows that similar molecular events accompany artificial and natural seed aging.
INTRODUCTION

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 both for the preservation of genetic resources and commercial applications. For orthodox or desiccation-tolerant seeds, low seed moisture content, low temperature or cryopreservation seem 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 QTL were identified affecting viability, which were located on different chromosomes. This behavior suggests that seed longevity is a multigenic trait including various seed traits as for example germination under various stresses or sucrose and seed oligosaccharides 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 a 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 dormancy might also be a factor that reduces seed longevity. Also, transgenic Arabidopsis seeds over-accumulating a heat stress transcription factor (HSF) 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 (PLDalpha1) 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 (PIMT), an enzyme repairing abnormal L-isoaspartyl (isoAsp) 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 as transcriptome profiling (Ogawa et al., 2003; Nakabayashi et al., 2005; reviewed in 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,b,c; Rossignol et al., 2006; Jorrín et al., 2007). In this way, previous proteomic studies
unveiled the requirements in terms of RNA and protein synthesis for Arabidopsis seed germination (Gallardo et al., 2001, 2002a,b; 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 the present 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 mechanisms of seed vigor loss during storage. Sensitivity of seeds to the 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,b; 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 this CDT for different times, up to seven days. 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 the 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 to natural aging conditions.

RESULTS AND DISCUSSION

The 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 (Ler) seeds for up to seven days (see “Materials and Methods”). The germination ability of each seed sample was assessed by germination assays (Fig. 1). The CDT led to a rapid decline of seed vigor, affecting both the speed, homogeneity, and the final extent of Arabidopsis seed germination (Fig. 1).

**Rationale of the Proteomic Approach**

To reveal molecular mechanisms associated with the loss of seed vigor induced by the CDT, a differential proteomic approach was carried out, under two different protocols. In the first we hypothesized that the 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 the 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 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 to 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, the present study allowed the identification of 87 novel proteins accumulates in Arabidopsis seeds (Supplemental Table S2).

**The Proteome Is Markedly Affected in Seeds Submitted to the CDT**

A typical 2D-gel corresponding to the control non-deteriorated seeds is presented in Figure 2A. Image analysis of 2D protein patterns from control and deteriorated seed samples
revealed 12 proteins spots that were more abundant in the deteriorated seeds, corresponding to ten 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 progress of the 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 the 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 nos 253, and 312) or decreased (spots nos 146, and 311) during progress of the CDT.

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

**The Glycolytic Pathway is Affected During Seed Aging**

Among the 12 proteins being more abundant in deteriorated seeds than in control seeds, four of them belong to the glycolytic pathway (Table I). Thus, the abundance of these protein spots, corresponding to glyceraldehyde-3-phosphate dehydrogenase (cGAPDH; E.C. 1.2.1.12; spots nos 253, 302 and NADP-GAPDH; E.C. 1.2.1.13; spot no 312) and to phosphoglucomutase (EC 2.7.5.1; spot no 305), significantly increased in seeds submitted to the 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 (3-PGA) (Baxter et al., 2007). Our results thereby
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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; E.C. 2.8.1.2; spot no 146; Figs. 2 and 3; Table II) was abundant in control high-vigor seeds (0 d). However, during the 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 or cyanide (Papenbrock and Schmidt, 2000a,b), 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 must therefore be controlled. Also, cyanide can inhibit the activity of heme proteins as peroxidases (Ellis and Dunford, 1968; Job and Ricard, 1975) and catalases (Tejera García et al., 2007). Further, 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 non-dormant sunflower 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 methionine and S-adenosylmethionine 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 plant (Cipollini and Gruner, 2007). The glucosinolates of Arabidopsis seeds are distinguished by their high concentration, unique aliphatic constituents and the 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 mobilization of sulfur for iron-sulfur cluster biosynthesis or repair (Pagani et al., 1984; Bonomi et al., 1985). Also, MST plays physiological role in the protection against oxidative stress, and particularly contributes to the maintenance of cellular redox homeostasis via the metabolic regulation of cysteine degradation (Nagahara and Katayama, 2005). In plants, the mobilization of sulfur for transport processes in older leaves was also proposed (Papenbrock and Schmidt, 2000a,b).

Our results revealed for the first time that a loss in seed vigor is associated with a decreased level of β-mercaptopyruvate sulfurtransferase, 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**

The present proteomic analysis revealed two protein spots (spots nos 254, and 255) progressively disappearing in seeds according to the time of CDT. These spots correspond to two isoforms of the RAB (Responsive to ABA) 18 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 dehydrin/RAB group of LEA proteins and seed longevity (Wechsberg et al., 1994). These proteins are inducible by dehydration and ABA (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 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 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 a large-scale of hydrophobic interactions such as 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 homologue are correlated with seed stress tolerance (Soeda et al., 2005). Thus, its transcript level increased during seed maturation, declined during priming (an invigoration treatment of seeds based upon their controlled hydration; Heydecker et al., 1973) and germination. Furthermore, the mRNA can be re-induced 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 the present proteomic study, we have not identified other dehydrins 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. Alternately, we cannot exclude the possibility that other LEA proteins are also involved in seed vigor, but that they are present in too low levels so that 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 the 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; Job et al., 2005; Rajjou et al., 2004, 2006a; http://www.seed-proteome.com). The results revealed that protein carbonylation strongly increased in deteriorated seeds, indicating the occurrence of ROS during the CDT. In agreement with previous data (Job et al., 2005; Rajjou et al., 2006a, 2007a,b), several polypeptides corresponding to the alpha- and beta-subunits of the 12S cruciferins (legumin-type seed storage proteins) were strongly carbonylated in deteriorated seeds compared to
control seeds. However, the present work also revealed that several other proteins ought to be oxidized in Arabidopsis seeds submitted to the CDT (Fig. 4; Table III). Among them, several isoforms of the Rubisco large subunit (spots nos 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 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 the Rubisco (Feller and Fischer, 1994). Finally, deterioration of Rubisco has also been observed during oxidative stress or ozone treatment (Pell et al., 1997). All these observations point out the hypersensitivity to oxidative stress of this protein, which is also found in the present study.

Many proteins with chaperon activities were also favored targets for oxidation (spots nos 1, 43, 90, 135, 136, 137, and 140; Table III). Among them, three HSP70 proteins (spots nos 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; Cabisco et al., 2000), presumably because they act as shields protecting other proteins against ROS damage (Cabisco et al., 2000). Other chaperon proteins associated with endoplasmic reticulum, as the luminal binding protein BiP (spots nos 1, and 135), calreticulin (spot no 140) and protein disulfide isomerase (PDI) (spot no 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 stress response and aging has been recently reviewed in human (Yoshida, 2007). Our results support this hypothesis in plants.

It is worth noting that three LEA protein isoforms (spots nos 60, 61, and 431), encoded by the At2g42560 gene, appeared to be more oxidized in deteriorated seeds than in control seeds.
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(Table III). Protein sequence of this LEA protein displays a strong homology with that of previously described seed biotinylated proteins from pea (Pisum sativum; Duval 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 proteins exhibits a characteristic biochemical feature in that they bind 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 metabolic quiescent state in dry seed by biotin deprivation (Alban et al., 2000; Job et al., 2001). It is also interesting to note the existence of an ortholog of this biotinylated LEA protein in Medicago truncatula seeds, whose accumulation level is strongly associated with reinduction of desiccation tolerance in radicles (Boudet et al., 2006). Hence, a specific carbonylation of this biotinylated LEA protein caused by the CDT could entail an alteration of Arabidopsis seed survival in the dry state.

Overall, our results document that the 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.

The CDT Exerts an Influence on Proteome Expression During Germination

The evolution of the seed proteome during germination was also analyzed 1-d post-imbibition for the control and deteriorated seeds. This stage corresponds to the germination sensu stricto of the Arabidopsis (Ler) high-vigor control seeds, as none of these seeds showed radicle protrusion at that time (Fig. 1). Out 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 storage protein precursors (spots nos 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-Ds-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 five 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 the CDT (spot no 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 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 a fast establishment of a vigorous seedling. Our present 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 therefore a very good candidate as a diagnostic marker of seed vigor.

Amongst proteins accumulating to lower amounts in germinating deteriorated seeds than in corresponding control seeds, two isoforms of the cytosolic O-acetylserine(thiol)lyase (OASTL; EC 2.5.1.47) encoded by At4g14880 gene were identified (spots nos 174, and 175) (Fig. 5; Table V). This enzyme forms a complex with serine 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 OASTL 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 as glutathione or phytochelatins, proteins, vitamins, cofactors as S-adenosylmethionine and hormones (Höfgen et al., 2001). All these sulfur compounds play fundamental roles in plant metabolism (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 to synthesize Cys can induce an irreparable loss of seed vigor owing to its general implication in metabolism and antioxidative potential in plants.

2-Alkenal reductase (2AER; EC 1.3.1.74) encoded by the At5g16970 gene also showed a strongly depressed accumulation in germinating deteriorated seeds (spot no 29; Table V). Interestingly, conditional over-expression of the 2AER gene in Arabidopsis results in increased salt tolerance during germination (Papdi et al., 2008). The 2-alkenal reductase possesses a NADPH-dependent oxidoreductase activity, which has been shown to play a key role in the detoxification of reactive carbonyls occurring during degradation of lipid peroxides, and hence in the protection of cells 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 the present 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 the CDT (Table V). Indeed, several proteins associated with translation, as initiation factor 4A-1 (eIF4A), elongation factor 1-gamma 2 (eEF-1-gamma 2), elongation factor 1B-gamma (eEF-1B gamma) and ribosomal protein 60S (spots nos 129, 128, 105, and 314) are less abundant in deteriorated seeds during germination sensu stricto (Table V). Moreover, we already observed in the present study that many other proteins involved in protein metabolism are altered by carbonylation in deteriorated seeds (spots nos 1, 43, 90, 135, 136, 137, and 140). Protein metabolism regroups several biological functions as protein folding, protein translocation, thermostolerance, oligomeric assembly and switching between active and inactive protein conformations. Our results demonstrate that simultaneous impairing of these functions is closely linked with the loss of seed vigor. Our conclusion is supported by a recent study showing that transgenic seeds over-accumulating a heat stress transcription factor (HSF), which enhances the accumulation of heat shock proteins (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 (reviewed by 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 of 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
[\(^{35}\text{S}\)]-Met incorporation declined dramatically in the deteriorated seed samples. For example, seeds deteriorated for three days of CDT presented an 8-fold decrease in [\(^{35}\text{S}\)]-Met incorporation compared with control seeds although under these conditions the aged seeds still kept good vigor with a maximum of 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 five and seven days had respectively a germination maximum of about 28% and 2% and showed an almost nil 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 the CDT, we characterized the neosynthesized proteome of three seed samples submitted to this treatment for 0 d, 2 d and 7 d. Radiolabeled proteins were separated by 2DE and revealed by autoradiography (Fig. 7). Translational activity of the non-deteriorated seeds (0 d) was high, as shown previously (Fig. 6). The analysis of this neosynthesized protein pattern revealed 1272 protein spots (Fig. 7A) out of which 217 proteins could be identified by using our Arabidopsis seed reference maps (Supplemental Table S1). These proteins are involved in a large number of plant metabolic processes, in cell division, 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 mRNA are translated during germination sensu stricto and play a fundamental role for the metabolic restart in the initialization of the
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germination program (Aspart et al., 1984; Rajjou et al., 2004). Deteriorated seeds submitted to
two days of CDT displayed a 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 non-deteriorated 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 evidenced in the
non-deteriorated 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 (Bewley and Black, 1994; Finkelstein, 1993; Cuming, 1999). It has been
shown that the seed maturation program can be re-induced during early step of seed
germination (Lane, 1991; Lopez-Molina et al., 2002; Rajjou et al., 2006a,b). This recruitment
of the late maturation program either by de novo transcription (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 an active metabolism. For a
longer time of CDT (7 d) seeds became almost unable to support de novo protein synthesis
(Fig. 7C). In summary, the present results clearly indicate that seed vigor is closely associated
with the ability of the seeds to re-induce the late maturation program during early stage of
germination.

Similar Events Occur During Accelerated and Natural Aging

There is still uncertainty as to whether the CDT mimics natural aging. This is a major
concern of seed companies because, for practical reasons, they rely on the CDT and
germination assays to predict seed storability (Delouche and Baskin, 1973). It was therefore of importance to compare the biochemical behavior of seeds submitted to the 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-year and 8-year-old, and presented a maximum of germination of about 45% and 23%, respectively. A third one, was 11-year-old, and did not germinate even after 30-d post-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 nos 146, and 7). Spot no 146 (Figs. 2, 3 and 8; Tables II and V) corresponding to β-mercaptopyruvate sulfurtransferase was abundant in non-deteriorated 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 no 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 the CDT (Figs. 6 and 7).

Overall, our data thereby provide the first molecular indication supporting the usefulness of the CDT for 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 by natural aging, caused large reduction 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 source, thereby limiting the substrate for protein synthesis. This hypothesis was tested by incubating CD-treated and control seeds in solutions of different amino acids, pyruvate or glucose.

We found that Asp, Glu, or Met at 1 M could not stimulate the germination of seeds submitted to the CDT. Also neither glucose nor pyruvate could stimulate the germination of the CD-treated 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 the CDT or following natural aging. To further explore this question, stored mRNAs were extracted from non-deteriorated and aged seeds and the translation potential of these mRNAs were 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, the present assay allowed only globally estimating the template activity of the extracted pool of stored mRNAs, and we cannot exclude at present the possibility that particular stored mRNAs playing a role in seed quality could be damaged 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.

**CONCLUSIONS**

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 presently analyzed 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 in 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 the present work, it appears that changes in the regulation of protein synthesis, post-translational modifications, and protein turnover are crucial determinants of age-related decline in the maintenance, repair, and survival of the seed. The controlled deterioration treatment (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 differentially deteriorated seed proteome in order to get 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 of which the level of accumulation varied as a consequence of the 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 unveiled essential mechanisms for seed vigor 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 lend 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 upstream mechanism to maintain seed vigor.

Another fundamental feature depicted 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-CD-treated 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 present experimental conditions. Since these seeds would require a functional translational machinery, we assume that their translational machinery is either 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 till e.g. DNA damage is repaired. Induction of DNA damage during seed ageing has been demonstrated for a long time (Osborne et al., 1980/81).

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). The present study realized on the model plant Arabidopsis enables to reach a better understanding of molecular mechanisms underlying loss of germination vigor during seed aging. Finally, for
the first time we demonstrated that the controlled deterioration treatment (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 presently characterized proteins in seed vigor by reverse genetics.

MATERIALS AND METHODS

Plant Material and Germination Experiments

Non-dormant seeds of Arabidopsis thaliana, accession Landsberg erecta (Ler), were used in all experiments. Germination assays were carried out at 25°C, with 16 h light/8 h 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.

Controlled Deterioration Treatment (CDT)

The 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 Montfoort, the Netherlands), 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 h, 4 h, 16 h, 1 d, 2 d, 3 d, 5 d 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 1-d post-imbibed seeds 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, Lyon, France), 4% (w/v) CHAPS (Amersham Biosciences), and 1% (v/v) Pharmalyte pH 3 to 10 carriers 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 (Mannheim, Germany); 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 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 7 h at 3,500 V. Proteins were then separated according to size. Prior to the second dimension, the gel strips were equilibrated for 2 x 30 min in 2 x 100 mL 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)
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was added to the first equilibration solution, and iodoacetamide [4% (w/v)] 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, 0.035% (v/v) 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 on 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 14 h at 100 V. Ten gels (200 x 250 x 1.0mm) 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 (non-deteriorated and deteriorated seeds). Two-dimensional gels were stained with silver nitrate according to Blum et al. (1987) for densitometric analyses. Image analysis was carried out with the ImageMaster 2D Elite version 4.01 software (Amersham Biosciences). Kinetics of protein accumulation were analyzed by non-linear regression analysis using exponential relationships and the 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 (DNPH) and immunological detection of the DNP adducts with monoclonal anti-DNP antibody (OxyBlot™ Oxidized Protein Detection Kit; Chemicon, France) 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 one day in the
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presence of $[^{35}S]$-Met (1.85 MBq; ICN Biomedicals, S.A.R.L.). Protein synthesis was measured by trichloroacetic acid (TCA) precipitation of aliquots of reaction mixtures spotted on Whatmann GF/C filters; after ten 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 2DE reference maps (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005; http://www.seed-proteome.com). In the present 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). Integrity of the rRNA and mRNA pools was analyzed, respectively, by gel electrophoresis and by using a wheat germ extract in vitro translation assay (Promega, USA) with $^{35}$S-labeled Met (ICN Biochemicals SARL 1.85 MBq).

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LEGENDS TO FIGURES

**Figure 1.** Influence of controlled deterioration treatment (CDT) on Arabidopsis seed germination. Seeds were submitted to the CDT for different periods (●, 0 d; ○, 4 h; ▲, 16 h; △, 1 d; ♦, 2 d; ◊, 3 d; ■, 5 d; □, 7 d) as described in “Materials and Methods”. The graph shows a representative experiment carried out three times in triplicate.

**Figure 2.** Influence of controlled deterioration treatment (CDT) on the proteome of Arabidopsis seeds. An equal amount (150 µg) of total soluble protein extracts was loaded in each gel. A, Silver-stained 2D gel of total soluble proteins from non-deteriorated seeds (0 d, control seeds). The indicated portions of the gel, (a, b, c, and d) are reproduced in panel B. B, Enlarged windows (a–d) of 2D gels as shown in A for non-deteriorated seeds (left), 3-d-deteriorated seeds (middle) and 7-d-deteriorated seeds (right). The seven labeled protein spots (spots nos 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; Job et al., 2005; Rajjou et al., 2004, 2006a; http://www.seed-proteome.com; see Tables I and II and Supplemental Table S2). Protein spot quantitation was carried out as described in "Materials and Methods," from at least five gels for each seed sample.

**Figure 3.** Dynamic evolution of protein spot abundance in Arabidopsis seeds submitted to the controlled deterioration treatment (CDT). The relative abundance of protein spots was calculated by dividing the normalized spot volumes in the deteriorated seeds (4 h, 16 h, 1 d, 2 d, 3 d, 5 d or 7 d) by the corresponding normalized spot volumes in the non-deteriorated seeds (0 d). A, Time courses of relative abundance increase in deteriorated seeds during CDT for the two protein spots (spots nos 253, and 312). B, Time courses of relative abundance decrease in deteriorated seeds during CDT for the two protein spots (spots nos 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 non-linear 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 (d). T_{1/2} values are listed in Tables I, II, IV and V.

**Figure 4.** Protein carbonyl patterns in Arabidopsis seeds following controlled deterioration treatment (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 non-deteriorated 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 2,4-dinitrophenylhydrazone (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. This figure shows that both the CDT and natural aging induce an oxidative stress on specific proteins, presumably through the generation of ROS.

**Figure 5.** Changes in protein accumulation patterns in deteriorated seeds during germination sensu stricto (1-d post-imbibition). An equal amount (150 µg) of total soluble protein extracts was loaded in each gel. Proteins were separated by 2D-gel electrophoresis. A representative silver-stained 2D gel of total soluble proteins from non-deteriorated dry mature seeds is presented in Figure 2A. The analysis was carried out on non-deteriorated seed samples (0 d,
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control seeds) and deteriorated seeds (3 d, 5 d and 7 d of CDT) imbibed in water for 1 d. The
18 labeled protein spots (spots nos 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; Job et al., 2005; Rajjou
et al., 2004, 2006a; http://www.seed-proteome.com; see Tables IV and V and Supplemental
Table S2). Protein spot quantitation was carried out as described in "Materials and Methods,"
from at least five gels for each seed sample.

Figure 6. Influence of the controlled deterioration treatment (CDT) and natural aging on de
novo protein synthesis during germination sensu stricto (1-d post-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 ten 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 d, 1 d, 2 d, 3 d, 5 d 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, 7-year-old seeds, 8-year-old seeds and 11-year-
old seeds).

Figure 7. Comparison of de novo protein synthesis patterns during germination sensu stricto
(1-d post-imbibition) of deteriorated seeds. A, Protein profiles of de novo synthesized proteins
in non-deteriorated 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 imbibition, submitted to 2D gel electrophoresis, and the radiolabeled proteins revealed as described in "Materials and Methods." The thirty three labeled protein spots were identified by mass spectrometry and by comparison with Arabidopsis seed protein reference maps (Gallardo et al., 2001, 2002a; Job et al., 2005; Rajjou et al., 2004, 2006a; http://www.seed-proteome.com; see Table VI and Supplemental Table S2). a, 12S seed storage protein precursor; b, alpha-subunit of 12S seed storage protein; c, beta-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).

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

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

| No. | Exp. MW (kDa) | Exp. pl | Arabidopsis Protein Name | Th. MW (kDa) | Theo. pl | AGI No. | % Cov. | Relative Abundance Ratio 7 d / 0 d | T1/2 (d) |
|-----|---------------|---------|--------------------------|--------------|----------|---------|-------|----------------------------------|----------|
| 7b  | 37.55         | 5.09    | 60S acidic ribosomal protein | 34.37        | 5.08     | At3g11250 | 24%   | 3.4 (±0.1)                      | >7       |
| 212c| 42.57         | 5.18    | Actin 2                   | 41.21        | 5.43     | At3g18780 | 33%   | 2.5 (±0.4)                      | >7       |
| 253c| 40.29         | 5.84    | Glyceraldehyde-3-phosphate dehydrogenase, cytosolic Peptidase M1 family protein | 36.99        | 6.34     | At3g04120 | 18%   | >16                             | >7       |
| 293c| 96.69         | 5.61    | Glyceraldehyde-3-phosphate dehydrogenase | 103.40       | 6.09     | At1g63770 | 4%    | >5.1                            | >7       |
| 302c| 40.34         | 6.03    | Glyceraldehyde-3-phosphate dehydrogenase | 36.90        | 7.21     | At1g13440 | 30%   | 7.7 (±1.4)                      | >7       |
| 305c| 68.25         | 5.70    | Phosphoglucomutase         | 63.46        | 5.57     | At1g70730 | 34%   | 2.2 (±0.1)                      | >7       |
| 308b| 28.52         | 5.89    | Alpha-cruciferin 12S (seed storage protein fragment) | 50.56        | 6.55     | At1g03880 | 10%   | 3.4 (±0.5)                      | >7       |
| 312b| 14.21         | 3.82    | NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (Fragment) | 53.04        | 6.61     | At2g24270 | 8%    | 6.0 (±0.3)                      | >7       |
| 320b| 57.36         | 5.82    | Tubulose bisphosphate arboxylase large chain | 53.47        | 6.25     | AtGc00490 | 24%   | >2.7                            | >7       |
| 321b| 57.36         | 5.84    | Tubulose bisphosphate arboxylase large chain | 53.47        | 6.25     | AtGc00490 | 28%   | >2.8                            | >7       |
| 322b| 35.21         | 5.20    | 60S acidic ribosomal protein | 33.65        | 4.93     | At2g40010 | 15%   | >4.4                            | >7       |
| 376b| 57.35         | 4.92    | Tubulin beta-8 chain       | 50.39        | 4.46     | At5g23860 | 9%    | 5.0 (±0.2)                      | 1.5 (±0.2) |

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

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

| No. | Exp. MW (kDa) | Exp. pI | Arabidopsis Protein Name | Th. MW (kDa) | Theo. pI | AGI No. | % Cov. | Relative Abundance Ratio 7 d / 0 d | T1/2 (d) |
|-----|---------------|---------|--------------------------|--------------|---------|---------|--------|----------------------------------|---------|
| 4a  | 57.35         | 4.89    | Tubulin beta 2 beta 3 chain Mercaptopyruvate sulfurrtransferase | 50.73        | 4.7     | At5g62700 | 35%    | 0.26 (±0.01) | 0.17 (±0.05) |
| 146b| 37.67         | 5.03    | Dehydrin                 | 41.89        | 5.95    | At1g79230 | 35%    | 0.33 (±0.05) | 1.12 (±0.33) |
| 254b| 22.1          | 4.96    | Dehydrin                 | 18.44        | 7.95    | At5g66400 | 6%     | 0.13 (±0.04) | 0.093 (±0.15) |
| 255b| 21.65         | 5.18    | Dehydrin                 | 18.44        | 7.95    | At5g66400 | 6%     | <0.22 | >7 |
| 304b| 69.3          | 5.56    | Phosphoglucomutase       | 63.44        | 5.73    | At1g70730 | 37%    | 0.30 (±0.04) | 0.37 (±0.12) |
| 311b| 14.1          | 3.2     | Beta-cruciferin 12S (seed storage protein fragment) | 21.20        | 6.19    | At4g28520 | 11%    | 0.19 (±0.01) | 0.56 (±0.04) |

*Protein numbering following Arabidopsis seed protein reference maps available on this web site: http://www.seed-proteome.com; *aListed proteins correspond to previously identified proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005); *bData obtained from densitometric analysis of individual spots from proteins in 2D gels stained with silver nitrate (see Figure 2A for an example of 2D gel: normalized spot volume in the deteriorated seeds (7 d of CDT) divided by the normalized spot volume in the non-deteriorated control seed (0 d of CDT); from five different gels and three independent extractions.
### Table III. Identification and relative carbonyl content of oxidized proteins during Arabidopsis seed aging.

| No. a | Exp. MW (kDa) | Exp. pI | Arabidopsis Protein Name | Th. MW (kDa) | Th. pI | AGI No. | % Cov. | Relative Abundance b d | Ratio 7 d/0 d |
|-------|---------------|---------|--------------------------|--------------|--------|---------|--------|------------------------|---------------|
| **Protein metabolism** |     |         |                          |              |        |         |        |                        |               |
| 140b  | 60.26         | 4.15    | Calreticulin 1 (Precursor) | 48.51        | 4.2    | At1g56340 | 15%   | 2.3                    |               |
| 1b    | 79.36         | 5.08    | dnaK-type molecular chaperone BiP | 71.17       | 5.08   | At5g52020 | 20%   | >100                   |               |
| 135b  | 80.5          | 5.04    | dnaK-type molecular chaperone BiP | 71.17       | 5.08   | At5g52020 | 20%   | >100                   |               |
| 136a  | 77.87         | 4.87    | Heat shock cognate 70 kDa protein 1 | 71.37       | 5.03   | At5g02500 | 19%   | >100                   |               |
| 43b   | 78.9          | 5.06    | Heat shock cognate 70 kDa protein 3 | 71.13       | 4.69   | At3g09440 | 15%   | >100                   |               |
| 137a  | 76.06         | 5.07    | Heat shock protein 70    | 71.37       | 5.03   | At3g12580 | 38%   | >100                   |               |
| 614a  | 68.68         | 5.83    | Aspartyl-tRNA synthetase  | 62.90       | 6.12   | At4g17750 | 39%   | 2.1                    |               |
| 90a   | 64.11         | 4.81    | Protein disulfide isomerase | 55.60       | 4.81   | At1g21750 | 39%   | 2.1                    |               |
| **Energy metabolism and Photosynthesis** |     |         |                          |              |        |         |        |                        |               |
| 109b  | 64.79         | 6.35    | Malate oxidoreductase or Malic enzyme | 64.28       | 6.32   | At2g19900 | 14%   | >100                   |               |
| 63b   | 57.09         | 6.11    | Isocitrate lyase         | 50.42       | 6.29   | At3g21720 | 30%   | >100                   |               |
| 10b   | 57.36         | 5.77    | Ribulose bisphosphate carboxylase large chain | 53.47       | 6.25   | At1g00490 | 30%   | >100                   |               |
| 319b  | 57.36         | 5.57    | Ribulose bisphosphate carboxylase large chain | 53.47       | 6.25   | At1g00490 | 26%   | >100                   |               |
| 320b  | 57.36         | 5.82    | Ribulose bisphosphate carboxylase large chain | 53.47       | 6.25   | At1g00490 | 21%   | >100                   |               |
| 321b  | 57.36         | 5.84    | Ribulose bisphosphate carboxylase large chain | 53.47       | 6.25   | At1g00490 | 30%   | >100                   |               |
| **Stress response** |     |         |                          |              |        |         |        |                        |               |
| 60b   | 66.68         | 5.74    | Late embryogenesis abundant (LEA) | 67.19       | 5.78   | At2g25600 | 25%   | 1.2                    |               |
| 61b   | 66.68         | 5.78    | Late embryogenesis abundant (LEA) | 67.19       | 5.78   | At2g25600 | 32%   | 1.5                    |               |
| 431b  | 66.68         | 5.79    | Late embryogenesis abundant (LEA) | 67.19       | 5.78   | At2g25600 | 32%   | 2.2                    |               |
| **Hydrolase** |     |         |                          |              |        |         |        |                        |               |
| 96b   | 64.62         | 6.08    | Glycosyl hydrolase family 1 protein | 57.83       | 6.02   | At3g21370 | 11%   | >100                   |               |
| 207a  | 64.96         | 5.92    | Glycosyl hydrolase family 1 protein | 57.83       | 6.02   | At3g21370 | 21%   | >100                   |               |
| 208a  | 64.96         | 6.23    | Glycosyl hydrolase family 1 protein | 57.83       | 6.02   | At3g21370 | 14%   | >100                   |               |
| **Seed storage proteins** |     |         |                          |              |        |         |        |                        |               |
| 35a   | 56.47         | 6.84    | 12S seed storage protein [Precursor] | 55.86       | 6.36   | At4g28520 | 16%   | >100                   |               |
| 70a   | 49.43         | 7.19    | 12S seed storage protein [Precursor] | 52.59       | 7.68   | At5g44120 | 22%   | >100                   |               |
| 71a   | 50.44         | 7.67    | 12S seed storage protein [Precursor] | 52.59       | 7.68   | At5g44120 | 34%   | >100                   |               |
| 110b  | 57            | 6.4     | 12S seed storage protein [Precursor] | 55.86       | 6.36   | At4g28520 | 19%   | >100                   |               |
| 111a  | 56.89         | 7.19    | 12S seed storage protein [Precursor] | 55.86       | 6.36   | At4g28520 | 16%   | >100                   |               |
| 271b  | 59.96         | 6.52    | 12S seed storage protein [Precursor] | 55.86       | 6.36   | At4g28520 | 30%   | >100                   |               |
| 239b  | 28.95         | 6.64    | Alpha-cruciferin 12S (seed storage protein fragment) | 48.03       | 6.56   | At1g03880 | 18%   | >100                   |               |
| 80a   | 32.64         | 5.85    | Alpha-cruciferin 12S (seed storage protein fragment) | 34.68       | 6.42   | At4g28520 | 33%   | 3.29                   |               |
| 82a   | 33.89         | 6.24    | Alpha-cruciferin 12S (seed storage protein fragment) | 34.68       | 6.42   | At4g28520 | 42%   | 1.6                    |               |
| 83a   | 34.35         | 6.42    | Alpha-cruciferin 12S (seed storage protein fragment) | 34.68       | 6.42   | At4g28520 | 33%   | 1                      |               |
| 84a   | 30.46         | 6.61    | Alpha-cruciferin 12S (seed storage protein fragment) | 31.75       | 6.49   | At5g44120 | 42%   | 2.1                    |               |
| 85a   | 27.2          | 6.5     | Alpha-cruciferin 12S (seed storage protein fragment) | 27.24       | 6.34   | At1g03880 | 32%   | 2.9                    |               |
| 118a  | 29.21         | 6.04    | Alpha-cruciferin 12S (seed storage protein fragment) | 34.68       | 6.42   | At4g28520 | 26%   | 1                      |               |
| 119a  | 30.66         | 6.16    | Alpha-cruciferin 12S (seed storage protein fragment) | 31.75       | 6.49   | At5g44120 | 28%   | 2.9                    |               |
| 134a  | 26.35         | 6.34    | Alpha-cruciferin 12S (seed storage protein fragment) | 27.24       | 6.34   | At1g03880 | 21%   | 3.9                    |               |
| 238a  | 29.42         | 6.5     | Alpha-cruciferin 12S (seed storage protein fragment) | 31.75       | 6.49   | At5g44120 | 28%   | 2.1                    |               |
| No. | Exp. MW (kDa) | Exp. pI | Arabidopsis Protein Name | Th. MW (kDa) | Th. pI | AGI No. | % Cov. | Relative Abundance \(^d\) | Ratio 7 d / 0 d |
|-----|---------------|---------|-------------------------|-------------|--------|---------|-------|------------------------|----------------|
| Seed storage proteins |
| 240\(^b\) | 27.28 | 6.74 | Alpha-cruciferin 12S (seed storage protein fragment) | 27.24 | 6.34 | At1g03880 | 16% | 3.57 |
| 241\(^b\) | 34.11 | 5.87 | Alpha-cruciferin 12S (seed storage protein fragment) | 34.68 | 6.42 | At4g28520 | 32% | 1.2 |
| 243\(^b\) | 34.92 | 6.62 | Alpha-cruciferin 12S (seed storage protein fragment) | 34.68 | 6.42 | At4g28520 | 19% | 1 |
| 278\(^b\) | 27.24 | 6.44 | Alpha-cruciferin 12S (seed storage protein fragment) | 31.75 | 6.49 | At5g44120 | 24% | >100 |
| 98\(^b\) | 18.08 | 6.12 | Beta-cruciferin 12S (seed storage protein fragment) | 20.8 | 7.03 | At1g03880 | 45% | 4.9 |
| 498\(^b\) | 22.82 | 8.85 | Beta-cruciferin 12S (seed storage protein fragment) | 21.2 | 6.19 | At4g28520 | 79% | 12.4 |
| 87\(^b\) | 18.43 | 6.36 | Beta-cruciferin 12S (seed storage protein fragment) | 20.8 | 7.03 | At1g03880 | 33% | >100 |
| 88\(^b\) | 22.82 | 8.68 | Beta-cruciferin 12S (seed storage protein fragment) | 21.2 | 6.19 | At4g28520 | 44% | 1.8 |
| 231\(^b\) | 19.71 | 8.82 | Beta-cruciferin 12S (seed storage protein fragment) | 21.2 | 6.19 | At4g28520 | 39% | >100 |
| 233\(^b\) | 18.45 | 9.1 | Beta-cruciferin 12S (seed storage protein fragment) | 20.84 | 9.06 | At5g44120 | 52% | >100 |
| 497\(^b\) | 18.45 | 6.12 | Beta-cruciferin 12S (seed storage protein fragment) | 21.2 | 6.19 | At4g28520 | 64% | >100 |
| 499\(^b\) | 20.67 | 8.85 | Beta-cruciferin 12S (seed storage protein fragment) | 20.84 | 9.06 | At5g44120 | 53% | 5.3 |

| Others processes |
|------------------|---------------|---------|-------------------------|-------------|--------|---------|-------|------------------------|----------------|
| 259\(^b\) | 27.01 | 6.1 | Expressed protein | 27.28 | 6.7 | At1g05510 | 21% | 5.7 |

\(^a\)Protein numbering following Arabidopsis seed protein reference maps available on this website: [http://www.seed-proteome.com](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; \(^d\)Data obtained from densitometric analysis of individual spots from carbonylated proteins in 2D gels revealed by anti-DNP immunoassay (examples are shown in Figure 4): normalized spot volume in the deteriorated seeds (7 d of CDT) divided by the normalized spot volume in the non-deteriorated control seeds (0 d of CDT) (ratio of carbonylation in deteriorated dry seeds (7 d of CDT) over carbonylation in non-deteriorated dry seed (0 d of CDT)), from three different and independent protein extractions; >100 means that the accumulation level of the corresponding carbonylated protein in the non-deteriorated dry seeds (0 d of CDT) was close to background.
Table IV. Arabidopsis proteins whose abundance was significantly greater in deteriorated seeds than in control seeds during germination *sensu stricto* (1-d post-imbibition)

| No. | Exp. MW (kDa) | Exp. pl | Arubidopsis Protein Name | Th. MW (kDa) | Theo. pl | AGI No. | % Cov. | Relative Abundance *d* | Ratio 7 d / 0 d | T1/2 (d) |
|-----|---------------|---------|-------------------------|--------------|---------|---------|--------|----------------------|----------------|---------|
| **Energy metabolism** | | | | | | | | | | | |
| 253° | 40.29 | 5.84 | Glyceraldehyde-3-phosphate dehydrogenase, cytosolic | 36.99 | 6.34 | At3g04120 | 18% | >6.2 | >7 |
| 302c | 40.34 | 6.03 | Glyceraldehyde-3-phosphate dehydrogenase | 36.90 | 7.21 | At1g13440 | 30% | >7.6 | >7 |
| 368b | 40.36 | 6.24 | Glyceraldehyde-3-phosphate dehydrogenase | 36.90 | 7.21 | At1g13440 | 17% | >4.5 | >7 |
| 369b | 40.33 | 6.20 | Glyceraldehyde-3-phosphate dehydrogenase | 36.90 | 7.21 | At1g13440 | 22% | >5.0 | >7 |
| 349b | 64.85 | 6.77 | Malate oxidoreductase | 64.24 | 6.68 | At2g19900 | 12% | >32.0 | >7 |
| 350b | 64.70 | 7.21 | Malate oxidoreductase | 64.24 | 6.68 | At2g19900 | 2% | >39.0 | >7 |
| **Amino acid metabolism** | | | | | | | | | | | |
| 359c | 56.90 | 5.57 | S-Adenosyl-L-homocysteine hydrolase | 53.36 | 5.83 | At4g13940 or At3g23810 | 13% | >20.0 | >7 |
| 370c | 41.66 | 6.50 | Glutamate dehydrogenase | 44.51 | 6.02 | At5g18170 or At3g03910 | 13% | >2.5 | >7 |
| **Seed storage proteins** | | | | | | | | | | | |
| 354b | 42.61 | 6.60 | 12S seed storage protein [Precursor] | 52.56 | 8.08 | At5g44120 | 25% | >9.2 | >7 |
| 177b | 45.47 | 6.07 | 12S seed storage protein [Precursor] | 48.03 | 6.56 | At1g03880 | 17% | >3.0 | >7 |
| 151b | 46.11 | 5.38 | Cupin family protein | 49.66 | 5.45 | At1g03890 | >6.0 | >7 |
| **Hydrolase and Protease** | | | | | | | | | | | |
| 378c | 80.28 | 5.77 | Subtilisin-like serine protease | 81.80 | 6.76 | At3g14067 | 19% | >3 | >7 |
| 360c | 64.96 | 5.75 | Glycosyl hydrolase family | 84.29 | 7.71 | At5g64570 | 14% | >3.0 | >7 |
| **Others processes** | | | | | | | | | | | |
| 139° | 40.07 | 5.56 | Reversibly glycosylated polypeptide | 40.7 | 5.61 | At3g02230 or At5g15650 | 7% | >2.5 | >7 |
| 377b | 38.42 | 6.58 | Potassium channel beta subunit | 36.52 | 7.49 | At1g04690 | 15% | >3.8 | >7 |
| 380c | 26.31 | 5.89 | Nucleoside diphosphate kinase II, chloroplast | 25.53 | 9.30 | At5g63310 | 12% | >3.8 | >7 |

*Protein numbering following Arabidopsis seed protein reference maps available on this web site: http://www.seed-proteome.com; °Listed proteins correspond to previously identified proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005); ‡Listed proteins correspond to proteins identified during this work; the peptide sequences determined are available in Supplemental Table S2; *Data obtained from densitometric analysis of individual spots from proteins in 2D gels stained with silver nitrate (see Figure 2A for an example of 2D gel): normalized spot volume in the deteriorated seeds (7 d of CDT) incubated 1 d in water divided by the normalized spot volume in the non-deteriorated control seeds (0 d of CDT) incubated 1 d in water; from five different gels and three independent extractions.
### Table V. Arabidopsis proteins whose abundance was significantly smaller in deteriorated seeds than in control seeds during germination sensu stricto (1 d post-imbibition)

| No. | Exp. MW (kDa) | Exp. pl | Arabidopsis Protein Name | Th. MW (kDa) | Theo. pl | AGI No. | % Cov. | Relative Abundance | T1/2 (d) |
|-----|---------------|---------|--------------------------|--------------|---------|---------|-------|------------------|----------|
| Translation and protein metabolism | | | | | | | | | |
| 128 | 47.45 | 5.45 | Elongation factor 1B-gamma | 46.66 | 5.36 | At1g09640 | 26% | <0.28 | >7 |
| 129 | 47.20 | 5.50 | Eukaryotic initiation factor 4A-1 | 46.70 | 5.47 | At3g13920 | 28% | <0.49 | >7 |
| 105 | 48.63 | 5.61 | Elongation factor 1-gamma 2 | 46.4 | 5.55 | At1g57720 | 41% | <0.5 | >7 |
| 314 | 37.54 | 5.22 | 60S acidic ribosomal protein P0-C | 34.37 | 4.78 | At2g40010 | 7% | <0.25 | >7 |
| 200 | 40.57 | 5.72 | Protein disulfide isomerase-like (PDIL) | 39.50 | 5.80 | At2g47470 | 20% | <0.44 | >7 |
| 364 | 65.22 | 5.56 | T-complex protein 1. theta subunit (TCP-1-Theta) | 58.92 | 5.01 | At3g03960 | 2% | <0.30 | >7 |
| Energy metabolism | | | | | | | | | |
| 39 | 38.52 | 6.23 | Glyceraldehyde-3-phosphate dehydrogenase | 36.91 | 6.62 | At3g04120 | 26% | <0.48 | >7 |
| 40 | 38.55 | 6.26 | Glyceraldehyde-3-phosphate dehydrogenase | 36.91 | 6.62 | At3g04120 | 27% | <0.44 | >7 |
| 307 | 40.30 | 5.87 | Glyceraldehyde-3-phosphate dehydrogenase | 36.91 | 6.62 | At3g04120 | 19% | <0.21 | >7 |
| 365 | 62.77 | 6.30 | Isocitrate lyase | 50.42 | 5.29 | At3g21720 | 16% | <0.21 | >7 |
| Cell detoxification and stress response | | | | | | | | | |
| 23 | 56.48 | 6.64 | Catalase | 56.93 | 6.63 | At4g35090 | 16% | <0.25 | >7 |
| 146 | 37.67 | 5.03 | Mercaptoerythritol sulfurtransferase | 38.13 | 5.81 | At5g16970 | 14% | <0.26 | >7 |
| 29 | 38.35 | 5.72 | 2-Alkenal reductase | 38.13 | 5.81 | At5g16970 | 14% | <0.26 | >7 |
| 284 | 25.34 | 5.43 | GSH-dependent Dehydroascorbate reductase | 23.62 | 5.79 | At4g46290 | 44% | <0.49 | >7 |
| Cell division | | | | | | | | | |
| 24 | 42.94 | 5.06 | Actin 7 | 41.73 | 5.31 | At5g09810 | 23% | <0.43 | >7 |
| 4 | 57.35 | 4.89 | Tubulin beta 2 beta 3 chain | 50.73 | 4.70 | At5g62700 | 35% | <0.32 | >7 |
| Seed storage proteins | | | | | | | | | |
| 504 | 60.87 | 6.20 | 12S seed storage protein [Precursor] | 55.86 | 6.36 | At4g28520 | 7% | <0.18 | >7 |
| 311 | 14.1 | 3.2 | Beta-cruciferin 12S (seed storage protein fragment) | 58.23 | 6.53 | At4g28520 | 11% | <0.21 | >7 |
| Others processes | | | | | | | | | |
| 182 | 50.76 | 5.53 | DEAD box RNA helicase | 48.38 | 5.49 | At5g11200 | 32% | <0.42 | >7 |
| 361 | 54.65 | 5.22 | 4-methyl-5(b-hydroxyethyl)-thiazole monophosphate biosynthesis protein | 41.84 | 5.08 | At3g14960 | 6% | <0.16 | >7 |
| 116 | 30.24 | 5.77 | Expressed protein | 28.78 | 5.92 | At3g45690 | 40% | <0.35 | >7 |

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

| No. | Exp. MW (kDa) | Exp. pl | Arabidopsis Protein Name | Th. MW (kDa) | Th. pl | AGI No. | % Cov. |
|-----|---------------|---------|--------------------------|--------------|--------|---------|--------|
| Translation and protein metabolism |                |         |                          |              |        |         |        |
| 127 | 93.92         | 5.89    | Elongation factor EF-2    | 94.25        | 5.89   | At1g60707 | 24%    |
| 9  | 17.94         | 5.50    | HSP 17.6                 | 17.83        | 5.22   | At5g12030 | 14%    |
| 45  | 76.38         | 5.24    | HSP 70                   | 70.91        | 5.30   | At1g16030 | 24%    |
| 138 | 76.06         | 5.07    | HSP 70                   | 71.37        | 5.30   | At3g12580 | 19%    |
| 190  | 101.62        | 5.82    | HSP101                  | 101.27       | 5.99   | At1g74310 | 16%    |
| 364  | 65.22         | 5.56    | T-complex protein 1. theta subunit (TCP-1-Theta) | 58.92       | 5.01   | At3g03960 | 2%     |
| 579  | 36.05         | 3.98    | Glycine-rich protein similar to P23 co-chaperone | 25.47       | 4.17   | At4g02450 | 24%    |
| Energy metabolism |                |         |                          |              |        |         |        |
| 44b | 25b           |         |                          |              |        |         |        |
| 1155c | 1155c        |         |                          |              |        |         |        |
| 739c | 392c          |         |                          |              |        |         |        |
| 84b | 83b           |         |                          |              |        |         |        |
| 80b | 78b           |         |                          |              |        |         |        |
| 69b | 69b           |         |                          |              |        |         |        |
| 271c | 271c          |         |                          |              |        |         |        |
| 241c | 241c          |         |                          |              |        |         |        |
| 392c | 392c          |         |                          |              |        |         |        |
| 739c | 739c          |         |                          |              |        |         |        |
| 1154c | 1154c         |         |                          |              |        |         |        |
| 1155c | 1155c        |         |                          |              |        |         |        |
| 25c | 23c           |         |                          |              |        |         |        |
| 44c | 16.14         | 7.42    |                          | 20.80        | 7.03   | At1g03880 | 7%     |

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Table VI. (Continued from previous page.)

| No.| Exp. MW (kDa) | Exp. pI | Arabidopsis Protein Name | Th. MW (kDa) | Th. pI | AGI No. | % Cov. |
|----|---------------|--------|--------------------------|--------------|--------|---------|--------|
| 87b | 18.43         | 6.36   | Beta-cruciferin 12S (seed storage protein fragment) | 20.80        | 7.03   | At1g03880 | 33%    |
| 88b | 22.82         | 8.68   | Beta-cruciferin 12S (seed storage protein fragment) | 21.20        | 6.19   | At4g28520 | 44%    |
| 267b| 23.44         | 5.58   | Beta-cruciferin 12S (seed storage protein fragment) | 20.84        | 9.06   | At5g44120 | 22%    |
| 705c| 11.75         | 8.90   | Beta-cruciferin 12S (seed storage protein fragment) | 21.20        | 6.19   | At5g03880 | 44%    |
| 1153c| 11.51        | 9.02   | Beta-cruciferin 12S (seed storage protein fragment) | 21.20        | 6.19   | At4g28520 | 39%    |
| 1156c| 10.68         | 9.05   | Beta-cruciferin 12S (seed storage protein fragment) | 20.84        | 9.06   | At5g44120 | 41%    |
| 246b| 29.45         | 6.70   | Storage proteins 7S      | 55.05        | 7.15   | At3g22640 | 28%    |
| 247b| 34.02         | 6.77   | Storage proteins 7S      | 55.05        | 7.15   | At3g22640 | 18%    |

*Protein numbering following Arabidopsis seed protein reference maps available on this web site: [http://www.seed-proteome.com](http://www.seed-proteome.com); Listed proteins correspond to previously identified proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004, 2006a; Job et al., 2005); Listed proteins correspond to proteins identified during this work; the peptide sequences determined are available in Supplemental Table S2.
Table VII. The effect of natural aging on the germination of Arabidopsis seeds

\( G_{\text{max}} \), maximum of germination.

| Seed samples      | \( G_{\text{max}} \) (%) | Standard deviation (%) |
|-------------------|---------------------------|------------------------|
| Freshly harvested seeds | 99.00                     | 1                      |
| 7-year-old seeds  | 45.33                     | 2.96                   |
| 8-year-old seeds  | 23.00                     | 2.52                   |
| 11-year-old seeds | 0.00                      | 0                      |
SUPPLEMENTAL DATA

Supplemental Table S1: Arabidopsis proteins whose de novo synthesis occurs during germination sensu stricto (1-d post-imbibition) of non-deteriorated control seeds (0d).

Supplemental Table S2: Peptide sequences identified by MS-MS sequencing and corresponding to novel proteins in Arabidopsis seeds identified in this work.
Figure 1. Influence of controlled deterioration treatment (CDT) on Arabidopsis seed germination. Seeds were submitted to CDT for different periods (●, 0 d; ○, 4 h; ▲, 16 h; △, 1 d; ◆, 2 d; ◇, 3 d; ■, 5 d; □, 7 d) as described in “Materials and Methods”. The graph shows a representative experiment carried out three times in triplicate.
Figure 2. Influence of controlled deterioration treatment (CDT) on the proteome of Arabidopsis seeds. An equal amount (150 µg) of total soluble protein extracts was loaded in each gel. A, Silver-stained 2D gel of total soluble proteins from non-deteriorated seeds (0 d, control seeds). The indicated portions of the gel, (a, b, c, and d) are reproduced in panel B. B, Enlarged windows (a–d) of 2D gels as shown in A for non-deteriorated seeds (left), 3-d-deteriorated seeds (middle) and 7-d-deteriorated seeds (right). The seven labeled protein spots (spots nos 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; Job et al., 2005; Rajjou et al., 2004, 2006a; http://www.seed-proteome.com; see Tables I and II and Supplemental Table S2). Protein spot quantitation was carried out as described in "Materials and Methods," from at least five gels for each seed sample.
Figure 3. Dynamic evolution of protein spot abundance in Arabidopsis seeds submitted to the controlled deterioration treatment (CDT). The relative abundance of protein spots was calculated by dividing the normalized spot volumes in the deteriorated seeds (4 h, 16 h, 1 d, 2 d, 3 d, 5 d or 7 d) by the corresponding normalized spot volumes in the non-deteriorated seeds (0 d). A, Time courses of relative abundance increase in deteriorated seeds during CDT for the two protein spots (spots nos 253, and 312). B, Time courses of relative abundance decrease in deteriorated seeds during CDT for the two protein spots (spots nos 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 non-linear 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 (d). T_{1/2} values are listed in Tables I, II, IV and V.
Figure 4. Protein carbonyl patterns in Arabidopsis seeds following controlled deterioration treatment (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 non-deteriorated 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 2,4-dinitrophenylhydrazone (DNP) after derivatization with the corresponding hydrazine, as described (Job et al., 2005). Proteins undergoing carboxylation are labeled with black arrows. They are listed in Table III. This figure shows that the CDT and natural aging induce an oxidative stress on specific proteins, presumably through the generation of ROS.
Figure 5. Changes in protein accumulation patterns in deteriorated seeds during germination sensu stricto (1-d post-imbibition). An equal amount (150 µg) of total soluble protein extracts was loaded in each gel. Proteins were separated by 2D-gel electrophoresis. A representative silver-stained 2D gel of total soluble proteins from non-deteriorated dry mature seeds is presented in Figure 2A. The analysis was carried out on non-deteriorated seed samples (0 d, control seeds) and deteriorated seeds (3 d, 5 d and 7 d of CDT) imbibed in water for 1 d. The 18 labeled protein spots (spots nos 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; Job et al., 2005; Rajjou et al., 2004, 2006a; http://www.seed-proteome.com; see Tables IV and V and Supplemental Table S2). Protein spot quantitation was carried out as described in "Materials and Methods," from at least five gels for each seed sample.
Figure 6. Influence of the controlled deterioration treatment (CDT) and natural aging on de novo protein synthesis during germination sensu stricto. 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 ten 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 d, 1 d, 2 d, 3 d, 5 d 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, 7-year-old seeds, 8-year-old seeds and 11-year-old seeds).
Figure 7. Comparison of de novo protein synthesis patterns during germination sensu stricto (1-d post-imbibition) of deteriorated seeds. A, Protein profiles of de novo synthesized proteins in non-deteriorated 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 [35S]-Met in the germination assays, as described in "Materials and Methods." Soluble proteins were extracted after 1-d imbibition, submitted to 2D gel electrophoresis, and the radiolabeled proteins revealed as described in "Materials and Methods." The thirty three labeled protein spots were identified by mass spectrometry and by comparison with Arabidopsis seed protein reference maps (Gallardo et al., 2001, 2002a; Job et al., 2005; Rajjou et al., 2004, 2006a; http://www.seed-proteome.com; see Table VI and Supplemental Table S2). a, 12S seed storage protein precursor; b, alpha-subunit of 12S seed storage protein; c, beta-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).
Figure 8. Similar protein pattern evolution during controlled deterioration treatment (CDT) or natural aging. An equal amount (150 µg) of total protein extracts was loaded in each gel. A representative silver-stained 2D gel of total proteins from non-deteriorated seeds (0 d, control seeds) is presented Figure 2A. A, Enlarged window of 2D gels as shown in 2Ab for non-deteriorated seeds. B, Enlarged window of 2D gels as shown in 2Ab for deteriorated seeds (7 d of CDT). C, Enlarged window of 2D gels as shown in 2Ab for freshly harvested seeds. D, Enlarged window of 2D gels as shown in 2Ab for 7-year-old seeds (natural aging).