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Patterns of Protein Oxidation in Arabidopsis Seeds and during Germination^{1[w]}

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Increased cellular levels of reactive oxygen species are known to occur during seed development and germination, but the consequences in terms of protein degradation are poorly characterized. In this work, protein carbonylation, which is an irreversible oxidation process leading to a loss of function of the modified proteins, has been analyzed by a proteomic approach during the first stages of Arabidopsis (*Arabidopsis thaliana*) seed germination. In the dry mature seeds, the legumin-type globulins (12S cruciferins) were the major targets. However, the acidic α -cruciferin subunits were carbonylated to a much higher extent than the basic (β) ones, consistent with a model in which the β -subunits are buried within the cruciferin molecules and the α -subunits are more exposed to the outside. During imbibition, various carbonylated proteins accumulated. This oxidation damage was not evenly distributed among seed proteins and targeted specific proteins as glycolytic enzymes, mitochondrial ATP synthase, chloroplastic ribulose biphosphate carboxylase large chain, aldose reductase, methionine synthase, translation factors, and several molecular chaperones. Although accumulation of carbonylated proteins is usually considered in the context of aging in a variety of model systems, this was clearly not the case for the Arabidopsis seeds since they germinated at a high rate and yielded vigorous plantlets. The results indicate that the observed specific changes in protein carbonylation patterns are probably required for counteracting and/or utilizing the production of reactive oxygen species caused by recovery of metabolic activity in the germinating seeds.

Reactive oxygen species (ROS) are involved in various aspects of seed physiology, where they support dual function being either cytotoxic or playing a role in development, dormancy breakage, and in defense against biotic and abiotic stresses (for review, see Bailly, 2004; Mittler et al., 2004). Thus, a number of studies have documented the production of H₂O₂, nitric oxide, hydroxyl radicals, and superoxide radicals during germination of various species (for review, see Bailly, 2004). During seed formation on the mother plant, desiccation damage is also largely suspected to be related to oxidative processes, as indicated by the observation that dry, mature, desiccation-tolerant seeds display high levels of ROS-scavenging enzymes (e.g. catalase and glutathione reductase) as well non-enzymatic antioxidant components such as glutathione and tocopherols (vitamin E; for review, see Bailly, 2004). Indeed, Arabidopsis (*Arabidopsis thaliana*) mutants impaired in vitamin E biosynthetic genes exhibit substantial reduced seed longevity and severe seedling growth defects during germination demonstrat-

ing that uncontrolled ROS production can lead to oxidative stress and cellular damage, resulting in seed deterioration and impeding germination and early seedling development (Sattler et al., 2004).

All biologically relevant macromolecules, i.e. nucleic acids, membrane lipids, and proteins, are susceptible to damage by ROS. Such oxidative damage accumulates over time during the life cycle of many organisms and has been suggested to be one possible cause of aging (Stadtman, 1992; Agarwal and Sohal, 1994). Protein carbonylation is a widely used marker of protein oxidation (Ballesteros et al., 2001; Das et al., 2001; Mostertz and Hecker, 2003; Johansson et al., 2004), and sensitive methods for its detection have been developed (Levine et al., 1990, 1994). It occurs by direct oxidative attack on Lys, Arg, Pro, or Thr residues of proteins, thus inhibiting or altering their activities and increasing their susceptibility toward proteolytic attack (Rivett and Levine, 1990; Berlett and Stadtman, 1997; Dukan et al., 2000; Dunlop et al., 2002). Plants accumulate large amounts of storage protein reserves in developing seeds for use as an initial source of reduced nitrogen by the germinating seedling. Furthermore, studies of the effect of transcriptional and translational inhibitors on Arabidopsis seed germination have underscored the importance of nonstorage proteins present in the dry mature seeds and of proteins that are synthesized de novo during imbibition for the success of germination (Rajjou et al., 2004). This raises the possibility that oxidative stress as occurs during maturation drying and germination can

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induce oxidation of seed proteins and in turn deleteriously affect germination vigor.

The model plant *Arabidopsis* can be used to answer this question on a genomic basis (Somerville and Koornneef, 2002). Here, we provide evidence that massive protein oxidation occurs during *Arabidopsis* seed maturation and germination. In the mature seeds, carbonylation mainly affected 12S cruciferins, the major seed storage proteins in *Arabidopsis*. Upon imbibition, oxidized 12S-cruciferin subunits disappeared steadily, reflecting their mobilization during plantlet establishment. In parallel, the carbonylation of many proteins occurred during imbibition. The results of this study demonstrate that protein oxidation damage during germination is a selective phenomenon. This is likely to have implications for the success of the germination process.

RESULTS

Time Course of Protein Carbonylation during Germination

The extent of protein carbonylation during *Arabidopsis* seed germination was assessed by using non-dormant seeds from the Landsberg *erecta* ecotype. On water and under optimal conditions (25°C), radicle protrusion started at 1.5 d of imbibition, and it took almost 1.8 d for 50% of the seeds to reach this phase. Germination was completed after 2-d imbibition. The protein content of dry mature seeds and germinating seeds was examined by SDS-PAGE. Profiles of seed proteins from dry mature seeds and from germinating seeds are presented in Figure 1. Protein detection using Coomassie Blue coloration disclosed three major

protein bands in the dry mature seeds, of about 34, 30, and 20 kD (Fig. 1A). They correspond respectively to the α - and β -subunits of the legumin-type globulins (also called 11S or 12S globulins and more specifically cruciferins in *Arabidopsis*; see later in Fig. 2 and Table I), which are the major seed storage proteins in *Arabidopsis* (Pang et al., 1988; Gallardo et al., 2001; Gruis et al., 2002). Mature 12S globulins are hexamers composed of six acidic (α) and six basic (β) polypeptides of about 11 to 12S (Adachi et al., 2003). The intensity of the cruciferin bands steadily and rapidly decreased during germination and then precipitously dropped after 2-d imbibition corresponding to seedling establishment. After 4-d imbibition, cruciferin subunits were hardly detectable (Fig. 1A).

In initial experiments, the time course of protein carbonylation was monitored by one-dimensional (1D) SDS-PAGE and western blotting using the 2,4-dinitrophenylhydrazine (DNPH) immunoassay. As shown in Figure 1B, protein carbonylation was detected in the mature seeds, principally affecting the α - and β -subunits of the 12S cruciferins. Very surprisingly, carbonylation of the α -subunits was much more intense (6-fold) than that of the β -subunits. For both subunit types, the amount of protein carbonylation steadily decreased during germination, with about one-half of the carbonylated α -subunits disappearing after 2-d imbibition (Fig. 1B).

Characterization of Carbonylated Proteins in Mature Dry Seeds by Proteomics

To identify more precisely possible targets for protein carbonylation in *Arabidopsis* seeds, we used a proteomic immunochemical approach (Levine et al.,

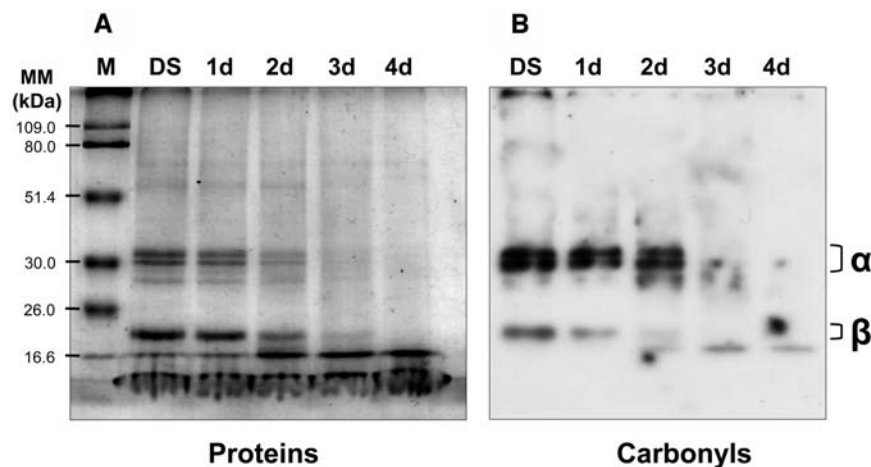


Figure 1. Detection of oxidatively modified proteins of *Arabidopsis* seeds during germination. Protein extracts from the dry mature seeds (DS) and from seed samples taken at the indicated times (d) during germination were prepared as described in "Materials and Methods" and analyzed by SDS-PAGE. Protein stain (A) and anti-DNP immunoassay (B) are shown. The approximate gel locations of acidic (α) and basic (β) 12S-cruciferin subunits are indicated with brackets. Gels were scanned, and the normalized volumes of the protein bands were determined as indicated in "Materials and Methods." From A, normalized volumes of α - and β -cruciferin chains for the mature seeds (lane DS) are 51.4 and 48.6, respectively. From B, normalized volumes of carbonylated α - and β -cruciferin subunits for the mature seeds (lane DS) are 85.8 and 14.2, respectively. Representative results are shown.

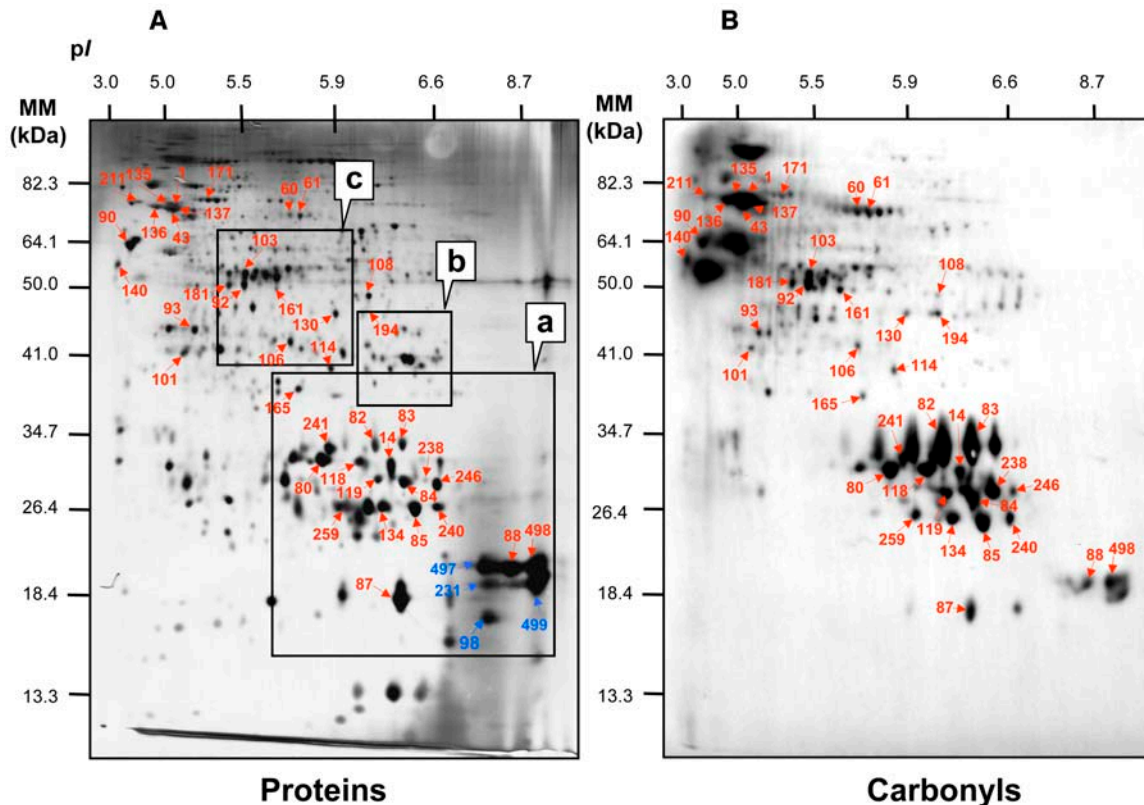


Figure 2. Two-dimensional profiles of protein abundance and oxidation in dry mature *Arabidopsis* seeds. Protein extracts were prepared as described in "Materials and Methods." Protein stain (A) and anti-DNP immunoassay (B) are shown. Proteins undergoing carbonylation are labeled with red arrows. They are listed in Table I. Major cruciferin β -subunits that are not carbonylated in the dry mature seeds are labeled with blue arrows: spot number 98, β -subunit of 12S cruciferin encoded by gene At1g03880; spot number 231, β -subunit of 12S cruciferin encoded by gene At4g28520; spot number 497, β -subunit of 12S cruciferin encoded by gene At4g28520; spot number 499, β -subunit of 12S cruciferin encoded by gene At5g44120. The indicated portions of the gel, a, b, and c, are reproduced in Figures 3 and 4. Representative results are shown.

1990, 1994). Carbonylated proteins were identified by matching the 2,4-dinitrophenylhydrazine (DNP)-derivatized protein spots to master gel maps of *Arabidopsis* seed proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004; <http://seed.proteome.free.fr>). As Figure 2 shows, this analysis allowed detection of several oxidized proteins in the dry mature seeds. Yet, since not all seed proteins are oxidized in the mature dry seeds (compare Fig. 2, A and B), protein oxidation during maturation/drying/storage targets specific proteins.

Consistent with the data in Figure 1, several polypeptides corresponding to the α - and β -subunits of 12S cruciferins were found to be carbonylated. Thus, spots numbers 14, 80, 82, 83, 118, and 241 (Fig. 2; Table I) were assigned by mass spectrometry analysis to the α -subunit of a 12S cruciferin encoded by the At4g28520 gene. These polypeptides presumably corresponded to the larger α -subunit protein band seen in Figure 1 since the predicted molecular mass of the α -subunit from this gene is 34.68 kD. Spots numbers 85, 134, and 240 (Fig. 2; Table I) corresponded to the α -subunit of a 12S-cruciferin isoform encoded by the gene At1g03880. In addition, spots numbers 84, 119, and 238 (Fig. 2; Table I) could be assigned to the α -subunit

of a third 12S cruciferin encoded by the gene At5g44120. All these six later polypeptides presumably accounted for the smaller α -subunit protein band evidenced by 1D SDS-PAGE (Fig. 1) since the predicted molecular masses of the α -subunits from the At1g03880 and At5g44120 genes are 27.24 and 29.11 kD, respectively. Furthermore, the data are consistent with the fact that three genes encode the 12S cruciferins in *Arabidopsis* (Pang et al., 1988). Carbonylation of 12S-cruciferin β -subunits could also be detected (Fig. 2; Table I), albeit to a much lower extent than seen for the α -subunits, a result consistent with those obtained by 1D gel electrophoresis (Fig. 1). One possibility to account for this difference in reactivity might be that the α - and β -cruciferin subunits contain different amounts of Lys, Arg, Pro, and Thr residues that are preferentially oxidized in proteins. However, for all three 12S cruciferins encoded by genes At4g28520, At1g03880, and At5g44120, this possibility seems unlikely since altogether these four residues account for about 16% to 17% and 20% to 22% of total amino acids in the corresponding α - and β -subunits, respectively. To decipher whether 12S-cruciferin β -subunits are potentially oxidizable, the dry mature seeds were submitted to

Table 1. Identification and relative carbonyl content of proteins oxidized during germination of Arabidopsis seed

Cov., Coverage; Exp., experimental; Theo., theoretical.

No.	Exp. Molecular Mass	Exp. pI	Theo. Molecular Mass	Theo. pI	Protein Name	AGI Locus Name	% Cov.	Relative Abundance ^a
	<i>kD</i>		<i>kD</i>					
Translation factors								
105 ^b	48.63	5.61	46.40	5.55	Elongation factor 1- γ 2	At1g57720	10	2.98
333 ^b	93.95	6.05	94.25	5.89	Elongation factor EF-2	At1g56070	20	7.83
129 ^b	47.20	5.50	46.70	5.47	Eukaryotic initiation factor 4A-1	At3g13920 or At1g72730	16	10.05
128 ^b	47.45	5.45	46.64	5.13	Elongation factor 1- β - γ	At1g09640	10	61.98
127 ^c	93.92	5.89	94.25	5.89	Elongation factor EF-2	At1g56070	31	>100
Glycolysis/Gluconeogenesis/Pentose phosphate pathway								
161 ^c	52.11	5.71	50.06	8.79	Dihydrolipoamide S-acetyltransferase	At3g25860	11	0.1
106 ^c	41.64	5.77	41.84	6.11	Alcohol dehydrogenase	At1g77120	22	0.32
103 ^c	57.95	5.58	47.72	5.54	Enolase	At2g36530	13	1
171 ^c	80.50	5.37	79.95	6.32	Transketolase	At3g60750	8	1
375 ^b	56.90	5.71	43.73	8.05	UDP-Glc pyrophosphorylase	At5g17310	46	3.09
197 ^c	40.9	6.77	38.37	7.46	Fru-bisphosphate aldolase	At2g36460	22	>100
198 ^c	40.7	6.89	38.37	7.46	Fru-bisphosphate aldolase	At2g36460	18	>100
36 ^c	40.42	6.44	36.99	6.34	Glyceraldehyde-3-phosphate dehydrogenase cytosolic	At3g04120 or At1g13440	34	>100
37 ^c	40.29	6.49	36.99	6.34	Glyceraldehyde-3-phosphate dehydrogenase cytosolic	At3g04120 or At1g13440	26	>100
39 ^b	38.52	6.23	36.99	6.34	Glyceraldehyde-3-phosphate dehydrogenase cytosolic	At3g04120 or At1g13440	25	>100
40 ^b	38.55	6.26	36.99	6.34	Glyceraldehyde-3-phosphate dehydrogenase cytosolic	At3g04120 or At1g13440	32	>100
Pyruvate metabolism								
109 ^c	64.79	6.35	64.28	6.32	Malate oxidoreductase or malic enzyme	At2g19900	14	6.27
349 ^b	64.79	6.44	64.26	6.73	Malate oxidoreductase or malic enzyme	At2g19900	8	29.57
296 ^b	96.35	5.37	98.23	5.53	Pyruvate-orthophosphate dikinase	At4g15530	5	>100
Citrate cycle (TCA cycle)								
114 ^c	39.56	5.87	35.57	6.11	Malate dehydrogenase cytoplasmic 1	At1g04410	21	0.12
130 ^c	47.75	5.84	45.73	6.52	Isocitrate dehydrogenase	At1g65930	18	1
290 ^b	96.69	5.88	98.13	6.36	Aconitate hydratase cytoplasmic	At4g35830	7	12.64
188 ^c	96.69	5.83	98.13	6.36	Aconitate hydratase cytoplasmic	At4g35830	9	41.05
26 ^c	96.38	5.75	98.15	5.79	Aconitate hydratase cytoplasmic	At2g05710	21	>100
Fatty acid biosynthesis								
165 ^c	38.32	5.78	41.20	9.36	Enoyl-[acyl-carrier-protein] reductase	At2g05990	36	0.06
194 ^b	45.50	6.15	50.40	8.16	3-Oxoacyl-[acyl- carrier-protein] synthase 1	At5g46290	24	1
Amino acid metabolism								
108 ^c	49.11	6.16	44.58	6.98	Indole-3-glycerol phosphate synthase	At2g04400	13	0.07
38 ^c	59.99	5.61	54.49	5.72	Aminopeptidase	At2g24200	27	2.03
160 ^c	82.27	5.94	84.34	6.47	5-Methyl-tetra- hydropteroyl-tri- Glu-homocysteine methyltransferase	At5g17920	12	12.1

(Table continues on following page.)

Table 1. (Continued from previous page.)

No.	Exp. Molecular Mass	Exp. pI	Theo. Molecular Mass	Theo. pI	Protein Name	AGI Locus Name	% Cov.	Relative Abundance ^a
	<i>kD</i>		<i>kD</i>					
206 ^c	82.05	6.09	84.34	6.47	5-Methyl-tetra- hydropteroyl-tri- Glu-homocysteine methyltransferase	At5g17920	16	47.53
295 ^b	96.42	5.43	103.45	6.48	Peptidase M1 family protein	At1g63770	5	>100
Seed storage proteins								
498 ^b	22.84	8.85	21.20	6.19	β -Cruciferin 12S (Seed storage protein fragment)	At4g28520	79	0.13
87 ^c	18.43	6.36	20.80	7.03	β -Cruciferin 12S (Seed storage protein fragment)	At1g03880	33	0.17
88 ^c	22.82	8.68	21.20	6.19	β -Cruciferin 12S (Seed storage protein fragment)	At4g28520	44	1
233 ^b	18.45	9.06	20.84	9.06	β -Cruciferin 12S (Seed storage protein fragment)	At5g44120	52	4.09
98 ^b	18.08	6.12	20.80	7.03	β -Cruciferin 12S (Seed storage protein fragment)	At1g03880	45	>100
241 ^b	34.11	5.87	34.68	6.42	α -Cruciferin 12S (Seed storage protein fragment)	At4g28520	32	0.07
14 ^c	30.46	6.30	34.68	6.42	α -Cruciferin 12S (Seed storage protein fragment)	At4g28520	15	0.14
83 ^c	34.35	6.42	34.68	6.42	α -Cruciferin 12S (Seed storage protein fragment)	At4g28520	31	0.14
82 ^c	33.89	6.24	34.68	6.42	α -Cruciferin 12S (Seed storage protein fragment)	At4g28520	39	0.17
80 ^c	32.64	5.85	34.68	6.42	α -Cruciferin 12S (Seed storage protein fragment)	At4g28520	31	0.19
134 ^c	26.35	6.34	27.24	6.34	α -Cruciferin 12S (Seed storage protein fragment)	At1g03880	12	0.29
84 ^b	30.46	6.61	29.11	6.49	α -Cruciferin 12S (Seed storage protein fragment)	At5g44120	55	0.39
118 ^c	29.21	6.04	34.68	6.42	α -Cruciferin 12S (Seed storage protein fragment)	At4g28520	21	0.41
85 ^c	27.20	6.50	27.24	6.34	α -Cruciferin 12S (Seed storage protein fragment)	At1g03880	24	0.48
240 ^b	27.28	6.74	27.24	6.34	α -Cruciferin 12S (Seed storage protein fragment)	At1g03880	16	1
119 ^c	30.27	6.10	29.11	6.49	α -Cruciferin 12S (Seed storage protein fragment)	At5g44120	27	1
238 ^b	29.42	6.50	29.11	6.49	α -Cruciferin 12S (Seed storage protein fragment)	At5g44120	28	1
111 ^c	56.89	7.19	55.86	6.36	12S seed storage protein [Precursor]	At4g28520	16	4.1
71 ^c	50.44	7.67	52.59	7.68	12S seed storage protein [Precursor]	At5g44120	34	6.76
70 ^c	49.43	7.19	52.59	7.68	12S seed storage protein [Precursor]	At5g44120	22	7.47
177 ^b	45.47	6.07	48.03	6.56	12S seed storage protein [Precursor]	At1g03880	25	48.26
442 ^b	50.54	6.71	52.59	7.68	12S seed storage protein [Precursor]	At5g44120	24	64.73
69 ^c	43.67	6.29	48.03	6.56	12S seed storage protein [Precursor]	At1g03880	18	>100
151 ^b	46.11	5.38	49.66	5.45	Cupin family protein	At1g03890	34	>100
246 ^b	29.45	6.70	55.05	7.15	Storage proteins 7S	At3g22640	25	1
Molecular chaperones								
90 ^c	64.11	4.81	55.60	4.81	Protein disulfide isomerase	At1g21750	39	0.29
1 ^c	79.36	5.08	71.17	5.08	dnaK-type molecular chaperone BiP	At5g42020	20	1

(Table continues on following page.)

Table 1. (Continued from previous page.)

No.	Exp. Molecular Mass	Exp. pI	Theo. Molecular Mass	Theo. pI	Protein Name	AGI Locus Name	% Cov.	Relative Abundance ^a
	<i>kD</i>		<i>kD</i>					
135 ^c	80.50	5.05	71.17	5.08	dnak-type molecular chaperone BiP	At5g42020	20	1
140 ^c	60.26	4.15	48.51	4.20	Calreticulin 1 (Precursor)	At1g56340	15	1
43 ^c	78.90	5.06	71.13	4.69	Heat shock cognate 70 kD	At3g09440	15	1
136 ^c	77.87	4.97	71.37	5.03	Heat shock cognate 70 kD	At5g02500	19	1
137 ^c	76.06	5.07	71.37	5.03	Hsp70	At3g12580	38	1
211 ^c	80.28	4.82	76.49	4.81	Hsp70	At4g24280	12	1
138 ^c	71.33	5.21	72.99	5.63	Hsp70	At5g09590	18	7.43
45 ^c	76.38	5.24	70.91	5.30	Hsp70	At1g16030	24	20.22
Stress response								
61 ^c	66.68	5.78	67.19	5.78	LEA	At2g42560	43	0.01
60 ^c	66.68	5.74	67.19	5.78	LEA	At2g42560	25	0.03
92 ^b	53.10	5.59	51.61	5.58	Jasmonate inducible protein-like	At2g33070	25	0.44
181 ^c	54.36	5.52	51.61	5.58	Jasmonate inducible protein-like	At2g33070	20	1
210 ^b	88.93	4.93	65.95	4.81	Low-temperature-induced 65-kD protein	At5g52300	18	6.79
131 ^b	38.48	6.22	36.38	6.23	Aldose reductase	At5g01670	20	>100
Hydrolase								
101 ^c	41.07	5.16	39.46	9.27	Hydrolase α/β -fold family protein	At3g54240	19	1
67 ^c	63.25	6.47	60.26	6.91	Glycosyl hydrolase family 1 protein	At3g09260	20	>100
68 ^c	63.47	6.56	60.26	6.91	Glycosyl hydrolase family 1 protein	At3g09260	17	>100
208 ^c	64.96	6.23	57.83	6.02	Glycosyl hydrolase family 1 protein	At3g21370	14	>100
216 ^b	65.26	6.08	57.83	6.02	Glycosyl hydrolase family 1 protein	At3g21370	26	>100
DNA metabolism								
297 ^b	97.40	5.20	89.38	4.88	Cell division cycle protein (CDC48)	At3g09840	23	>100
186 ^c	96.47	5.78	102.78	10.05	DNA topoisomerase I	At5g55300	11	>100
187 ^c	96.47	5.81	102.78	10.05	DNA topoisomerase I	At5g55300	11	>100
Others processes								
259 ^b	27.01	6.10	27.28	6.70	Expressed protein	At1g05510	21	1
93 ^c	42.55	5.27	41.96	5.26	Actin 7	At5g09810	21	0.09
104 ^c	58.87	5.48	54.13	5.13	ATP synthase β -chain (mitochondrial precursor)	At5g08670 or At5g08680 or At5g08690	24	5.5
10 ^c	57.36	5.77	53.47	6.25	Rubisco large chain	AtCg00490	30	2.98
320 ^b	57.36	5.82	53.47	6.25	Rubisco large chain	AtCg00490	19	15.13
319 ^b	57.36	5.57	53.47	6.25	Rubisco large chain	AtCg00490	26	18.62
321 ^b	57.36	5.84	53.47	6.25	Rubisco large chain	AtCg00490	23	>100

^aData obtained from densitometric analysis of individual spots from carbonylated proteins in 2D gels revealed by anti-DNP immunoassay (an example is shown in Fig. 2B); normalized spot volume in the Arabidopsis seeds incubated for 2 d in water divided by the normalized spot volume in the dry mature seeds (ratio of carbonylation at 2 d over carbonylation in dry seeds), from three different gels and independent extractions; >100 means that the accumulation level of the corresponding carbonylated protein in the mature dry seeds was close to background. ^bListed proteins correspond to proteins identified during this work; the peptide sequences determined are available in the supplemental data. ^cListed proteins correspond to previously identified proteins (Gallardo et al., 2001, 2002a; Rajjou et al., 2004).

a controlled deterioration test (also called accelerated aging), in which seeds are stored at high relative humidity and temperature (Delouche and Baskin, 1973). This test, which has been described for Arabidopsis (Tesnier et al., 2002; Clercx et al., 2004; Sattler

et al., 2004), entails an oxidative stress responsible for seed vigor loss (Khan et al., 1996; Goel et al., 2003; Calucci et al., 2004). In agreement with this finding, none of the seeds from the aged Arabidopsis seed lot prepared in this manner germinated after 11-d

imbibition (data not shown). It is noted that, in this study, the revelation of carbonylated proteins on the nitrocellulose blots with the peroxidase-conjugated anti-rabbit antibodies (Fig. 3, C and D) was carried out for a shorter time than in Figure 2B, to reveal all possible changes induced by the controlled deterioration test. As Figure 3 shows, controlled deterioration of the dry mature seeds was associated with a strong increase in the extent of carbonylation of 12S-cruciferin β -subunits, up to a similar level of that of the α -subunits. Therefore, from a comparison of the data in Figures 2 and 3, we conclude that in dry mature *Arabidopsis* seeds, the β -subunits of 12S cruciferins are much less carbonylated than the corresponding α -subunits (Fig. 2), although both types of subunits are potentially equally oxidizable (Fig. 3).

In addition to the 12S-cruciferin subunits, several other proteins ought to be oxidized in the dry mature *Arabidopsis* seeds (Fig. 2). The most prominent of these carbonylated proteins, e.g. heat shock proteins (spots nos. 43, 45, 136, 137, 138, and 211), late embryogenesis abundant (LEA) proteins (spots nos. 60 and 61), enolase (spot no. 103), or jasmonate-inducible protein (spots nos. 92 and 181), are listed in Table I.

Evolution of the Oxidized Seed Proteome during Germination

To reveal changes both in the total proteome and in the oxidized proteome during germination, protein

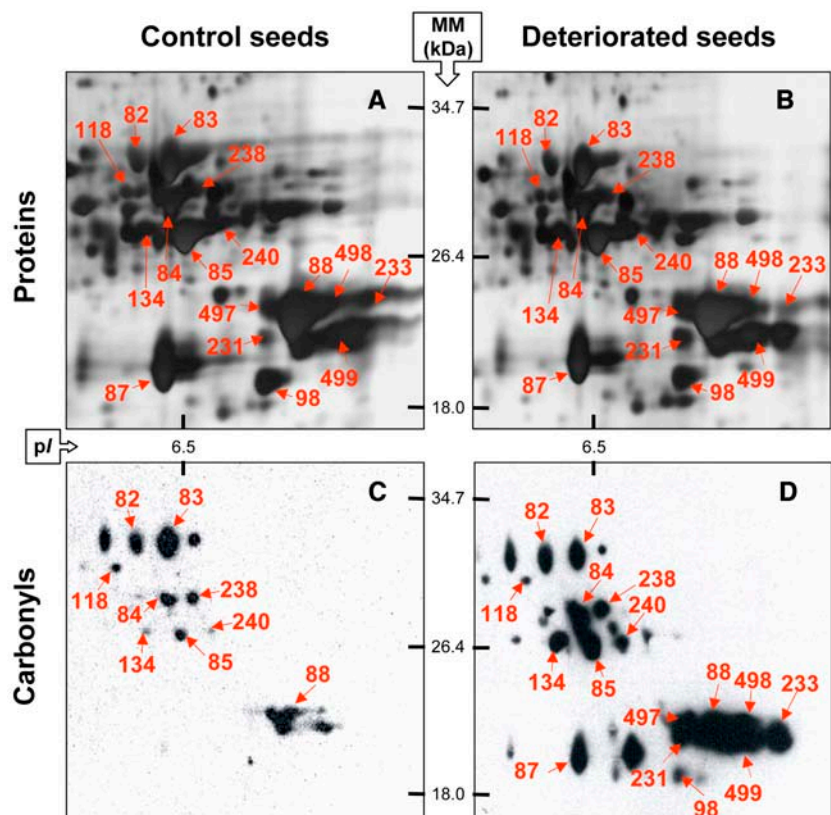
extracts from dry mature seeds imbibed for 1 and 2 d were submitted to two-dimensional (2D) gel electrophoresis and the proteins revealed either by silver nitrate coloration (total proteins) or by western blotting using the anti-DNP immunoassay (carbonylated proteins).

A number of carbonylated proteins were evidenced, of which the accumulation level either remained constant or varied during germination. These proteins are listed in Table I, and experimental data are shown in enlarged windows of 2D gels (Fig. 4, A–C).

In agreement with the data in Figure 1, a drastic drop in carbonylated 12S-cruciferin subunits was observed during germination, particularly concerning the larger α -subunits encoded by the At4g28520 gene (spots nos. 14, 80, 82, 83, and 241; Fig. 4A; Table I).

The most prominent carbonylated proteins whose level increased during germination are the Rubisco large chain (spots nos. 10, 319, 320, and 321; Fig. 4C; Table I), heat shock protein of 70 kD (Hsp70) chaperones (spots nos. 45 and 138; Table I), glycoside hydrolase family 1 proteins (spots nos. 67, 68, 208, and 216; Table I), cytosolic glyceraldehyde-3-phosphate dehydrogenase (spots nos. 36, 37, 39, and 40; Fig. 4B; Table I), Fru-1,6-bisphosphate aldolase (spots nos. 197 and 198; Fig. 4B; Table I), aminopeptidases (spots nos. 38 and 295; Table I), the mitochondrial F_0F_1 -ATP synthase β -subunit (spot no. 104; Fig. 4C; Table I), and several translation initiation and elongation factors (spots nos. 105, 128, and 129; Fig. 4C; Table I). These data

Figure 3. Increased protein carbonyl levels in β -subunits of 12S cruciferins after controlled deterioration. Protein extracts were prepared as described in “Materials and Methods” from the dry mature seeds (Control seeds) and seeds submitted to a controlled deterioration assay (Deteriorated seeds) and analyzed by 2D-PAGE. Protein stains (A and B) and anti-DNP immunoassays (C and D) are shown. The labeled proteins are listed in Table I. Protein spots numbers 82, 83, 238, 84, 240, 85, 134, and 118 correspond to α -subunits of 12S cruciferins. Protein spots numbers 87, 88, 98, 233, 231, 497, 498, and 499 correspond to β -subunits of 12S cruciferins. The portions of the 2D gels shown correspond to window “a” in Figure 2A. Representative results are shown.



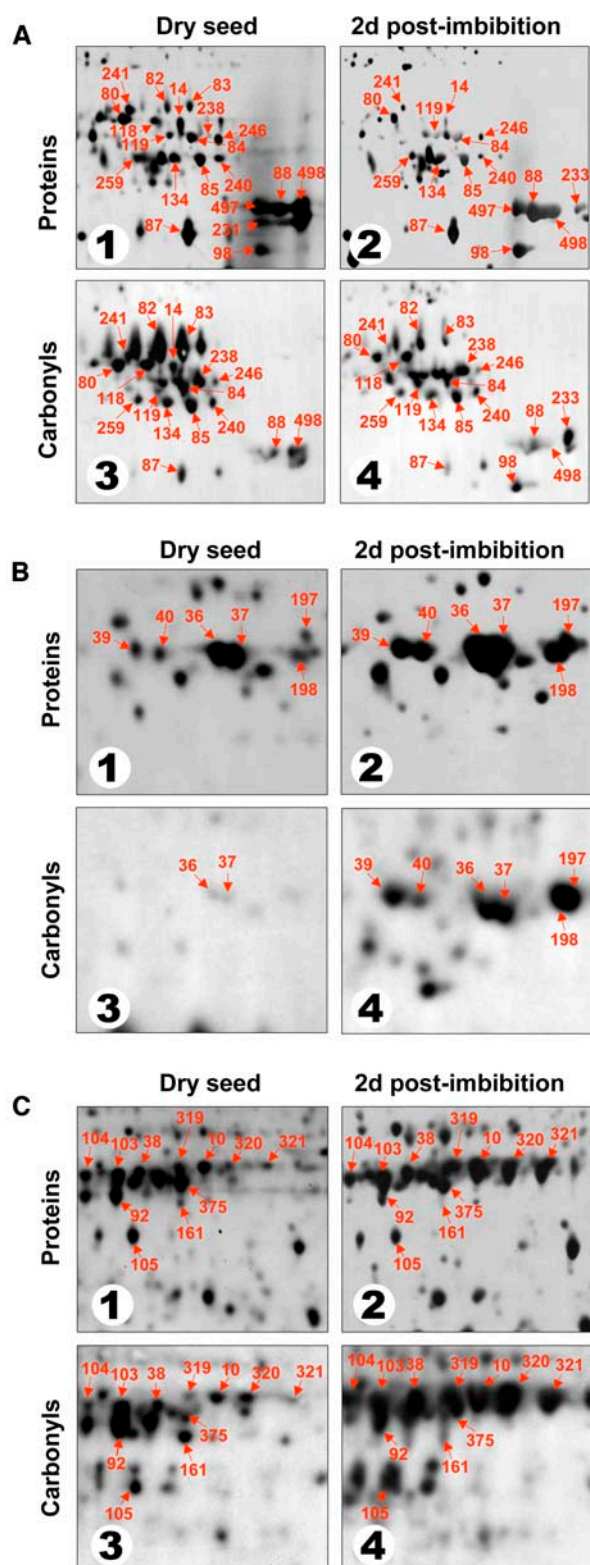


Figure 4. Detection of oxidatively modified proteins of Arabidopsis seeds after 2-d imbibition. Protein extracts from the dry mature seeds (Dry seed) and from seed samples taken after 2-d imbibition during germination (2 d postimbibition) were prepared as described in "Materials and Methods" and analyzed by 2D-PAGE. Protein stains (sections 1 and 2) and anti-DNP immunoassays (sections 3 and 4) are

document that protein carbonylation can occur in several cell subcellular compartments during germination, namely, plastids, mitochondria, and cytosol.

As for the dry mature seeds (Fig. 2), it is clear that not all proteins became carbonylated during germination (Fig. 4, A–C), demonstrating that protein oxidation does not occur at random during this developmental process. This is also clearly demonstrated by the different susceptibilities of individual isoforms of some of the target enzymes to oxidation, e.g. aconitase (Table I) and cytosolic GAPDH (compare the carbonylation and protein levels of spots nos. 36, 37, 39, and 40; Fig. 4B; Table I). Thus, as in other systems, carbonylation appears to be highly specific in Arabidopsis seeds.

DISCUSSION

This work demonstrates that Arabidopsis seed proteins are sensitive to oxidative damage during development and germination. This sensitivity was revealed by the detection of high levels of oxidative modification of seed proteins, as inferred by monitoring carbonyl groups with a DNP immunoassay. Since targets for carbonylation have not previously been investigated in seed tissues, many of the carbonylated proteins identified here (e.g. the 12S-cruciferin subunits, aldose reductase, or the LEA proteins) denote new proteins compared to oxidation-sensitive proteins described in other systems. Nevertheless, Hsp70 chaperones, aconitase, translation initiation and elongation factors, ATP synthase β -subunit, actin, and GAPDH have been shown previously to be highly sensitive to oxidation either in bacterial, yeast (*Saccharomyces cerevisiae*), or animal cells (Tamarit et al., 1998; Cabiscol et al., 2000; Dalle-Donne et al., 2001; Das et al., 2001; Shenton and Grant, 2003; England and Cotter, 2004; Reverter-Branchat et al., 2004; Shanmuganathan et al., 2004), pointing out common features in various living systems. Also, the fact that carbonylation affects the plastidic Rubisco and the β -subunit of mitochondrial F_0F_1 -ATP synthase both in seeds (this work) and in leaves (Johansson et al., 2004) suggests common features of protein oxidation in different tissues of plants.

shown. The labeled proteins are listed in Table I. A, Spots numbers 80, 241, 82, 14, 83, 238, 84, 240, 85, 134, 119, and 118, α -subunits of 12S cruciferins; spots numbers 87, 88, 98, 498, and 233, β -subunits of 12S cruciferins. The portion of the 2D gels shown corresponds to window "a" in Figure 2A. B, Spots numbers 39, 40, 36, and 37, cytosolic GAPDH; spots numbers 197 and 198, Fru-bisphosphate aldolase. The portion of the 2D gels shown corresponds to window "b" in Figure 2A. C, Spots numbers 319, 10, 320, and 321, Rubisco large chain; spot number 104, ATP synthase β -chain; spot number 103, enolase; spot number 38, aminopeptidase; spot number 92, jasmonate-inducible protein like; spot number 105, elongation factor 1- γ 2; spot number 161, dihydrolipoamide *S*-acetyl transferase; spot number 375, UDP-Glc pyrophosphorylase. The portion of the 2D gels shown corresponds to window "c" in Figure 2A.

Apart from the seed-specific proteins, the patterns of protein oxidation observed in this work are strikingly reminiscent of those described for *Escherichia coli* or yeast cells exposed to oxidative stress (Tamarit et al., 1998; Cabiscol et al., 2000; Reverter-Branchat et al., 2004; Shanmuganathan et al., 2004), lending further support to the contention that oxidative stress normally accompanies seed development and germination (Bailly, 2004).

The most prominent carbonylated proteins in the dry mature *Arabidopsis* seeds were found to correspond to the 12S-cruciferin subunits. However, an intriguing result of this study was that the α - and β -cruciferin subunits were not carbonylated to the same extent in the dry mature seeds or during germination. Yet, both types of subunits were shown to be potentially equally sensitive to carbonylation as demonstrated by subjecting the dry mature *Arabidopsis* seeds to a deterioration treatment (Fig. 3) that caused seed vigor loss by excessive accumulation of oxidative damage (Khan et al., 1996; Goel et al., 2003; Calucci et al., 2004). The 12S globulin hexamers are formed via a multistep pathway, each subunit being the product of a small gene family. Precursor proteins derived from these genes (of approximately 50 kD) are synthesized on rough endoplasmic reticulum and assembled into trimers in the lumen. Then the trimers traverse the protein secretory system to the protein storage vacuoles. Their accumulation in these organelles is accompanied by the posttranslational cleavage of the proglobulin subunits at a conserved Asn-Gly peptide bond, giving rise to the mature α - and β -chains, of approximately 30 and 20 kD, respectively. Cleavage of the trimers specifically gives rise to the formation of the hexamers in the vacuoles (Jung et al., 1998; Gruis et al., 2004). The crystal structure of the soybean (*Glycine max*) 12S globulin homohexamer (glycinin) and of the soybean proglycinin homotrimer (Adachi et al., 2001, 2003) revealed several disordered regions that are concentrated within the α -subunits and correspond to hydrophilic polypeptide regions exhibiting extensive sequence variability (Shutov et al., 2003). Furthermore, the variable regions are highly susceptible to limited proteolysis both in vivo and in vitro, suggesting a model in which the β -chains are buried within the interior of the 12S globulin molecules whereas the α -chains are more exposed to the outside (Castellani et al., 2000; Schlereth et al., 2000; Shutov et al., 2003). This structural model provides therefore an explanation for our present results showing a marked difference in protein carbonyl levels for the α - and β -cruciferin chains and lends further support that the structural features established in vitro for the 12S globulins also hold in vivo.

The protein carbonyl levels of the largest α -subunits encoded by At4g28520 gene (spots nos. 14, 80, 82, 83, 118, and 241; Table I) were quite similar in the viable mature dry seeds and in the dry mature seeds submitted to controlled deterioration (Fig. 3), suggesting that these subunits are fully carbonylated during

seed maturation. By contrast, the carbonyl level of the smaller cruciferin α -subunits encoded by genes At1g03880 (spots nos. 85, 134, and 240) and At5g44120 (spots nos. 84, 119, and 238) increased substantially during seed deterioration (Fig. 3), indicating that these polypeptides were not fully carbonylated during maturation. Since there is no significant difference in the expression profiles of the 12S-cruciferin genes of *Arabidopsis* during seed maturation (Pang et al., 1988), the results suggest that the susceptibility of the variable regions of the 12S-cruciferin isoforms to ROS might differ for structural reasons. It is noted that the procruciferins, which are still present in small amounts in dry mature *Arabidopsis* seeds (Gallardo et al., 2001; Gruis et al., 2004), underwent carbonylation during germination. Based on the structure of pro- and mature forms of legumin-type globulins (Adachi et al., 2001, 2003), it is possible that this feature results from the exposure of polypeptide bonds normally protected within the mature hexamer structure but now accessible to carbonylation by ROS. A similar explanation has been proposed to account for the mobilization of propolypeptide forms of 12S cruciferins during *Arabidopsis* seed germination (Gruis et al., 2004). In summary, carbonylation of cruciferin polypeptides seems to be highly dependent on cruciferin structure, reinforcing the finding that protein oxidation does not occur at random in seeds.

Besides 12S-cruciferin subunits, the dry mature *Arabidopsis* seeds were found to contain several carbonylated proteins. Furthermore, during germination there was a marked change in the oxidized proteome, disclosing new carbonylated proteins. Several of them are involved in glycolysis/gluconeogenesis pathways (Table I), which are the most predominant biochemical pathways during seed germination (Thomas and ap Rees, 1972; Zhang et al., 2004). This feature may be indicative of a need to control soluble sugar levels during germination since the levels of Glc and other sugars have been shown to affect the speed of seed germination in *Arabidopsis* (Dekkers et al., 2004; Gibson, 2005). Specific oxidation of glycolytic enzymes has also been reported in *E. coli* (Tamarit et al., 1998), yeast (Cabiscol et al., 2000; Shenton and Grant, 2003; Reverter-Branchat et al., 2004; Shanmuganathan et al., 2004), and HL-60 cells (England and Cotter, 2004) in response to oxidative stress. It has been proposed previously that blocking glycolysis could be beneficial during conditions of oxidative stress since it would result in an increased flux of Glc equivalents through the pentose phosphate pathway leading to the generation of NADPH (Ravichandran et al., 1994; Schuppe-Koistinen et al., 1994; Godon et al., 1998; Cabiscol et al., 2000; Shenton and Grant 2003; Shanmuganathan et al., 2004). This could provide the reducing power for antioxidant enzymes, including the thioredoxin and GSH/glutaredoxin systems (Holmgren, 1989). It should be noted that the NADP/thioredoxin system composed of NADPH, thioredoxin *h*, and NADP-thioredoxin reductase plays

a crucial role in seed germination since it functions in the reduction of the major storage proteins in seeds, converting disulfide S-S bonds to the reduced SH state, thereby leading to increased solubility and mobilization (Lozano et al., 1996; Wong et al., 2004a). In this context, it is also worth noting that many of the carbonylated proteins in Table I have previously been identified as thioredoxin targets in wheat (*Triticum aestivum*) seeds (Wong et al., 2004b), including aconitase, actin, aldolase, mitochondrial ATP synthase β -chain, elongation factors, enolase, globulin subunits, cytosolic GAPDH, Hsp70s, Met synthase, protein disulfide isomerase, pyruvate-orthophosphate dikinase, and UDP-Glc pyrophosphorylase. The results lend further support for the existence of a link between ROS and redox regulatory events catalyzed by thioredoxin in seeds (Wong et al., 2004a), as is well documented for H_2O_2 , glutaredoxin, and thioredoxin in *E. coli* (Zheng et al., 1998, 2001).

In the tricarboxylic acid cycle, aconitase catalyzes the isomerization of citrate to isocitrate. This enzyme appears to be particularly susceptible to oxidase damage (Das et al., 2001), presumably because of the presence of a $[4Fe-4S]^{2+}$ cluster at its active site, which can result in the release of free iron (Gardner and Fridovich, 1992) and, in turn, trigger a cascade of oxidative modifications in macromolecules via the Fenton reaction.

It is interesting to note the specific carbonylation of cobalamin-independent Met synthase during seed germination (Table I). This enzyme that catalyzes the methylation of homocysteine to Met was previously shown to be the target of protein oxidation in *E. coli* subjected to oxidative stress (Hondorp and Matthews, 2004). Arabidopsis contains three functional isoforms of cobalamin-independent Met synthase. One isoform is present in plastids and is involved in the de novo synthesis of Met in this compartment. The two others are present in the cytosol and are involved in the regeneration of Met from homocysteine produced in the course of the activated methyl cycle (Ravanel et al., 2004). These cytosolic isoforms accumulate in dry mature Arabidopsis seeds, and their level further increases during early germination (Gallardo et al., 2002b; Ravanel et al., 2004). Since in plants the synthesis of AdoMet is the major route for Met metabolism, with 80% of this amino acid being engaged in this reaction (Giovanelli et al., 1985), a reduction in the cytosolic Met synthase activity would likely be beneficial to cope with oxidative stress as more Cys could become available for GSH production. A similar explanation has been proposed to account for the carbonylation of AdoMet synthetase in yeast in response to copper-induced oxidative stress (Shanmuganathan et al., 2004). Alternatively, decreasing the AdoMet level might provide a means for maintaining a sustained generation of ROS during germination since this molecule behaves as a very potent antioxidant (Caro and Cederbaum, 2004). There is increasing evidence suggesting that the production of ROS is beneficial for the germinating process

by facilitating radicle protrusion or by protecting the embryo against pathogen attack (Schopfer et al., 2001; Bailly, 2004). It is noted that aldose reductase, which is presumed to be involved in detoxification of ROS in plants exposed to oxidative stress (for review, see Bartels, 2001), underwent carbonylation during germination. Therefore, specific carbonylation of this enzyme might be another mechanism to regulate the accumulation of ROS during germination.

As in *E. coli* and yeast cells exposed to an oxidative stress (Tamarit et al., 1998; Cabisco et al., 2000; Shenton and Grant, 2003) a range of translation factors were found to undergo carbonylation during Arabidopsis seed germination (Table I). Since protein synthesis in yeast cells is readily inhibited by treatment with H_2O_2 , it has been proposed that such an inhibition may provide a means of preventing continued gene expression under potentially error-prone conditions (Shenton and Grant, 2003). Indeed, in nonproliferating senescent bacteria protein oxidation is dictated by the accuracy of the ribosomes (Ballesteros et al., 2001).

Among major targets of oxidized proteins in Arabidopsis seeds, a large number of molecular chaperones (heat shock proteins, calreticulin, DnaK) were found (Table I). Such molecular chaperones were also identified as major targets for carbonylation in yeast and bacterial cells exposed to oxidative stress (Tamarit et al., 1998; Cabisco et al., 2000; Costa et al., 2002; Reverter-Branchat et al., 2004; Shanmuganathan et al., 2004), and it was suggested that they may act as shields protecting proteins from oxidative damage.

A recent work described the progression and specificity of protein oxidation in the life cycle of Arabidopsis (Johansson et al., 2004). The authors observed an initial increase in protein oxidation during the first 20 d of the life cycle followed by a drastic reduction in protein carbonylation prior to bolting and flower development. Our present results complement this study since the previous work did not investigate seed development and germination in the plant life cycle. Since protein carbonyl groups are not repaired and alter protein function, accumulated protein damage caused by ROS may be a central feature of aging (for review, see Berlett and Stadtman, 1997). That protein carbonylation is not synonymous to aging in developing Arabidopsis seeds is demonstrated by the fact that the presently used dry mature seeds displayed high vigor, germinating synchronously under standard conditions in about 2 d following the start of imbibition and yielding vigorous plantlets. Thus, our present results support the proposal of Johansson et al. (2004) that the progression of oxidative damage in the life cycle of plants and animals is fundamentally different.

What then might be the role of protein carbonylation in seed physiology? Since carbonylation of proteins increases their susceptibility to proteolytic cleavage (Rivett and Levine, 1990; Berlett and Stadtman, 1997; Dukan et al., 2000; Dunlop et al., 2002), one possibility

might be that carbonylation of 12S-cruciferin subunits occurring during seed development facilitates their mobilization during germination. Once the mobilization process is initiated upon seed imbibition, further oxidation of 12S-cruciferin subunits (e.g. of the β -subunits) would not be necessary for propagation of the mobilization process. A key question is whether the carbonylation events occurring during germination play a role in seedling establishment or if they are merely the consequence of cells undergoing intense metabolism activity in the awakening from quiescence. In view of the specificity of protein oxidation presently observed and the fact that the patterns of oxidized proteins closely resemble those of bacterial or yeast cells exposed to oxidative stress, it appears more likely that protein carbonylation does not simply reflect secondary epiphenomena but is used as a means to adapt embryo metabolism to the oxidative conditions encountered during germination. In future work, it would be of interest to correlate seed vigor and extent of protein oxidation in seeds of varying quality and to understand the molecular basis of the specificity of protein carbonylation in developing and germinating seeds. This would allow characterization of novel markers of seed vigor.

MATERIALS AND METHODS

Plant Material and Germination Experiments

Nondormant seeds of *Arabidopsis* (*Arabidopsis thaliana*), accession Landsberg *erecta*, were used in all experiments. Germination assays were carried out at 25°C, with 16 h light/8 h dark daily, on three sheets of absorbent paper (Roundfilter paper circles, Ø 45 mm; Schleicher & Schuell, Dassel, Germany) wetted with 1.3 mL of distilled water, in covered plastic boxes (Ø 50 mm). A seed was regarded as germinated when the radicle protruded through the seed coat.

Controlled Deterioration Test

The controlled deterioration test was performed as described (18). Seeds were equilibrated at 85% relative humidity (20°C), and day 0 controls were immediately dried back at 32% relative humidity. Treatment was done by storing the seeds (at 85% relative humidity) for 7 d at 40°C. Then these seeds were also dried back at 32% relative humidity (20°C) during 3 d.

Preparation of Total Protein Extracts

Total protein extracts were prepared from dry mature viable and deteriorated seeds and from viable seeds at different stages of germination. Following grinding of seeds using mortar and pestle (150 mg representing approximately 8,400 seeds) in liquid nitrogen, total proteins were extracted at 2°C in 1.2 mL of thiourea/urea lysis buffer (Harder et al., 1999) containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, and 1% (v/v) Pharmalyte, pH 3 to 10, carrier ampholytes (Amersham Biosciences, Orsay, France). This extraction buffer also contained 18 mM Tris-HCl (Trizma HCl; Sigma, St Quentin Fallavier, France), 14 mM Trizma base (Sigma), the protease inhibitor cocktail complete Mini from Roche Diagnostics GmbH (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) was added, and the protein extracts were stirred for 20 min at 4°C and then centrifuged (35,000g, 10 min) at 4°C. The supernatant was submitted to a second clarifying centrifugation as above. The final supernatant corresponded to the total protein extract. Protein concentrations in the various extracts were measured according to Bradford (1976). Bovine serum albumin was used as a standard.

SDS-PAGE

SDS-PAGE of seed protein extracts (5 μ g protein) was performed with 12% (w/v) polyacrylamide resolving gels, as described by Laemmli (1970).

Two-Dimensional Electrophoresis

Proteins were analyzed by 2D gel electrophoresis as described (Görg et al., 1987; Rajjou et al., 2004). Isoelectrofocusing was carried out with protein samples with an equivalent to an extract of 105 seeds, corresponding to about 150 μ g 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; Amersham Biosciences). The second dimension was carried out in 10% SDS-polyacrylamide gels (Rajjou et al., 2004). Ten gels (200 \times 250 \times 1.0 mm) were run in parallel (Isodalt system from Amersham Biosciences). For each condition analyzed, 2D gels were made in triplicate and from two independent protein extractions.

Protein Staining and Analysis of Gels

One-dimensional gels were stained with the GelCode blue stain from Pierce (Rockford, IL). Two-dimensional gels were stained with silver nitrate according either to Blum et al. (1987) for densitometric analyses or to Shevchenko et al. (1996) for the mass spectrometry analyses. Stained gels were scanned with the UMAX Powerlook III scanner equipped with the MagicScan version 4.5 from UMAX Data Systems (Amersham Biosciences). Image analysis was carried out with the ImageMaster 2D Elite version 4.01 software (Amersham Biosciences), according to the instruction booklet ImageMaster 2D Elite from Amersham Biosciences. After spot detection and background subtraction (mode: lowest on boundary), 2D gels were aligned, matched, and the quantitative determination of the spot volumes was performed (mode: total spot volume normalization). In this method, the volume of each spot is divided by the total volume of all of the spots in the gel.

Detection of Carbonylated Proteins and Western Blotting

The appearance of carbonyl groups in proteins was analyzed by immunodetection of protein-bound DNP after derivatization with the corresponding hydrazine, as described (Talent et al., 1998; Korolainen et al., 2002) with slight modifications. SDS was added to the protein extract (100 μ L; 10 μ g/mL) at a final concentration of 0.8%. Following dialysis, four volumes of 10 mM DNPH (Sigma)/2 M HCl were added. Samples were agitated for 30 min at room temperature, and five volumes of 20/80 ice-cold TCA-acetone containing 1 mM DTT were added to each sample. The samples were centrifuged for 15 min at 15,000g at 4°C. The precipitated protein was then washed three times with 1 mL of 1:1 (v/v) ethanol:ethyl acetate and resolubilized in the thiourea/urea lysis buffer containing 2% (v/v) Triton X-100 and 20 mM DTT. Proteins were separated by 1D- or 2D-PAGE as above and transferred to nitrocellulose sheets (Bio-Rad, Marnes La Coquette, France) using standard procedures. Blots were rinsed twice for 5 min in 50 mM Tris-HCl, 150 mM NaCl, pH 7.5 (TBS), then incubated for 1 h at 25°C in Blocking Solution (Roche Diagnostics GmbH). After incubation for 1 h with rabbit anti-DNP antibodies (Serologicals, Norcross, GA) in TBS, blots were washed four times in TBS containing 0.05% (v/v) Tween 20 and incubated for 1 h in anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Sigma). Bound antibodies were visualized by using the ECL kit (Roche Diagnostics). Relative protein carbonyl levels were quantitated by densitometric analyses of the blots as described above.

Protein Identification by Mass Spectrometry

Identification of the proteins characterized in this work was obtained by mass spectrometry. Silver-stained protein spots of interest were obtained from at least three different 2D gels. The spots were excised from 2D SDS-PAGE gels with sterile tips and put in 1.5-mL sterile tubes. Each spot was rinsed then reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and incubated overnight at 37°C with 12.5 ng/ μ L trypsin (sequencing grade; Roche Diagnostics) in 25 mM NH_4HCO_3 as described (24). The tryptic fragments were extracted, dried, reconstituted with 2% (v/v) acetonitrile, 0.1% formic acid, and sonicated for 10 min. Analysis of tryptic peptides by tandem mass spectrometry was performed on a nanoelectrospray ionization quadrupole

time-of-flight hybrid mass spectrometer (Q-TOF Ultima Global; Waters Micro-mass, Manchester, UK) coupled with a nano-HPLC (Cap-LC; Waters). The samples were loaded and desalted on a C18 precolumn (LC-Packings PepMap C18, 5 μ m, 100 Å, 300 μ m \times 5 mm) at a flow rate of 20 μ L/min isocratically with 0.1% formic acid. The peptides were separated on a C18 column (Atlantis dC18, 3 μ m, 75- μ m \times 150-mm Nano Ease; Waters). After a wash with solvent A (water/acetonitrile 98/2 (v/v), 0.1% formic acid), a linear gradient from 5% to 60% of solvent B (water/acetonitrile 20/80 (v/v), 0.1% formic acid) was developed over 80 min at a flow rate of 180 nL/min. The Q-TOF spectrometer was operated in the Data Dependent Analysis mode using a 1-s mass spectrometry (MS) survey scan on three different precursor ions. The peptide masses and sequences obtained were either matched automatically to proteins in a nonredundant database (NCBI) using the Mascot MS/MS Ions Search algorithm (<http://www.matrixscience.com>) or blasted manually against the current databases. They are available as supplemental data in the online version of this article.

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