The Plant Journal (2007) **50**, 452–465 doi: 10.1111/j.1365-313X.2007.03063.x

ROS production and protein oxidation as a novel mechanism for seed dormancy alleviation

Krystyna Oracz[1,2], Hayat El-Maarouf Bouteau[2], Jill M. Farrant[3], Keren Cooper[3], Maya Belghazi[4], Claudette Job[5], Dominique Job[5], Françoise Corbineau[2] and Christophe Bailly[2,*]

[1]*Department of Plant Physiology, Warsaw Agricultural University, Nowoursynowska 159, 02-776 Warsaw, Poland,*

[2]*Université Pierre et Marie Curie-Paris 6, EA 2388 Physiologie des Semences, Le Raphaël, Site d'Ivry, Boîte 152, 4 Place Jussieu, Paris F-75005 France,*

[3]*Department of Molecular and Cell Biology, University of Cape Town, Private Bag 7701 Rondebosch, South Africa,*

[4]*Institut National de la Recherche Agronomique, Unité Mixte de Recherche 6175, Service de Spectrométrie de Masse pour la Protéomique, Nouzilly, F-37380 France, and*

[5]*Centre National de la Recherche Scientifique/Bayer CropScience Joint Laboratory, Unité Mixte de Recherche 2847, Bayer CropScience, Lyon, F-69283 France*

Received 17 November 2006; revised 21 December 2006; accepted 4 January 2007
*For correspondence (fax +33 1 44 27 59 27; e-mail bailly@ccr.jussieu.fr).

Summary

At harvest, sunflower (*Helianthus annuus* L.) seeds are dormant and unable to germinate at temperatures below 15°C. Seed storage in the dry state, known as after-ripening, is associated with an alleviation of embryonic dormancy allowing subsequent germination at suboptimal temperatures. To identify the process by which dormancy is broken during after-ripening, we focused on the role of reactive oxygen species (ROS) in this phenomenon. After-ripening entailed a progressive accumulation of ROS, namely superoxide anions and hydrogen peroxide, in cells of embryonic axes. This accumulation, which was investigated at the cellular level by electron microscopy, occurred concomitantly with lipid peroxidation and oxidation (carbonylation) of specific embryo proteins. Incubation of dormant seeds for 3 h in the presence of hydrogen cyanide (a compound that breaks dormancy) or methylviologen (a ROS-generating compound) also released dormancy and caused the oxidation of a specific set of embryo proteins. From these observations, we propose a novel mechanism for seed dormancy alleviation. This mechanism involves ROS production and targeted changes in protein carbonylation patterns.

Keywords: seed dormancy, after-ripening, reactive oxygen species, proteome analysis, carbonylation, sunflower.

Introduction

Seed dormancy, defined as the failure of viable mature seeds to germinate under favorable conditions, is assumed to be an important adaptive trait in nature, enabling seeds to remain quiescent until the conditions for germination and seedling establishment become favorable (Bewley, 1997; Bewley and Black, 1994; Finch-Savage and Leubner-Metzger, 2006). This trait can have an embryo and/or a coat component, hence the terms 'embryo' and 'coat' dormancy to distinguish between these two mechanisms. Under natural conditions, release of dormancy generally occurs during after-ripening (storage in dry conditions) or during stratification (imbibition at low temperature), which result in widening of the conditions allowing seed germination (Baskin and Baskin, 1998; Bewley, 1997; Bewley and Black, 1994; Donohue *et al.*, 2005; Finch-Savage and Leubner-Metzger, 2006; Koornneef *et al.*, 2002).

After-ripening is an intriguing phenomenon as it occurs at low seed moisture contents (MC), generally <0.10 g H_2O/g dry weight (DW). Under these extreme conditions, water is generally not available for biochemical reactions, and very little is known about the cellular and molecular mechanisms involved in this process. However, changes in gene expression and/or protein synthesis during after-ripening have been shown to occur in seeds of *Nicotiana tabacum*

(Leubner-Metzger, 2005), *Nicotiana plumbaginifolia* (Bove *et al.*, 2005) and *Arabidopsis thaliana* (Cadman *et al.*, 2006; Chibani *et al.*, 2006), which presumably reflects the existence of hydrated pockets within cells or tissues of the mature seeds (Leubner-Metzger, 2005). Non-enzymatic reactions are also likely to occur during dry storage of seeds, such as lipid peroxidation (McDonald, 1999; Priestley, 1986; Wilson and McDonald, 1986) or the Amadori and Maillard reactions associated with free radical production and oxidation processes (Esashi *et al.*, 1993; Murthy and Sun, 2000; Murthy *et al.*, 2003; Sun and Leopold, 1995). Furthermore, several studies have documented the production of reactive oxygen species (ROS) during seed storage in the dry state (Bucharov and Gantcheff, 1984; Hendry, 1993; McDonald, 1999; Pukacka and Ratajczak, 2005). ROS can react with virtually all biological molecules including lipids, DNA and proteins. Because proteins have numerous biological functions, their oxidation may result in modification of their enzymatic and binding properties and lead to diverse functional changes. Oxidation of proteins can occur through a number of different mechanisms, such as the formation of disulfide cross-links and glycoxidation adducts, nitration of tyrosine residues, and carbonylation of specific amino acid residues (Davies, 2005). Recent studies have indicated that protein oxidation is not necessarily a deleterious phenomenon in plants (Job *et al.*, 2005; Johansson *et al.*, 2004). Moreover, cellular ROS may show some selectivity with respect to their targets. For example, H_2O_2, which is an oxidant, can react with specific molecules at specific sites (Davies, 2005; Halliwell and Gutteridge, 1999). Importantly, ROS have been invoked to play a role in cellular signaling (for review, see Bailly, 2004), raising the hypothesis that these compounds can facilitate the shift from a dormant to a non-dormant status in seeds.

Sunflower (*Helianthus annuus*) seeds provide an excellent system for studying dormancy because they are deeply dormant at harvest, but this dormancy is progressively lost during dry storage (Corbineau *et al.*, 1990). Their dormancy results from both seed coat- and embryo-imposed dormancy, the latter being involved in the failure to germinate at 10–15°C. However, the molecular mechanisms of embryo dormancy and of its release during after-ripening are still largely unknown.

The objective of this study was to investigate whether the production of ROS and lipid autoxidation occur after harvest, and whether this can, in turn, bring about modifications in seed protein oxidation patterns that facilitate alleviation of seed dormancy. Moreover, in order to assess a putative causal association between ROS production, protein oxidation and dormancy alleviation, dormant sunflower seeds were imbibed in the presence of hydrogen cyanide, a compound that breaks dormancy (Bethke *et al.*, 2006; Bogatek *et al.*, 1991; Esashi *et al.*, 1991; Taylorson and Hendricks, 1973), or in the presence of methylviologen (MV), a ROS-generating compound (Slooten *et al.*, 1995). Although MV has not yet been reported as a dormancy-breaking chemical, we have used it here as a tool to assess the proposition that ROS generation is involved in dormancy alleviation in this species. The general finding is that dormancy release, both in dry and imbibed states, is associated with ROS production and the carbonylation of specific embryo proteins.

Results

Seed germination

Figure 1 shows the germination behavior of dormant and non-dormant de-coated (naked) sunflower seeds at various temperatures. At 15, 20 and 25°C, dormant seeds germinated slowly and reached 80–90% germination within 6–8 days (Figure 1a). Only about 20% of dormant naked seeds were able to germinate at 10°C within 10 days (Figure 1a), but dry storage markedly enhanced their germination at all temperatures tested, and, in particular, they became able to fully germinate at 10°C within 4 days (Figure 1b).

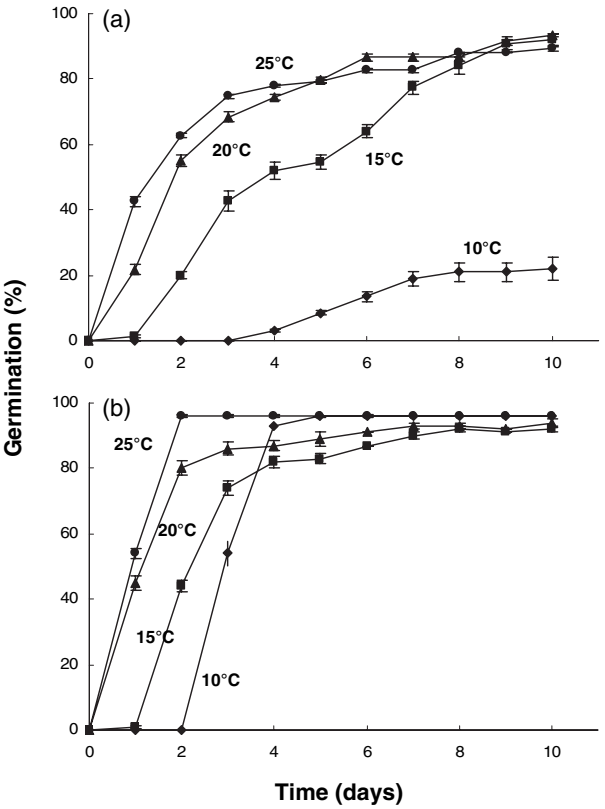

Figure 1. Germination of dormant and non-dormant sunflower embryos (i.e. naked seeds without pericarp) at various temperatures.
(a) Dormant embryos (immediately after harvest).
(b) Non-dormant embryos (after 2 months of dry storage).
Values are means of four replicates ± SD.

ROS production during dry storage

To characterize ROS production during after-ripening, hydrogen peroxide and superoxide anion contents were determined in axes (see Figure 2) excised from dormant and non-dormant seeds (Table 1). Dormancy release was associated with a marked enhancement in both compounds, as the level of H_2O_2 doubled and that of $O_2^{\cdot-}$ increased by about 50%. Dry storage was also associated with a slight but significant increase in malondialdehyde (MDA) content, indicating the occurrence of lipid peroxidation (Table 1).

Table 1 Hydrogen peroxide, superoxide and malondialdehyde (MDA) contents in dry dormant and non-dormant sunflower axes

	H_2O_2 (μmol g DW^{-1})	$O_2^{\cdot-}$ (μmol g DW^{-1})	MDA (μmol g DW^{-1})
Dormant axes	1.37 ± 0.21	5.29 ± 0.25	76.75 ± 3.65
Non-dormant axes	2.10 ± 0.14	8.66 ± 0.51	90.62 ± 0.72

Values are means of five replicates ± SD.

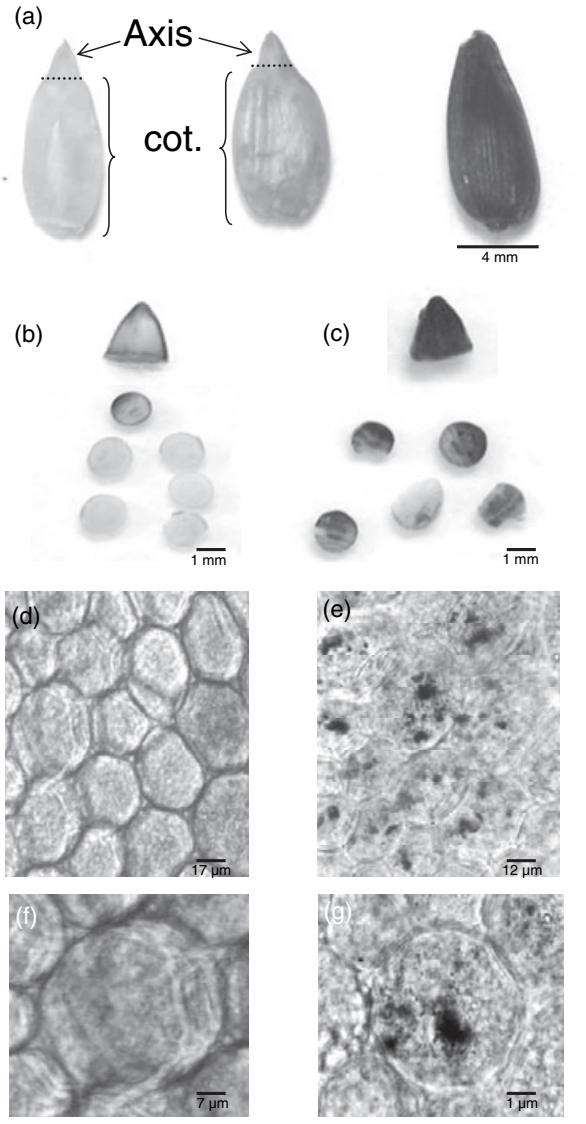

Figure 2. Localization of $O_2^{\cdot-}$ in sunflower embryonic axes.
(a) Whole seed (right), embryo (center) and half-embryo (showing only one cotyledon, left) of sunflower, showing axis and cotyledon (cot.).
(b, c) Whole axes and hand-cut sections of dormant (b) and non-dormant (c) sunflower axes. The superoxide anions were visualized with NBT staining.
(d, f) and (e, g) Sections of dormant and non-dormant axes, respectively, stained with NBT and viewed by light microscopy. The superoxide anion is detected as formazan precipitates within the cytoplasm of the cells of non-dormant axes (e, g). No marked coloration was visible in the cells of dormant axes (d, f).
Scale is indicated on bars within the figures.

In situ accumulation of $O_2^{\cdot-}$ in whole seeds (embryos) and in sections through axes during dry storage is shown in Figure 2. At the whole-tissue level, accumulation of formazans occurred almost homogeneously in axes of non-dormant seeds (Figure 2c). In contrast, staining of dormant axes by nitroblue tetrazolium (NBT) was only very slight (Figure 2b). Transverse sections through the axes confirmed the presence of formazan deposits at the cellular level. In dormant axes, no NBT precipitation was visible within the cells (Figure 2d,f), whereas numerous dark spots indicated the presence of superoxide anion within the cells of non-dormant axes (Figure 2e,g).

The detection of hydrogen peroxide was carried out by $CeCl_3$ staining and revelation by transmission electron microscopy (Bestwick *et al.*, 1997; Pellinen *et al.*, 2002). Cells from axes of both dormant and non-dormant seeds were filled with large lipid bodies and smaller protein bodies, which somewhat occluded observation of the cytoplasm (Figure 3a,b). However, in the presence of $CeCl_3$, H_2O_2 was clearly evident as electron dense (cerium perhydroxide) spots (arrowed) within the cytoplasm of the cells (Figure 3c–f).While some H_2O_2 could be detected in dormant seeds (Figure 3c,e), there was much higher staining in the cytoplasm of cells from axes of non-dormant seeds, revealing endogenous H_2O_2 production mainly in the non-dormant tissues (Figure 3d,f).

To determine whether ROS accumulation during dry storage caused seed dormancy alleviation, and was not simply a side-effect accompanying seed storage, whole dormant sunflower seeds were stored at 75% RH (relative humidity) or 5% RH at 25°C for various durations, and the H_2O_2 content in the axes was determined (Table 2). Storage of seeds at 75% RH was associated with a progressive reduction of dormancy, whereas dormancy was maintained when seeds were stored at 5% RH. After 6 weeks of storage at 25°C and 75% RH, dormancy was lost, as these seeds germinated at 10°C almost as fast as the non-dormant ones (i.e. the seeds obtained after 2 months of dry storage at 25°C and 60% RH; Table 3 and Figure 1b). Under these conditions,

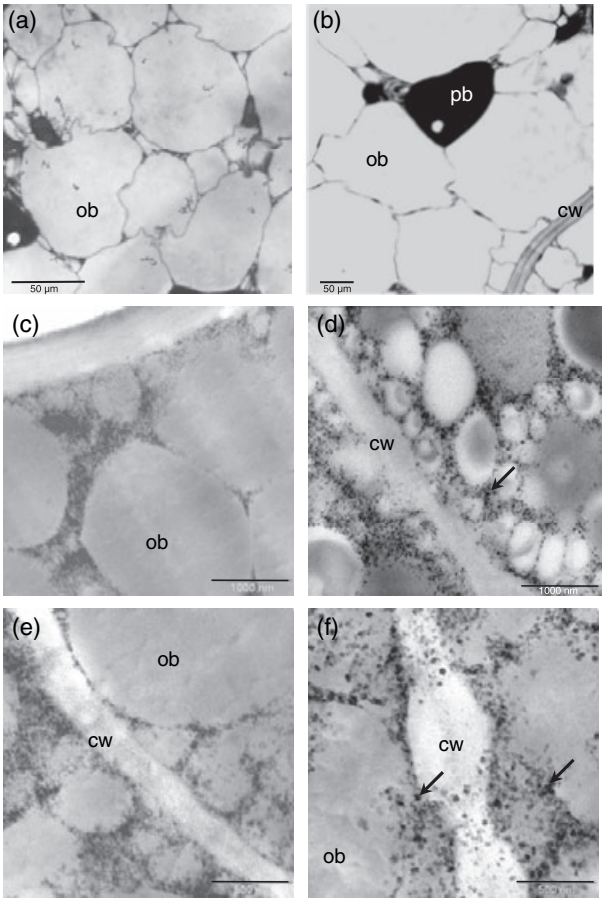

Figure 3. Subcellular localization of H_2O_2 in sunflower embryonic axes.
(a, b) Dormant and non-dormant axes, respectively, viewed by TEM without H_2O_2 staining (sections not infiltrated by $CeCl_3$).
(c, e) and (d, f) Dormant and non-dormant axes, respectively, viewed by TEM with $CeCl_3$-staining. H_2O_2 is visualized as black spots corresponding to electron-dense cerium perhydroxide precipitates (arrows). Abbreviations: cw, cell wall; ob, oil body; pb, protein body. Scale bars represent 50 µm (a,b), 1000 nm (c,d) and 500 nm (e,f).

Table 2 Germination after 7 days at 10°C and hydrogen peroxide content of axes of dormant sunflower seeds stored for 3 and 6 weeks under 75% and 5% RH at 25°C

Duration and conditions of storage	Germination (%) at 10°C after 7 days	H_2O_2 content (µmol g DW^{-1})
None (after harvest)	19 ± 2.2	1.37 ± 0.21
3 weeks, 75% RH	42 ± 1.1	2.01 ± 0.12
3 weeks, 5% RH	22 ± 1.5	1.41 ± 0.04
6 weeks, 75% RH	70 ± 2.0	2.21 ± 0.22
6 weeks, 5% RH	21 ± 0.5	1.40 ± 0.03

Values are means of four (germination) or five (H_2O_2) replicates ± SD

the hydrogen peroxide content of the embryos increased from 1.3 to 2.2 µmol g DW^{-1}, whereas this content remained unchanged when dormant seeds were stored for the same duration but at 5% RH, i.e. conditions that do not permit seed dormancy release (Table 3).

Protein carbonylation during dry after-ripening

To determine whether ROS production during after-ripening could be associated with protein oxidation (carbonylation), one-dimensional (1D) and two-dimensional (2D) PAGE of seed protein extracts were performed, and the presence of carbonyl groups was detected by Western blotting using the 2,4-dinitrophenylhydrazine (DNPH) immunoassay (Korolainen *et al.*, 2002).

The protein patterns obtained by 1D gel electrophoresis of soluble proteins from dormant and non-dormant embryonic axes are shown in Figure 4. Coomassie blue staining showed the soluble proteins from both dormant and non-dormant embryonic axes to be very similar. Soluble proteins from dormant axes exhibited three faint carbonylated bands of about 75, 60 and 55 kDa (Figure 4). In contrast, the extent of protein carbonylation was much higher in soluble proteins from non-dormant axes, and a number of new carbonylated bands were detected (Figure 4).

The protein targets of carbonylation during after-ripening were characterized by 2D gel electrophoresis and LC/MS–MS analyses. The 2D protein patterns of soluble proteins extracted from dormant and non-dormant axes were very similar (Figure 5a,c). However, in agreement with the data obtained by 1D gel electrophoresis (Figure 4), after-ripening was associated with increased carbonylation of several proteins (red arrows and red circles in Figure 5). There was also a decrease in the carbonylation level of some proteins during this process (yellow arrows in Figure 5). Finally, some carbonylated proteins remained at a constant level during after-ripening (green arrows in Figure 5). It is clear that not all seed proteins detected by silver staining were carbonylated, testifying to the specificity of protein oxidation during after-ripening.

Twenty spots representing carbonylated proteins were excised from 2D gels and analyzed by mass spectrometry. Identification failed for 12 proteins. Table 2 and Table S1 list the oxidized proteins that were identified by this approach, mainly by sequence homology with proteins from species other than sunflower. However, several proteins could be identified using the sunflower EST collections (http://cgpdb.ucdavis.edu/; http://genoplante-info.infobiogen.fr/), highlighting the usefulness of such collections as a tool for proteomics in the absence of extensive genomic sequence information. The data showed that the carbonylation level of elongation factor 2 (EF2, spot number 3 in Figure 5), pyruvate orthophosphate dikinase (PPDK, spot number 4 in Figure 5) and a protein corresponding to a sunflower EST, which has a strong homology with a 7S globulin of *Sesamum indicum* and with various vicilin-like proteins (spot number 15 in Figure 5) increased during dry after-

Table 3 Identification of carbonylated proteins in embryo axes of dry dormant and non-dormant sunflower seeds

Spot number	Accession	Protein name	MW (kDa)	pI	Change in carbonylation during after-ripening
3	gi|6056373	Elongation factor EF-2 (*Arabidopsis thaliana*)	93.9	6.1	Increase
4	gi|3024423	Pyruvate, phosphate dikinase, chloroplast precursor (*Flaveria brownie*)	93.9	5.1	Increase
7	gi|217855	81 kDa heat shock protein (*Arabidopsis thaliana*)	84.0	4.9	Constant
9	gi|2827002	HSP70 (*Triticum aestivum*)	73.4	5.0	Constant
12	gi|22273	Enolase (*Zea mays*)	60.0	5.5	Constant
15	gi|58735933	DH0AQA1ZD02RM1 HaDevS1, *Helianthus annuus* 7S globulin	60.0	6.9	Increase
18	gi|22460345	QHF6E13.yg.ab1 QH_EFGHJ sunflower RHA280, *Helianthus annuus* cDNA clone (≥gi|1458098) globulin-like protein (*Daucus carota*)	38.0	5.2	Constant
46	gi|32530040	DH0AB43ZF02RM1 HaDevR1, *Helianthus annuus* cDNA clone HaDevR143F02, mRNA sequence ≥gi|82621184|gb|ABB86280.1| proteasome-like protein alpha subunit-like (*Solanum tuberosum*)	37.0	6.6	Decrease

Peptide sequences were identified by MS-MS sequencing, see Table S1 for the peptide sequences. Experimental molecular weight (MW) and pI are indicated.

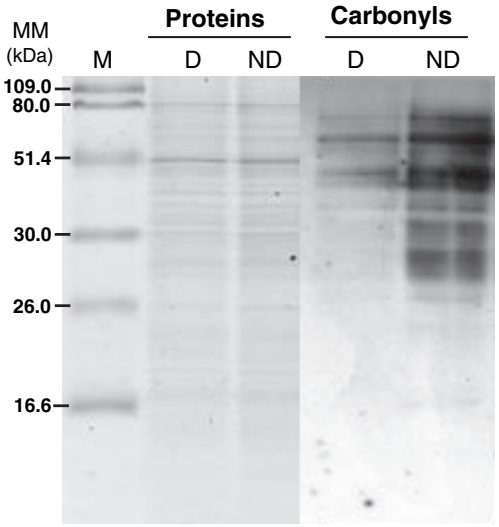

Figure 4. One-dimensional PAGE of oxidatively modified proteins from axes of dry dormant and non-dormant sunflower seeds.
Protein stain (proteins) and anti-DNP immunoassay (carbonyls) are shown. D, dormant; ND, non-dormant; M, markers; MM, molecular mass.

ripening. In contrast, seed dormancy alleviation during dry storage was associated with decreased carbonylation of several proteins including a 20S proteasome α-subunit (spot number 46 in Figure 5). Proteins whose carbonylation level remained constant during dry storage included 81 and 70 kDa heat shock proteins (HSPs, spot numbers 7 and 9 in Figure 5), enolase (spot number 12 in Figure 5) and a protein with strong homology to a globulin-like protein from carrot (*Daucus carota*) (spot number 18 in Figure 5).

Effect of cyanide and methylviologen on the protein oxidation pattern

To further document a link between protein oxidation and release of seed dormancy, dormant sunflower seeds were imbibed in the presence of hydrogen cyanide, a compound known to break dormancy in many species (Bethke *et al.*, 2006; Bogatek *et al.*, 1991; Côme *et al.*, 1988; Esashi *et al.*, 1991; Taylorson and Hendricks, 1973). A 3 h imbibition of the dormant sunflower seeds in the presence of 1 mM hydrogen cyanide efficiently released dormancy (Figure 6). Furthermore, this incubation triggered the carbonylation of specific proteins in dormant axes only (e.g. the blue circles in Figure 7). This carbonylation pattern was specific as it was not seen with non-dormant seeds incubated either in water or in the presence of hydrogen cyanide (Figure 7d). To further assess the correlation between ROS accumulation, protein carbonylation and break of dormancy, dormant and non-dormant seeds were imbibed for 3 h in the presence of methylviologen, a compound known to induce ROS production (Slooten *et al.*, 1995). After this treatment, the H_2O_2 content of dormant axes was 2.7 ± 0.11 μmol g DW^{-1}, whereas it was 2.0 ± 0.13 μmol g DW^{-1} when dormant seeds were imbibed on water (data not shown). Figure 6 shows that this compound was very effective in promoting dormancy release in sunflower seeds, lending further support to the hypothesis that dormancy release is associated with the generation of ROS. Remarkably, not only did this compound break dormancy, but this also entailed the same specific pattern of protein oxidation as seen with hydrogen cyanide (blue circle in Figure 7). Oxyblots also revealed that imbibition of dormant and non-dormant embryos on water

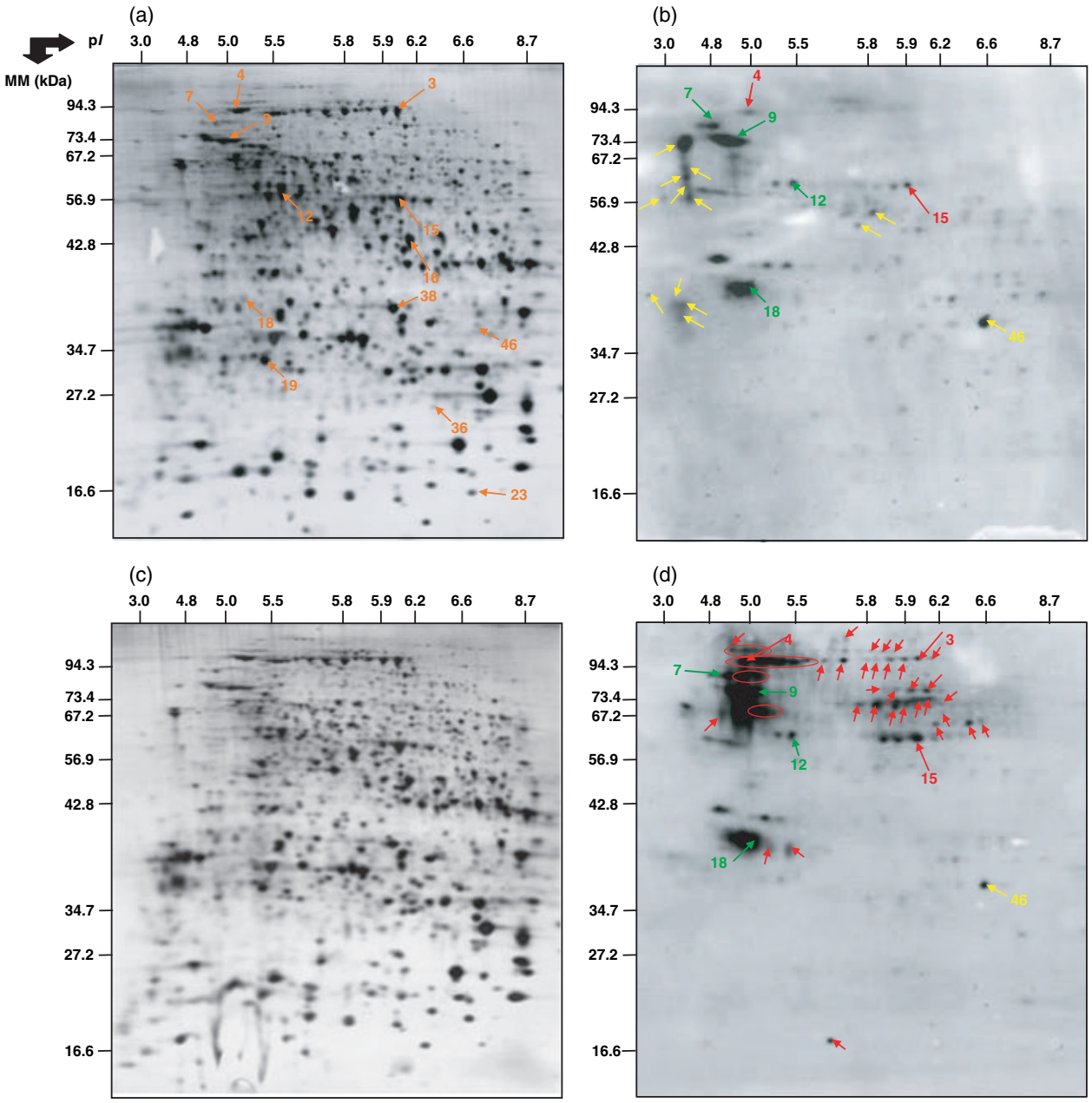

Figure 5. Two-dimensional profiles of protein abundance and oxidation in axes of dry dormant and non-dormant sunflower seeds.
(a, b) Dormant axes; (c, d) non-dormant axes.
Protein stains (a, c) and anti-DNP immunoassays (b, d) are shown. Proteins undergoing increased carbonylation during after-ripening are labeled with red arrows, those for which the carbonylation level remained constant during after-ripening are labeled with green arrows, and those for which the carbonylation level decreased during after-ripening are labeled by yellow arrows. Numbers indicated on the arrows correspond to the proteins that have been identified by mass spectrometry (listed in Table 3).

was associated with an increase in protein carbonylation (compare Figures 5 and 7). However, the level of protein oxidation was higher in non-dormant axes, which displayed many new oxidized proteins ranging from approximately 15–40 kDa (Figure 7b). Conversely, around 20 proteins that were found to be carbonylated during imbibition in dormant axes were not detected in non-dormant axes (Figure 7a, yellow circles). Carbonylated proteins identified by mass spectroscopy are listed in Table 4. EF2 (spot number 3), PPDK (spot number 4), 81 and 70 kDa HSP (spot numbers 7 and 9), enolase (spot number 12) and 7S globulin (spot number 15), previously identified in dry-after ripened seeds, were also carbonylated in the axes of all imbibed seeds (Figure 7, Tables 3 and 4). The globulin-like protein (spot

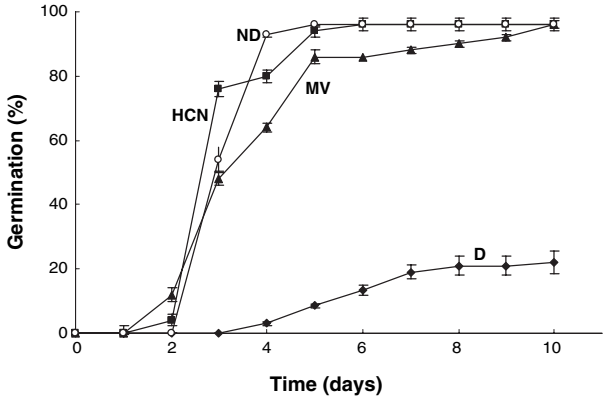

Figure 6. Effect of a 3 h treatment by 1 mM hydrogen cyanide (HCN) and 0.1 mM methylviologen (MV) on the germination of dormant sunflower embryos (D) at 10°C. ND, non-dormant embryos. Means of four replicates ± SD.

number 18) was also always carbonylated in imbibed axes, but the level of oxidation appeared to be stronger in non-dormant axes isolated from seeds imbibed on water (Figure 7b). Treatment of imbibed dormant seeds with cyanide and MV also resulted in the carbonylation of proteins not detected in dry after-ripened seeds. For example, proteins that show homology with 11S storage protein (spot number 36 in Figure 7) and epoxide hydrolase (spot number 38 in Figure 7) were detected in dormant seeds but did not appear in the axes of non-dormant seeds treated with cyanide and MV. Other proteins sharing homology with an 11S globulin precursor, an alcohol dehydrogenase and a basic 2S albumin (spot numbers 19, 16 and 23, respectively, in Figure 7) were oxidized in axes of all seeds able to germinate. Spot number 21 was detected only in imbibed non-dormant seeds (Figure 7b,d,f), and this protein was found to be similar to dehydrin (Table 4).

Discussion

Dormancy is a characteristic feature of sunflower seeds at their harvest. This dormancy exhibits both seed coat and embryo components, and progressively appears during seed development on the mother plant (Corbineau *et al.*, 1990). The seed-coat-imposed dormancy acts through the effects of phenolics on oxygen availability for the embryo, and is therefore expressed to a greater extent at high temperatures, while the embryo-imposed dormancy is likely to be involved in the restriction of germination at temperatures below 15°C (Corbineau *et al.*, 1990; Gay *et al.*, 1991). In agreement, dormant naked seeds assayed immediately after harvest only germinated very poorly at 10°C (Figure 1a), and after-ripening in dry conditions (20°C, 60% RH, 2 months) resulted in a widening of the temperatures permitting germination (Figure 1b).

Our results show that after-ripening is associated with an accumulation of superoxide anions and hydrogen peroxide in the embryonic axes (Table 1, Figures 2 and 3). $O_2^{\cdot-}$ appeared as formazan precipitates within the cells (Figure 2), and ultrastructural studies showed that hydrogen peroxide accumulated in the cytoplasm of embryonic axis cells (Figure 3). By storing the seeds at various RH, it was possible to modulate the extent of dormancy release (Table 2). This suggests that the water status within the embryo cell is likely to play a critical role in the process of after-ripening, and shows that hydrogen peroxide accumulation is tightly associated with the breaking of embryo dormancy as this compound only accumulated under conditions associated with dormancy release. Thus a causal link between ROS production, or at least H_2O_2 accumulation, and after-ripening is likely to exist, and this process is not just related to seed storage. This demonstrates that, in sunflower seeds, ROS production in the dry state is initiated after harvest, and that, in agreement with previous proposals that ROS can act as cell messengers (reviewed in Bailly, 2004), these molecules could therefore act as a signal to allow dormancy release and favor subsequent seed germination. However, it is also likely that prolonged storage of seeds in the dry state would be associated with a sustained production of ROS, which would lead to oxidative stress and to the related deteriorative events known to occur during seed aging (Bailly, 2004).

In plants, and more especially in seeds, ROS may originate from the mitochondrial respiratory chain or be produced through the action of enzymes such as NADPH oxidase (Bailly, 2004). However, in dry tissues, such as in mature orthodox seeds, respiration is prevented and enzymes are presumably not active, which suggests the occurrence of other mechanisms for ROS production. At low moisture content, non-enzymatic reactions are known to occur, such as the Amadori and Maillard reactions (Priestley, 1986; Sun and Leopold, 1995) and lipid peroxidation (McDonald, 1999). Enzymatic oxidation of lipids through lipoxygenase is also possible when water activity is as low as 0.4 (Drapron, 1985). Using model systems, such as those based on oil encapsulation in a glassy matrix, it has been demonstrated that glasses do not prevent oxygen diffusion, thus allowing autoxidation reactions of lipids (Andersen and Skibsted, 2002; Andersen *et al.*, 2000; Nelson and Labuza, 1992). As the MDA content increased during dormancy alleviation (Table 1), we assume that after-ripening is associated with lipid peroxidation, which could be a consequence of ROS accumulation and may, in turn, be involved in free radical formation.

To test the hypothesis that ROS accumulation during after-ripening is involved in dormancy alleviation, and to assign a possible mechanism whereby this might occur, we characterized the oxidized proteome of embryonic axes from dormant and non-dormant seeds. Protein carbonyla-

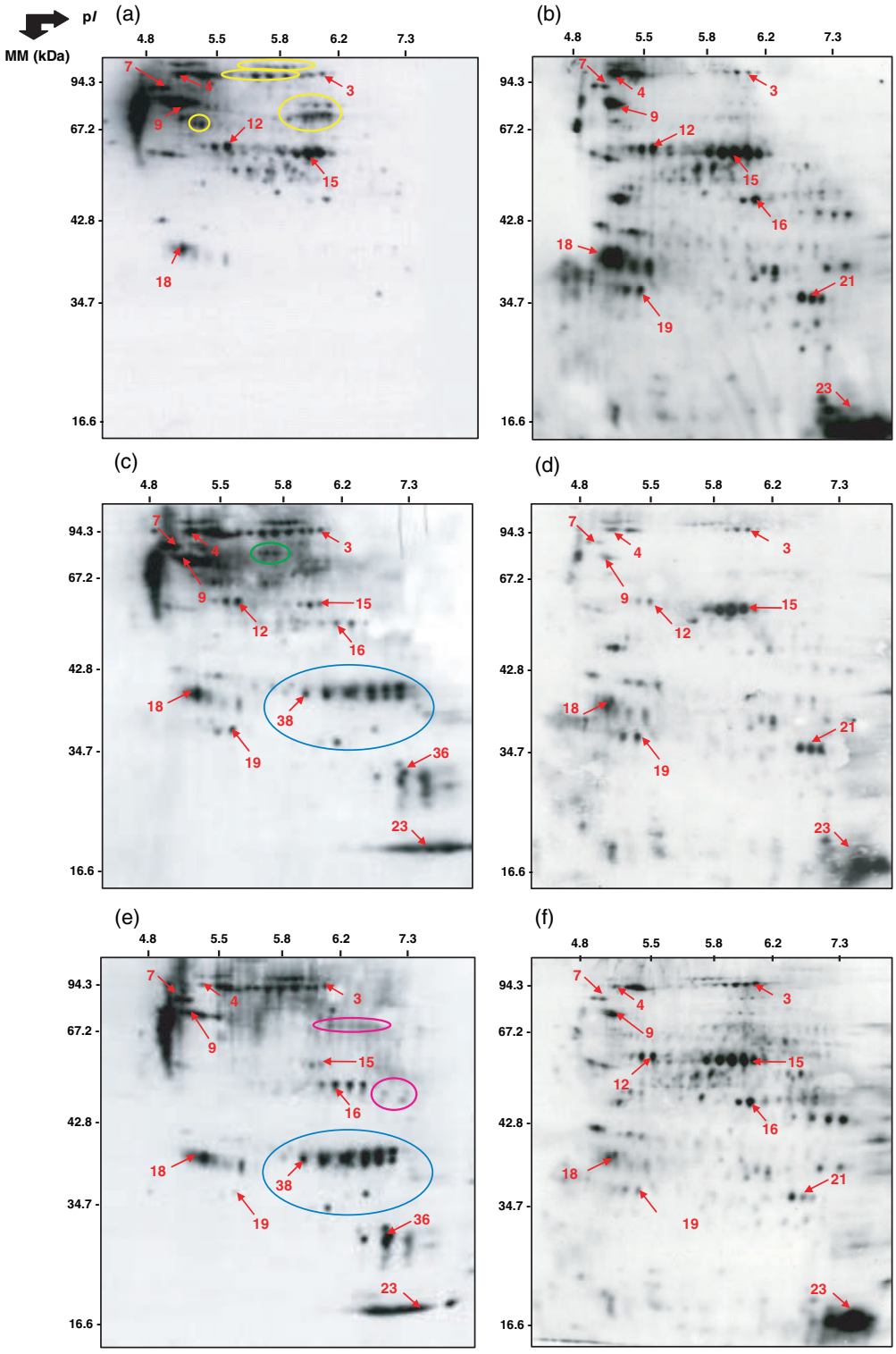

Figure 7. Two-dimensional profiles of protein oxidation in axes of dormant and non-dormant sunflower seeds after 3 h imbibition at 10°C under various conditions. (a) Dormant axes and (b) non-dormant axes imbibed on water; (c) dormant axes and (d) non-dormant axes imbibed in the presence of 1 mM HCN; (e) dormant axes and (f) non-dormant axes imbibed in the presence of 0.1 mM methylviologen.
Numbers indicated on the arrows correspond to proteins that have been identified by mass spectrometry (see Tables 3 and 4). Yellow circles, proteins carbonylated in dormant axes but not in non-dormant axes during imbibition on water. Blue circles, proteins specifically carbonylated in the presence of hydrogen cyanide or methylviologen. Green and purple circles, proteins specifically carbonylated in the presence of cyanide or methylviologen, respectively, during imbibition of dormant axes.

Table 4 Identification of carbonylated proteins in embryo axes of dormant and non-dormant sunflower seeds imbibed for 3 h on water (W) or in the presence of hydrogen cyanide (CN) or methylviologen (MV) at 10°C

					Carbonylation					
Spot number	Accession	Protein name	MW (kDa)	pI	D W	D CN	D MV	ND W	ND CN	ND MV
3	See Table 3	Elongation factor EF-2	93.9	6.1	+	+	+	+	+	+
4	See Table 3	Pyruvate, phosphate dikinase, chloroplast precursor	93.9	5.1	+	+	+	+	+	+
7	See Table 3	81 kDa heat shock protein	84.0	4.9	+	+	+	+	+	+
9	See Table 3	HSP70	73.4	5.0	+	+	+	+	+	+
12	See Table 3	Enolase	60.0	5.5	+	+	+	+	+	+
15	See Table 3	7S globulin	60.0	6.9	+	+	+	+	+	+
16	gi|71793966	alcohol dehydrogenase (*Alnus glutinosa*)	42.8	6.2	–	+	+	+	+	+
18	See Table 3	Globulin-like protein	38.0	5.2	+	+	+	++	+	+
19	gi|22459294	QHF12M17.yg.ab1 QH_EFGHJ sunflower RHA280 *Helianthus annuus* cDNA clone QHF12M17, mRNA sequence ≥gi|81238594|gb|ABB60055.1| 11S globulin precursor isoform 4 (*Sesamum indicum*)	34.0	5.5	–	+	+	+	+	+
21	gi|22462073	QHG17D11.yg.ab1 QH_EFGHJ sunflower RHA280 *Helianthus annuus* cDNA clone QHG17D11, mRNA sequence ≥gi|27526460|emb|CAC80712.1| putative dehydrin (*Helianthus petiolaris*)	36.0	6.5	–	–	–	+	+	+
23	gi|27526481	Basic 2S albumin (*Helianthus annuus*)	16.6	7.0	–	+	+	+	+	+
36	gi|22310301	QHA17G24.yg.ab1 QH_ABCDI sunflower RHA801 *Helianthus annuus* cDNA clone QHA17G24, mRNA sequence ≥gi|4127629|emb|CAA76572.1| 11S storage protein (*Coffea arabica*)	21.0	7.3	–	+	+	–	–	–
38	gi|22393902	QHI21H20.yg.ab1 QH_ABCDI sunflower RHA801 *Helianthus annuus* cDNA clone QHI21H20, mRNA sequence ≥gi|5302785|emb|CAB46034.1| putative epoxide hydrolase (*Arabidopsis thaliana*)	40.0	5.8	–	+	+	–	–	–

D, dormant seeds; ND, non-dormant seeds. Peptide sequences were identified by MS-MS sequencing; see Table 3 for characteristics of spots 3, 4, 7, 9, 12, 15 and 18, and Table S1 for the peptide sequences. Experimental molecular weight (MW) and pI are indicated.

tion results from oxidative attack on Arg, Lys, Pro or Thr residues of proteins, which can affect enzyme activities or alter susceptibility of the modified proteins to proteolysis (Berlett and Stadtman, 1997; Davies, 2005; Dunlop *et al.*, 2002). Our present data clearly indicate the occurrence of carbonylation of specific embryonic proteins during after-ripening of sunflower seeds (Figures 4 and 5). An alternative possibility to account for our results could be a redistribution of carbonylated proteins in the various protein fractions (i.e. soluble and non-soluble proteins) because of cell structural changes occurring during after-ripening that would render the carbonylated proteins more soluble from the non-dormant axes than from the corresponding dormant ones. However, the fact that no marked ultrastructural changes occurred during after-ripening (Figure 3a,b) and that the soluble proteomes revealed by silver nitrate staining were very similar for the dormant and non-dormant axes does not favor this idea (Figure 5). We suggest that, in the sunflower seed system, protein carbonylation may result from an accumulation of ROS themselves, and from accumulated lipid peroxidation products such as MDA (Table 1), which is known to react with lysine residues to form carbonyl derivatives (Burcham and Kuhan, 1996; Liu and Wang, 2005). In animals, protein carbonylation has been widely used as a measurement of oxidative damage, and it has been shown to increase in aging tissues (Dalle-Donne *et al.*, 2003; Ding *et al.*, 2006; Nyström, 2005). In marked contrast, protein carbonylation may not be an inevitable consequence of tissue aging in plants. Thus, in Arabidopsis, patterns of protein carbonylation vary widely during

progression of the life cycle, and the extent of protein carbonylation drops abruptly prior to the vegetative to reproductive transition (Johansson *et al.*, 2004). Carbonylation of numerous proteins also occurs during Arabidopsis seed germination, although the germinated seeds gave rise to vigorous plantlets (Job *et al.*, 2005).

The present data document the effectiveness of both hydrogen cyanide and methylviologen in breaking sunflower seed dormancy (Figure 6). Hydrogen cyanide has already been shown to alleviate seed dormancy in several species, including Arabidopsis (Bethke *et al.*, 2006; Bogatek *et al.*, 1991; Côme *et al.*, 1988; Esashi *et al.*, 1991; Taylorson and Hendricks, 1973). Methylviologen, on the other hand, is a compound known to generate oxidative stress in plants in the light through the photosynthetic electron transport chain (Xiong *et al.*, 2007), as well as in the dark, but by an unknown mechanism (Slooten *et al.*, 1995). However, its utilization as a compound that breaks seed dormancy has not been reported to date, and the advantage of using methylviologen in our study is that it supplies evidence that ROS probably play a fundamental role in seed dormancy release.

The oxyblots presented in Figure 7 clearly show that carbonylation of specific proteins occurred upon treating the dormant sunflower seeds with either hydrogen cyanide or methylviologen (e.g. proteins shown in the blue circles in Figure 7). This specific carbonylation pattern was not seen by incubating dormant seeds in water only (Figure 7a), although this latter incubation also resulted in an increase in protein carbonylation compared with the dry seeds (compare Figures 5b and 7a). It is also interesting that the pattern of protein carbonylation is similar in all of the non-dormant axes (Figure 7), regardless of the treatment. This has significance in the present experiments and in interpreting the data as they provide treatment controls illustrating the reproducibly of the technique and the biological behavior. In summary, seed dormancy release during dry storage and imbibition in the presence of hydrogen cyanide or methylviologen was associated with the appearance of specific carbonylation patterns, although these patterns differed when dormancy was broken in dry and in imbibed state conditions (compare Figures 5 and 7). It would nevertheless be interesting to further characterize the specific features of the carbonylated proteome during breaking of dormancy by carrying out time-course experiments during the after-ripening process. The similarity of the effects of HCN and MV on the oxidized proteome also suggests that these compounds share a common mechanism for triggering dormancy release in imbibed dormant seeds.

The role of protein oxidation in dormancy alleviation could be discussed in relation to the nature of the carbonylated proteins presently identified. EF2, PPDK and 7S globulin (spot numbers 3, 4 and 15, respectively, in Figure 5, Table 2 and Table S1) exhibited an increased carbonylation level during after-ripening. EF2 catalyzes peptidyl-tRNA translocation of the ribosome during the elongation phase of protein translation. Its level is known to decrease dramatically during wheat seed development, particularly during the desiccation phase (Gallie *et al.*, 1998). Carbonylation of EF2 during storage in the dry state after completion of seed development might therefore terminate protein synthesis that is associated strictly with developmental processes. PPDK plays a role in photosynthesis in C4 plants but its function in C3 plants is not fully elucidated. Interestingly, this enzyme appears to be absent from many dicot seeds, including Arabidopsis, with the remarkable exception of sunflower seeds (Chastain *et al.*, 2006). As PPDK is presumed to be involved in seed development rather than in seed germination (Chastain *et al.*, 2006), its carbonylation during after-ripening could provide a means to down-regulate some residual enzyme activity. In this context, it has been shown in rice that, as seed development progresses towards late maturation, the enzyme undergoes post-translational down-regulation in terms of activity and amount via regulatory phosphorylation (PPDK inactivation) and protein degradation (Chastain *et al.*, 2006). Carbonylation of storage proteins has previously been reported in dry mature Arabidopsis seeds, and it was suggested that carbonylation of these proteins facilitates their mobilization during germination (Job *et al.*, 2005). Thus, in sunflower seeds, breaking of dormancy in the dry state may be associated with preparation for storage protein mobilization.

The carbonylation level of some proteins decreased during after-ripening. For example, this was the case for a 20S proteasome α-subunit (spot number 46; Figure 5, Table 2 and Table S1). This observation is consistent with data showing a requirement of for proteasome activity in sunflower embryos for both the breaking of dormancy by ethylene and the progression of germination (Borghetti *et al.*, 2002).

Proteins that were similarly oxidized in dry and imbibed dormant and non-dormant sunflower axes were also characterized. Among them, HSP 70 and 81 (spot numbers 7 and 9, Figures 5 and 7, Tables 3 and 4 and Table S1) and enolase (spot number 12, Figures 5 and 7, Tables 3 and 4 and Table S1) were found. Molecular chaperones are known to be targets of carbonylation in yeast and bacteria challenged by oxidative stress (Cabiscol *et al.*, 2000; Tamarit *et al.*, 1998). Thus, it is possible that carbonylation of these HSPs in both dormant and non-dormant sunflower seeds reflects the occurrence of an oxidative stress during the desiccation phase of seed development. It has been suggested that the protective role these proteins play is as shields protecting other proteins against ROS damage (Cabiscol *et al.*, 2000). Enolase catalyzes the dehydration of 2-phosphoglycerate to PEP and is therefore the second enzyme involved in metabolism, after PPDK, that has been found to be oxidized in sunflower axes. This result is consistent with the observation that enolase is one of the most prominent carbonylated proteins in dry mature Arabidopsis seeds (Job *et al.*, 2005).

The use of hydrogen cyanide or methylviologen also allowed identification of carbonylated proteins that were specifically associated with breaking of dormancy in the imbibed state. This was the case for alcohol dehydrogenase (ADH, spot number 16, Figure 7, Table 4 and Table S1), which was carbonylated in all axes of seeds undergoing germination. Consistent with this, the beneficial effect of alcohol on breaking the dormancy of seeds (Cohn *et al.*, 1989; Corbineau *et al.*, 1991) or buds (Claassens *et al.*, 2005) is well documented. Two identified proteins, globulin precursor isoform 4 (spot number 19 Figure 7, Table 4 and Table S1) and a basic 2S albumin (spot number 23, Figure 7, Table 4 and Table S1) exhibited the same pattern of oxidation as ADH, supporting the finding that germination requires the oxidation of specific reserve proteins. A putative epoxide hydrolase was found to be carbonylated in dormant axes treated by hydrogen cyanide and methylviologen (spot number 38, Figure 7, Table 4 and Table S1). Epoxide hydrolases catalyze the conversion of epoxides to diols. The known functions of such enzymes include detoxification of xenobiotics, drug metabolism, synthesis of signaling compounds, intermediary metabolism and responses to oxidative stress (Newman *et al.*, 2005). In plants, epoxide hydrolases are thought to participate in general defense systems (Newman *et al.*, 2005). Therefore, the specific carbonylation of this protein is consistent with the occurrence of oxidative stress during dormancy alleviation.

In conclusion, taken together, our results allow us to propose a novel mechanism for seed dormancy release. This mechanism involves a change in proteome oxidation, resulting from an accumulation of ROS during after-ripening. Hence, ROS accumulation appears to be a key signal governing cell activity during after-ripening. Furthermore, this hypothesis may provide a more general model to account for breaking of seed dormancy in view of the similar results obtained during breaking of dormancy in the dry state and the imbibed state.

Experimental procedures

Plant material

Two batches of sunflower (*Helianthus annuus* L., cv LG5665) seeds, harvested in 2004 and 2005 near Montélimar (Drôme, France) and purchased from Limagrain (www.limagrain.com), were used in this study. Seeds were dormant at harvest and their moisture content was approximately 4% on a fresh weight (FW) basis. After-ripening was performed by placing the seeds after harvest at 25°C and 60% relative humidity (RH) for 2 months (Corbineau *et al.*, 1990). Seeds were also placed at 25°C over saturated solutions of NaCl and $ZnCl_2$ in tightly closed jars, giving RH of 75\% and 5%, respectively (Vertucci and Roos, 1993). All the results presented in this study represent means of the data obtained from seeds harvested in both 2004 and 2005.

Germination tests

Germination assays were performed with naked seeds (i.e. without pericarp) in darkness in 9 cm Petri dishes (25 seeds per dish, eight replicates) placed on a layer of cotton wool moistened with deionized water. A seed was considered as germinated when the radicle had elongated to 2–3 mm. Germination counts were made daily for 10 days.

Cyanide and methylviologen treatments

The treatment of sunflower embryos by gaseous 1 mM HCN was carried out as described by Bogatek *et al.* (1991). Naked dry seeds were placed in a tightly closed glass jar (500 ml volume) on a layer of sterile cotton wool moistened with deionized water (50 seeds per jar). A glass tube containing 5 ml of 0.1 M KCN solution placed in the jar was used as a source of gaseous HCN, which was produced by acidifying the KCN solution with 5 ml of lactic acid (10% v/v). After 3 h of treatment in darkness at 10°C, the jars were opened and gaseous cyanide released, and the seeds were rinsed carefully three times with deionized water before germination tests or biochemical analyses. Treatment by methylviologen was carried out by placing naked seeds in darkness at 10°C on a cotton wool moistened with a solution of 0.1 mM MV for 3 h. Embryos were also rinsed with deionized water after treatment.

Determination of superoxide anion content

Superoxide anion content was determined according to the method developed by Elstner and Heupel (1976). Axes (0.2 g FW) were ground in 4 ml of sodium phosphate buffer (pH 7.8, 50 mM) at 4°C. The extracts were centrifuged at 16 000 ***g*** for 15 min, and the resulting supernatants were used for $O_2^{\cdot-}$ determination. The supernatant (1 ml) was first incubated at 25°C for 30 min in the presence of 1 mM hydroxylamine hydrochloride in 50 mM sodium phosphate buffer (pH 7.8). Then, 0.5 ml of this reaction mixture was incubated with 0.5 ml of 17 mM sulfanilamide and 0.5 ml of 7 mM 2-naphtylamine at 25°C for 30 min. The absorbance was measured at 540 nm after centrifugation at 13 000 ***g*** for 10 min. A calibration curve was established using sodium nitrite. The results are expressed as μmol g^{-1} DW of seeds and correspond to the means of measurements carried out on five extracts $\pm$ SD.

In situ *localization of superoxide anion*

Whole axes and hand-cut sections of axes were incubated in 6 mM nitroblue tetrazolium (NBT) in 10 mM Tris-HCl buffer (pH 7.4) at room temperature for 30 min. Superoxide anion was visualized as deposits of dark-blue insoluble formazan compounds (Beyer and Fridovich, 1987).

Determination of hydrogen peroxide content

The H_2O_2 content of excised axes was determined according to the method described by O'Kane *et al.* (1996). Axes (0.5 g FW) were ground in a mortar and homogenized with 5 ml of 0.2 M perchloric acid. After 15 min of centrifugation at 13 000 ***g*** at 4°C, the resulting supernatant was neutralized to pH 7.5 with 4 M KOH and then centrifuged at 1000 ***g*** for 3 min at the same temperature. The supernatant was immediately used for spectrophotometric determination of H_2O_2 at 590 nm using a peroxidase-based assay. The reaction mixture contained 12 mM 3-dimethylaminobenzoic acid in 0.375 M

phosphate buffer (pH 6.5), 1.3 mM 3-methyl-2-benzothiazolidone hydrazone, 20 μl (0.25 U) horseradish peroxidase (Sigma; http://www.sigmaaldrich.com/) and 50 μl of the collected supernatant to a total volume of 1.5 ml. The reaction was started by the addition of peroxidase. Increase in absorbance at 590 nm was monitored after 5 min at 25°C and compared with the absorbance obtained with known amounts of H_2O_2. The results are expressed as nmol H_2O_2 g^{-1} DW and correspond to means of the values obtained with five different extracts ± SD.

Subcellular localization of hydrogen peroxide

The localization of H_2O_2 was determined by $CeCl_3$ staining as described by Bestwick *et al.* (1997). Sections of axes (approximately 5 mm^3) were imbibed in 50 mM MOPS buffer (pH 7.2) containing 5 mM $CeCl_3$ under vacuum until they were fully infiltrated. $CeCl_3$-treated sections and control sections (without staining) were fixed in 6% glutaraldehyde in 25 mM sodium phosphate buffer (pH 7.2) for 24 h at room temperature, washed in the same buffer and post-fixed in 1% osmium tetroxide in 25 mM sodium phosphate buffer (pH 7.2) for 18 h at room temperature. After several washes in double-distilled water, the tissues were progressively dehydrated in ethanol, then soaked in propylene oxide and embedded in Araldite (Sigma). The blocks were sectioned with glass knives at 120 nm using a Reichert Ultractu S (Leica, www.leica-microsystems.com), stained with lead citrate and 2% uranyl acetate (Reynolds, 1963), and viewed with a LEO912 transmission electron microscope (Leo Electron Microscopy, www.stm.zeiss.com).

Malondialdehyde measurements

Lipid peroxidation was evaluated by measuring malondialdehyde (MDA) content from 0.5 g FW of embryonic axes, according to the method of Heath and Parker (1968). The results are expressed as μmol g^{-1} DW of seeds and correspond to means of measurements carried out with five extracts ± SD.

Preparation of protein extracts

Axes from dry dormant and non-dormant seeds (140 mg FW, corresponding approximately to 30 axes) were ground in liquid nitrogen using a mortar and pestle. Soluble proteins were extracted from the resulting powder at 4°C in 1.0 ml of a buffer containing 10 mM HEPES, 1 mM EDTA, the protease inhibitor cocktail 'complete Mini' from Roche Molecular Biochemicals, 60 U DNAse I (Roche Diagnostics, www.roche-applied-science.com), and 6 U RNAse A (Sigma, www.sigmaaldrich.com). After 10 min at 4°C, 20 mM dithiothreitol (DTT) was added, and the protein extracts were stirred for 20 min at 4°C and then centrifuged (20 000 ***g***, 15 min) at 4°C. The final supernatant corresponded to the soluble protein extract. Protein concentrations in the various extracts were measured according to the method described by Bradford (1976) using a Bio-Rad assay kit (http://www.bio-rad.com/). Bovine serum albumin was used as a standard.

One- and two-dimensional electrophoresis

One-dimensional SDS–PAGE of seed protein extracts (5 μg protein) was performed using 12% w/v polyacrylamide resolving gels, as described by Laemmli (1970).

Proteins were also analyzed by two-dimensional gel electrophoresis as described previously (Görg *et al.*, 1987; Job *et al.*, 2005). Isoelectrofocusing (100 μg protein) was carried out using gel strips forming an immobilized non-linear pH gradient from 3 to 10 (Immobiline DryStrip, pH 3–10 NL, 18 cm; Amersham Pharmacia Biotech; http://www5.amershambiosciences.com/). Strips were rehydrated for 14 h at 22°C with the thiourea/urea lysis buffer as described previously (Harder *et al.*, 1999), containing 2% v/v Triton X-100, 20 mM dithiothreitol and the protein extracts. Isoelectrofocusing was performed at 22°C in the Multiphor II system (Amersham Pharmacia Biotech) for 1 h at 300 V and 7 h at 3500 V. Then, the gel strips were equilibrated for 2 × 20 min in 2x 100 ml of equilibration solution containing 6 M urea, 30% v/v glycerol, 2.5% w/v SDS, 0.15 M bis-Tris and 0.1 M HCl (Görg *et al.*, 1987; Harder *et al.*, 1999). DTT (50 mM) was added to the first equilibration solution, and iodoacetamide (4% w/v) was added to the second (Harder *et al.*, 1999). Separation in the second dimension was carried out in polyacrylamide gels (10% w/v acrylamide, 0.33% w/v piperazidine diacrylamide, 0.18 M Trizma base, 0.166 M HCl, 0.07% w/v ammonium persulfate and 0.035% v/v Temed). Electrophoresis was performed at 10°C in a buffer (pH 8.3) containing 25 mM Trizma base, 200 mM taurine and 0.1% w/v SDS for 1 h at 35 V and for 14 h at 110 V. Ten gels (200 × 250 × 1.0 mm) were run in parallel (Isodalt system; Amersham Pharmacia Biotech). For each treatment analyzed, 2D gels were run in triplicate.

One-dimensional gels were stained with the GelCode blue stain from Pierce (www.piercenet.com). Two-dimensional gels were stained with silver nitrate according to the methods described by Blum *et al.* (1987) for densitometric analyses or Shevchenko *et al.* (1996) for mass spectrometry analyses. Stained gels were scanned with a UMAX Powerlook III scanner equipped with MagicScan version 4.5 from UMAX Data Systems (Amersham Biosciences, www.amershambiosciences.com).

Detection of carbonylated proteins and Western blotting

The appearance of carbonyl groups in proteins was analyzed by immunodetection of 2,4-dinitrophenylhydrazone (DNP)-derivatized protein as described previously (Job *et al.*, 2005; Korolainen *et al.*, 2002). SDS was added to the protein extract (100 μl, 10 μg $μl^{-1}$) to 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 15 000 ***g*** at 4°C. The precipitated protein was then washed three times with ice-cold acetone containing 1 mM DTT, then 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 SDS–PAGE as described above, and transferred to nitrocellulose sheets (Bio-Rad) using standard procedures. Carbonylated proteins were revealed by incubation with rabbit anti-DNP antibodies (Chemicon, www.chemicon.com) followed by incubation with anti-rabbit secondary antibodies conjugated to horseradish peroxidase (Sigma) and detection with the ECL kit (Roche Diagnostics) (Job *et al.*, 2005). Relative protein carbonyl levels were quantitated by densitometric analyses of the blots as described above.

Protein identification by mass spectrometry

Bands and spots of interest were excised from 1D and 2D SDS–PAGE gels using sterile tips and placed in 1.5 ml sterile tubes. Each

polyacrylamide piece was rinsed with water, then reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and incubated overnight at 37°C with 12.5 ng μl^{-1} trypsin (sequencing grade; Roche Diagnostics) in 25 mM NH_4HCO_3. 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 Micromass, www.waters.com) coupled with a nano-HPLC (Cap-LC; Waters Micromass). The samples were loaded and desalted on a C18 pre-column (LC-Packings PepMap C18, 5 μm, 100 Å, 300 μm × 5 mm; Dionex Corp., www.dionex.com) at a flow rate of 20 $\mu l\ min^{-1}$ isocratically with 0.1% formic acid. The peptides were separated on a C18 column (Atlantis dC18, 3 μm, 75 μm × 150 mm Nano Ease; Waters). After washing 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 $nl\ min^{-1}$. The Q-TOF spectrometer was operated in data-dependent analysis mode using a 1 sec mass spectrometry (MS) survey scan on three different precursor ions. The peptide masses and sequences obtained were either matched automatically to proteins in a non-redundant database National Center for Biotechnology Information, NCBI, www.ncbi.nlm.nih.gov using the Mascot MS/MS ions search algorithm (http://www.matrixscience.com) or manual BLAST searches were performed against the current databases. (NCBI, Swiss-Prot, http://expasy.org/sprot/)

Supplementary material

The following supplementary material is available for this article online:

Table S1 Identification of carbonylated proteins in embryo axes of dormant and non-dormant sunflower seeds.

This material is available as part of the online article from http://www.blackwell-synergy.com.

References

Andersen, M.L. and Skibsted, L.H. (2002) Detection of early events in lipid oxidation by electron spin resonance spectroscopy. *Eur. J. Lipid Sci. Technol.* **104**, 65–68.

Andersen, A.B., Risbo, J., Andersen, M.L. and Skibsted, L.H. (2000) Oxygen permeation through an oil-encapsulating glassy food matrix studied by ESR line broadening using a nitroxyl spin probe. *Food Chem.* **70**, 499–508.

Bailly, C. (2004) Active oxygen species and antioxidants in seed biology. *Seed Sci. Res.* **14**, 93–107.

Baskin, J.M. and Baskin, C.C. (1998) *Seeds: Ecology, Biogeography and Evolution of Dormancy and Germination*. San Diego, CA: Academic Press.

Berlett, B.S. and Stadtman, E.R. (1997) Protein oxidation in aging, disease and oxidative stress. *J. Biol. Chem.* **272**, 20313–20316.

Bestwick, C.S., Brown, I.R., Benneth, M.H.R. and Mansfield, J.W. (1997) Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *phaseolicola*. *Plant Cell*, **9**, 209–221.

Bethke, P.C., Libourel, I.G., Reinohl, V. and Jones, R.L. (2006) Sodium nitroprusside, cyanide, nitrite, and nitrate break Arabidopsis seed dormancy in a nitric oxide-dependent manner. *Planta*, **223**, 805–812.

Bewley, J.D. (1997) Seed germination and dormancy. *Plant Cell*, **9**, 1055–1066.

Bewley, J.D. and Black, M. (1994) *Seeds. Physiology of Development and Germination*, 2nd edn. New York: Plenum Press.

Beyer, W.F. and Fridovich, I. (1987) Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. *Anal. Biochem.* **161**, 559–566.

Blum, H., Beier, H. and Gross, H.J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis*, **8**, 93–99.

Bogatek, R., Dziewanowska, K. and Lewak, S. (1991) Hydrogen cyanide and embryonal dormancy in apple seeds. *Physiol. Plant.* **83**, 417–421.

Borghetti, F., Nakamura Noda, F. and Martins de Sá, C. (2002) Possible involvement of proteasome activity in ethylene-induced germination of dormant sunflower embryos. *Braz. J. Plant Physiol.* **14**, 125–131.

Bove, J., Lucas, P., Godin, B., Oge, L., Jullien, M. and Grappin, P. (2005) Gene expression analysis by cDNA-AFLP highlights a set of new signaling networks and translational control during seed dormancy breaking in *Nicotiana plumbaginifolia*. *Plant Mol. Biol.* **57**, 593–612.

Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein–dye binding. *Anal. Biochem.* **72**, 248–254.

Bucharov, P. and Gantcheff, T. (1984) Influence of accelerated and natural aging on free radical levels in soybean seeds. *Physiol. Plant.* **60**, 53–56.

Burcham, P.C. and Kuhan, Y.T. (1996) Introduction of Carbonyl groups into proteins by the lipid peroxidation product, malondialdehyde. *Biochem. Biophys. Res. Commun* **220**, 996–1001.

Cabiscol, E., Piulats, E., Echave, P., Herrero, E. and Ros, J. (2000) Oxidative stress promotes specific protein damage in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **275**, 27393–27398.

Cadman, C.S.C., Toorop, P.E., Hilhorst, H.W.M. and Finch-Savage, W.E. (2006) Gene expression profiles of Arabidopsis Cvi seeds during dormancy cycling indicate a common underlying dormancy control mechanism. *Plant J.* **46**, 805–822.

Chastain, C., Heck, J.W., Colquhoun, T.A., Voge, D.G. and Gu, X.Y. (2006) Posttranslational regulation of pyruvate, orthophosphate dikinase in developing rice (*Oryza sativa*) seeds. *Planta*, **224**, 924–934.

Chibani, K., Ali-Rachedi, S., Job, C., Job, D., Jullien, M. and Grappin, P. (2006) Proteomic analysis of seed dormancy in Arabidopsis. *Plant Physiol.* **142**, 1493–1510.

Claassens, M.M., Verhees, J., van der Plas, L.H., van der Krol, A.R. and Vreugdenhil, D. (2005) Ethanol breaks dormancy of the potato tuber apical bud. *J. Exp. Bot.* **56**, 2515–2525.

Cohn, M.A., Jones, K.L., Chiles, L.A. and Church, D.F. (1989) Seed dormancy in red rice. 7. Structure–activity studies of germination stimulants. *Plant Physiol.* **89**, 879–882.

Côme, D., Corbineau, F. and Lecat, S. (1988) Some aspects of metabolic regulation of cereal seed germination and dormancy. *Seed Sci. Technol.* **16**, 175–186.

Corbineau, F., Bagniol, S. and Côme, D. (1990) Sunflower (*Helianthus annuus* L.) seed dormancy and its regulation by ethylene. *Isr. J. Bot.* **39**, 313–325.

Corbineau, F., Gouble, B., Lecat, S. and Côme, D. (1991) Stimulation of germination of dormant oat (*Avena sativa* L.) seeds by ethanol and other alcohols. *Seed Sci. Res.* **1**, 21–28.

Dalle-Donne, I., Giustarini, D., Colombo, R., Rossi, R. and Milzani, A. (2003) Protein carbonylation in human diseases. *Trends Mol. Med.* **9**, 169–176.

Davies, M.J. (2005) The oxidative environment and protein damage. *Biochim. Biophys. Acta*, **1703**, 93–109.

Ding, Q., Dimayuga, E. and Keller, J.N. (2006) Proteasome regulation of oxidative stress in aging and age-related diseases of the CNS. *Antioxid. Redox Signal.* **8**, 163–172.

Donohue, K., Dorn, L., Griffith, C., Kim, E., Aguilera, A., Polisetty, C.R. and Schmitt, J. (2005) Niche construction through germination cueing: life-history responses to timing of germination in *Arabidopsis thaliana*. *Evolution* **59**, 771–785.

Drapron, D. (1985) Enzyme activity as a function of water activity. In *Properties of Water in Food* (Simatos, D. and Multon, J.L., eds). Dordrecht: Martinus Nijhoff Publishers, pp. 171–190.

Dunlop, R.A., Rodgers, K.J. and Dean, R.T. (2002) Recent development in the intracellular degradation of oxidized proteins. *Free Radic. Biol. Med.* **33**, 894–906.

Elstner, E.F. and Heupel, A. (1976) Inhibition of nitrite formation from hydroxylammoniumchloride: a simple assay for superoxide dismutase. *Anal. Biochem.* **70**, 616–620.

Esashi, Y., Isuzugawa, K., Matsuyama, H. and Hasegawa, R. (1991) Endogenous evolution of HCN during pre-germination periods in many seed species. *Physiol. Plant.* **83**, 27–33.

Esashi, Y., Ogasawara, M., Górecki, R. and Leopold, A.C. (1993) Possible mechanisms of afterripening in *Xanthium* seeds. *Physiol. Plant.* **87**, 359–364.

Finch-Savage, W.E and Leubner-Metzger, G. (2006) Seed dormancy and the control of germination. *New Phytol.* **171**, 501–523.

Gallie, D.R., Le, H., Caldwell, C. and Browning, K.S. (1998) Analysis of translation elongation factors from wheat during development and following heat shock. *Biochem. Biophys. Res. Commun.* **245**, 295–300.

Gay, C., Corbineau, F. and Côme, D. (1991) Effects of temperature and oxygen on seed germination and seedling growth in sunflower (*Helianthus annuus* L.). *Environ. Exp. Bot.* **31**, 193–200.

Görg, A., Postel, W., Weser, J., Günther, S., Strahler, J.R., Hanash, S.M. and Somerlot, L. (1987) Elimination of point streaking on silver stained two-dimensional gels by addition of iodoacetamide to the equilibration buffer. *Electrophoresis*, **8**, 122–124.

Halliwell, B. and Gutteridge, J.M.C. (1999) *Free Radicals in Biology and Medicine*, 3rd edn. New York: Oxford University Press.

Harder, A., Wildgruber, R., Nawrocki, A., Fey, S.J., Larsen, P.M. and Görg, A. (1999) Comparison of yeast cell protein solubilization procedures for two-dimensional electrophoresis. *Electrophoresis*, **20**, 826–829.

Heath, R.L. and Parker, L. (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoechiometry of fatty acid peroxidation. *Arch. Biochem. Biophys.* **125**, 189–198.

Hendry, G.A.F. (1993) Oxygen, free radical processes and seed longevity. *Seed Sci. Res.* **3**, 141–153.

Job, C., Rajjou, L., Lovigny, Y., Belghazi, M. and Job, D. (2005) Patterns of protein oxidation in Arabidopsis seeds and during germination. *Plant Physiol.* **138**, 790–802.

Johansson, E., Olsson, O. and Nyström, T. (2004) Progression and specificity of protein oxidation in the life cycle of *Arabidopsis thaliana*. *J. Biol. Chem.* **279**, 22204–22208.

Koornneef, M., Bentsink, L. and Hilhorst, H. (2002) Seed dormancy and germination. *Curr. Opin. Plant Biol.* **5**, 33–36.

Korolainen, M.A., Goldsteins, G., Alafuzoff, I., Koistinaho, J. and Pirttila, T. (2002) Proteomic analysis of protein oxidation in Alzheimer's disease brain. *Electrophoresis*, **23**, 3428–3433.

Laemmli, U.K. (1970) Cleavage of structural protein during the assembly of the head of the bacteriophage T4. *Nature*, **227**, 680–685.

Leubner-Metzger, G. (2005) Beta-1,3-glucanase gene expression in low-hydrated seeds as a mechanism for dormancy release during tobacco after-ripening. *Plant J.* **41**, 133–145.

Liu, W. and Wang, J.Y. (2005) Modification of protein by polyunsaturated fatty acid ester peroxidation products. *Biochim. Biophys. Acta.* **1752**, 93–98.

McDonald, M.B. (1999) Seed deterioration: physiology, repair and assessment. *Seed Sci. Technol.* **27**, 177–237.

Murthy, U.M.N. and Sun, W.Q. (2000) Protein modification by Amadori and Maillard reactions during seed storage: roles of sugar hydrolysis and lipid peroxidation. *J. Exp. Bot.* **51**, 1221–1228.

Murthy, U.M.N., Kumar, P.P. and Sun, W.Q. (2003) Mechanisms of seed ageing under different storage conditions for *Vigna radiata* (L.) Wilczek: lipid peroxidation, sugar hydrolysis, Maillard reactions and their relationship to glass state transition. *J. Exp. Bot.* **54**, 1057–1067.

Nelson, K.A. and Labuza, T.P. (1992) Relationship between water and lipid oxidation rates: water activity and glass transition theory. In *Lipid Oxidation in Foods* (St Angelo, A.J., ed). Washington, DC: American Chemical Society, pp. 93–103.

Newman, J.W., Morisseau, C. and Hammock, B.D. (2005) Epoxide hydrolases: their roles and interactions with lipid metabolism. *Prog. Lipid Res.* **44**, 1–51.

Nyström, T. (2005) Role of oxidative carbonylation in protein quality control and senescence. *EMBO J.* **24**, 1311–1317.

O'Kane, D., Gill, V., Boyd, P. and Burdon, R. (1996) Chilling, oxidative stress and antioxidant responses in *Arabidopsis thaliana* callus. *Planta*, **198**, 371–377.

Pellinen, R.I., Korhonen, M.S., Tauriainen, A.A., Palva, E.T. and Kangasjärvi, J. (2002) Hydrogen peroxide activates cell death and defense gene expression in birch. *Plant Physiol.* **130**, 549–560.

Priestley, D.A. (1986) *Seed Aging. Implications for Seed Storage and Persistence in the Soil*. Ithaca, NY: Cornell University Press.

Pukacka, S. and Ratajczak, E. (2005) Production and scavenging of reactive oxygen species in *Fagus sylvatica* seeds during storage at varied temperature and humidity. *J. Plant Physiol.* **162**, 873–885.

Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208–212.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850–858.

Slooten, L., Capiau, K., Van Camp, W., Van Montagu, M., Sybesma, C. and Inzé, D. (1995) Factors affecting the enhancement of oxidative stress tolerance in transgenic tobacco overexpressing manganese superoxide dismutase in the chloroplasts. *Plant Physiol.* **107**, 737–750.

Sun, W.Q. and Leopold, A.C. (1995) The Maillard reaction and oxidative stress during aging of soybean seeds. *Physiol. Plant.* **94**, 94–104.

Tamarit, J., Cabiscol, E. and Ros, J. (1998) Identification of the major oxidatively damaged proteins in *Escherichia coli* cells exposed to oxidative stress. *J. Biol. Chem.* **273**, 3027–3032.

Taylorson, R.B. and Hendricks, S.B. (1973) Promotion of seed germination by cyanide. *Plant Physiol.* **52**, 23–27.

Vertucci, C.W. and Roos, E.G. (1993) Theoretical basis of protocols for seed storage II. The influence of temperature on optimal moisture levels. *Seed Sci. Res.* **3**, 201–213.

Wilson, D.O. and McDonald, M.B. (1986) The lipid peroxidation model of seed aging. *Seed Sci. Technol.* **14**, 269–300.

Xiong, Y., Contento, A.L., Nguyen, P.Q. and and. Bassham, D.C. (2007) Degradation of oxidized proteins by autophagy during oxidative stress in Arabidopsis. *Plant Physiol.* **143**, 291–299.

