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Targeted mRNA Oxidation Regulates Sunflower Seed Dormancy Alleviation during Dry After-Ripening [C][W]

Jérémie Bazin,[a] Nicolas Langlade,[b] Patrick Vincourt,[b] Sandrine Arribat,[c] Sandrine Balzergue,[c] Hayat El-Maarouf-Bouteau,[a,1] and Christophe Bailly[a,1,2]

[a] UR5 EAC7180 Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, Université Paris 06, 75005 Paris, France

[b] Laboratoire Interactions Plantes Microorganismes, Institut National de la Recherche Agronomique, 31326 Castanet Tolosan, France

[c] Equipe Génomique Fonctionnelle d'Arabidopsis, Unité de Recherche en Génomique Végétale, Unité Mixte de Recherche, Institut National de la Recherche Agronomique 1165, Université d'Evry Val d'Essonne, ERL Centre National de la Recherche Scientifique 8196, F-91057 Evry Cedex, France

After-ripening is the mechanism by which dormant seeds become nondormant during their dry storage after harvest. The absence of free water in mature seeds does not allow detectable metabolism; thus, the processes associated with dormancy release under these conditions are largely unknown. We show here that sunflower (*Helianthus annuus*) seed alleviation of dormancy during after-ripening is associated with mRNA oxidation and that this oxidation is prevented when seeds are maintained dormant. In vitro approaches demonstrate that mRNA oxidation results in artifacts in cDNA–amplified fragment length polymorphim analysis and alters protein translation. The oxidation of transcripts is not random but selective, and, using microarrays, we identified 24 stored mRNAs that became highly oxidized during after-ripening. Oxidized transcripts mainly correspond to genes involved in responses to stress and in cell signaling. Among them, protein phosphatase 2C PPH1, mitogen-activated protein kinase phosphatase 1, and phenyl ammonia lyase 1 were identified. We propose that targeted mRNA oxidation during dry after-ripening of dormant seeds could be a process that governs cell signaling toward germination in the early steps of seed imbibition.

INTRODUCTION

How can a resting, but living, organism undergo dramatic cell reprogramming in the absence of free cellular water? Although resting forms of anhydrobiotic organisms exist in both animal and plant kingdoms, this intriguing question is particularly relevant in the case of orthodox seeds (i.e., seeds that tolerate a final dessiccation step in their developmental program and whose moisture content (MC) at shedding is generally below 0.1 g water g dry weight $[DW]^{-1}$). At harvest, seeds of many orthodox species are dormant (i.e., unable to germinate in apparently appropriate environmental conditions). This evolutionary trait results from an adaptation to climatic constraints: it allows species survival through unfavorable seasons and enables seeds to remain quiescent until the conditions for germination and seedling establishment become favorable (Finch-Savage and Leubner-Metzger, 2006; Donohue et al., 2010). For many species, dormancy can be alleviated after a prolonged period of seed storage in the dry state, so-called dry after-ripening, which results in a widening of the environmental conditions that permit germination. However, it is worthwhile to note that dry after-ripening is generally effective in a range of seed MC between ~5% and 15 to 18% fresh weight basis, which corresponds to region 2 of sorption isotherms (i.e., weakly bound water), at that it can be prevented at very low MC (Bazin et al., 2011). Nevertheless, various authors have proposed that changes in gene expression patterns occur during dry after-ripening, in MC conditions allowing dormancy alleviation, and that this might explain the acquisition of the potential to germinate (Bove et al., 2005; Leubner-Metzger, 2005; Cadman et al., 2006; Leymarie et al., 2007).

In anhydrobiotic conditions of after-ripening, nonenzymatic mechanisms would be good candidates for playing a role in seed dormancy release. Oxygen can diffuse within glasses, such as the vitreous cytoplasm at low seed moisture levels, and ultimately lead to reactive oxygen species (ROS) accumulation, as has been shown by Oracz et al. (2007). These authors demonstrated that ROS caused lipid peroxidation and carbonylation of a specific subset of proteins that were associated with seed dormancy release. However, lipids and proteins are not the only targets of oxidative modifications by ROS. Nucleic acids, in

[1] These authors contributed equally to this work.

[2] Address correspondence to christophe.bailly@upmc.fr.

The authors responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) are: Hayat El-Maarouf-Bouteau (hayat.bouteau@upmc.fr) and Christophe Bailly (christophe.bailly@upmc.fr).

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particular, are very sensitive to free radicals. Oxidative DNA damage causes mutations, which have been associated with many heritable diseases and aging in mammals (Radak and Boldogh, 2010). RNA can also be oxidized by ROS, particularly by hydroxyl radicals, and RNA has been shown to be much more vulnerable to oxidative damage than DNA (Kong and Lin, 2010). The most prevalent oxidized base of RNA is guanine, whose one-electron oxidation gives 8-oxo-7,8-dihydroguanine (8-OHG). RNA oxidation has been cited as a key event in neurodegenerative processes, such as Alzheimer's or Parkinson's diseases (Nunomura et al., 1999; Zhang et al., 1999). It has been proposed that RNA containing guanine could have antioxidant functions, thus preventing oxidative damage to genomic DNA (Martinet et al., 2005; Kong and Lin, 2010). Moreover, mRNA oxidation is suspected to cause premature termination of translation and, thus, to affect protein synthesis (Shan et al., 2003; Tanaka et al., 2007). Interestingly, recent studies (Shan et al., 2003; Chang et al., 2008) have proposed that RNA oxidation is not random but highly selective, as some mRNAs were more susceptible to oxidative damage, thus providing a possible regulatory mechanism of cell functioning under physiological conditions.

Dry seeds accumulate a large amount of translatable mRNA (i.e., stored mRNA; Dure and Waters, 1965), which is produced during seed development and used upon imbibition to ensure translation of the proteins required for completing germination (Rajjou et al., 2004; Nakabayashi et al., 2005; Kimura and Nambara, 2010). It has been shown that de novo transcription is not essential for early stages of germination (Rajjou et al., 2004), which suggests that the pool of stored proteins or of mRNA that is translated into proteins during the early steps of seed imbibition governs dormancy expression and germination potential. Similarly, degradation of specific mRNAs has also long been implicated in the breaking of seed dormancy (Ingle and Hageman, 1964; Beevers and Splittstoesser, 1968; Wurzburger and Leshem, 1971; Dyer, 1993), and recently it has been demonstrated that degradation of a specific subset of mRNAs might be a prerequisite to germination (Howell et al., 2009; Xu and Chua, 2009). Consequently, modifications of stored mRNA during after-ripening would have the potential to regulate mRNA translation and/or mRNA degradation in the early steps of seed imbibition and then to subsequently program cell functioning toward germination or dormancy maintenance.

More specifically, we postulate that stored mRNA can be oxidized during seed after-ripening and that this process may be involved in the regulation of dormancy. After demonstrating that changes in transcript profile can occur during sunflower (*Helianthus annuus*) seed after-ripening, using cDNA–amplified fragment length polymorphim (cDNA-AFLP), we show here that some mRNAs are oxidized during after-ripening and that this is associated with dormancy release. Sunflower microarray chips allowed us to identify the oxidized mRNA and to demonstrate that this process is selective. Consequences of mRNA oxidation have been studied, and our data may help to explain the observed changes in transcript abundance in the dry state that we obtained using cDNA-AFLP. We propose mRNA oxidation as a mechanism of seed dormancy alleviation, which emphasizes the role of stored mRNAs and of their posttranscriptional modification in this process.

RESULTS

Seed Germination

Figure 1A shows the germination after 7 d at 10°C of sunflower embryos after different periods of storage at 25°C under 5, 60, and 75% RH. Such RH gave embryo MCs of 0.025, 0.04, and 0.06 g water g DW^{-1}, respectively. After harvest (i.e., after 0 week of after ripening), only 25% of embryos were able to germinate within 7 d at 10°C, but dry storage at 60 and 75% RH progressively induced the release of dormancy as shown by the increase of germination percentage after 7 d at 10°C (Figure 1A). Calculation of $DSDS_{50}$ (days of seeds dry storage required to reach a 50% rate of germination) gives values of 4.5 and 9.5 d at 75 and 60% HR, respectively (Figure 1A). This shows that dormancy release occurred more rapidly at 75% than at 60% RH and that low RH (5%) prevented dormancy release since even after 42 d at this RH, embryo germination did not exceed 35% at 10°C (Figure 1A). However, embryos stored at 60% RH germinated more slowly than the ones stored at 75% RH, as the

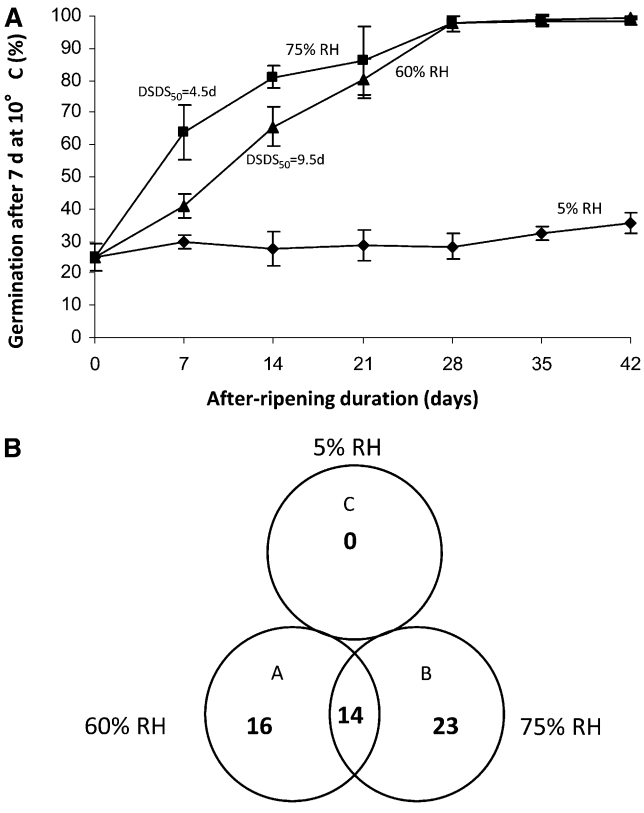

Figure 1. Effect of Relative Humidity on Dormancy Release and Gene Expression.

(A) Germination of sunflower embryos after 7 d at 10°C following various durations of after-ripening at 25°C and 5% (diamonds), 60% (triangles), and 75% RH (squares). Values are means of four replicates ± SD.

(B) Venn diagram showing the distribution of differentially expressed TDFs accumulated in embryonic axis of embryos after-ripened for 42 d at 25°C under 5% (group A), 60% (group B), and 75% RH (group C).

P50 values (time to obtain 50% germination at 10°C) were 2.2 and 1.1 d, respectively.

Transcriptome Analysis during After-Ripening

In order to assess if changes in mRNA abundance might occur during seed dry after-ripening, we performed a global transcriptomic analysis using the cDNA-AFLP method with dry embryonic axes excised from embryos that were stored after harvest under different RH. We compared mRNA of embryonic axes from dormant seeds with those from dormant embryos stored for 6 weeks under 5, 60, and 75% RH (Figure 1B). Results presented were obtained using 24 different primer combinations among the 256 available and therefore represent the analysis of 1/10 of the entire sunflower seed transcriptome. A relatively large number (53) of reproducible transcript-derived fragments (TDFs) were differentially accumulated in embryos that were stored at 60 and 75% RH, and the higher the RH, the higher the number of TDF (Figure 1B). When embryos were stored under 5% RH, a condition that prevents dormancy release (Figure 1A), cDNA-AFLP analysis did not reveal any accumulation of TDFs (Figure 1B). As shown on Venn diagrams (Figure 1B), 16 TDFs increased in the embryonic axes under 60% RH (group A), 23 under 75% (group B), 14 under both 60 and 75% RH (group A+B), and 0 under 5% RH (group C). Differences between groups A and B are explained by the differences of germination speed of embryos belonging to the two groups (embryos stored at 60% RH germinate more slowly at 10°C than embryos stored at 75% RH). However, one must underline that most of the group A TDFs are also present in group B (14 among 16). Genes belonging to the AB group, which may be related to dormancy release, have been cloned and their sequence similarities have been investigated using the BLASTn and/or BLASTp algorithm (http://blast.ncbi.nlm.nih.gov). Gene comparison allowed identification of TDF showing high sequence similarity to genes with known or putative functions that include, for example, genes coding for an aspartic protease, a ubiquitin conjugating enzyme, a heat shock transcription factor (*Arabidopsis thaliana* HsfA5), and a putative senescence-associated protein (see Supplemental Table 1 online). Although cDNA-AFLP data suggested accumulation of some transcripts in the dry state, we postulated that the observed changes of TDF pattern might also result from artifacts resulting from oxidative modifications of mRNA.

Effect of mRNA Oxidation on TDF Polymorphism and in Vitro Translation

To assess whether oxidation of mRNA can induce false-positive results on cDNA-AFLP analysis, we oxidized extracted mRNA using the iron-ascorbate system. This system generates hydroxyl radicals (HO·) in vitro through a modified Fenton reaction (see Methods). We therefore oxidized extracted mRNA templates from dormant seeds with increasing intensities of oxidative conditions (increasing H_2O_2 concentrations). Quantification of the marker of RNA oxidation, 8-OHG, in mRNA extracts showed that it progressively increased from a basal level of ~100 pg μg RNA^{-1} in the absence of H_2O_2 to ~600 pg μg RNA^{-1} when H_2O_2 reached 10 μM in the iron ascorbate system (Figure 2A). The oxidative treatment of RNAs did not alter their overall integrity, as shown by the mRNA pattern on agarose gels, which did not reveal any apparent degradation (Figure 2B). Figure 2C is an example of a portion of silver-stained cDNA-AFLP gel after in vitro oxidation of mRNA. Polymorphism of TDF was evidenced at the lowest H_2O_2 concentration (i.e., 0.1 μM; Figure 2C) and at all the concentrations tested (see arrows in Figure 2C). Size and occurrence of TDFs appeared to be a random process since the various replicates performed revealed various TDF patterns, but polymorphism was generally much more pronounced at the higher H_2O_2 concentration. Moreover, whatever the combinations of primers that were used for cDNA-AFLP analysis, in vitro oxidation of mRNA caused TDF polymorphism. Using the same combination of primers with independently oxidized RNA templates leads to nonreproducible TDF polymorphism (see Supplemental Figure 1 online), thus showing the randomization of mRNA oxidation in vitro.

The iron-ascorbate system was also used to investigate the effect of mRNA oxidation on in vitro translation, using a rabbit reticulocyte lysate system. Efficiency of translation was assessed by measuring the amount of radioactivity incorporated into the proteins newly synthesized from extracted and oxidized mRNA. Incorporation of radioactivity into proteins decreased when H_2O_2 concentration into the mRNA oxidation system increased (Figure 3A). It was ~6200 cpm μg $protein^{-1}$ in the absence of H_2O_2 (control) but only 3000 cpm μg $protein^{-1}$ in the presence of 10 μM H_2O_2 (Figure 3A). Translated proteins were separated using two-dimensional (2D) electrophoresis to determine whether the decrease in translation efficiency resulted from a global alteration of translation of all mRNAs or if some transcripts were more subject to translation errors. In agreement with the radioactivity incorporation data (Figure 3A), autoradiography showed a global decline of spot intensities when H_2O_2 concentration increased (Figure 3B). Indeed, whereas 121 ± 12 spots were visible on gels obtained with control mRNA, only 91 ± 9 and 42 ± 13 were revealed on gels from mRNA treated with 1 and 10 μM H_2O_2, respectively (Figure 3B). Figure 3C shows the effect of mRNA oxidation on synthesis of abundant (a) or rare (b) proteins. It demonstrates that rare proteins disappeared when mRNAs were treated with 1 μM H_2O_2, whereas the amount of abundant proteins decreased only with 10 μM treatment (Figure 3C). Analysis of several replicates of this experiment demonstrated that in vitro oxidation of mRNA always induced a decrease of protein synthesis, but it was not possible to identify specific targets of RNA oxidation in vitro.

In Vivo Oxidation of mRNA during Dry After-Ripening

The amount of the nucleic acid oxidation marker 8-OHG was quantified in total RNA and mRNA extracted from dormant and nondormant embryos (dormant embryos stored for 8 weeks under 60% RH) and from storage control embryos (dormant embryos stored for 8 weeks under 5% RH). Figure 4 shows that the level of 8-OHG in total RNA did not vary among the samples. However, when using poly(A)-RNA purified from total RNA, we observed a significant increase, from ~150 to 220 pg 8-OHG μg RNA^{-1} in mRNA extracted from nondormant embryos only (Figure 4). Although the 8-OHG content of mRNA increased slightly

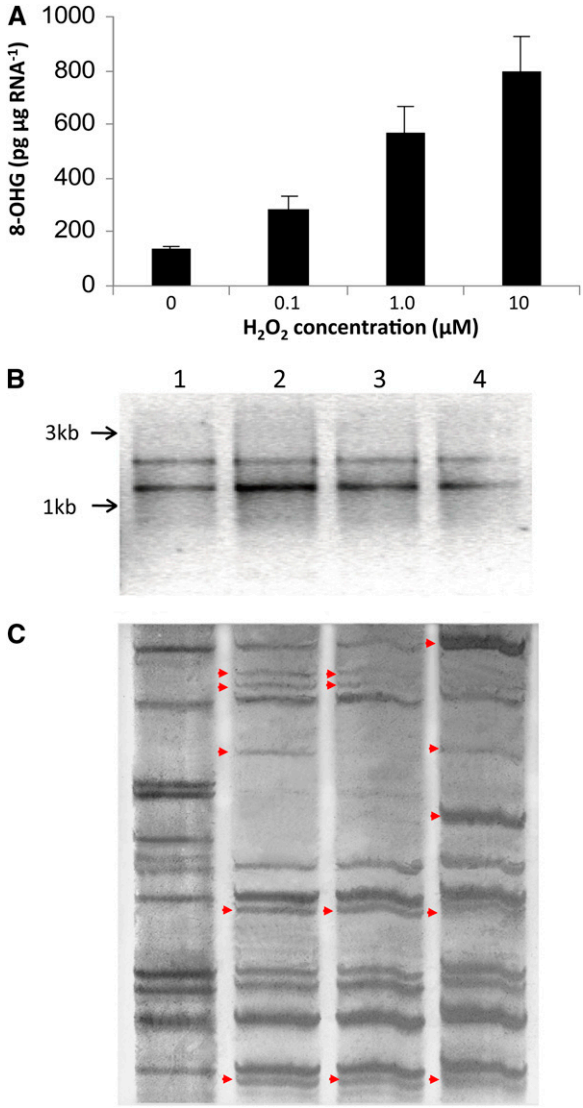

Figure 2. Effect of mRNA Oxidation on cDNA-AFLP Efficiency.

(A) 8-OHG of mRNA oxidized in vitro with the iron-ascorbate system in the presence of 0, 0.1, 1, and 10 μM H_2O_2. Values are mean of three replicates ± SD.

(B) Ethidium bromide–stained agarose gel showing the absence of RNA degradation during the treatment. Lane 1, control RNA (0 μM); lanes 2 to 4, RNA oxidized in the presence of 0.1, 1, and 10 μM H_2O_2. Arrows indicate 3- and 1-kb RNA weight.

(C) Portion of a silver-stained cDNA-AFLP gel obtained with mRNA treated as in **(A)**. Lane 1, control RNA (0 μM); lanes 2 to 4, RNA oxidized in the presence of 0.1, 1, and 10 μM H_2O_2. Primers used were Eco2 and Mse6 (see Supplemental Table 2 online). Arrowheads indicate polymorphisms. [See online article for color version of this figure.]

during seed storage at 5% RH, it did not significantly differ from the one measured in dormant embryos (Figure 4). In order to assess the relationship between mRNA oxidation and dormancy alleviation, dormant seeds from another seed batch were stored at 20°C and under four RH ranging from 5 to 75% for 30 d. Figure 5A shows that dormancy alleviation at 20°C was faster when seeds were stored under 50.5% RH than when they were stored under 75 and 33% RH. As at 25°C, 5% RH prevented dormancy release. The germination rate following after-ripening tracked well with the amount of 8-OHG of mRNA, the faster the germination at 10°C, the higher the level of oxidation of mRNA (Figure 5B).

Identification of Oxidized Transcripts

Oxidized transcripts were isolated using an immunoprecipitation protocol, and this protocol is specific for oxidized mRNA (see Supplemental Figure 2 online). In order to identify the oxidized mRNAs isolated by immunoprecipitation, we performed DNA microarray analysis using the Affymetrix Gene Chip array. Four arrays, corresponding to two independent biological replicates of oxidized mRNA extracted from dormant and nondormant embryos were performed. It is worth noting that the data obtained from the two independent biological replicates show that a specific subset of mRNA is oxidized during dry after-ripening. This is shown in Supplemental Figure 3 online, which presents statistical analyses and demonstrates the high reproducibility between the replicates. Comparison between oxidized mRNA fractions of dormant and nondormant embryonic axes revealed that 463 transcripts were differentially oxidized (see Supplemental Data Set 1 online). A large fraction of transcripts displayed only a slight variation of intensity (i.e., with a Log2 ratio of intensity < 1) (Figure 6A). Therefore, we only paid attention to the transcripts that presented highly significant differences of expression that we arbitrary associated with values of Log2 intensity ratio > 1.5 (Figure 6A). This subset contained 24 transcripts only present in the nondormant fraction (Figure 6), with relative Log2 intensity ratio ranging from 1.5 to 4, which indicates that oxidation of these transcripts increased from 3- to 16-fold during dry after-ripening.

Nucleotide sequences of the 24 oxidized transcripts more abundant in nondormant embryonic axes were compared to determine whether sensitivity of mRNA to oxidation could result from the presence of specific motifs or sequences. Supplemental Figure 4 online shows that no common specific sequence was evident among the 24 transcripts. We also assessed the possibility that the guanine content of the 24 transcripts would be higher than in nonoxidized transcripts, since our method of detection of oxidation is based on the presence of 8-OHG, the oxidation product of guanine. Guanine content of the 24 transcripts was therefore compared with the one found in a set of 50 nonoxidized transcripts. No significant difference in guanine content was present, as this value was 22.5% ± 2.9% in oxidized transcripts and 23.1% ± 3.6% in nonoxidized transcripts. Finally, we also determined whether the oxidation of a specific subset of transcripts in nondormant embryos might result from an increase in the amount of these transcripts during after-ripening or to their higher representation among the mRNA population. This was not the case since the total amount of the transcripts that we identified as becoming oxidized did not change during dormancy alleviation (see Supplemental Figure 5 online). This is also shown Figure 5B: the *y* axis shows the relative abundance of total transcripts (oxidized and nonoxidized) in

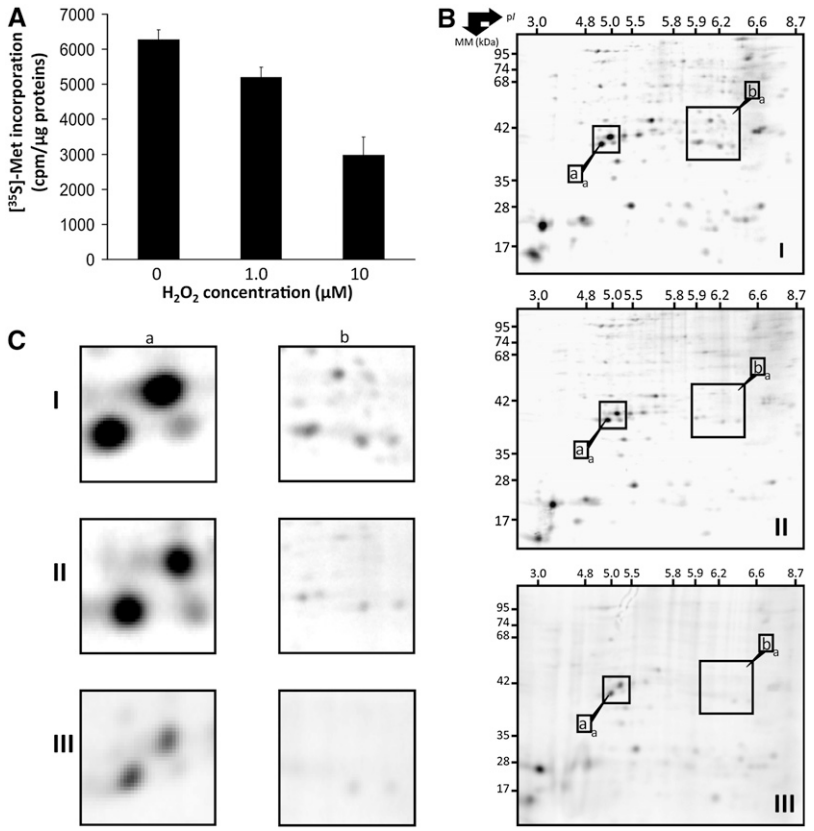

Figure 3. Effect of mRNA Oxidation on Protein Synthesis.

(A) Measurement of radioactivity (^{35}S-Met) incorporation into proteins after in vitro translation of differentially oxidized mRNA using the iron-ascorbate system. Values are mean of three replicates ± SD.

(B) Autoradiography of 2D electrophoresis gels obtained with in vitro–translated proteins translated from differentially oxidized mRNA. I, Control RNA (0 µM H_2O_2); II, RNA oxidized with 1 µM H_2O_2; III, RNA oxidized with 10 µM H_2O_2. a and b correspond to abundant and nonabundant proteins, respectively.

(C) Enlargements of indicated portions of the 2D gels from **(B)** showing the decrease of spot intensity for abundant (a) and nonabundant (b) proteins.

dormant and nondormant axes, and the *x* axis the relative abundance of oxidized transcripts. Although this figure demonstrates that the ratios of total transcripts were different in dormant and nondormant axes, it also shows that the oxidized transcripts did not correspond to the most represented transcripts in the nondormant fraction. This is shown in Supplemental Figure 5 online, which indicates that the level of expression of the oxidized transcripts ranged from ~1 to 2 of Log2 (intensity) value only.

Microarray analysis allowed the identification of the 24 oxidized transcripts specifically found in nondormant embryonic axes (Table 1). They have been clustered using gene ontology tools (Provart and Zhu, 2003) that distinguished three different classes: metabolic processes, response to stress and transport, and six unidentified transcripts. Clustering revealed that a majority of oxidized transcripts can be related to response to stress (Table 1). In this largest group, we identified, for example, genes belonging to the cytochrome P450 gene family, a protein phosphatase PP2C, a homogentisate phytyltransferase, and a key enzyme of the phenylpropanoid pathway (phenylalanine ammonia-lyase 1 [PAL1]).

In addition, the expression of the 18 identified transcripts that were found to be highly oxidized during after-ripening was followed during imbibition at 10°C of dormant and nondormant embryos (Table 1). Our transcriptomic data show that the relative abundance of most of these transcripts was significantly lower in nondormant embryonic axis than in dormant axis. At 24 h of imbibition, three of the transcripts were not significantly differentially expressed in dormant and nondormant axes when only one of them, UDP-glucoronosyl/UDP-glucosyl transferase family protein chr1, showed a higher expression in nondormant embryos (Table 1). All the others were either not detectable or less expressed in nondormant embryos (Table 1).

DISCUSSION

Seed dry after-ripening is an intriguing but critical process for survival and propagation of crops and wild species since it results in a progressive dormancy release that subsequently allows germination (Baskin and Baskin, 2004; Finch-Savage and

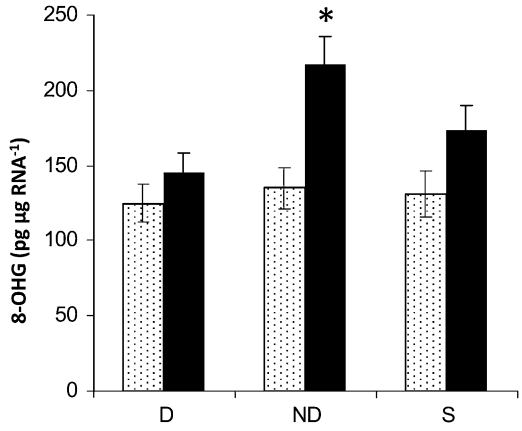

Figure 4. Quantification of Oxidized RNA.

8-OHG content of total RNA (dotted bars) and poly(A)-RNA (black bars) isolated from embryonic axis of dormant seeds (D), nondormant seeds after-ripened for 6 weeks at 25°C under 60% RH (ND), or 5% RH (storage control, S). Asterisk indicates a statistically significant difference (*t* test, $P < 0.05$) between ND sample and D and S samples. Values are means of five biological replicates ± SD.

Leubner-Metzger, 2006; Finch-Savage et al., 2007). The kinetics of sunflower embryo dormancy release can nevertheless be modulated by the RH applied during seed storage: low RH (i.e., 5% RH in this study) inhibited this process, at least during the time of the experiment, whereas dormancy release occurred within 5 to 6 weeks when seeds were stored above 60% RH (Figure 1), in agreement with the results presented by Bazin et al. (2011).

As dormancy alleviation is a major change in cell functioning, various authors have proposed that changes in gene expression could occur during dry after-ripening using a candidate gene approach (Leubner-Metzger, 2005) or global transcriptomic analyses (Bove et al., 2005; Cadman et al., 2006; Leymarie et al., 2007). In our model system, we show that dormancy alleviation was associated with changes in transcript abundance as suggested by results of cDNA-AFLP analysis showing an accumulation of TDF during after-ripening (Figure 1B). Importantly, our data demonstrate that seed MC, as modulated by the ambient RH, plays a critical role in expression of TDF, since low RH prevented this accumulation and that TDF patterns at 60 and 75% RH were different (Figure 1B). This would suggest that MC fine-tuned mRNA abundance in dry tissues since 5, 60, and 75% RH gave MC of 0.025, 0.04, and 0.06 g water g DW^{-1}, respectively. These results are in agreement with thermodynamic data showing that the nature of the mechanisms involved in dormancy release depends on seed MC even in the dry state (Bazin et al., 2011). They also must be considered with regards to the effect of seed MC on ROS generation in dry seeds, since the lowest RH (i.e., 5%) prevented both ROS (Oracz et al., 2007) and transcript accumulation. Using a limited number of primer combinations, we analyzed 10% of the entire transcriptome of sunflower seeds and found that 39 TDFs were differentially expressed in dormant and nondormant embryos, with 14 being more specific to dormancy alleviation (Figure 1B). This number is relatively high compared with the data obtained by Leymarie et al. (2007) and Bove et al. (2005), who showed that few transcripts increased in abundance during after-ripening, whereas a higher number decreased, as is also the case in *Arabidopsis* (Finch-Savage et al., 2007).

Our data, and those previously cited, therefore suggest that the abundance of numerous transcripts could change during dry after-ripening, which raises the question of the mechanisms underlying such changes in a medium devoid of free water. In these drastic conditions, the possibility of active gene transcription is difficult to explain since at low seed MC, the cytoplasm vitrifies, and its glassy structure prevents molecular diffusion and chemical reactions (Buitink and Leprince, 2004). However, mRNA abundance is also regulated at the posttranscriptional level by mechanisms such as RNA silencing or mRNA processing (Floris et al., 2009). Compared with DNA, RNA is much more prone to oxidative modifications, which is related to its single-stranded structure, relatively lesser association with protective proteins, and even cellular distribution (Li et al., 2006; Kong and Lin, 2010). In contrast with metabolic modifications, which require a hydrated system, oxidation of biomolecules can occur in anhydrobiosis. For example, Oracz et al. (2007) demonstrated that protein carbonylation resulting from ROS accumulation was associated with sunflower seed dormancy release during dry after-ripening.

We therefore postulated that oxidation of mRNA may contribute to explaining the changes in apparent gene transcription during seed after-ripening shown in this study and by various authors. Indeed, in vitro mRNA oxidation randomly induced several polymorphic TDFs on cDNA-AFLP gels (Figure 2; see Supplemental Figure 1 online). These results are in accordance with those of Gong et al. (2006), who showed that submillimolar H_2O_2 treatment of *Escherichia coli* cultures induced RNA oxidation and produced cDNA polymorphism. RNA bases can be directly oxidized by hydroxyl radicals, and 8-OHG is the most abundant RNA lesion, as guanine has the lowest oxidation potential (Nunomura et al., 2006). A direct consequence of mRNA oxidation would be an alteration of translation. Oxidized

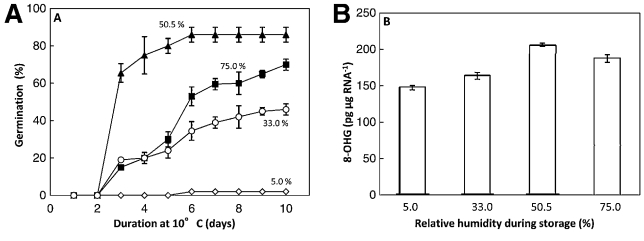

Figure 5. Effect of Relative Humidity during After-Ripening on Oxidation of mRNAs.

(A) Germination of sunflower embryos at 10°C following 30 d of after-ripening at 20°C and 5% (open diamonds), 33% (open circles), 75% (squares), and 50.5% (triangles) RH. Values are means of four replicates ± SD.

(B) 8-OHG content of mRNA isolated from embryonic axis of dormant seeds after-ripened for 30 d at 20°C under 5.0, 33, 50.5, and 75% RH. Values are means of five biological replicates ± SD.

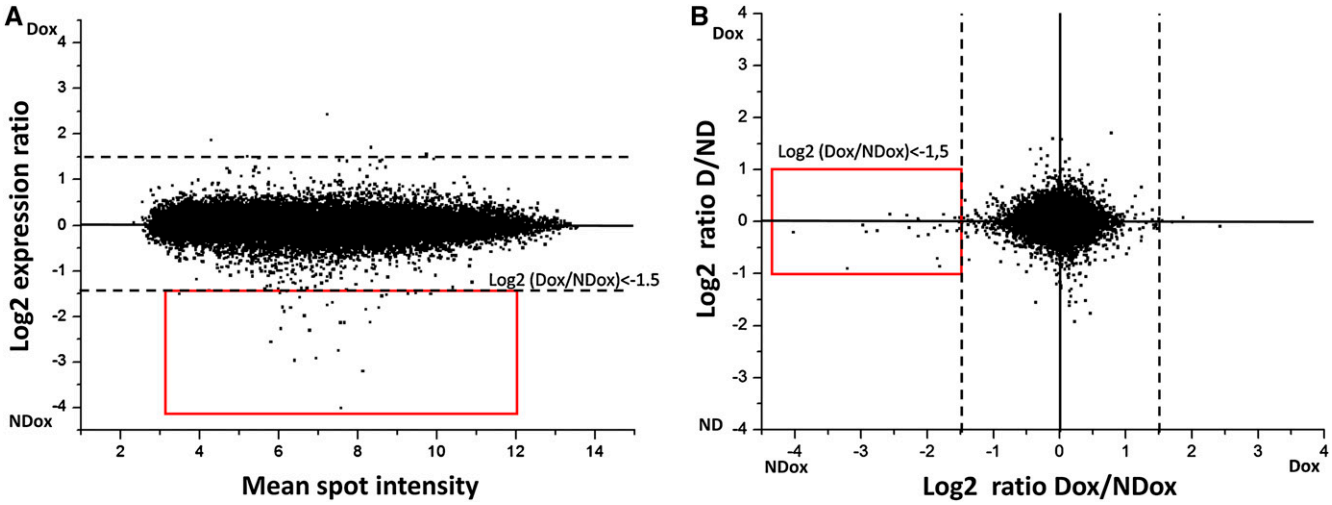

Figure 6. Microarray Analysis of Oxidized mRNAs.

(A) MA plot of oxidized mRNAs obtained after normalization of 8-OHG–containing fraction of mRNA extracted from axes of dormant (Dox) and nondormant (NDox) sunflower embryos. The rectangle indicates the transcripts specifically oxidized (Log2 > 1.5) in nondormant embryos.

(B) Plot of the D/ND Log2 intensity ratio of probes (total transcriptome) versus the Dox/NDox Log2 (oxidized transcriptome; i.e., 8-OHG–containing fraction). The rectangle indicates probes that show a Dox/NDox log2 ratio > 1.5 (i.e., the subset of transcripts more highly oxidized during after-ripening).

Data were obtained by analysis of the transcriptome data sets from four arrays per condition (two biological replicates × two technical replicates).

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bases in mRNA cause ribosome stalling on the transcript, leading to decreased protein expression (Shan et al., 2003) and to the production of short polypeptides because of premature termination or translation error-induced degradation (Tanaka et al., 2007). This putative effect of mRNA oxidation on translation efficiency has been demonstrated here using an in vitro system since we show that increasing levels of mRNA oxidation decreased protein synthesis, thus altering the protein pattern (Figure 3). If mRNA oxidation occurs in vivo during seed after-ripening and is directed toward a specific subset of transcripts, it would possibly play a role in the regulation of de novo protein synthesis in the early steps of seed imbibition and could control the potential for a seed to germinate or to remain dormant.

This hypothesis was assessed by monitoring changes in the amount of the RNA oxidation marker, 8-OHG, during after-ripening and by identifying the oxidized mRNAs. We show that the amount of 8-OHG of poly(A)-RNA increased by 50% during dormancy alleviation, but this increase was not detectable when using the total RNA population (Figure 4). This indicates that mRNAs are more sensitive to oxidation than other RNA species in our system. Noticeably, mRNA oxidation level did not increase significantly when seeds were stored at low RH (storage control, Figure 4), which suggests that mRNA oxidation was strictly associated with the acquisition of germination potential. The close relationship between mRNA oxidation and dormancy alleviation is also shown Figure 5. At another temperature of after-ripening (20°C), dormancy release occurs faster at 50.5% RH, in agreement with Bazin et al. (2011), and these conditions of after-ripening are associated with higher accumulation of 8-OHG. At 5% RH, ROS accumulation (Oracz et al., 2007) and rate of mRNA oxidation are reduced to such an extent that it does not permit acquisition of germination capacity. RNA oxidation under physiological conditions has been demonstrated in animals. For example, RNA oxidation in human neuronal cells is suspected to play a role in the case of age-related neurological diseases such as Alzheimer's and Parkinson's (Nunomura et al., 1999; Zhang et al., 1999). Compared with pathological situations, such as Alzheimer's disease where mRNA oxidation levels rise up to 30 to 70% from the basal level (Kong and Lin, 2010), the increase in mRNA oxidation during dormancy alleviation was relatively low (Figure 4), but after-ripening is not a deleterious process.

The characterization of the oxidized transcriptome (8-OHG fraction) of dormant and nondormant embryonic axis demonstrates the high reproducibility between biological replicates, which shows that mRNA oxidation is not random but directed toward a specific subset of 24 mRNAs (see Supplemental Figure 3 and Supplemental Data Set 1 online). This selectivity does not result from artifactual or side effects since we show that there is no common specific sequence within the oxidized transcripts (see Supplemental Figure 4 online) and that their amount of guanine, which is converted to 8-OHG, is similar to that of non-oxidized transcripts. We also show that the oxidation of specific transcripts was not related to their content: microarrays analyses of global transcriptome of dormant and nondormant embryos did not permit to fit the amount of a particular transcript with its level of oxidation (Figure 6; see Supplemental Figure 5 online). Similar selectivity of mRNA oxidation has also been observed in mammalian cells (Shan et al., 2003, 2007; Chang et al., 2008; Vascotto et al., 2009; Kong and Lin, 2010), but the mechanisms

Table 1. Gene Ontology Classification of the Oxidized Transcripts According to Classical Gene Ontology Categories Using the Web-Based Tool Classification Superviewer (http://bar.utoronto.ca) with Normalized Class Score Option

Heliagene Accession No. (www.heliagene.org)	Annotation (TAIR Accession No. or IPR)	GO: Biological Processes	Log2 Ratio NDox/Dox	Log2 Ratio ND/D
		Metabolic processes		
HuCL14020C001	AT5G05860.1	UGT76C2 (UDP-glucosyl transferase 76C2)	1.53	ND
HuCX944565	AT1G05680.1	UDP-glucoronosyl/UDP-glucosyl transferase family protein chr1	1.71	3.46
HuCL00146C001	AT3G12120.2	FAD2 (FATTY ACID DESATURASE 2) chr3	1.77	ND
HuCL07863C001	AT1G22430.2	Oxidoreductase/zinc ion binding	2.78	1.27
		Response to stress		
HuBQ967737	AT3G55270.1	MKP1 (MKP1); MAP kinase phosphatase	1.50	1
HuCL02340C001	AT4G36220.1	FAH1 (FERULATE-5-HYDROXYLASE 1)	1.50	1.29
HuCL01764C002	AT4G02930.1	Elongation factor Tu	1.60	ND
HuCL10608C001	AT5G52450.1	MATE efflux protein-related chr5	1.75	ND
HuCL07092C001	AT4G31940.1	CYP82C4 (cytochrome P450, family 82, subfamily C, polypeptide 4)	1.81	1.55
HuCL08462C001	AT3G26210.1	CYP71B23 (cytochrome P450, family 71, subfamily B, polypeptide 23)	1.93	1.18
HuCL00001C830	AT2G37040.1	PAL1	2.18	NS
HuCL20634C001	AT5G25120.1	CYP71B11 (cytochrome P450, family 71, subfamily B, polypeptide 11)	2.95	ND
HuCL13928C001	AT5G35715.1	CYP71B8 (cytochrome P450, family 71, subfamily B, polypeptide 8)	3.25	1.35
HuAJ539665	AT4G27800.3	Protein phosphatase 2C PPH1/PP2C PPH1 (PPH1)	3.92	NS
HuCL02924C001	AT1G13080.1	CYP71B2 (CYTOCHROME P450 71B2)	4.07	NS
		Transport		
HuCL11402C001	AT5G40780.2	LHT1 (LYSINE HISTIDINE TRANSPORTER1)	1.87	ND
HuCL00974C001	AT1G15520	ABC-2–type transporter	1.87	1
HuCL11043C001	AT2G18950.1	Homogentisate phytyltransferase	2.33	ND
		Unknown		
HuBQ976517		Unknown protein	1.54	–
HuAJ412623		Unknown protein	1.66	–
HuCD857474		Unknown protein	1.72	–
HuCL00722C002		Unknown protein	1.76	–
HuBQ913694		Unknown protein	2.60	–
HuCX944517		Unknown protein	2.90	–

Mean Log2 (intensity) values of oxidized transcripts from nondormant (NDox) and dormant (Dox) embryonic axes and of transcripts from nondormant (ND) and dormant (D) embryonic axes imbibed for 24 h at 10°C. TAIR, The Arabidopsis Information Resource; IPR, InterPro Records; GO, Gene Ontology; ND, not detectable; NS, not significant.

involved in this are unknown. Selective oxidation of mRNAs could be related to their cellular location (i.e., their distance from the site of ROS production) or more probably to their functional state within the messenger ribonucleoproteins (mRNPs). Recently it has been shown that mRNPs exist in three functional states in plants, as is the case in mammalian cells (Weber et al., 2008) (i.e., translated mRNPs, stored mRNPs [stress granules], and mRNPs under degradation [P-bodies]). The oxidation of mRNA might depend on this functional state. For example, the so-called stress granules are initiated under oxidative stress, and they have been proposed to serve as sorting sites for mRNA to ensure its appropriate storage (Kedersha and Anderson, 2002). RNA in stress granules could be less sensitive to oxidation, but mRNPs have not been characterized in seeds yet. This set of data brings a new look on the putative role of stored mRNA, also called long-lived mRNA, in seed dormancy. We hypothesize that the selective oxidation of a subset of stored mRNA might be a prerequisite for germination. As shown previously, mRNA oxidation will lead to selective translation following imbibition and to loss of normal protein level and/or protein function, but this process can also address the oxidized mRNA to P-bodies, the site of their active degradation (Sheth and Parker, 2006). Transcriptomic data obtained during seed imbibition moreover show that most of the transcripts oxidized during after-ripening were less abundant in nondormant embryos than in dormant ones (Table 1). This suggests that mRNA oxidation could selectively lead to their decay by a mechanism that remains to be elucidated. Understanding the involvement of this process in dormancy would require a detailed timescale analysis of the translated RNA fraction during germination. Interestingly, recent data have underlined the role of mRNA decay in plants as a possible regulatory mechanism of plant development (Narsai et al., 2007; Belostotsky and Sieburth, 2009), and our results bring novel insights sustaining this hypothesis.

Comparisons of oxidized transcriptomes from dormant and nondormant embryos revealed that the oxidation level of 463

transcripts was statistically different between both fractions (Figure 6; see Supplemental Data Set 1 online). Our protocol allowed us to identify the transcripts that were significantly oxidized (i.e., that contained a large amount of 8-OHG). Therefore, 24 transcripts that presented the highest variations only were analyzed (i.e., their Log2 ratio value was higher than 1.5) (Figure 6, Table 1). A large majority of highly oxidized transcripts corresponded to genes associated with response to stress. Interestingly, this gene ontology (i.e., genes associated with stress) has already been associated with regulation of seed dormancy (Carrera et al., 2008; Bentsink et al., 2010). However, these genes are not related to the ones that were previously associated with after-ripening phenotypes in *Arabidopsis*, such as *aba1-1* and *abi1-1* (Carrera et al., 2008), *cho1* (Yano et al., 2009), or *rbohB* (Müller et al., 2009). However, it is difficult to distinguish between after-ripening phenotypes and the action of after-ripening, which is evaluated by the ability to suppress dormancy after imbibition. We nevertheless emphasize that oxidation, a nonenzymatic process occurring in the dry state, is surprisingly directed toward transcripts often previously identified as putative players in seed dormancy or related metabolic pathways (hormones and ROS).

The role of mRNA oxidation in the release of dormancy can therefore be discussed with regards to the nature of the oxidized transcripts, since the corresponding proteins will not be translated during early imbibition. The PP2C PPH1 transcript showed a strong increase in oxidation level during after-ripening (Table 1). PP2Cs are ubiquitous protein phosphatases found in all eukaryotes and include 76 members in *Arabidopsis* (Kerk, 2006); they have been involved in abscisic acid (ABA), jasmonate, and ethylene responses (Meyer et al., 1994; Sheen, 1998; Schweighofer et al., 2007). PPH1 has been shown to be involved in growth and development of *Myxococcus xanthus* (Treuner-Lange et al., 2001) and in state transitions in *Arabidopsis* (Shapiguzov et al., 2010). MPK1, a mitogen-activated protein kinase, is involved in tolerance to stress (Ulm et al., 2002) and in repression of salicylic acid synthesis (Bartels et al., 2009). MPK1 is also induced by ABA (Blanco et al., 2006). Putative involvement of ABA signaling–related pathways in dormancy release in this system is also underlined by the identification of the ABC-2–type transporter (AT1G15520). This is a PDR-type ABC transporter that has been shown to mediate cellular uptake of ABA (Kang et al., 2010). Several transcripts of genes coding for members of the CYP71 subfamily of cytochrome P450 have been shown to be oxidized (Table 1). Cytochrome P450 are heme-containing proteins that are involved in various oxidative reactions and play a role in the synthesis of lipophilic compounds, especially in the responses to abiotic and biotic stresses (Narusaka et al., 2004). The CYP71 subfamily contains 22 members (Nelson et al., 1996), but their physiological functions remain unclear and their putative role in seed dormancy will require further investigation. *PAL1* is one of the genes, among four in *Arabidopsis*, coding for PAL, an enzyme that catalyzes the first step of the phenylpropanoid pathway and is considered as being a key regulation point between primary and secondary metabolism (Olsen et al., 2008; Huang et al., 2010). PAL activity regulates the content of the antioxidant flavonoids (Lepiniec et al., 2006). *Homogentisate phytyltransferase* transcripts were also oxidized. This gene, which controls the accumulation of tocopherols (Havaux et al., 2005), a major antioxidant in plants, has been shown to play a role seed longevity (Sattler et al., 2004). With regards to the role of ROS in seed germination, it worth noting that the major pathways of antioxidant compounds become altered during after-ripening. The decrease in PAL1 and homogentisate phytyltransferase could therefore participate to the accumulation of ROS that occurs during imbibition seed of nondormant embryos (Oracz et al., 2007). Future studies will aim to predict whether the proteins corresponding to the oxidized mRNA play a detrimental role in seed germination. Identification of oxidized transcripts in seeds of the model species *Arabidopsis* would help to design genetic approaches, using mutants, to identify new genes involved in dormancy regulation.

This study elucidates a potential mechanism of seed dormancy alleviation that fits well with the absence of active metabolism in anhydrobiosis. Targeted mRNA oxidation degradation could play a role in the early steps of seed imbibition that governs the process toward germination. Selective alteration of translation could fine-tune the cell signaling pathway that controls dormancy. Further understanding of the mechanisms allowing selective mRNA oxidation and identification of this process in other plant developmental processes or in plant responses to stress will be a challenge for the future. RNA oxidation should also be considered more widely with regards to the regulation of other developmental processes in plants or in their response to environmental stresses.

METHODS

Plant Material and After-Ripening Treatments

Two batches of sunflower (*Helianthus annuus* cv LG5665) seeds harvested in 2008 and 2009 near Montélimar (Drôme, France) and purchased from Limagrain were used in this study. At harvest, seeds were dormant and were stored at −30°C until use to maintain their dormancy. After-ripening was performed by placing the dormant seeds at 25°C over saturated solutions of $ZnCl_2$, NH_4NO_3, and NaCl in tightly closed jars giving RHs of 5, 60, and 75%, respectively (Vertucci and Roos, 1993). Another seed batch, harvested in the same location in 2010, was used in the experiment shown Figure 5. Dormant seeds of this batch were stored at 20°C over saturated solutions of $ZnCl_2$, $CaCl_2$, $Ca(NO_3)_2$, and NaCl in tightly closed jars giving RHs of 5, 33, 50.5, and 75%, respectively (Vertucci and Roos, 1993).

Germination Tests

Germination assays were performed at 10°C with naked seeds (i.e., seeds without pericarp, so-called embryos) in darkness in 9-cm Petri dishes (25 seeds per dish, four 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 to 3 mm. Germination counts were made daily for 7 d. The days of seeds dry storage required to reach 50% germination ($DSDS_{50}$) was calculated as the duration of after-ripening necessary to reach 50% germination at 10°C.

RNA Extraction and Poly(A) RNA Purification

Isolated embryonic axes were frozen in liquid nitrogen and stored at −80°C until use. For each extract, 25 axes were ground to a fine powder in liquid nitrogen, and total RNA was extracted by a hot phenol procedure as previously described by Oracz et al. (2008), according to Verwoerd et al.

(1989). All solutions and water were Chelex100-pretreated to eliminate metal ions that may oxidize RNA during extraction. RNA concentration was determined spectrophotometrically at 260 nm using a Nanovue spectrophotometer (GE Healthcare). Poly(A)-RNA was purified using the dynabeads oligo(dT) mRNA purification kit (Invitrogen) according to the manufacturer's instructions.

In Vitro Oxidation of RNA

Ten micrograms of total or poly(A)-RNA were oxidized in a mixture containing 0.1, 1, or 10 μM of hydrogen peroxide, 2 μM ferrous chloride ($FeCl_2$; Sigma Aldrich), and 2 μM ascorbate (Sigma-Aldrich) in 100 mM HEPES buffer, pH 7.2. The mixture was incubated for 1 h at 37°C, and the reaction was stopped by adding 10 mM EDTA and immediately cooled on ice. Oxidized RNA was concentrated by ethanol/sodium acetate precipitation and dissolved in diethylpyrocarbonate-treated water.

cDNA-AFLP

One microgram of poly(A)-RNA from embryonic axes or from in vitro oxidation treatment was used for cDNA-AFLP analysis according to Leymarie et al. (2007). All oligonucleotides were obtained from Eurogentec, and amplifications were performed using a personal mastercycler (Eppendorf). Fragments were separated on a sequencing polyacrylamide gel and were visualized by silver staining according to Bassam et al. (1991).

Isolation and Sequencing of the TDFs

Fragments were eluted from silver-stained gels using the procedure of Frost and Guggenheim (1999) with the modifications described by Leymarie et al. (2007). Amplifications were performed with the appropriate primers (see Supplemental Table 2 online) with 5 μL of this solution diluted 10-fold. PCR reactions were initiated at 94°C for 1 min followed by 35 cycles at 94°C for 1 min, 52°C for 1 min, 72°C for 1 min, and terminated at 72°C for 10 min.

Cloning was performed from fresh PCR products with the pCR 2.1-TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. After plasmid purification using a miniprep Plasmix kit (Talent), the insert size was checked by PCR amplification using M13 primers and the insert sequenced by Genomexpress. Similarity studies were performed with BLAST program using the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov/BLAST/) and the Compositae Genome Project Database (http://cgpdb.ucdavis.edu). Sequences of TDFs are referenced in the GenBank database with accessions numbers provided in Supplemental Table 1 online.

In Vitro Translation and 2D Electrophoresis

In vitro translations were done using 2 μg of oxidized and nonoxidized mRNA using the rabbit reticulocytes lysate system kit (Promega) following the manufacturer's instructions. After translation reaction, 40 μL of translation products were acetone/trichloroacetic acid (TCA) precipitated, washed two times in acetone 100%, and resuspended in 500 μL of isolectric focusing (IEF) buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% ampholines 3-10 [Bio-Rad], and 20 mM DTT). Protein synthesis was measured by TCA precipitation of aliquots of reaction mixtures spotted on Whatmann GF/C filters; after eight washing steps in cold 5% TCA and 0.04 M sodium pyrophosphate and two washing steps in absolute methanol, filters were dried and counted for radioactivity in a liquid scintillation counter. The 2D electrophoresis protocol was adapted from O'Farrell (1975). IEF was performed using immobilized pH gradient strips (7 cm, pH 4 to 7; ReadyStrips; Bio-Rad). Protein IEF was performed with 130 μL of protein extract using a Bio-Rad Protean IEF cell at 20°C as follows: active rehydration at 50 V for 12 h, 200 V constant voltage for 15 min, 500 V constant voltage for 15 min, 1000 V linear increase in voltage for 30 min, 5000 V low increase in voltage for 30 min, and 5000 V constant voltage until a total of 10,000 V/h was reached. Immobilized pH gradient strips were stored at −20°C until use. The strips were washed for 10 min in equilibration buffer (6 M urea, 20% glycerol, 0.375 M Tris-HCl, pH 8.8, and 2% SDS) containing 130 mM DTT followed by washing with equilibration buffer containing 135 mM iodoacetamide for 10 min. SDS-PAGE was performed using 10% polyacrylamide gels at 25 V for 30 min and then 125 V for 2 h in a Bio-Rad MiniProtean III. Gels were stained with Coomassie Brilliant Blue and dried in a gel dryer (model 583; Bio-Rad) for 1 h at 80°C prior to exposure to a phosphor screen (GE Healthcare) for 72 h. Screens were scanned using the Storm 840 scanner, and images were analyzed using ImageMaster Platinium (GE Healthcare).

8-OHG Quantification

8-OHG was quantified in mRNA samples using the OxiSelect Oxidative RNA Damage ELISA kit (Cell Biolabs) with minor modifications. Purified mRNA from different samples was digested to nucleosides using nuclease P1 and alkaline phosphatase treatment. Ten micrograms of mRNA were incubated for 2 h at 37°C with 10 units of nuclease P1 (New England Biolabs) in 20 mM sodium acetate buffer, pH 5.2, following by 1 h incubation at 37°C with 10 units of shrimp alkaline phosphatase (Promega) in 100 mM Tris-HCl, pH 7.5. The reaction mixture was then centrifuged (2 min, 13,000*g*, 4°C), and 50 μL of supernatant was used for 8-OHG determination with competitive ELISA assay according to the manufacturer's instructions. Standard curves were done using pure 8-OHG according to the manufacturer's instructions. Each sample was analyzed in duplicate on ELISA microplates, and values presented are mean of five biological replicates ± SD.

Oxidized RNA Immunoprecipitation

Oxidized RNA was immunoprecipitated according to Shan et al. (2003). mRNA (2.5 μg) was incubated at 65°C for 2 min and immediately placed on ice to remove secondary structures. mRNA was then incubated with 2 μg of antibody 15A3 (QED Bioscience) at room temperature for 2 h in 200 μL 1× PBS. For negative control trials, the primary antibody was omitted (see Supplemental Figure 2 online). Washed immobilized Protein G gel beads (20 μL; Pierce) were added and incubated at 4°C for an additional 15-h period. The beads were washed four times with PBS and 0.04% (v/v) Nonidet P-40 (Roche Products). Immunoprecipitated RNA was then extracted from the pellet by adding in the following order: 300 μL of 1× PBS with 0.04% Nonidet P-40, 30 μL of 10% (w/v) SDS, and 300 μL of PCI (phenol:chloroform:isoamyl alcohol, 25:24:1). The mixture was incubated during 15 min at 37°C, homogenized every 5 min, and separated to aqueous phase and organic phase by spinning at 14,000 rpm for 5 min. The aqueous phase was ethanol/salt precipitated in the presence of 2 μL of 5 mg mL^{-1} glycogen as coprecipitant. RNA was then resuspended in 15 μL of diethylpyrocarbonate water and 2× 1 μL was used for quantification using the Nanodrop spectrophotometer (Thermo Scientific). After quantification, a volume corresponding to 150 ng of RNA was concentrated to 3 μL for microarray analysis.

Array Hybridization on Affymetrix GeneChip Array

Array data obtained by analysis of the transcriptome data sets were from four arrays per condition (two biological replicates of 400 embryos × two technical replicates). All RNA samples were checked for their integrity on the Agilent 2100 bioanalyzer according to the manufacturer's instructions (Agilent Technologies). Six to forty nanograms of oxidized RNA or 100 ng of total RNA was used to synthesize fragmented and biotin-labeled single-stranded DNAs with the GeneChip WT cDNA synthesis and amplification kit and GeneChip WT terminal labeling kit (Affymetrix).

Quantification of the single-stranded DNAs was performed with NanoDrop (Spectrophotometer ND1000; Thermo Fisher Scientific) after cleanup by the Sample Cleanup Module (Affymetrix). Single-stranded cDNA (5.5 μg) was fragmented and labeled followed by hybridization for 16 h at 45°C to the Affymetrix GeneChip sunflower microarray developed by an international consortium of Syngenta Seeds, Biogemma, Institut National de la Recherche Agronomique (Toulouse, France; P. Vincourt), the University of Georgia (Athens, GA; S. Knapp), and the University of British Columbia (Vancouver, Canada; L. Rieseberg). The array consists of 2.6 million oligonucleotide probes, representing 284,000 ESTs mainly from sunflower and some from other inbred lines. After hybridization, the arrays were washed with two different buffers (stringent: 6× SSPE and 0.01% Tween 20; and nonstringent: 100 mM MES, 0.1 M [Na^+], and 0.01% Tween 20) and stained with a complex solution including Streptavidin R-Phycoerythrin conjugate (Invitrogen/Molecular Probes) and antistreptavidin biotinylated antibody (Vector Laboratories). The washing and staining steps were performed in a GeneChip Fluidics Station 450 (Affymetrix). The Affymetrix GeneChip sunflower Genome Arrays were finally scanned with the GeneChip Scanner 3000 7G piloted by the GeneChip launcher. All these steps were performed on an Affymetrix platform at Institut National de la Recherche Agronomique–Unité de Recherche en Génomique Végétale. The .raw CEL files were imported in R software for data analysis. All raw and normalized data are available through the CATdb database (AFFY_Dormance_totalRNA_\sunflower and AFFY_Dormance_mRNAoxidized_sunflower; Gagnot et al., 2008) and from the Gene Expression Omnibus repository at NCBI (Barrett et al., 2007) under accession numbers GSE23046 and GSE23048.

Data Analysis

The data were normalized with the rma algorithm (Irizarry et al., 2003), available in the Bioconductor package (Gentleman et al., 2004). To determine differentially expressed genes, we performed a usual two-group *t* test that assumes equal variance between groups. The variance of the gene expression per group is a homoscedastic variance, where genes displaying extremes of variance (too small or too large) were excluded. The genes excluded were those with a specific variance/common variance ratio smaller than the α-quantile of a χ^2 distribution of one degree of liberty or greater than the 1-α-quantile of a χ^2 distribution of one degree of liberty with α equal to 0.0001. This filter was done for each condition and not across conditions. The number of excluded genes was 784 (Dox versus NDox) and 799 (D versus ND). The raw P values were adjusted by the Bonferroni method, which controls the family-wise error rate (Ge et al., 2003). A gene was declared differentially expressed if the Bonferroni P value is < 0.05. For gene ontology studies, *Arabidopsis thaliana* accession numbers of annotated sunflower genes were submitted to functional classification using the Classification Superviewer tool (http://bar.utoronto.ca) with normalization algorithm (Provart and Zhu, 2003).

Accession Numbers

Accession numbers are provided in Table 1 and in Supplemental Table 1 online. Microarray data have been deposited with the Gene Expression Omnibus repository at NCBI (Barrett et al., 2007) under accession numbers GSE23046 and GSE23048.

Supplemental Data

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

Supplemental Figure 1. Portions of Silver-Stained cDNA-AFLP Gels Obtained with the Same Primer Combination Amplification of cDNA from Independently in Vitro–Oxidized mRNA.

Supplemental Figure 2. RNA Gel Electrophoresis Showing Anti 8-OHG Immunoprecipitation Selectivity.

Supplemental Figure 3. Reproducibility of Microarray Data from 8-OHG Fraction of Dormant and Nondormant Embryonic Axes.

Supplemental Figure 4. Geneious Software Sequence Alignment of the 24 Oxidized Transcripts Obtained Using the ClustalW Algorithm.

Supplemental Figure 5. Mean Log2 (Intensity) Values of the 24 Oxidized Transcripts in Dormant and Nondormant Total Transcriptome.

Supplemental Table 1. Differentially Expressed TDFs during After-Ripening of Sunflower Seeds at 60 and 75% RH (Group A+B).

Supplemental Table 2. Primers Used in This Study.

Supplemental Data Set 1. Microarray Data Showing Differentially Oxidized Transcripts during Dry After-Ripening.

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AUTHOR CONTRIBUTIONS

J.B. performed research and analyzed data. N.L. and P.V. contributed new analytic tools. S.B. and S.A. performed research and analyzed data. H.E.-M.-B. contributed new analytic tools, designed the research, and analyzed data. C.B. designed the research, analyzed data, and wrote the article.

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