

Reactive oxygen species and seed germination

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Abstract: Reactive oxygen species (ROS) are continuously produced by the metabolically active cells of seeds, and apparently play important roles in biological processes such as germination and dormancy. Germination and ROS accumulation appear to be linked, and seed germination success may be closely associated with internal ROS contents and the activities of ROS-scavenging systems. Although ROS were long considered hazardous molecules, their functions as cell signaling compounds are now well established and widely studied in plants. In seeds, ROS have important roles in endosperm weakening, the mobilization of seed reserves, protection against pathogens, and programmed cell death. ROS may also function as messengers or transmitters of environmental cues during seed germination. Little is currently known, however, about ROS biochemistry or their functions or the signaling pathways during these processes, which are to be considered in the present review.

Key words: endosperm weakening; ROS signaling; ROS-scavenging systems; seed dormancy

Introduction

Our view of the role of reactive oxygen species (ROS) has greatly changed in light of recent studies. The derivatives of the reduction of oxygen as a superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), or singlet oxygen (${}_1O^2$), were initially viewed as hazardous compounds, but they are now recognized as important molecules in plant biological processes. ROS appear to be central components of plant adaptations to biotic and abiotic stress, exacerbating cell damage as well as signaling the activation of defense responses (Miller et al. 2008; Shetty et al. 2008). The investigation of ROS as important biological molecules (and not simply as hazardous compounds) started after workers became aware of their crucial roles in pathogenesis, in which ROS are involved in processes such as the hypersensitive response of plant-pathogen incompatible interactions; limiting pathogen infection by reinforcing plant cell walls and/or killing pathogens directly; and in signaling acquired resistance as reviewed by Shetty et al. (2008).

Although high concentrations of ROS can lead to phytotoxicity, relatively low levels can be used as acclimating signals, so that ROS represent important signaling molecules under conditions of abiotic stress (Gill & Tuteja 2010; Miller et al. 2008). ROS functions, their plant biochemistry production sites, and scavenging antioxidant defense machinery in response to abiotic stress conditions have been reviewed (see Miller et al. 2008 and Gill & Tuteja 2010), and their signaling

functions as well as their roles in plant growth and development are now quite well documented (see Ahmad et al. 2008; del Rio et al. 2006; Gapper & Dolan 2006; Tanou et al. 2010). These topics will therefore not be the focus of this review, which will concentrate on the roles of ROS and ROS-scavenging systems in seed germination. According to a number of recent works (see reviews by Bailly et al. 2008 and El-Maarouf-Bouteau & Bailly 2008), the roles of ROS in seeds are not as negative as previously thought, and ROS appear to play key signaling roles in seed biological process such as germination and dormancy release (Barba-Espín et al. 2011; Leymarie et al. 2012; Oracz et al. 2009).

ROS production sites in seeds

Sources of ROS production are dependent on molecular mobility and cytoplasmic viscosity that govern the occurrence and rates of metabolic reactions (Walters, 1998). Seed moisture content is high at the early stages of seed development in orthodox seeds, such as during embryogenesis and seed filling (El-Maarouf-Bouteau & Bailly 2008). After that, and during the desiccation or maturation drying phase, seeds suffer dramatic water losses (El-Maarouf-Bouteau & Bailly 2008). Accordingly, ROS sources fluctuate substantially from the beginning of embryogenesis to the end of germination, and both processes vary in seed tissues according to their cell hydration states (Bailly et al. 2008).

In dry seeds, enzyme activities are extremely reduced (Vertucci et al. 1995) and ROS probably orig-

inate from non-enzymatic reactions such as lipid peroxidation that occur even with very low moisture contents (McDonald 1999) and from Amadori and Maillard reactions (Sun & Leopold 1995). In hydrated seeds, however, all metabolically activate compartments may become sources of ROS, such as glyoxysomes (by the catabolism of lipids), peroxisomes (by the catabolism of purines), mitochondria (through respiratory activity), chloroplasts (by electron transfer in photosystems), and plasma membranes (by NADPH oxidase) (Bailly 2004).

The major sources of ROS production (such as H_2O_2) in hydrated seeds during germination as well as the ROS targets can be attributed to mitochondrial activity (Noctor et al. 2007), since the resumption of respiration in imbibed seeds can lead to electron leakage and increased production of ROS (El-Maarouf-Bouteau & Bailly 2008). Pergo & Ishii-Iwamoto (2011) noted ROS production in *Ipomea triloba* seeds as soon as mitochondrial respiration resumed. The mitochondrial electron-transfer chain (ETC) harbors electrons with sufficient free energy to directly reduce O_2 , and this change is considered the unavoidable primary source of mitochondrial ROS (Rhoads et al. 2006). Complexes I and II of the mitochondrial ETCs are known sites of O_2^- production, which is further reduced by superoxide dismutase (SOD) dismutation to H_2O_2 (Rodriguez-Serrano et al. 2009). Approximately 2–3% of oxygen used by the mitochondria is diverted to superoxide and H_2O_2 formation (Puntarulo et al. 1998). H_2O_2 can further react with reduced Fe^{2+} and Cu^{2+} to produce $\cdot OH$, which can penetrate membranes and leave the mitochondrion (Rhoads et al. 2006).

In terms of chloroplasts, the oxygen generated during photosynthesis can accept electrons passing through the photosystems, resulting in O_2^- formation (Gill & Tuteja 2010). The presence of ROS production centers such as triplet Chl and the ETCs in PSI and PSII make chloroplasts important sites of ROS production (O_2^- , 1O_2 and H_2O_2) (Gill & Tuteja 2010). However, while chloroplasts can generate ROS at the beginning of seed development, they rapidly become non-functional (El-Maarouf-Bouteau & Bailly 2008). Due to the mobilization and metabolism of lipid reserves during seed germination, glyoxysomes (a type of peroxysome) are also an important ROS production site in seeds, leading to O_2^- and H_2O_2 formation by glycolate oxidase (Gill & Tuteja 2010). Additionally, fatty acid β -oxidation, the enzymatic reactions of flavin oxidases, and the deprotonation of O_2^- are other metabolic processes responsible for H_2O_2 generation in peroxisomes (see review by Gill & Tuteja 2010). Moreover, $NO\cdot$ radical production also occurs in peroxisomes (Gill & Tuteja 2010).

Another site of ROS production is the plasma membrane, through the activities of NADPH oxidases that transfer electrons from cytoplasmic NADPH to oxygen (Grant & Loake 2000). As was recently suggested by Gill & Tuteja (2010), other important sources of H_2O_2 (in the apoplast of plant cells) are the detoxification reactions catalyzed by the cytochrome P450 in the cytoplasm and the endoplasmic reticulum, pH-

dependent cell wall-peroxidases, germin-like oxalate oxidases, and amine oxidases, which have received little attention until recently.

ROS signaling pathways and functions in seeds

Many studies have shown that the transition from a quiescent seed to a metabolically active organism is associated with ROS generation. The production of hydrogen peroxide during the early imbibition period of seeds, as well as nitric oxide, hydroxyl radicals and superoxide radicals have been observed during seed germination in various species (see El-Maarouf-Bouteau & Bailly 2008).

In order to be able to play a role in cell functioning ROS must reach cellular targets – a process in which cytoplasmic viscosity could play a key role in signaling vs the deleterious roles of ROS (Bailly et al. 2008). In a dry state, ROS targets must be close to their production sites, while in hydrated seeds the free water allows the ROS (or their longer-living forms, such as H_2O_2) to travel and reach targets farther from their production sites (Bailly et al. 2008).

Little is known about where and how ROS are sensed when exerting a specific effect. The expressions of large numbers of genes are sensitive to ROS produced in different subcellular compartments (Neill et al. 2002). A given ROS can selectively interact with a target molecule that perceives increases in ROS concentrations and then translate that information into alterations of gene expression (Laloi et al. 2004). Transcriptional activity may be altered by the oxidation of certain components of the signaling pathway that go on to activate transcription factors, or ROS may directly modify redox-sensitive transcription factors (Laloi et al. 2004). Both indirect (Kovtun et al. 2000) and direct (Mou et al. 2003) ROS-activation of transcription factors have been seen in plants, although most of information refers to H_2O_2 . Hydrogen peroxide is linked to pathogen-induced resistance where, upon infection, plasma membrane NADPH oxidase is activated and produces O_2^- that is subsequently converted to H_2O_2 via spontaneous dismutation or via SOD activity (Desikan et al. 1996). In this process, H_2O_2 acts as toxic compound to both plant cells and pathogens, killing infected cells and inhibiting pathogen spread; H_2O_2 can also serve as a substrate in peroxidative cross-linking reactions of cell walls, thus reinforcing cell resistance to infection (Levine et al. 1996). The signaling role of H_2O_2 has also been proposed in plant abiotic stress acclimation, although its functions in seeds during stress are still largely unknown. Maize seedlings injected with H_2O_2 and menadione (a superoxide-generation compound) became more tolerant to chilling stress, partly due to an enhanced antioxidant system that prevents the accumulation of ROS during stress (Prasad 1996). Thermotolerance in mustard seedlings induced by salicylic acid or heat acclimation was correlated with a transient peak in H_2O_2 (Dat et al. 1998). Partial exposure to excess light or H_2O_2 injections induced pro-

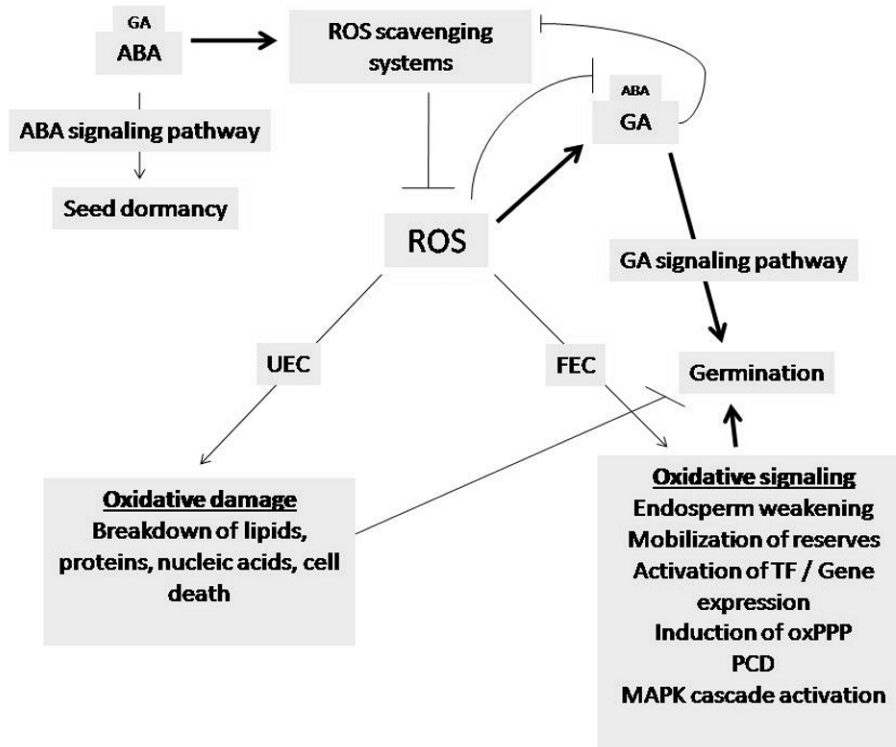


Fig. 1. Role of ROS in dormancy release and germination (modified from El-Maarouf-Bouteau & Bailly, 2008). Low GA/ABA ratios are related to low ROS levels. Under high GA/ABA ratios, ROS levels increase, leading to 1) oxidative damage under unfavorable environmental conditions (UEC) and seed germination inhibition, or 2) oxidative signaling under favorable environmental conditions (FEC) and seed germination.

tection against subsequent excess light-induced photobleaching in *Arabidopsis* leaves, which was correlated with the H₂O₂-dependent expression of the APX cytosolic gene (Karpinski et al. 1999). These results show that H₂O₂ is part of the signaling cascade that induces specific gene expression, making the study of ROS in seed signaling pathways highly interesting.

Since cells possess enzymatic systems that are capable of producing superoxides (although no cellular system is known to exclusively generate H₂O₂) information about the importance of O₂⁻ as a mediator of cellular processes has been emerging (Buetler et al. 2004). It is known that O₂⁻ serves as a growth stimulus in bacteria and yeast and similar findings have been made with eukaryotic cells, where SOD expression (which is modulated by superoxide production) was found to be cell cycle-dependent (Buetler et al. 2004). Additionally, O₂⁻ has been seen to play a role in cell death and in plant defense responses (Doke et al. 1994; Jabs et al. 1996; Wisniewski et al. 1999).

The role of ROS have been well recognized in seeds in endosperm weakening, mobilization of seed reserves, protection against pathogens, and programmed cell death (PCD) – as reviewed by El-Maarouf-Bouteau & Bailly (2008) and presented in Fig. 1. A micropylar endosperm covers the radical tip in some seeds and constitutes a mechanical barrier to radicle protrusion. The role of ROS in endosperm weakening has been proposed in analogy to its involvement in cell wall loosening in growing tissues (Liszak et al.

2004). ROS can break down cell wall-polysaccharides (Schweikert et al. 2002), regulate cellular calcium channels (Carol & Dolan 2006), and mediate auxin functions in cell growth (Schopfer et al. 2002). In reserve mobilization processes, ROS can have a role in breaking down polysaccharides (as mentioned above), DNA, RNA, and fatty acids (Buetler et al. 2004) and in protein carbonylation (which increases protein susceptibility to proteolytic cleavage) – thus aiding the mobilization of those molecules during germination (Job et al. 2005). In protecting against pathogens, ROS may be used as weapons (as toxic compounds) or they may trigger hypersensitive reactions in the PCD of infected sites (as discussed earlier) (Grant & Loake 2000). ROS appears to induce plant cell death by initiating transductional pathways rather than through direct poisoning due to phytotoxicity (Van Breusegem et al. 2001). In this process, ROS trigger signal transduction cascades that lead to cell death by, for example, inhibiting *de novo* protein synthesis, blocking calcium fluxes, kinase/phosphatase activities, and oxidative bursts (Van Breusegem et al. 2001). Both O₂⁻ and H₂O₂ are indisputably involved in genetically programmed cell death in plants (Buetler et al. 2004; Van Breusegem et al. 2001; Wisniewski et al. 1999).

ROS involvements in PCD during seed germination and seedling establishment have been verified in the aleurone layer of cereal grains and are related to H₂O₂ interactions with GA and ABA hormones (Fath et al.

2001, 2002). It is known that GA promotes germination and post-germination processes that are otherwise inhibited by ABA. While the activities of ROS scavenging enzymes are down-regulated by GA, they are maintained by ABA, which suggests a ROS link to PCD execution. Müller et al. (2009) reported direct *in vivo* evidence for hydroxyl radical ($\cdot\text{OH}$)-mediated cell wall loosening during plant seed germination and seedling growth. These authors showed that wall polysaccharides are oxidized *in vivo* by the developmentally regulated action of apoplastic $\cdot\text{OH}$ in radicles and endosperm caps. Both the production (by the Fenton reaction) and action of $\cdot\text{OH}$ increased during endosperm weakening and radicle elongation and were inhibited by ABA (a germination-inhibiting hormone); these effects were reversed by GA, demonstrating a positive role for $\cdot\text{OH}$ in cell wall loosening during seed germination (Müller et al. 2009). These authors also reported a slight relation between hydroxyl radical production sites, cell wall polysaccharides attack targets, and radicle growth – reinforcing the idea that plant cell wall loosening by $\cdot\text{OH}$ is an action controlled by this type of ROS.

The signaling roles of ROS in seed germination and dormancy have been well documented. During the germination phase *sensu stricto* (phase II of the full process of germination), which involves the activation of a regulatory system controlled by intrinsic (i.e. dormancy) and extrinsic (i.e. environmental conditions) factors, ROS generation and accumulation increases in various species (see the review by Bailly et al. 2008). In addition to the roles discussed above (endosperm weakening, mobilization of seed reserves, protection against pathogens, and PCD), ROS have been proposed as messengers or transmitters of environmental cues during seed germination (Bailly et al. 2008). The generation of ROS in stressful germination situations (excessive heat, cold, or UV, or hypoxia stresses) would prevent radicle emergence, and the role of ROS would therefore reside at the interface between signaling and deleterious effects (Bailly et al. 2008). ROS are currently recognized as important factors in seed dormancy [see the reviews by Bailly et al. (2008) and El-Maarouf-Bouteau & Bailly (2008)]. ROS production and accumulation by embryos leads to dormancy alleviation (which has been confirmed by the stimulation of seed germination by exogenous H_2O_2 in various species) (Bailly et al. 2008). The role of ROS in seed dormancy control is due their interaction with plant hormones that have central functions in seed dormancy and germination (such as ABA, GA, ethylene, auxin and brassinosteroids) [see Bailly et al. (2008) and El-Maarouf-Bouteau & Bailly (2008)].

The roles of the ROS in seed dormancy and germination has been confirmed by recent research. The germination of sunflower (Oracz et al. 2009) and *Arabidopsis thaliana* (Leymarie et al. 2012) seeds is associated with the accumulation of superoxide and hydrogen peroxide in the embryonic axes and radicle respectively. In *A. thaliana*, H_2O_2 production was double in germinating seeds – which was attributed to the activation

of the mechanisms involved in ROS production. Leymarie et al. (2012) likewise verified the presence of an efficient cell-to-cell ROS-propagation system in germinating seeds: O_2^- accumulated specifically in the radicle of the embryo in non-dormant seeds, whereas dormant seeds accumulated this compound irregularly. Accordingly, the increased activity of NADPH-oxidase (a superoxide radical source) in germinating seeds (Oracz et al. 2009) reinforces the role of ROS in seed germination. Müller et al. (2009) noted the importance of NADPH-oxidase *AtrbohB* in germinating *Arabidopsis* seeds after-ripening. *AtrbohB* mutants fail to after-ripen and show reduced protein oxidation; the inhibition of superoxide production by diphenylene iodonium leads to delays in *Arabidopsis* and cress seed germination and cress endosperm weakening. According to these authors, ROS produced by *AtrbohB* during after-ripening (the process by which dormant seeds become non-dormant during dry storage after harvesting) could act via ABA signaling or post-translational protein modifications. A role for ROS in dormancy alleviation during after-ripening was also seen in sunflower seeds, and was associated with mRNA oxidation; this oxidation does not occur while the seeds are dormant (Bazil et al. 2011).

In addition to ROS production stimulation by specific sites (such as NADPH-oxidase), modulations of ROS-scavenging activities may also be a source of ROS modulation during the germination process. Oracz et al. (2009) reported inhibited CAT and SOD activities (which may increase ROS accumulation) in germinating seeds. Leymarie et al. (2012), however, did not observe any differences in SOD activity between germinating and non-germinating seeds, and only a slight decrease in CAT activity was seen. These results therefore show species-specific variations of ROS accumulation processes during seed germination. Variations in the amounts and times of ROS production were also reported by Leymarie et al. (2012), suggesting that ROS takes on different roles during germination.

ROS may also function in breaking seed dormancy and in germination by activating the oxidative phase of the pentose phosphate pathway (Barba-Espín et al. 2011). The exogenous application of H_2O_2 in pea seeds has been observed to induce carbonylation of enzymes involved in the regeneration of reducing power during glycolysis, which stimulated the oxidative phase of the pentose phosphate pathway (oxPPP) (Barba-Espín et al. 2011). In addition to maintaining redox potentials necessary to protect against oxidative stress (Kruger & von Schaewen, 2003), activation of the oxPPP has been implicated in breaking seed dormancy (Finch-Savage et al. 2007). Additionally, Barba-Espín et al. (2011) noted a significant correlation between internal H_2O_2 contents and gene expression linked to mitogen-activated protein kinase (MAPKs). MAPK cascades have important roles in regulating plant growth and development by affecting cell division and the actions of hormones during seed germination (Xu et al. 2010), so that variations in seed

H₂O₂ contents could induce germination by activating those cascades (Barba-Espín et al. 2011).

Interestingly, Bazin et al. (2011) did not observe ROS accumulation in barley embryos during after-ripening, but reported interactions between ROS and seed hormones. Exogenous H₂O₂ partially alleviated seed dormancy through a small increase in embryonic ABA content related to a slight induction of transcription associated with primary dormancy (*HvNCED*). H₂O₂ treatment did not affect ABA sensitivity but up-regulated the expression of *HvExpA11* (GA induced gene), inhibited the expression of *HvGA2ox3* involved in GA catabolism, and enhanced the expression of *HvGA2ox1* (which is implicated in GA synthesis). H₂O₂ was therefore implicated in dormancy alleviation in barley through the activation of GA signaling and synthesis rather than by repressing ABA signaling.

It is important to stress that in order to activate signaling molecules ROS must be regulated by an efficient ROS scavenging system that tightly regulates its concentrations. Systems of ROS-scavenging have been exhaustively discussed and will therefore not be covered in this review (see Gill & Tuteja 2010).

ROS, the environment, and seed germination

Seed are subject to all types of environmental stress conditions. Interestingly, the germination responses of seeds to environmental factors are closely related to ROS production and accumulation. Both biotic and abiotic stresses are known to induce increased ROS production in seeds. The phytotoxic effects of allelochemicals on germination (delaying or preventing seed germination), for example, are associated with increases in lipid peroxidation, ROS accumulation, and alterations in the activities of antioxidant enzymes (Oracz et al. 2007; Pergo & Ishii-Iwamoto 2011). Strictly regulated concentrations of ROS are currently viewed as being essential for germination (the oxidative window) (Bailly et al. 2008). Cellular antioxidant systems maintain intracellular redox homeostasis, preventing the accumulation of toxic amounts of ROS while allowing ROS-mediated signaling to occur (Foyer & Noctor 2009). The importance of the coupled activities of SOD and CAT in maintaining the oxidative status of seeds during germination was suggested by Pergo & Ishii-Iwamoto (2011). We recently confirmed the central role of APX in assuring seed germination when exposed to toxic levels of arsenic (As) and zinc (Zn) (Gomes et al. 2012).

The deleterious role of ROS in seeds is due to their high reactivity with biomolecules, and the shift from a signaling to a deleterious mode is related to the accumulation of these ROS above threshold levels (Bailly et al. 2008). Unregulated ROS molecules can chemically react with nucleic acids and affect the genetic code of the embryo and react with seed storage compounds such as proteins and lipids, thus compromising the germination process (Kranmer & Colville 2011). Slightly enhanced levels of oxidative stress have been observed to stimulate germination (Lefevre et al. 2009), however,

as germination processes are strictly regulated by ROS concentrations (Bailly et al. 2008).

Another process in which ROS are seen as having a central role is the dehydration of orthodox seeds (desiccation). Oxidative processes and free radical accumulations have frequently been reported during desiccation, accompanied by lipid peroxidation and substantial seed tissue damage (Corbineau et al. 2004; Kermode & Savage 2002). According to Côme & Corbineau (1996) various ROS-protective mechanisms become progressively unable to impede ROS damage to cell structures during water loss and subsequent rehydration. Accordingly, increased activities of ROS-enzymatic scavenging enzymes (glutathione reductase – GR, SOD and CAT) have been seen during dehydration in both tolerant and sensitive tissues, although these activities are sustained upon rehydration only in tolerant tissues (Farrant et al. 2004). The acquisition of desiccation tolerance has been seen to be closely related to the activation of antioxidant systems (Bailly et al. 2008). Decreased H₂O₂ contents in sunflower seeds during maturation-drying were attributed to both reductions of mitochondrial activities and to an efficient antioxidant defense system controlling ROS accumulation during water loss (Lehner et al. 2006). Dried tolerant bean seeds showed high CAT and GR and low SOD and APX activities, while the opposite situation was seen in immature dried seeds (Bailly et al. 2001), suggesting that the acquisition of drying tolerance is also related to reorientation of the enzymatic ROS-scavenging systems (Bailly et al. 2008).

Similar to the situation seen during desiccation, ROS are also involved in the loss of seed viability, and the free radical hypothesis of aging is widely accepted (Bailly et al. 2008; Rajjou et al. 2008). Viability losses during storage result from accumulations of ROS and its associated damage and a decrease in antioxidant mechanisms, which lead to oxidative damage during seed imbibition (Bailly et al. 2008). Losses of ROS-scavenging enzyme activities, the occurrence of oxidative reactions leading to membrane damage and lipid peroxidation have been reported with progressive seed aging (Bailly et al. 1996; Munné-Bosch et al. 2011; Pukacka & Ratajczak 2007). However, viability losses are not always associated with lipid peroxidation and may be due to oxidative damage to proteins and nucleic acids (Bailly et al. 2008). Rajjou et al. (2008) reported that oxidative stress, which can induce protein modifications through carbonylation, provoked decreases in seed vigor. These authors observed the carbonylation of isoforms of LEA proteins that are associated with the *in vivo* binding of the vitamin biotin (Job et al. 2001) – a fundamental molecular cofactor of housekeeping enzymes involved in carboxylation, decarboxylation, and transcarboxylation reactions (Rajjou et al. 2008). For a review of molecular and biochemical features involved in seed germination vigor, see Rajjou et al. (2012). Although our comprehension of the factors affecting seed viability is very important, little is yet known about ROS functions during seed aging. The development of new methodologies and improvements in present tech-

niques of ROS evaluation in seeds, as proposed by Bailly et al. (2011), should allow a better understanding of this subject in the near future.

Conclusions

ROS are important molecules that play central roles in plant signaling, as for example in dormancy alleviation and seed germination. These molecules interact with seed hormones such as ABA and GA and influence ABA/GA signaling pathways, and also serve as environmental sensors that allow seeds to germinate (or not) in response to environmental conditions. Little is currently known, however, about ROS signaling pathways or their biological roles as harmful substances in seeds. Further experiments in this area will be required to furnish a comprehensive view of the ROS-mediated signaling cascades and their functions in seeds, especially in the events controlling germination and dormancy release.

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