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Commentary

Die or survive? - redox changes as seed viability markers

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Global climatic changes endanger the survival of many species, so the preservation of their seeds in gene banks and their propagation and re-establishment in natural habitats is a major task. The conservation of genetic diversity is also important for plant breeding, since the wild relatives of crops can be used as sources of genes affecting tolerance to biotic and abiotic stresses. Because of the importance of this genetic diversity, the seeds of landraces and old cultivars are also preserved in gene banks for breeding purposes. However, during the storage of seeds, their germination ability decreases as they undergo ageing (Shaban 2013), which limits our ability to maintain seeds indefinitely. This process of ageing depends on many factors, including the genetic background and the developmental and environmental conditions, as shown by Nagel *et al.* (2014) in this issue of *Plant, Cell & Environment*.

Among the environmental factors influencing the germination ability of seeds, the temperature, relative air humidity and oxygen concentration at which they are maintained are very important (Bailly 2004). Higher values of temperature, humidity and oxygen concentration promote the formation of reactive oxygen species (ROS) in seeds, leading to damage to vital macromolecules including proteins, lipids and nucleic acids. Besides these harmful effects, ROS also participate in redox signalling, ensuring normal seed development, dormancy and germination (Bailly 2004). In seeds, the mitochondria (through the respiratory electron transport chain) and peroxisomes (via glycolate oxidase and xanthine oxidase) are the main sources of ROS, but ROS may also be produced at other sites by NADPH oxidases and during the non-enzymatic autooxidation of lipids (Bailly 2004). The maintenance of an

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/pce.12515

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appropriate redox balance can decrease seed ageing and preserve longevity, a balance that is ensured by various antioxidants which remove excess ROS.

The role of ROS and antioxidants in seed ageing has been studied in various plant species by controlled deterioration treatments (Bailly 2004), and their involvement was demonstrated at the levels of transcripts, proteins and metabolites (Rajjou *et al.* 2008, Chen *et al.* 2013, Demirkaya 2013). Interestingly, transcriptome analysis of pea seeds subjected to ageing at 50°C indicated that the expression of genes related to oxidative stress was altered prior to any sign of viability loss (Chen *et al.* 2013). At the proteome level, the oxidation of proteins through carbonylation indicated the presence of ROS during ageing at 40°C in Arabidopsis seeds, which could be the result of a decrease in the amount of the antioxidant defence-related proteins (cysteine synthase, 2-alkenal reductase) (Rajjou *et al.* 2008). In pepper seeds aged at 60°C seed viability levels were negatively correlated with mean germination time, lipid peroxidation and electrolyte leakage, and positively associated with antioxidant enzyme activities (e.g. superoxide dismutase, catalase, peroxidase) (Demirkaya 2013). Similarly, high temperature treatment (40°C) of soybean seeds also resulted in greater membrane damage (Xin *et al.* 2014), which was accompanied by poor development of the mitochondria, as well as decreased antioxidant enzyme activities (superoxide dismutase, and enzymes of the ascorbate-glutathione cycle), total ascorbate and glutathione contents, and ratios of the reduced and oxidised forms of these compounds in the mitochondria. Similar changes were also observed in tomato seeds aged at high humidity (20°C/75% RH and 30°C/45% RH) (De Vos *et al.* 1994). These investigations indicate that the reduction of antioxidant capacity has a significant contribution to the loss of seed viability during ageing.

Most studies on the ageing of seeds have only measured antioxidant concentrations in order to predict changes in their germination ability. However, the characterization of the redox environment by determining the half-cell reduction potential of the redox couples and measuring their reducing capacity (i.e the concentration of the reduced form) is much more appropriate for this purpose (Schafer & Buettner 2001). The half-cell reduction potential can be calculated using the Nernst-equation from the standard reduction potential of a redox couple (measured under standard conditions: 1 molal solution, 10^5 Pa, 298 K, pH=0), which should be corrected depending on the actual non-standard environmental conditions, concentrations of the reduced and oxidised forms of a redox pair, and the stoichiometry of the redox reaction (e.g. glutathione disulphide (GSSG) is formed from 2 glutathione (GSH) molecules). There are many redox couples in living cells which together maintain the redox environment. Among them, the GSH/GSSG couple is very important, and the half-cell

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reduction potential of the GSSG/GSH couple ($E_{GSSG/2GSH}$) was found to correlate with the biological status of the cell: about -240 mV is linked to cell proliferation, about -200 mV is correlated with cellular differentiation, and about -170 mV indicates apoptosis in human and animal cell lines (Schafer & Buettner 2001). A similar model was also described for seeds using four plant species (Kranner *et al.* 2006). The reducing capacity of glutathione depends on the concentration of the reduced form, and determines how effectively GSH can contribute to the buffering of the cellular redox environment. For example, at higher GSH concentrations, this reducing capacity is greater, which means the $E_{GSSG/2GSH}$ value is more negative at the same percent of GSSG. It should be mentioned that the binding of GSH to proteins (i.e. glutathionylation) has a great influence on the reducing capacity of GSH. On the other hand, (de)glutathionylation of proteins (or GSH-dependent thiol/disulphide conversion of their cysteine residues) determines protein structure and activity, a process controlled by the cellular redox state. In addition, compartment-specific differences in the redox state occur in the cells of seeds, such that transfer of proteins from one cell organelle to another may lead to protein (de)activation (Bailly 2004; Kocsy *et al.* 2013).

Changes in both the concentration of GSH and in the $E_{GSSG/2GSH}$ value have been previously described as a universal stress marker in plant seeds (Kranner *et al.* 2006). As a further development of this approach, the mathematically combined half-cell reduction potentials of the low-molecular-weight thiols were also found to be appropriate for monitoring seed ageing in *Lathyrus vernus* (Birtić *et al.* 2011). All these parameters, which are appropriate for the characterization of the redox environment, were also used by Nagel *et al.* (2014) for the monitoring of seed viability in barley.

Although several genetic, biochemical and physiological parameters were found in previous studies to be associated with seed ageing and longevity, in most experiments only one or a few genotypes were examined, which does not allow general conclusions to be drawn on the role of these parameters in seed viability. In contrast, Nagel *et al.* (2014) investigated the seeds of 175 barley genotypes from four continents, an experimental system appropriate for determining broad relationships. The phylogenetic analysis of these genotypes indicated an overlap with the population structure and origin of the genotypes, the breeding status (landrace or cultivar), and the row characteristics of the spikes. It also demonstrated the great variability of the barley gene pool examined. The effect of long-term storage (35 years) was investigated using 50 barley genotypes from four continents, and the percentage of normal seedlings (typical young seedlings consisting of a radicle (embryonic root), hypocotyl (embryonic shoot), and cotyledons (seed leaves), and developing like seedlings from non-

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aged seeds without any retardation in growth or visible damage) varied between 91% and 46%, indicating the contribution of genotypic effect to seed ageing and longevity. Besides the effect of genetic background on germination ability, the influence of the conditions under which the maternal plants developed was also studied by comparing plants grown in two fields (152 and 160 genotypes) with different nutrient supplies. A comparison of two artificial ageing methods revealed that controlled deterioration at 45°C and 60% RH for 15 days was less destructive to seed viability than accelerated ageing at 43°C and 100 % RH for 3 days. As well, lower nutrient supplies to the maternal plant resulted in a smaller percentage of total germination and normal seedlings, an effect which was increased by artificial ageing. This observation indicates that the effects of development and environment on ageing are additive.

The authors then determined the chromosomal loci that respond to environmental effects during seed development and to ageing conditions using genome-wide association mapping on 160 genotypes. For the percentage of normal seedlings, 63 marker-trait associations were found at 55 loci, while 44 associations were detected at 36 loci for the percentage of total germination, of which 28 were identical between the two groups. The analysis of markers related to seed development and ageing indicated a connection with abiotic and biotic stress responses. This may be a redox change-dependent relationship, since redox regulation has a central role during both development and stress responses in various organs of plants (Bailly 2004; Considine & Foyer 2014; Kocsy *et al.* 2013).

Besides their mapping work, Nagel *et al.* (2014) also investigated the possible link between various biochemical markers and seed ageing and longevity. There was no correlation between seed deterioration and the concentration of storage compounds (oil, starch and proteins) or lipid-soluble antioxidants (tocochromanols). However, changes in the amounts and redox states of the water-soluble antioxidant GSH and related low-molecular-weight thiols were found to be related to seed viability and longevity. During the controlled deterioration of barley at 13% and 18% moisture content at 44°C for 41 and 7 days, respectively, the amount of total glutathione and the percentage of total germination gradually decreased in all six genotypes examined. The $E_{GSSG}/_{2GSH}$ value increased over time, and in most cases reached a range of between -180 and -160 mV, values where a loss of seed viability is known to occur (Kranner *et al.* 2006). Similarly, after storage for 7-14 years at ambient temperature, a great loss in viability was accompanied by an increase in the $E_{GSSG}/_{2GSH}$ value to a similar range, a change that was not observed after cold storage. This indicates that ageing induced by long-term storage at ambient temperature or by controlled deterioration is associated with a shift in the $E_{GSSG}/_{2GSH}$ value to more positive ones. As with

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the GSH/GSSG couple, the half-cell reduction potentials of the other three low-molecular-weight thiol/thiol disulphide couples studied exhibited an inverse relationship with seed viability. A similar relationship was found for the mathematically combined half-cell reduction potential of all four investigated thiols, which gives an even better prediction of the cellular redox state than the $E_{GSSG}/_{2GSH}$ value relating only to the GSH/GSSG couple.

For the characterization of the redox environment, the $E_{GSSG}/_{2GSH}$ value is important, but the concentration of GSH should be also presented since the reducing capacity of GSH can be characterised by this value. The concentration of GSH showed a close correlation with germination ability in barley (Nagel *et al.* 2014), and a similar relationship was found for the percentage of GSSG. Besides the GSH and GSSG concentrations, the $E_{GSSG}/_{2GSH}$ value also depends on the pH of the seed cells, which is taken into account in the Nernst equation. Nagel et al. (2014) found indirect evidence for the acidification of the cytoplasm during artificial ageing of seeds. If the $E_{GSSG/2GSH}$ value was calculated using a lower pH of 7 and 6.8 instead of the average value of 7.3 taken from the literature (Kranner *et al.* 2006), there was a horizontal shift from negative to positive $E_{GSSG/2GSH}$ values. The sigmoidal curve obtained by plotting the $E_{GSSG}/_{2GSH}$ value against the percentage of total germination resulted in a similar shift during the loss of seed viability, which led to the conclusion of a decrease in seed pH (Kranner *et al.* 2006; Nagel *et al.* 2014).

Based on previous results on the control of growth and development in whole plants (Considine and Foyer 2014; Kocsy *et al.* 2013) and on the viability and germination in seeds (Bailly *et al.* 2004), it can be assumed that a network of interactions between ROS, antioxidants and hormones is involved in the mediation of the combined effect of genetic background, and developmental and environmental conditions on seed ageing and longevity. While the role of hormones during seed germination has been extensively studied, demonstrating that abscisic acid has a negative and gibberellins a positive effect on germination ability (Miransari & Smith 2014), work on their influence on seed ageing and longevity has only just started. Both the overexpression of a gene affecting gibberellin synthesis and treatment with gibberellin increased seed longevity in Arabidopsis by inducing mucilage formation on the seed surface, a mechanism which was independent of abscisic acid (Bueso *et al.* 2014). Combining this observation with the results of Nagel *et al.* (2014) a model for the interaction of redox and hormonal control during seed storage can be proposed (Fig. 1). The genetic background-, development- and environment-dependent interactions between ROS, antioxidants and hormones affect the redox state of the seeds which can be characterised by the $E_{GSSG/2GSH}$ value. A shift to more oxidising conditions could be

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advantageous for seed longevity up to a certain level, as it may ensure reduced metabolism and increased activation of protective mechanisms through redox-signalling events that control the activity of redox-responsive genes and proteins which contribute to the preservation of seed viability. However, after a critical range is achieved, a further oxidation of the cellular redox environment could lead to the reduction in germination that occurs during long storage or artificial ageing. According to earlier investigations (Birtic *et al.* 2011; Kranner *et al.* 2006) and the present study by Nagel *et al.* (2014), changes in the $E_{GSSG/2GSH}$ value may be a good indicator of seed viability. In general, a small increase in $E_{GSSG/2GSH}$ up to -180 mV may indicate the adaptation of seeds to the storage conditions, values between -160 mV and -180 mV show a loss in viability, while values higher than -160 mV indicate that the seeds have died. Based on the present model, the study of plant hormones and their interaction with the redox system in the control of seed ageing and longevity in barley could be an interesting continuation of the work of Nagel *et al.* (2014).

The preparation of this commentary was supported by the Hungarian Scientific Research Fund (OTKA K83642).

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Fig. 1. Schematic model of the mediation of genetic, developmental and environmental effects through the interaction of hormonal and redox regulation on seed viability. The genetic background, developmental conditions during the growth of the maternal plant and

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environmental parameters during seed storage all affect levels of hormones, reactive oxygen species (ROS) and antioxidants. Their interaction leads to the modification of the redox state, which is indicated by changes in the half-cell reduction of the glutathione disulphide/glutathione couple ($E_{GSSG}/_{2GSH}$). A small increase in the $E_{GSSG}/_{2GSH}$ value up to -180 mV may indicate the adaptation of the seeds to storage conditions, values between -160 mV and -180 mV show a loss in viability, while values greater than -160 mV indicate that the seeds have died.

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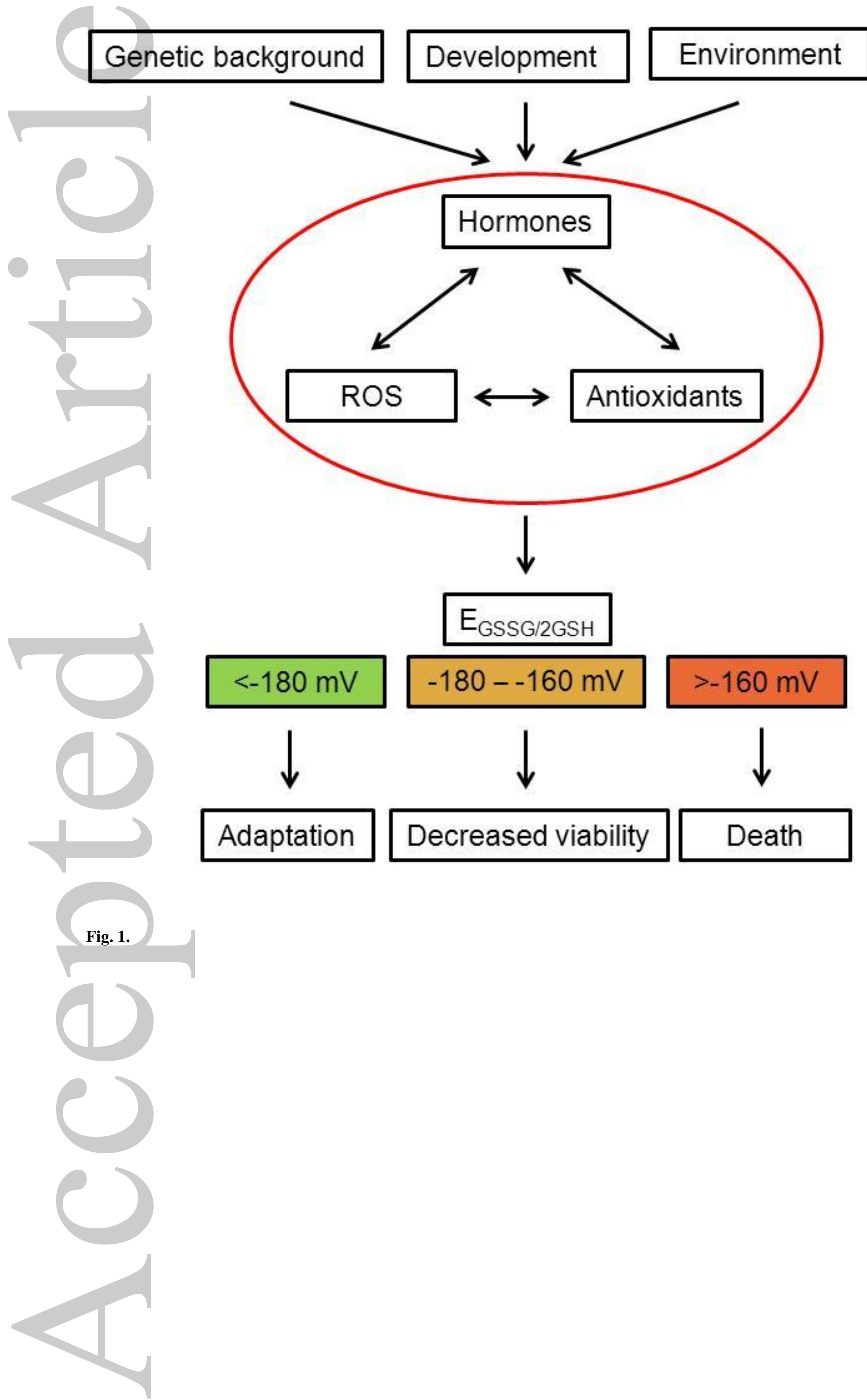

Fig. 1.

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