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Review

Tansley review

What is stress? Concepts, definitions and applications in seed science

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Summary

'Stresses' that impact upon seeds can affect plant reproduction and productivity, and, hence, agriculture and biodiversity. In the absence of a clear definition of plant stress, we relate concepts from physics, medicine and psychology to stresses that are specific to seeds. Potential 'eustresses' that enhance function and 'distresses' that have harmful effects are considered in relation to the seed life cycle. Taking a triphasic biomedical stress concept published in 1936, the 'General Adaptation Syndrome', to the molecular level, the 'alarm' response is defined by post-translational modifications and stress signalling through cross-talk between reactive oxygen and nitrogen species, and seed hormones, that result in modifications to the transcriptome. Protection, repair, acclimation and adaptation are viewed as the 'building blocks' of the 'resistance' response, which, in seeds, are the basis for their longevity over centuries. When protection and repair mechanisms eventually fail, depending on dose and time of exposure to stress, cell death and, ultimately, seed death are the result, corresponding to 'exhaustion'. This proposed seed stress concept may have wider applicability to plants in general.

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'All Ding' sind Gift, und nichts ohn' Gift; allein die Dosis macht, daß ein Ding kein Gift ist':
All things are poison and nothing is without poison, only the dose permits something not to be poisonous'

Paracelsus Philippus Aureolus Theophrastus Bombastus von Hohenheim (1493–1541)

I. Definitions of stress

'Stress' or 'pressure' was introduced into the theory of elasticity as an amount of force for a given unit area (Cauchy, 1821). When sufficient force is applied to material, the material bends and the change in length is termed 'strain'. With increasing stress, the initially linear relationship between stress and strain becomes nonlinear until the proportionality limit, after which the material deforms elastically (it can bend back), then plastically (it cannot bend back) until it ruptures (Fig. 1a). Since the 1930s, biologists have attempted to apply this terminology to biological systems, albeit the nature of the stresses will vary between nonliving materials and organisms (Levitt, 1972). Compared with mechanics, the stress–strain terminology becomes confused, because an initial stress typically leads to a chain of strains, but these are often referred to as stresses. Fig. 1(c) gives an example of the intricately linked responses of a plant to water deprivation, where the low soil water potential is viewed as the initial stress. All further effects would be strains according to the terminology in mechanics. Strains can lead to damage, but, unlike in nonliving materials, they can also provoke responses of the plant to prevent or repair damage. By analogy with mechanics, an 'elastic response' would involve reversible damage that can be repaired, so that function and viability are maintained, whereas a 'plastic response' may comprise irreversible damage as a result of the failure of repair mechanisms, reaching the ultimate breaking point with plant death.

A commonly accepted stress concept in the biomedical sciences is the 'General Adaptation Syndrome' (GAS) of the endocrinologist Hans Selye (1936). The GAS comprises three phases (Fig. 1b). When a threat or stressor is identified or realized, the body is in a state of 'alarm': for example, mammals produce adrenaline. If the stress persists, the organism enters into the 'resistance' phase where it attempts to cope using mechanisms of stress protection and defence. In the 'exhaustion' phase, the organism's resources are eventually depleted and the organism is unable to maintain normal function. The initial autonomic nervous system symptoms, such as sweating and raised heart rate, may reappear. Long-term damage may occur as the capacity of the glands and the immune system are exhausted and can manifest itself in illnesses. Selye also distinguished two types of stress, 'eustress' and 'distress', and these were later introduced into psychology (Lazarus, 1966). Eustresses enhance function, for example through training or challenging work, whereas distresses refer to persistent stresses that are not resolved through coping or adaptation and may lead to illnesses, for example escape (anxiety) or withdrawal (depression) behaviour.

Plant stress has been defined by Lichtenthaler (1996) as 'any unfavourable condition or substance that affects or blocks a plant's metabolism, growth or development', by Strasser as 'a condition caused by factors that tend to alter an equilibrium', and by Larcher as 'changes in physiology that occur when species are exposed to extraordinary unfavourable conditions that need not represent a threat to life but will induce an alarm response' (reviewed in Gaspar *et al.*, 2002). Equivalent to 'stress' and 'strain' in mechanics, plant scientists often use 'stress factor' and 'stress'. Irrespective of terminology, stress factors (or stresses) coming from outside need to be distinguished from stresses (or strains) within an organism. We shall distinguish external stress factors from internal stresses whenever possible, except for commonly used jargon; for example, we use 'stress response' rather than 'stress factor response'. Factors that induce stress can be 'biotic', resulting from living organisms, such as fungi and insects, or 'abiotic', resulting from nonliving factors, such as drought, extreme temperatures, salinity and pollutants, for example heavy metals. The balance between tolerance and sensitivity may determine whether a stress factor has a positive (eustress) or negative (distress) effect. For example, water deficit causes distress for vegetative tissues of vascular plants (except for resurrection plants) and is lethal below the permanent wilting point, whereas water deficit above the permanent wilting point or for short periods of time may induce hardening (Table 1). In addition, short-term and long-term (persisting) stresses need to be distinguished, as well as 'low stress events' that can be partially compensated for by acclimation, adaptation and repair, and strong or chronic stress events that cause considerable damage and may lead to cell and plant death (Gordon, 1992; Lichtenthaler, 1996). Hence, a plant's response to stress will vary with increasing duration and severity of stress.

Despite the long-standing interest of plant scientists in stress concepts, surprisingly little attention has been given to seeds. A seed contains a new miniature plant in the form of the embryo (Fig. 2) which, on germination, produces the next plant generation (Bewley, 1997). As a result of their essential role in plant reproduction, one would intuitively expect that plants have evolved mechanisms that protect their seeds from stress. Indeed, in the dry, quiescent state, protected by their seed coat, many seeds are exceptionally tolerant of stress factors, such as temperature extremes, that are lethal to adult plants (Table 1). By contrast, seeds may be highly vulnerable to stresses at other developmental stages (Fig. 3), such as during seed development on the mother plant (e.g. drought), or during germination (e.g.

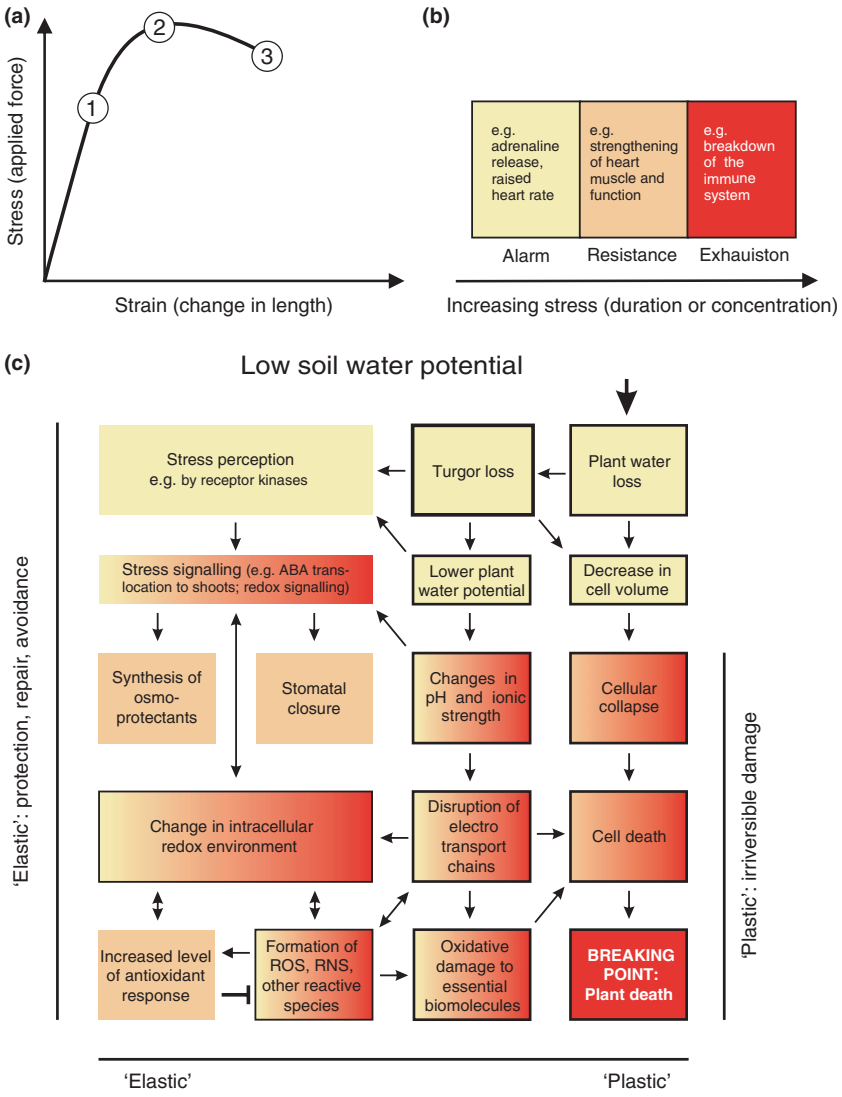

Fig. 1 Can stress concepts from physics and medicine be applied to plants? (a) Simplified scheme of material stress following the law $\sigma = F/A$, where σ is 'stress' and F is the force acting over an area A. The change in length in response to the applied pressure is termed 'strain'. Plotting stress against strain shows an initial linear relationship in which the slope is equivalent to the modulus of elasticity, until the proportionality limit (1), and thereafter the relationship is nonlinear. When the elastic limit (2) is exceeded, the material deforms plastically until the rupture point (3) is reached. (b) Selye's 'General Adaptation Syndrome' defines human stress for medical purposes. Three phases of stress response include alarm (yellow), resistance (orange) and exhaustion (red); see text for details. (c) In biological systems, the term 'stress' is often used to describe what would correspond to a 'strain' according to the definition used in materials science. The flow chart is an extremely simplified example of the intricately linked effects of a 'stress', water deprivation, to give examples of strains (bold lines around boxes) that evoke responses of the plant (no lines) and intermediate processes that have elements of strain and response (thin lines). The responses of the plant can feed back downstream and upstream into the system, leading to resistance based on protection and repair. The individual processes are also assigned the colours yellow, orange and red according to 'alarm', 'resistance' and 'exhaustion'. Two or three colours within one box indicate that the process corresponds to more than one of the phases in Selye's stress concept.

pathogen attack). These variations in stress tolerance that coincide with developmental switches make seeds very attractive models to study stress. In this article, we review the current literature on stress in seeds and consider the above concepts where appropriate, proposing a novel stress concept for seeds based on the GAS.

II. The seed life cycle revisited in view of the eustress–distress concept

1. Seed maturation

Seed morphology (Fig. 2) and physiology vary greatly between taxa. However, seeds of different species may encounter common eustresses and distresses during their life cycles (Fig. 3). In 'orthodox' (i.e. desiccation-tolerant) seeds (Roberts, 1973), produced by the majority of higher plants, desiccation during maturation is the first severe stress experienced. However, maturation drying induces a set of protection mechanisms that prepare the seed for survival in the dry state. These include osmoprotectants, carbohydrates and proteins [Late Embryogenesis Abundant (LEA) proteins and Heat Shock Proteins (HSP)] that are conducive to the formation of an intracellular glass, and antioxidants (Hoekstra *et al.*, 2001; Buitink & Leprince, 2004). Hence, maturation drying has the characteristics of a eustress, resulting in a dry, quiescent seed that can survive adverse conditions.

'Recalcitrant' seeds, mostly produced by trees, do not undergo maturation drying and are desiccation sensitive (Berjak & Pammenter, 2008). They are shed at high seed water content (WC) and remain metabolically active until they germinate. Recalcitrant seeds form soil seedling banks rather than seed banks, representing a different ecological strategy. Their high WC makes them intolerant of freezing temperatures, and they lose viability below a critical WC.

Table 1 Examples of potential abiotic stress factors and their effects on whole plants and orthodox seeds, classified according to the eustress–distress concept

	Effect on whole plants		Effect on orthodox seeds	
Stress factor	Distress	Eustress	Distress	Eustress
Water deficit	Lethal below the permanent wilting point (Hsiao, 1973)	Above the permanent wilting point may induce hardening, for example in *Zea mays* leaves (Chazen & Neumann, 1994)	Stressful in the final phases of germination, for example impairment of protein synthesis and axis elongation in *Phaseolus vulgaris* seeds (Dasgupta *et al.*, 1982)	Induces protection mechanisms during maturation drying (Hoekstra *et al.*, 2001)
Temperature	Extreme temperature may be lethal, for example heat stress in *Triticum aestivum* resulted in leaf senescence (Harding *et al.*, 1990)	May induce hardening, for example acclimation of *Spinacea oleracea* to cold stress (Somersalo & Krause, 1989)	Temperature extremes may be lethal after imbibition in *Brassica napus* seeds (Gusta *et al.*, 2006)	Extreme cold/heat may alleviate or induce dormancy (Finch-Savage & Leubner-Metzger, 2006)
Fire	Lethal to most vegetative tissues of nonpyrophytes (Tyler, 1996)	Competitive advantage for pyrophytes due to removal of competitors, for example in the Chaparral (Tyler, 1996)	Lethal to seeds unless protected within the soil, for example seeds of *Acacia* and *Grevilla* (Auld & Denham, 2006)	Smoke may be required to break dormancy, for example in *Hibbertia* (Dixon *et al.*, 1995)
Nutrients	Imbalances may cause malfunction/ malformation, for example iron deficiency leading to chlorosis in rice (Jolley *et al.*, 1996)	Deficit may stimulate root growth, for example lateral root proliferation in Arabidopsis in nitrate-rich patches (Zhang & Forde, 1998)	Deficiency or excess may cause malfunction, for example excess copper inhibits germination of rice seeds (Ahsan *et al.*, 2007a)	High concentrations of certain nutrients may break dormancy, for example NO_3 breaks dormancy in *Sisymbrium officinale* (Hilhorst, 1990)
Wind	May cause mechanical damage and excessive transpiration (Ancelin *et al.*, 2004)	May reinforce supporting vasculature, for example in Arabidopsis (Antosiewicz *et al.*, 1997)	Potential mechanical stress	May be essential to seed dispersal, for example in *Tragopogon dubius* (Greene & Johnson, 1989)
Contamination, for example by nonessential heavy metals	Toxic to nontolerant plants, for example can result in sterility in rice contaminated by arsenic (Wells & Gilmour, 1977)	Competitive advantage for heavy metal-tolerant plants and hyperaccumulators with specific adaptations, for example in the arsenic hyperaccumulator *Pteris vittata* (Zhao *et al.*, 2002)	High concentrations are toxic to seeds, for example Cd is toxic to rice seeds, reducing viability (Ahsan *et al.*, 2007b)	At low concentrations, some heavy metals may enhance germination or induce dormancy (citations in Kranner & Colville, 2010)

Therefore, desiccation and freezing clearly cause distress. In addition, severe stresses on the mother plant will generally cause distress for both orthodox and recalcitrant seeds (Table 1; Fig. 3). Stresses at early stages of seed development can even result in seed abortion (Cheikh & Jones, 1994).

2. Dormancy

Dormancy is a key trait of many seeds that allows persistence in soil seed banks for extended periods of time, after which germination is completed only when environmental conditions are favourable for the establishment of a new plant generation (Finch-Savage & Leubner-Metzger, 2006), that is when the impact of environmental stresses is minimal. Following this line of reasoning, dormancy could be seen as part of a genetically programmed 'resistance phase' according to Selye's concept (see Section V). 'Primary dormancy' is induced during seed maturation and depends on the balance between abscisic acid (ABA), promoting dormancy, and gibberellic acid (GA), promoting germination. Primary dormancy is released during dry after-ripening or by dormancy-breaking environmental cues in an imbibed state. Secondary dormancy is a reversible state that some seeds with nondeep physiological dormancy cycle in and out of, depending on environmental conditions and, again,

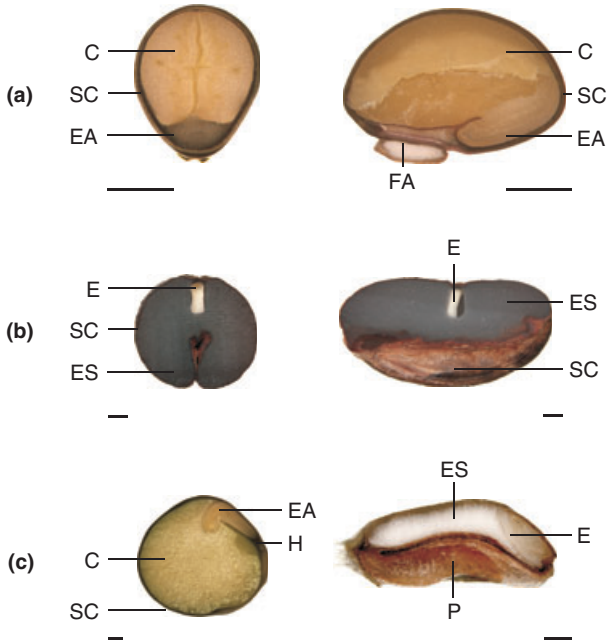

Fig. 2 Examples of the variation in seed anatomy across taxa. A typical seed consists of the embryo, the endosperm, one (monocotyledons) or two cotyledons (dicotyledons), or seed leaves, and the seed coat. (a) Cross- and longitudinal sections, respectively, of a *Tephrosia cordata* seed (Leguminosae). (b) Cross- and longitudinal sections, respectively, of a *Phoenix dactylifera* seed (palm; Arecaceae); (c) Longitudinal sections of a *Pisum sativum* seed (garden pea; Leguminosae; left image) and a *Triticum aestivum* seed (wheat; Poaceae; right image). Nutrients for the embryo can be stored in the endosperm, which is the triploid product of double fertilization and can be rich in starch, oil and protein; in other cases, however, the endosperm is absorbed by the embryo during seed development and the cotyledons develop into storage tissues. The seed coat, or testa, develops from the integument(s) that surround(s) the nucellus, and can vary considerably in texture and thickness from very thick, as in a coconut, to papery, as in a garden pea. Correspondingly, the seed coat can form an unyielding barrier or be not much of a barrier at all, depending on the species. C, cotyledon; E, embryo; EA, embryonic axis; ES, endosperm; FA, funicular aril; H, hilum; P, pericarp; SC, seed coat. Scale bars represent 1 mm. Images: Dr Wolfgang Stuppy, Hannelore Morales and Elly Vaes; copyright Royal Botanic Gardens, Kew, UK.

on ABA concentration (Finch-Savage & Leubner-Metzger, 2006). Most dormancy-breaking cues impose stress, such as chilling, heat shock, passage through the visceral organs of fruit eaters or fire, which would cause distress on the whole-plant level (Table 1), but eustress for seeds as they alleviate dormancy.

3. Persistence in soil seed banks

In soil seed banks, seeds are subjected to biotic and abiotic stress factors, including pathogens, temperature extremes (freezing or heat), salinity and heavy metals (Kranner & Colville, 2010), which may induce both eustress and distress (Table 1). For example, seeds are exposed to hydration–

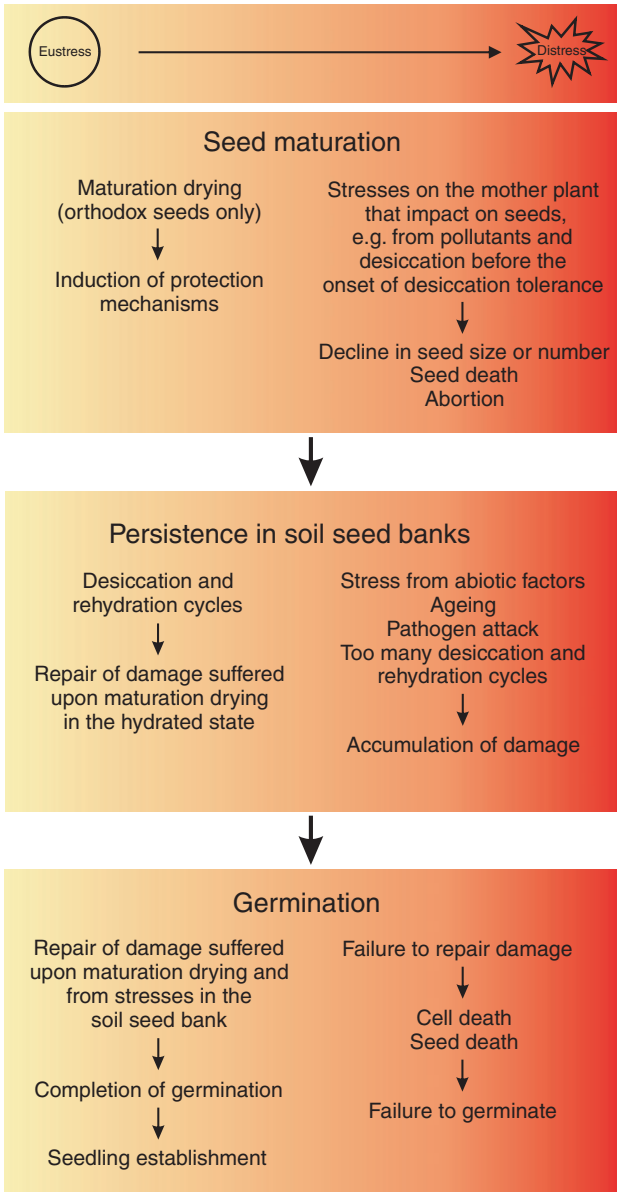

Fig. 3 Stresses that accompany the seed life cycle, viewed through the eustress–distress concept. Except for the distresses that affect orthodox seeds through their effects on the mother plant, seed maturation and dormancy appear to be commonly accompanied by eustresses that prepare the seed for survival based on the protection mechanisms induced in response to environmental cues. Persistence in the soil seed bank is accompanied by distresses and eustresses. Germination and the first stages of seedling establishment are amongst the most vulnerable stages of plant development, and distresses generally include all biotic and abiotic factors experienced by mature plants. If seeds and seedlings acclimate to these stresses, a distress may become a eustress again. Hence, whether a stress factor causes eustress or distress depends on the specific circumstances, and has genetic components, indicative of adaptation. This scheme refers to orthodox seeds. Recalcitrant seeds do not undergo maturation drying. Hence, the eustresses on maturation are not relevant and recalcitrant seeds are only subjected to the abiotic and biotic stress factors in the middle box until they germinate.

rehydration cycles as the soil WC changes (except for seeds with water-impermeable coats, e.g. many wild legumes). Rehydration contains elements of eustress, as it allows repair processes to be activated, for example, repair of damaged DNA, proteins, membranes and mitochondria via stored mRNAs (see Section V). Several hydration–rehydration cycles can impose eustress, improving seed vigour (i.e. the mean time to germination; Dubrovsky, 1996), but, with increasing number of cycles, can cause distress, decreasing seed viability (Berrie & Drennan, 1971). Longer-term submergence in water during flooding will also cause distress, limiting oxygen availability to the seed and causing hypoxia and anoxia (Borisjuk & Rolletschek, 2009), and may decrease germinability (Ismail *et al.*, 2008). However, seeds of species from frequently flooded habitats, such as tidal salt marshes, have adapted to tolerate hypoxia, for example those of the halophyte *Suaeda maritima* (Wetson *et al.*, 2008). Additional distress factors that accompany flooding include the dispersal of water-borne pathogens, such as *Pythium phragmitis* (Nechwatal & Mendgen, 2005). Ultimately, distresses that seeds experience in soil seed banks, or during storage for human use, will induce ageing (see Section VI) that will become evident when vigour and germinability are compromised.

4. Germination

Germination of orthodox seeds starts with the uptake of water and is completed when the radicle protrudes and cell division has started (Bewley, 1997). Rapid imbibition can induce stress during the transition of membranes from a rigid gel phase to a liquid crystalline phase, resulting in solute leakage. On full rehydration, leakage ceases without apparent damage (Hoekstra *et al.*, 1999), except in sensitive species or aged seeds where rapid water uptake causes imbibitional damage (Hoekstra *et al.*, 1999; Neya *et al.*, 2004), a distress. Moreover, if the damage accumulated between seed maturation and germination is too great, repair processes may be impaired (Bray & Dasgupta, 1976; Sen & Osborne, 1977; Elder *et al.*, 1987), resulting in loss of vigour and viability (see Section VI). Once a seed is committed to forming a seedling and desiccation tolerance is lost, it becomes vulnerable to desiccation (Bewley, 1997) and freezing (Gusta *et al.*, 2006), which now cause distress.

III. Common denominators of many stresses: reactive oxygen and nitrogen species

1. The multifaceted roles of reactive oxygen and nitrogen species in exerting distress, eustress or no stress

All abiotic and biotic stresses that impair photosynthetic and respiratory electron transport increase the production of reactive oxygen species (ROS) (Halliwell, 2006; De Gara *et al.*, 2010; Table 2). In seeds, photosynthetic activity declines with progressing maturation (El-Maarouf-Bouteau & Bailly, 2008), so that the probability of plastidial ROS formation decreases, and respiration will be a major source of ROS production in all phases of the seed life cycle until limited by seed WC (see Section IV). Excess ROS can induce oxidation and depolymerization of nucleic acids, breakage of peptide bonds, oxidation of carbonyl, thiol (SH, or sulphydryl) groups and Fe–S clusters in proteins, as well as oxidation of membrane lipids, polysaccharides and polyunsaturated fatty acids (PUFAs), leading to loss of cell function, cell death and, ultimately, seed death (see Sections V and VI). Reactive nitrogen species (RNS) can also cause distress by damaging cellular structures and, together with ROS, cause 'nitrosative' stress (Table 2). Excessive ROS formation can be partly prevented via controlled uncoupling of electron flow from phosphorylation in mitochondria via the alternative oxidase and uncoupling proteins (Jarmuszkiewicz, 2001; Borecký & Vercesi, 2005), and by the dissipation of excess light energy as heat by carotenoids in the photosynthetic apparatus. ROS levels are controlled by ROS-processing enzymes and low-molecular-weight antioxidants (details in Supporting Information, Table S1), which very likely work together (Foyer & Noctor, 2005).

Reactive oxygen species and RNS are also key components of signalling networks, through which they regulate developmental processes, causing eustress, or no stress at all (Table 2). Germinating seeds produce ROS in the apoplast, where they are involved in cell wall loosening, regulation of growth and development, and pathogen defence. For example, apoplastic hydroxyl radicals ($^{\cdot}OH$) in cress radicles and endosperm caps break dormancy by *in vivo* scission of cell wall polysaccharides at specific sites, acting via ABA or protein carbonylation (Müller *et al.*, 2009a,b). In pea seeds, elevated production of extracellular superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) during germination has been suggested to defend the emerging seedling against pathogens and, together with the roles of ROS in growth and development, may contribute to successful seedling establishment (Schopfer *et al.*, 2001; Kranner *et al.*, 2010c). Similarly, certain types of oxidative modifications, for example protein carbonylation, are implicated in seed ageing, causing distress (Rajjou *et al.*, 2008), but also in redox signalling required for germination, exerting eustress. In sunflower seeds, protein carbonylation correlates with dormancy alleviation (Oracz *et al.*, 2007); Arabidopsis lines deficient in NADPH oxidase (a membrane-bound complex involved in $O_2^{\cdot-}$ production) display reduced protein carbonylation and fail to after-ripen (Müller *et al.*, 2009a); and carbonylation of reserve proteins increases their susceptibility to proteolytic cleavage, enabling their mobilization during germination (Job *et al.*, 2005). Hence, stress tolerance involves keeping ROS and RNS at safe levels, whilst allowing signalling to

Table 2 Examples of reactive oxygen species (ROS) and reactive nitrogen species (RNS) production, potential damage caused and seed-relevant roles in signalling

Reactive species[1]	Typical reaction mechanism	Biological half-life (order of magnitude)	Potential damage	Role in signalling in seeds	Seed-related references
Superoxide anion[2] $O_2^{\cdot-}$	$^3O_2 + \bar{e} \rightarrow O_2^{\cdot-}$	μs	Oxidation of protein Fe–S centres, protein SH groups Reduction of quinones and transition metal complexes of Fe^{3+} and Cu^{2+}, thus affecting the activity of metal-containing enzymes Reactions with unsaturated lipids	Dormancy breaking during seed after-ripening, wound response and germination	Müller *et al.* (2009a) Roach *et al.* (2010) Kranner *et al.* (2010c)
Hydroxyl radical[3] $^{\cdot}OH$	$H_2O_2 + O_2^{\cdot-} \rightarrow {}^3O_2 + {}^{\cdot}OH + {}^{-}OH$ $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + {}^{-}OH$ (Fenton and Haber–Weiss reactions)	ns	Oxidation of proteins, DNA, lipids and carbohydrates	Endosperm loosening during seed germination	Müller *et al.* (2009b)
Hydrogen peroxide[4] H_2O_2	$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + {}^3O_2$	ms	Oxidation of protein SH groups	Growth, differentiation, pathogen response, wound response	El-Maarouf-Bouteau & Bailly (2008)
Singlet oxygen[5] $^1O_2$	Excited pigment + $^3O_2 \rightarrow {}^1O_2$ + pigment $^{\cdot}OH + O_2^{\cdot-} \rightarrow {}^1O_2 + {}^{-}OH$	μs	Oxidation of proteins, DNA, lipids	Early imbibition, wound response	Kranner & Birtic (2005) Chen *et al.* (2003)
Nitric oxide[6] (or nitrogen monoxide) $NO^{\cdot}$	$NO^{\cdot}$ reacts with a wide range of targets, including protein and nonprotein thiols, $O_2^{\cdot-}$ and Fe	s	*S*-nitrosylation of protein SH groups	Dormancy breaking Antioxidant effects, protecting membranes and lipoproteins from oxidation Control of Fe uptake and homeostasis Oxygen sensing required for maintenance of steady-state oxygen levels	Matakiadis *et al.* (2009) Liu *et al.* (2009) Beligni *et al.* (2002) Jasid *et al.* (2008) Borisjuk & Rolletschek (2009)
Peroxynitrite $ONOO^-$	$O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^-$	s	Oxidation of proteins, DNA, lipids Nitration of protein tyrosine residues Displacement of iron from Fe–S proteins		Alamillo & García-Olmedo (2001)

[1]ROS are unavoidable byproducts of aerobic metabolism, for example in electron transport chains. In respiration, ROS are produced by one-, two- or three-electron reductions of ground state oxygen (or triplet oxygen; $^3O_2$) that generally accompany the four-electron reduction of $^3O_2$ to H_2O during respiration. $O_2^{\cdot-}$, $^{\cdot}OH$, hydroperoxyl (protonated superoxide, $HO_2^{\cdot}$) and $NO^{\cdot}$ are free radicals, whereas H_2O_2, $^1O_2$, ozone (O_3) and O_2NOO^- are not free radicals but are more reactive than $^3O_2$.

[2]In mitochondria, $O_2^{\cdot-}$ is formed in complexes I and III of the electron transport chain and in complex II during reverse electron transport. Cytosolic enzymes (e.g. nitropropane dioxygenase; galactose oxidase; xanthine oxidase) can also transfer single electrons from a substrate onto $^3O_2$ to produce $O_2^{\cdot-}$. In the cell wall and plasma membrane, NAD(P)H oxidases [or respiratory burst oxidase homologues (Rboh; Müller *et al.*, 2009a], peroxidases and tyrosinases (Minibayeva *et al.*, 2009; Li *et al.*, 2010) can form extracellular $O_2^{\cdot-}$. Auto-oxidation of some reduced compounds (e.g. flavins; pteridines; diphenols; ferredoxin) can also transfer single electrons to $^3O_2$ to produce $O_2^{\cdot-}$.

[3]Hydroxyl radicals are the most reactive chemical species.

[4]In addition to $O_2^{\cdot-}$ dismutation, certain oxidases (e.g. glycolate oxidase; glucose oxidase; urate oxidase; oxalate oxidase; amino acid oxidases) can produce H_2O_2. During photorespiration, peroxisomes also generate H_2O_2 from the β-oxidation of fatty acids (Foyer & Noctor, 2005). Moreover, H_2O_2 is produced in the cell wall (Bolwell *et al.*, 2002).

[5]Singlet oxygen is mainly produced in the photosynthetic apparatus. Light energy trapped by chlorophyll molecules can be transferred to $^3O_2$, forming $^1O_2$, which plants counteract by regulating energy distribution between the light-harvesting complexes and by the use of certain carotenoids, which can quench both the triplet chlorophyll state and $^1O_2$. $^1O_2$ can attack polyunsaturated fatty acid side chains to form lipid peroxides, which can oxidize chloroplast molecules and trigger cell death (Wagner *et al.*, 2004).

[6]Production of $NO^{\cdot}$ increases in actively growing tissues, such as embryonic axes (Simontacchi *et al.*, 2004). $NO^{\cdot}$ is involved in the stress response, such as stomatal closure and plant–pathogen interactions (Wilson *et al.*, 2008). $NO^{\cdot}$ can reduce ROS toxicity and lipid peroxidation, acting as a chain-breaking antioxidant to scavenge peroxyl radicals (Kröncke *et al.*, 1997).

occur, or, as Halliwell (2006) stated, 'free radicals are not all bad, nor antioxidants all good', in agreement with the notion of Paracelsus that it is the dose that makes the poison.

2. Interaction between ROS, RNS and seed hormones

Interactions of ROS and RNS with seed hormones have been best investigated in relation to dormancy. Although excessive ROS production is deleterious, an 'oxidative window' may exist in which the generation of strictly regulated ROS concentrations is required for germination (Bailly *et al.*, 2008). H_2O_2 releases dormancy, partly by degrading endogenous inhibitors, such as ABA (Bailly, 2004), and through the activation of ERF1, a component of the ethylene signalling pathways (Oracz *et al.*, 2009). Dormancy alleviation in Arabidopsis also involves responses of aleurone cells to nitric oxide ($NO^{\cdot}$), GA and ABA, with $NO^{\cdot}$ upstream of GA in a signalling pathway (Bethke *et al.*, 2007) and $NO^{\cdot}$ participating in ABA catabolism, a requirement for dormancy breaking (Liu *et al.*, 2009). A recent model proposes that a heterodimeric protein complex exists that promotes germination by destabilizing DELLA proteins that block transcription. The abundance of one monomer is influenced by ABA and the other by GA (Penfield & King, 2009). This complex also regulates GA and ABA metabolism in seeds, creating the feedback necessary to balance dormancy and germination.

3. The intracellular redox environment

Under continuous stress, antioxidant recycling typically fails, resulting in increased ROS production and a shift in antioxidant redox state towards more oxidizing conditions (Schafer & Buettner, 2001). A shift in the glutathione (GSH) half-cell reduction potential ($E_{GSSG/2GSH}$) towards more positive values, viewed as representative of the 'intracellular redox environment' (i.e. the sum of all half-cell reduction potentials of all intracellular redox couples), occurs during the life cycle of human cells until a state of intracellular oxidation is reached at which a cell undergoes programmed cell death (PCD) (Schafer & Buettner, 2001). In agreement with this concept, $E_{GSSG/2GSH}$ increases towards more oxidizing values as seed lots lose viability (Kranner *et al.*, 2006). These changes in the intracellular redox environment generally impact on redox signalling with downstream effects, such as further disruption of electron transport chains, resulting in more ROS production and damage to macromolecules (Fig. 1c), as well as post-translational protein modification leading to changes in protein function (see Section IV).

IV. Alarm

Considerable progress in biochemistry and molecular biology has been made since the 1930s. Here, we take the GAS to the molecular level, considering stress signalling, post-translational and transcriptional modifications that enable and support a functional protection and repair machinery in the alarm and resistance phases, and the failure of protection and repair leading to cell death and, ultimately, seed death in the exhaustion phase (Fig. 4).

1. Stress perception, ROS production and signalling

All three phases of the GAS-based seed stress model (Fig. 4) will involve signalling, but stress perception and transduction are key when stress commences, that is in the alarm phase, and are discussed here. Stress can be perceived by membrane-bound receptor proteins, such as receptor kinases, and/or by perceiving the first effects of damage (Fig. 1c). The characterization of the stress receptor proteins in plants is only starting (Hirayama & Shinozaki, 2010); for example, a plasma membrane His Kinase ATHK1 has recently been identified that senses osmotic stress during maturation in Arabidopsis seeds. Plants could also perceive stress nonspecifically through changes in membrane potentials or osmotic pressure that affect ion fluxes and trigger post-translational modifications and ROS production (Xiong *et al.*, 2002). For example, plants could sense heat through changes in membrane fluidity (Wahid *et al.*, 2007). In Arabidopsis seeds, altered expression of 1-Cys peroxiredoxin may act as a sensor for unfavourable environmental conditions (Haslekås *et al.*, 2003).

The signalling network composed of ROS, RNS, antioxidants and hormones in hydrated seeds will largely resemble that in vegetative tissues, where the presence of free water allows molecular trafficking. The mechanisms of stress signalling in higher plants have been reviewed by others (Møller *et al.*, 2007; Baena-González & Sheen, 2008; Kudla *et al.*, 2010). We focus on seed-specific issues, such as on the mechanisms by which ROS are formed and travel, and how stress is sensed and transduced in desiccated seeds. Interestingly, seeds at WCs as low as 7% apparently perceive environmental cues, such as those required for dormancy breaking (Finch-Savage *et al.*, 2007), implying that signalling pathways are operative in desiccated seeds. In addition, transcription and translation have been reported in desiccated seeds (Chibani *et al.*, 2006). Furthermore, deterioration of desiccated seeds and seeds in the glassy state has been associated with oxidative damage (Bailly, 2004; El-Maarouf-Bouteau & Bailly, 2008), again arguing for the existence of ROS-producing processes at low WCs. Metabolic ROS formation in the glassy state is unlikely, although it cannot be excluded with certainty that metabolic reactions continue at very slow rates in highly viscous liquids, including glasses. In addition, it would be naive to assume that desiccated seeds are exempt from oxidative modification in the presence of atmospheric oxygen. ROS production will probably result from nonenzymatic

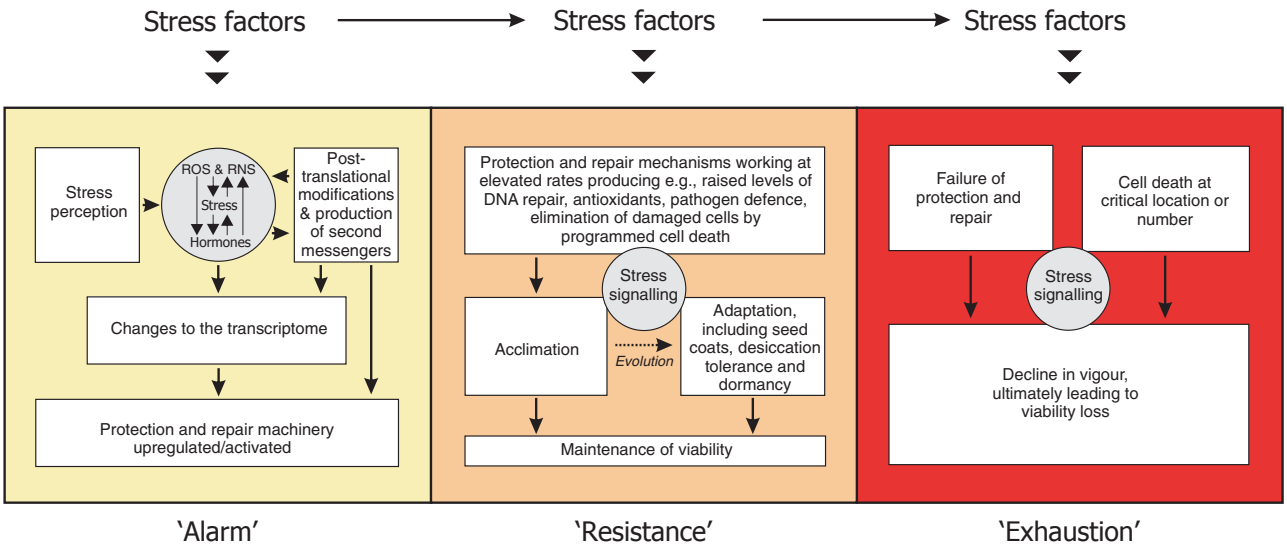

Fig. 4 Proposed seed stress concept. Taking the General Adaptation Syndrome to the molecular level, we propose a novel stress concept for seeds. We suggest that the alarm phase involves stress perception and transduction through the reactive oxygen species (ROS)–reactive nitrogen species (RNS)–hormone signalling network, post-translational modifications of macromolecules and alterations to the transcriptome so that the protection and repair machinery becomes activated and upregulated, respectively, in response to the perception of a stress and/or the initial damage caused. Under continuing stress (time or severity), the resistance phase is reached when sufficient gene products required for protection and repair are produced to maintain viability. Resistance includes inducible protection (e.g. upregulated antioxidants that protect macromolecules from further damage) and repair mechanisms (e.g. synthesis of DNA repair enzymes), the elimination of redundant cells that are damaged beyond repair (programmed cell death, PCD), characteristic of multicellular organisms, and pathogen defence. In seeds, desiccation tolerance, primary dormancy and the presence of a protective seed coat could be seen as constitutive protection mechanisms that enable long-term survival. The exhaustion phase is defined by the increasing failure of protection and repair mechanisms. For an individual cell, PCD and necrotic cell death will be the 'breaking point'. In multicellular organisms, PCD could be seen as part of the resistance phase, but, when cells die in critical numbers, or at a critical location (e.g. in the embryo), the ultimate breaking point is reached with seed death.

mechanisms, such as those of Amadori and Maillard reactions (Sun & Leopold, 1995) and lipid peroxidation, which even continue *post mortem* in archaeological material (Evershed *et al.*, 1997). Furthermore, proton mobility can vary within a desiccated seed as a result of the existence of hydrated pockets in which some metabolic activity may be possible (Leubner-Metzger, 2005). El-Maarouf-Bouteau & Bailly (2008) considered that ROS could be sensed and then involved in the regulation of cell signalling in the dry state, and stable free radicals may also accumulate during dry storage and be released on imbibition.

ROS and RNS signalling in higher plants includes the activation of the MAPK (mitogen-activated protein kinase) cascade, inhibition of phosphatases and activation of Ca^{2+} channels and Ca^{2+}-binding proteins (Møller *et al.*, 2007). Short-lived ROS, such as $^{\cdot}OH$, can react with receptor proteins close to their production site, whereas long-lived ROS, such as H_2O_2, can reach targets far from their production site (Møller *et al.*, 2007). Therefore, damage to macromolecules and ROS signalling in the dry state are more likely mediated by short-lived ROS, whereas both short- and long-lived ROS, together with $NO^{\cdot}$ stored as S-nitrosothiols (see Section V), will participate in signalling in the hydrated state.

2. Damage to macromolecules

In seeds, damage to macromolecular structures, such as lipid peroxidation, without viability loss has been reported during maturation, germination and ageing of orthodox seeds and desiccation of recalcitrant seeds (Hendry *et al.*, 1992; Chaitanya & Naithani, 1994; Pukacka & Ratajczak, 2007a). Therefore, a small number of modified molecules could be key elements of signalling cascades that induce repair and protection mechanisms. Damage to seed nucleic acids includes single-strand DNA breaks caused by direct ROS attack of deoxyribose units or by covalent modification of bases (Bray & West, 2005), changes in DNA content (Sen & Osborne, 1974, 1977) and DNA fragmentation (Osborne, 2000). Double-strand breaks result in a loss of genetic information if homologous recombination and nonhomologous end-joining repair pathways are not initiated (Bray & West, 2005; Waterworth *et al.*, 2007). Following the accumulation of DNA damage, plants activate ATM (ataxia telangiectasia mutated) and ATR (ATM- and RAD3-related) protein kinases. These kinases phosphorylate proteins, resulting in the activation of DNA stress checkpoints that control the recruitment of repair mechanisms and arrest or delay the cell cycle (Waterworth

et al., 2007; Culligan & Britt, 2008). The PUFAs of seed storage and membrane lipids are other prime targets for oxidative damage. Lipid peroxidation involves both non-enzymatic oxidation of PUFAs and their enzymatic oxidation by lipoxygenases (LOXs) and α-dioxygenases, resulting in lipid hydroperoxides, oxygenated fatty acids and ROS, propagating the chain reaction.

Proteins are further targets for oxidative modification. They scavenge an estimated 50–75% of reactive radicals and can retain a 'fingerprint' of an initial oxidative insult (Davies *et al.*, 1999). The thiol group of free Cys residues is particularly prone to oxidation, for example to sulphenic acid (P-SOH). Re-reduction of P-SOH is possible, but further oxidation to sulphinic ($P\text{-}SO_2H$) and/or sulphonic ($P\text{-}SO_3H$) acids is irreversible. Other irreversible protein modifications induced by ROS and RNS include di-Tyr formation, protein–protein cross-linking, and Lys and Arg carbonylation, and are associated with changes in the tertiary structure and permanent loss of function, which may lead to the degradation of the damaged proteins or their progressive accumulation (Colville & Kranner, 2010). The oxidative stress that accompanies the onset of many stresses, such as seed ageing and maturation drying, also changes the intracellular redox environment and, further downstream, impacts on the redox regulation of proteins with consequences for seed germination and ageing.

3. Post-translational protein modification

Reversible modification of protein thiols is involved in the regulation of protein function, and may also protect proteins from irreversible damage (Colville & Kranner, 2010). Reversible modifications participate in the regulation of protein function, in which Cys residues cycle between the oxidized and reduced state. Free Cys residues can form inter- or intramolecular disulphides, S-nitrosothiols and mixed disulphides with GSH, termed protein *S*-glutathionylation. The resulting mixed disulphides can be reversed by enzymatic systems, such as thioredoxin, glutaredoxin and protein disulphide isomerase, using GSH or NADPH as reducing equivalents. In orthodox seeds, protein thiol–disulphide conversion and *S*-glutathionylation appear to be targeted responses to maturation drying to protect protein thiols from auto-oxidation and to store GSH (Colville & Kranner, 2010). Examples are the *S*-glutathionylation of the acyl carrier protein in *Spinacia oleracea* (Butt & Ohlrogge, 1991) and the formation of mixed disulphides and *S*-glutathionylation in wheat (De Gara *et al.*, 2003; Rhazi *et al.*, 2003). Protein thiol content also declined in orthodox *Acer platanoides* seeds during maturation drying, but was unchanged in a recalcitrant *Acer pseudoplatanus* seed lot (Pukacka & Ratajczak, 2007a), further suggesting that protein thiol–disulphide conversions are a protection mechanism in orthodox seeds. Similarly, *S*-nitrosothiols, resulting from the reaction of $NO^{\cdot}$ with thiol groups, can be viewed as a form of stored $NO^{\cdot}$ (Lindermayr & Durner, 2007) that can accumulate at the onset of stress, such as during maturation drying, to be remobilized for signalling on germination. In summary, post-translational protein modifications, such as thiol–disulphide conversions, can act as protection mechanisms that are initiated when stress commences.

4. Transcriptional regulation

Protein modification, metabolite composition, genetic and epigenetic regulation, including changes in nucleosome distribution, histone modification, DNA methylation and npcRNA (nonprotein-coding RNA) all appear to participate in the abiotic stress response in plants (Urano *et al.*, 2010). In both vegetative tissues and seeds, gene transcripts may be divided into 'early responsive' genes involved in initial protection and repair, corresponding to alarm, and 'late responsive' genes involved in stress acclimation (Buitink *et al.*, 2006; Yun *et al.*, 2010), corresponding to resistance. For example, *Mt*SNF4b participates in the regulation of the early defence responses of *Medicago truncatula* seeds (Bolingue *et al.*, 2010), and genes linked to metabolism, stress response and reserve catabolism are upregulated in germinating sugar beet seeds exposed to multiple abiotic stress factors (Pestsova *et al.*, 2008). In addition, exposure to multiple stress factors can accelerate the expression of stress-related genes during seed maturation (Wan *et al.*, 2008).

Transcription factors (TFs) are key components of stress signalling pathways, controlling gene expression by acting as switches for regulatory cascades. Fluxes in transcript patterns of TFs have been described in soybean (Jones *et al.*, 2010) and Arabidopsis (Le *et al.*, 2010) during seed development, suggesting that TFs are important for controlling stage-specific biological events during seed formation. The interactions between DNA methylation, small RNAs and silencing of transposable elements (Mosher & Melnyk, 2010) will also probably contribute to the seed stress response in the alarm phase, and need to be unravelled by future research. Taken together, the activation and upregulation of protection and repair through the regulation of gene expression, in conjunction with post-translational modifications, prepare seeds for the survival of stressful environments.

V. Resistance

The ability of seeds to resist adverse environmental conditions is based on generally applicable protection and repair mechanisms, but also on multifunctional traits, such as the presence of a seed coat, desiccation tolerance and dormancy. The seed coat partly protects the seed from invading pathogens as a mechanical barrier and through inclusions of toxic compounds (Moïse *et al.*, 2005), intracellular glasses slow down the rate of deteriorative reactions (Sun, 1997; Buitink

& Leprince, 2004) and dormancy permits seed survival for extended periods of time until environmental conditions are favourable for plant establishment (Finch-Savage & Leubner-Metzger, 2006). These innate traits could be viewed as genetically programmed, constitutive protection mechanisms in the resistance phase, that is adaptations (Fig. 4).

1. Repair of macromolecules and protection from further damage

In the resistance phase, repair and protection are sufficient to maintain viability and, when the stress is alleviated, the organism can recover. For a seed, successful resistance is revealed when it can germinate following stress. Seed germination generally depends on repair, because nucleic acids, proteins and lipids are inevitably subject to desiccation-induced oxidative damage during seed maturation drying, and also during seed ageing (Kranner *et al.*, 2006; Bailly *et al.*, 2008; Rajjou *et al.*, 2008). Pathways of DNA repair that are operative in the resistance phase, or following stress removal, include base excision repair, nucleotide excision repair and mismatch repair, where the intact complementary strand acts as a template, although repair itself can generate ROS (Bray & West, 2005). During early seed imbibition, protein synthesis is reactivated and DNA repair is initiated (Sen & Osborne, 1974, 1977; Bray & Dasgupta, 1976). These early repair mechanisms are the most probable explanation for the beneficial effects of hydro- and osmo-priming (Sen & Osborne, 1974). Furthermore, thiol–disulphide conversions of proteins are reversed during seed imbibition. For example, rehydration resulted in the rapid reduction of glutathione disulphide (GSSG) and protein-bound glutathione (PSSG) to GSH and thiolated proteins, respectively, in pea (Kranner & Grill, 1993), spinach (Butt & Ohlrogge, 1991) and wheat (De Gara *et al.*, 2003; Rhazi *et al.*, 2003) seeds. In addition, proteases were activated by the reduction of their disulphide bonds to degrade reserve proteins such as glutenins (Yano *et al.*, 2001). Similarly, the $NO^{\cdot}$ stored in S-nitrosothiols can be remobilized through reduction by ascorbate (Asc), GSH or thioredoxin metal-induced haemolytic cleavage, making $NO^{\cdot}$ available for signalling during germination. Hence, protein thiol–disulphide conversions activated in the alarm phase provide protection of proteins from auto-oxidation on increasing stress in the resistance phase; when the stress is released, disulphides are converted to thiols to regain protein function.

Following the up-regulation of gene expression for antioxidant synthesis in the alarm phase, the synthesis machinery now works at elevated rates, that is transcription and translation are enhanced so that sufficient gene products accumulate that protect macromolecules from further oxidative damage. Seeds generally activate their antioxidant systems on rehydration, for example wheat, pine and cress seeds (De Gara *et al.*, 1997; Tommasi *et al.*, 2001; Müller *et al.*, 2010), which could also be viewed as adaptations to the stresses that accompany maturation drying. The seeds of tocopherol-deficient Arabidopsis mutants showed severe defects during germination and seedling growth, reinforcing the importance of antioxidant protection (Sattler *et al.*, 2004).

2. Pathogen defence

As a result of their nutritional value, seeds are attractive to seed predators and require highly expressed pathogen defence. For example, the extracellular ROS production that accompanies seed imbibition and early seedling development (Schopfer *et al.*, 2001; Kranner *et al.*, 2010c) could be involved in pathogen defence. An immediate, transient burst of $O_2^{\cdot -}$ and H_2O_2 occurred within 30 min of imbibition of pea seeds and, later, coinciding with radicle elongation, a second increase in $O_2^{\cdot -}$ production (Kranner *et al.*, 2010c). Apoplastic $O_2^{\cdot -}$ generally plays a role in developmental processes (Gapper & Dolan, 2006) and probably contributes to successful seed germination, seedling growth and development. Similarly, excision of embryonic axes from *Castanea sativa* seeds caused a burst of $O_2^{\cdot -}$ production within 5 min after excision, with putative roles in wound response, regeneration and growth following mechanical injury (Roach *et al.*, 2008, 2010). Lipid peroxidation byproducts can also be involved in the regulation and expression of defence genes, for example phytoprostanes, malondialdehyde, 12-oxophytodienoate and other small α,β-unsaturated carbonyl group-containing molecules in tomato (Thoma *et al.*, 2003) and Arabidopsis (Stintzi *et al.*, 2001) leaves and in germinating Arabidopsis seeds (Sattler *et al.*, 2006). In peanut and almond seeds, LOXs are involved in defence signalling after infection with *Aspergillus* (Tsitsigiannis *et al.*, 2005; Mita *et al.*, 2007).

3. Programmed cell death

Excess ROS and events that damage macromolecules also produce secondary toxic messengers that feed into PCD pathways (Fig. 5). For an individual cell, cell death will be the ultimate 'breaking point'. However, PCD allows the selective elimination of unwanted cells or cells that have been damaged beyond repair, giving multicellular organisms control over cellular development and a mechanism of defence against infections and diseases (Hengartner, 2000; Samejima & Earnshaw, 2005). Therefore, for a seed, PCD will be vital in the resistance phase, and has been observed during seed ageing before the onset of viability loss (Kranner *et al.*, 2006, 2010a).

Autophagy ('self-eating') is a form of PCD universal to eukaryotic cells by which cell contents are digested in vac-

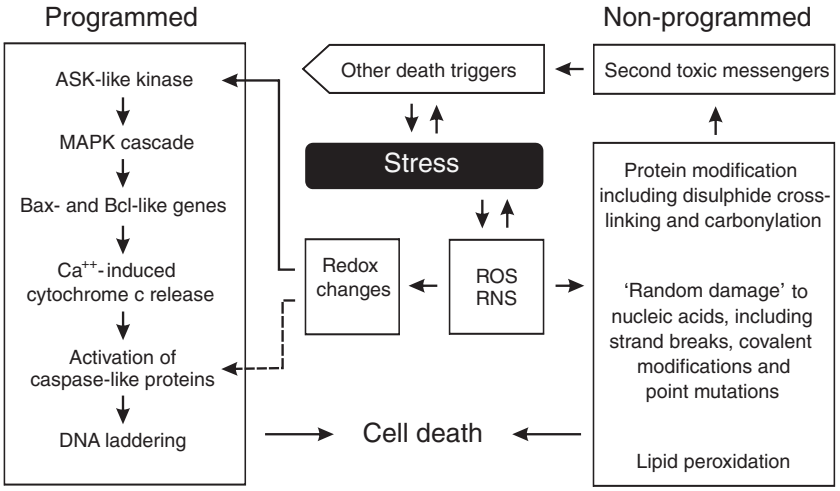

Fig. 5 Simplified scheme of mechanisms that contribute to cell death. Stress is envisaged to elicit a chain of interlinked proximate causes and effects, with cell death being the ultimate effect. Reactive oxygen and nitrogen species (ROS and RNS) and other 'death triggers' can be formed as a result of stress, but they will also be the cause of more stress. It seems unlikely that cell death results from only programmed or only nonprogrammed cell death, as links between the two processes exist, here exemplified for second toxic messengers (such as the lipid peroxidation byproducts 4-hydroxy nonenal, A1- and B1-phytoprostane; Thoma *et al.*, 2003), which may be produced through nonprogrammed events, but can trigger programmed cell death (PCD) through the activation of the MAPK (mitogen-activated protein kinase) cascade, which is implicated in animal and plant PCD. The nonexclusive scheme for PCD on the left is a simplification of the working model published by Kranner *et al.* (2006), suggesting that intracellular redox changes can activate the MAPK cascade. ASK (apoptosis-stimulating kinase) kinases, Bcl (B-cell lymphoma) and Bax (BCL-2-associated X protein) genes are involved in apoptosis in humans; their plant orthologues have not yet been identified, but a BAX inhibitor occurs in both animals and plants that is an ancient cell death suppressor (citations in Kranner *et al.*, 2006). PCD is generally initiated by a series of signalling events that may involve ROS and redox changes or other death triggers in the 'initiation phase'. In the 'effector phase', changes in the mitochondrial ion channels result in the release of cytochrome *c* into the cytoplasm. Cytochrome *c* can then activate caspase-like proteins (cysteine-dependent aspartate-specific proteases, such as *meta*- or *para*-caspases; Elbaz *et al.*, 2002), resulting in the degradation of key structural proteins, nucleic acids and the cytoskeleton, and the activation of DNases in the 'effector phase'. DNases cleave DNA into fragments of lengths corresponding to *c.* 180 base pairs, the so-called 'DNA ladder' (Hengartner, 2000; Elbaz *et al.*, 2002). Scheme modified after Kranner *et al.* (2010a).

uoles to degrade damaged or toxic components or to reclaim cellular materials (Bassham, 2007). Microautophagy (invagination of the tonoplast to deliver small pieces of cytoplasm to the vacuole) appears to accompany seed germination, facilitating the degradation of starch granules and storage proteins in the vacuole (Toyooka *et al.*, 2001). Autophagy is strongly induced by oxidative stress (Bassham, 2007), and Arabidopsis plants defective in autophagy are hypersensitive to ROS-producing agents (Xiong *et al.*, 2007) as a result of the accumulation of oxidized proteins, which cannot be efficiently degraded. Hence, autophagy contributes towards ordered cell dismantling during PCD, contributing to the survival of the whole organism (Bozhkov & Jansson, 2007).

In summary, protection and repair mechanisms that enable acclimation and adaptation are key elements of the resistance phase (Fig. 4).

VI. Exhaustion

1. Failure of protection and repair mechanisms

In the exhaustion phase, repair and protection fail and, when the stress is alleviated, the organism cannot recover, or only with severe physiological impairments. Following continuous stresses, such as those experienced in soil seed banks, or during seed storage, oxidative damage progresses as antioxidant recycling pathways break down. Irreversible damage also occurs during the lethal desiccation of recalcitrant seeds that causes uncontrolled ROS production (Varghese & Naithani, 2002). Selected examples of antioxidant breakdown during seed death, resulting from ageing of orthodox seeds include GSH in pea (Kranner *et al.*, 2006) and *Suaeda maritima* (Seal *et al.*, 2010) seeds; tocopherol in *Pinus sylvestris* (Tammela *et al.*, 2005) and *Suaeda maritima* (Seal *et al.*, 2010) seeds; Asc and tocopherol in *Fagus sylvatica* seeds (Pukacka & Ratajczak, 2007b); and during desiccation of recalcitrant seeds, superoxide dismutase (SOD) in *Shorea robusta* seeds (Chaitanya & Naithani, 1994); and SOD, tocopherol, Asc, glutathione reductase and guaiacol peroxidase in *Quercus robur* seeds (Hendry *et al.*, 1992).

Insufficient antioxidant control allows the accumulation of oxidative damage to macromolecules, contributing to seed deterioration. Seed viability loss correlates with severe lipid peroxidation (Hendry *et al.*, 1992; Chaitanya & Naithani, 1994; Tammela *et al.*, 2005; Pukacka & Ratajczak, 2007b),

changes in the content and composition of phospholipids, PUFAs and triacylglycerols (Gidrol *et al.*, 1989; Tammela *et al.*, 2005) and high levels of irreversible protein modification, such as carbonylation (Rajjou *et al.*, 2008). Lipid peroxidation, together with protein damage, causes loss of membrane integrity (Tammela *et al.*, 2005; Pukacka & Ratajczak, 2007b), contributing to cell death in aged orthodox and desiccated recalcitrant seeds, and in seeds that are prone to imbibitional injury after stressful events (Hoekstra *et al.*, 1999; Neya *et al.*, 2004). Moreover, on imbibition of aged, nonviable seeds, activation of DNA repair enzymes fails (Elder *et al.*, 1987), RNA and protein synthesis decrease (Bray & Dasgupta, 1976; Sen & Osborne, 1977), DNA double-strand breaks accumulate because of the failure of repair pathways (Bray & West, 2005), DNA and RNA integrity are lost (Kranner *et al.*, 2010a) and storage reserves cannot be mobilized (Kranner *et al.*, 2010b).

2. Cell death leading to viability loss

Severe oxidative damage to proteins, lipids and nucleic acids can lead to necrotic cell death and can also feed into PCD pathways (Fig. 5). The elimination of damaged cells by PCD may be part of the resistance response, provided that sufficient cells remain viable to allow survival; however, if too many cells die, in particular in the embryo, the whole seed will die. Deterioration of nucleic acids was observed during prolonged dry storage (Boubriak *et al.*, 2000; Osborne, 2000), and PCD was also associated with seed death (Osborne, 2000; Kranner *et al.*, 2006). Seed WC has a profound effect on longevity, with potential consequences regarding the mechanisms of cell death. In gene banks, orthodox seeds are stored at low WC and temperatures (e.g. at −20°C, or in liquid nitrogen), maintaining a glassy state. In agriculture, air-dried seeds of crop species are stored at higher relative humidities, with a viscous rather than glassy cytoplasm, and in soil seed banks, seeds may undergo hydration–rehydration cycles in accordance with changing environmental conditions (Mickelson & Grey, 2006). In 'wet' seeds, both PCD and nonprogrammed cell death are likely to operate, but, in the glassy state, direct oxidative damage is likely to be more prominent because of the limited molecular mobility of the cytoplasm and lack of water for biochemical processes.

DNA laddering is a hallmark of the final or execution phase of PCD (Hengartner, 2000), and occured during the ageing of pea seeds with a highly viscous cytoplasm (12% WC). Two scenarios have been discussed that cause DNA laddering, one leading to DNA laddering through the established PCD pathways and the other through an alternative pathway by which caspase-like proteins are activated by a series of nanoswitches (Kranner *et al.*, 2006) – chemical reactions between adjacent molecules operating on a nanometre scale (Schafer & Buettner, 2001). Triggered by a highly oxidative intracellular redox environment, caspase-like proteins could be activated through nanoswitches based on thiol–disulphide conversions, and DNA laddering would be the result. Such a redox-driven activation of caspase-like proteins could be part of PCD or necrotic cell death (Fig. 5). RNA degradation may also be associated with PCD (Xu & Hanson, 2000). PCD-associated rRNA fragmentation may lead to changes in the structure of rRNA and ribosomes (Nadano & Sato, 2000) and, as a result, impact upon the translational apparatus and protein synthesis during cell death.

3. Seed stress and death: a chain of causes and effects

We suggest that there is no simple relationship between cause and effect in processes that involve autocatalytic cascades and free radical chain reactions (Fig. 5), which can produce second toxic messengers, inducing other, or the same, cascades again (Kranner *et al.*, 2010a). For example, lipid peroxidation byproducts that result from oxidative stress could be viewed as products of nonprogammed events, but can also become second toxic messengers that induce PCD. A stress factor (the cause) can result in the production of compounds (the effect) which then become the cause for the subsequent reaction, resulting in a chain of causes and effects, a hallmark of oxidative stress pathways. Hence, stress from external environmental factors could be viewed as the initial cause for cascades of biochemical reactions (i.e. a series of stresses) that result in cell death as the final effect. However, the accumulation of dead cells can cause further stress for the organism. In addition, deteriorative processes, such as Maillard reactions, can contribute to cell death, but also proceed in decaying, archaeological material (Evershed *et al.*, 1997), and so the same process can be the cause and the effect. In summary, we envisage the loss of macromolecular integrity leading to cell death as a central part of the exhaustion phase, containing elements of both cause and effect.

VII. Conclusions

1. Bell-shaped types of stress responses confuse the diagnosis of stress

Inducible protectants, in particular antioxidants, are frequently used as stress markers. However, their concentrations often display bell-shaped patterns, with raised concentrations in the alarm and resistance phases and a decline in the exhaustion phase (Fig. 6a). Therefore, their use as stress markers is fraught with difficulties. Many studies present data for only a few selected time points, with the risk of drawing the wrong conclusions. If one sampling point is for the unstressed control and the other is taken at the end of

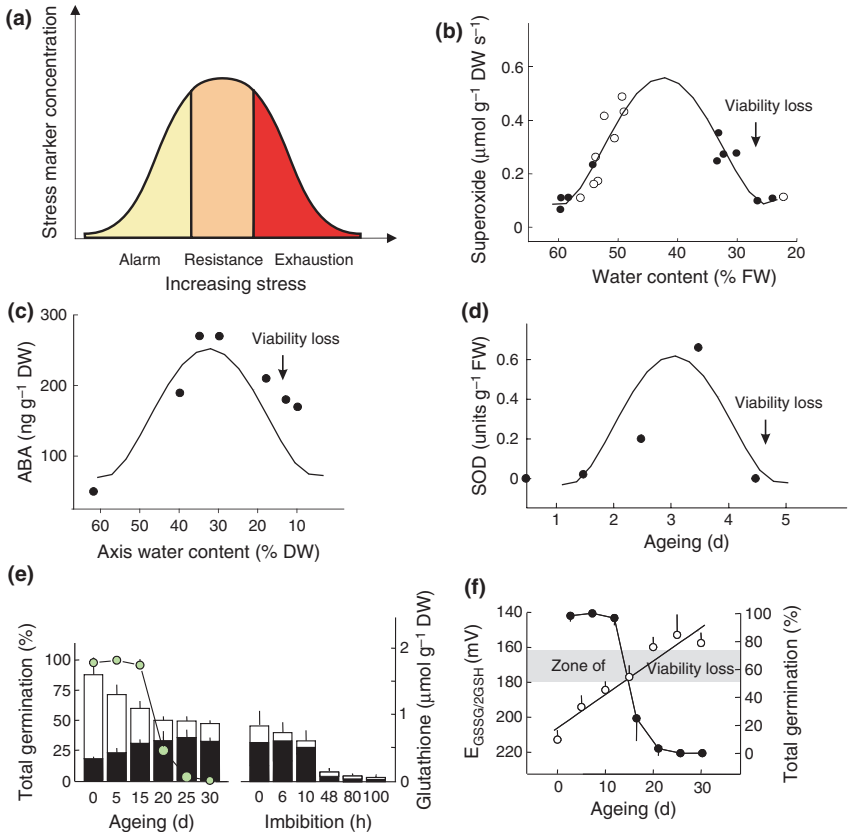

Fig. 6 Examples of bell-shaped stress responses. (a) Schematic model of the relationship between the severity of stress and the stress response. Some compounds, for example inducible cellular protectants, show a bell-shaped response to increasing stress, where the transiently enhanced production of a protectant (often used as a stress marker) could be viewed as equivalent to the resistance phase, and its decline could be viewed as exhaustion, indicative of the breakdown of the synthesizing machinery responsible for the production of protective compounds. (b) Extracellular $O_2^{\cdot-}$ production by the embryonic axes of recalcitrant *Castanea sativa* seeds in response to desiccation. Viability was lost between 30% and 20% water content (WC) when excised, isolated embryonic axes were desiccated (closed circles; data recalculated from Roach *et al.*, 2008) or when they were isolated after desiccation of intact seeds (open symbols; data are courtesy of Thomas Roach). (c) Production of abscisic acid (ABA) in response to desiccation in isolated embryonic axes excised from recalcitrant *Quercus robur* seeds. In this study, lipid peroxidation was associated with viability loss, reinforcing the intricate relationship between reactive oxygen species (ROS) and hormones in stress signalling (Finch-Savage *et al.*, 1996). (d) Enzyme activity of superoxide dismutase (SOD) in response to ageing of *Shorea robusta* seeds (Chaitanya & Naithani, 1994). (e) Glutathione disulphide (GSSG; black bars) increased with the duration of ageing (left graph; modified from Kranner *et al.*, 2006; circles denote total germination; data for imbibition are courtesy of Simona Birtić). When seeds that had been aged for 30 d were imbibed in the germination test (right graph), none germinated, and reduced glutathione (GSH; white bars) could not be recovered from GSSG, revealing an overall bell-shaped response of the stress marker GSSG, where no clear assessment can be made about the state of the seed at a given GSSG concentration; for example, seeds with 500 nmol GSSG could be viable (in the resistance phase) or dead (in the exhaustion phase). (f) Plotting seed viability (expressed as total germination; closed circles) against the glutathione half-cell reduction potential ($E_{GSSG/2GSH}$; open circles) recalculated from the data in (e) shows that bell-shaped responses can be linearized (graph modified after Kranner & Birtić, 2005), so that alarm, resistance and exhaustion can be assigned values; for example, seeds in the phase between −180 and −160 mV $E_{GSSG/2GSH}$ enter the exhaustion phase in which their cells progressively undergo programmed cell death (PCD).

the exhaustion phase, the system may appear not to have changed, whereas a more detailed sampling strategy would have revealed an initial increase in the stress marker, followed by a decrease, consistent with alarm and exhaustion, respectively. Sampling points in the middle of the alarm and the exhaustion phase may result in the same values for stress marker concentration, hindering the correct interpretation of the stress response. In other words, at the same stress marker concentration, a seed may be highly viable, preparing itself for survival, or it may be dead. Hence, we believe that data collection at numerous intervals over appropriate time courses is critical for correct interpretation. Selected examples of bell-shaped responses include extracellular ROS production (Fig. 6b) and intracellular ABA production (Fig. 6c) in isolated embryonic axes of recalcitrant seeds in response to desiccation, with increased production in mildly stressed axes and a decline in lethally stressed axes, and the pleiotropic patterns of SOD (Fig. 6d) and GSSG (Fig. 6e) in ageing seeds, reflecting both the adaptive and the detrimental stages of the stress response.

2. Can we put a number on stress?

The use of the concentrations of damaged compounds or the byproducts of damage as stress markers may be more successful as their decline or increase can be linear or exponential. However, a comparison of stress responses between species can still be difficult; for example DNA deterioration will accompany the exhaustion phase, but DNA concentrations vary greatly between and even within species as a result of the variation in ploidy, so that only semiquantitative data can be provided that reflect the experimental conditions.

More elegantly, the antioxidant redox state can be used to linearize bell-shaped curves (Fig. 6e,f) and many authors have appropriately used the ratios between oxidized and reduced forms. It is important to note that the redox state of concentration-dependent redox couples, such as GSSG/GSH (2 mol of GSH are converted into 1 mol of GSSG) can be defined more accurately by their half-cell reduction potentials and the concentrations of the reduced species, rather than ratios alone. Schafer & Buettner (2001) detailed the relationship between $E_{GSSG/2GSH}$ and the life span of human cells, demonstrating that $E_{GSSG/2GSH}$ allows a better definition of the zone of viability loss than ratios or percentage GSSG (as a percentage of GSH + GSSG). At values of $E_{GSSG/2GSH}$ between −180 and −160 mV, human cells undergo PCD and, at increasingly more positive (i.e. more oxidizing) values, necrotic cell death. This model was re-evaluated for a wide range of species across 13 plant and fungal orders, including vegetative tissues of lower plants and fungi, higher plant leaves and roots, and seeds. It was confirmed that the zone of cell viability loss, corresponding to the late resistance and exhaustion phases, is in the range of $E_{GSSG/2GSH}$ values between −180 and −160 mV for 92% of all investigated cases (Kranner *et al.*, 2006). More universally applicable stress markers that allow the classification of stress responses according to the phases of alarm, resistance and exhaustion will enable a better understanding of stress, as argued for by Lord Kelvin: 'when you can measure what you are speaking about and express it in numbers you know something about it; but when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meagre and unsatisfactory kind' (Kelvin, 1883).

3. Closing remarks

This review is intended to provoke thought about the nature of stress and how to measure and assess stress correctly. Whether a stress factor causes eustress, distress or no stress will depend on the organism concerned, its state of acclimation and adaptation, and the severity and duration of stress. We have proposed a modification of the GAS concept of Selye (1936) to reflect the progress in seed molecular biology and biochemistry over the last few decades. The outstanding stress resistance at certain stages in the seed life cycle and their vulnerability at other stages make seeds very attractive for the development of stress concepts. However, seeds represent particularly challenging models, because multifunctional traits, such as quiescence, dormancy and desiccation tolerance, are difficult to interpret in terms of stress response. Nonetheless, we believe that our GAS-based stress concept is applicable to this intricate model system, which suggests that it has wider relevance for plants in general.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Detoxification of reactive oxygen species (ROS)

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