

## Extremophyte adaptations to salt and water deficit stress

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**Abstract.** Plants that can survive and even thrive in extreme environments (extremophytes) are likely treasure boxes of plant adaptations to environmental stresses. These species represent excellent models for understanding mechanisms of stress tolerance that may not be present in stress-sensitive species, as well as for identifying genetic determinants to develop stress-tolerant crops. This special issue of *Functional Plant Biology* focuses on physiological and molecular processes that enable extremophytes to naturally survive high levels of salt or desiccation.

The world population is expected to reach above 9 billion by 2050, with most of this population growth occurring in developing countries (United Nations, World Population Prospects: The 2012 Revision). In contrast, global food production is declining, largely due to the adverse effects of global warming, exacerbated by poor agricultural practices and human-related disturbances. Drought and salinity have long been major factors affecting crop productivity and in the coming years these abiotic stressors are predicted to increase so significantly that conventional agricultural practices are likely to have been abandoned, particularly in Africa, by 2050 (Thornton *et al.* 2010; Dai 2013). To provide food security for future generations, it is imperative to develop strategies to generate crops that are able to grow productively in increasingly extreme environments.

Extremophile plants ('extremophytes') by definition are species that are able to tolerate, and even thrive in extreme environments where few other plant species, and certainly no conventional crops, are able to grow. These species are excellent models to understand key mechanisms associated with tolerance of abiotic stresses and can serve as a source of genes that could be used for breeding crops with improved tolerance to abiotic stresses. This special issue of *Functional Plant Biology* brings together a collection of papers on physiological and molecular mechanisms used by a selection of salt-tolerant extremophytes (or halophytes) and desiccation-tolerant extremophytes (commonly called resurrection plants) in their adaptations to saline and arid environments respectively.

### Salt-tolerant extremophytes

The global rise in salinisation of land due to clearance of vegetation and irrigation is of great concern due to the fact that salt stress is one of the most serious abiotic stresses limiting crop production (Flowers and Yeo 1995; Flowers *et al.* 2010a; Shabala 2013). Approximately 10% of the Earth's land surface,

including one-third of irrigated regions, currently suffers from salinisation, the economic cost being estimated at about US\$27 billion (Qadir *et al.* 2014). Clearly, the risk to global food security from soil salinisation is great, and the improvement of crop tolerance to salt stress is a major research and agrobiotechnological goal. Most research into understanding mechanisms of plant tolerance to salt stress, and into identifying stress tolerance determinants that could be used for crop improvement, has been performed on species such as the model plant *Arabidopsis thaliana*, which are generally stress-sensitive. Although such studies have yielded a large body of knowledge concerning the plethora of physiological, biochemical and molecular mechanisms that are activated in response to salt stress, these salt-sensitive species are unlikely to possess many salt stress tolerance mechanisms found in naturally salt-tolerant plants. This factor has likely contributed to the difficulty in identifying stress tolerance genes, which can be applied commercially in breeding procedures (Bressan *et al.* 2001). On the other hand, halophytes that are able to grow and reproduce in saline growth media, could represent a treasure trove of genes for improving crop tolerance to salt and for developing halophyte-based agriculture (Bressan *et al.* 2013; Shabala 2013; Cheeseman 2015; Ventura *et al.* 2015). Although the definition of a halophyte depends upon the threshold salt concentration used for that definition, it has been estimated that there are 350 known species that can tolerate at least 200 mM salt (Flowers *et al.* 2010a).

Halophytes have evolved several means of tolerating highly saline environments including tight control of salt uptake, compartmentalisation of Na<sup>+</sup> and Cl<sup>−</sup> ions (the predominant ions in saline soils) mostly in vacuoles for osmotic adjustment, the production of a variety of compatible solutes to adjust the osmotic potential of the cytoplasm and protect proteins from denaturation, and mechanisms to scavenge salt-induced production of reactive oxygen species (Flowers and Colmer 2008; Flowers *et al.* 2010a; Bressan *et al.* 2013; Flowers *et al.*

2015). Many of these mechanisms are also employed by salt-sensitive glycophytes, and thus plants exhibit a continuum of salt tolerance from the most sensitive glycophytes for whom 25 mM NaCl is toxic to the most tolerant halophytes that can survive up to 1M NaCl (Flowers *et al.* 2010a, 2010b). In addition, some halophytes have evolved specialised salt glands that excrete salt or epidermal salt bladders that sequester large quantities of salt (Shabala *et al.* 2014).

In this special issue, five papers examine several aspects of salt-tolerant extremophyte biology. The issue opens with two papers that examine salt tolerance mechanisms in halophytic relatives of *Arabidopsis thaliana* from the *Brassicaceae* family. In particular, *Eutrema salsugineum* (formally *Thellungiella salsuginea* and *Thellungiella halophila*) and *Schrenkiella parvula* (formally *Thellungiella parvula* and *Eutrema parvulum*) have emerged as excellent models for comparative analysis of stress tolerance mechanisms between closely related species (Amtmann 2009; Zhu *et al.* 2015). The first article by Ozfidan-Konakci *et al.* (2016) (pp. 575–589) reviews several mechanisms of salt tolerance found in halophytes with particular reference to *Eutrema salsugineum*, including ion compartmentalisation, osmotic adjustment, antioxidant defence and stomatal control. This is followed by a survey of the various halophytes located around the Salt Lake area of Turkey incorporating existing knowledge regarding physiological and molecular mechanisms of salt tolerance in these species. *S. parvula* is one of the species found at Salt Lake, and its genome sequence is available thereby making it an attractive halophytic model for comparison with *E. salsugineum*, whose genome has also been sequenced, and with *Arabidopsis*. Thus, the final part of the review examines our current understanding of stress tolerance gleaned from comparative analyses of these three *Brassicaceae*.

The second paper dealing with extremophyte *Arabidopsis* relatives investigates natural variation among 14 different *E. salsugineum* and two *Thellungiella* spp. accessions (Lee *et al.* 2016; pp. 590–606). Natural variation in salt tolerance has been demonstrated for glycophytes but the report by Lee *et al.* is the first to examine natural variation in a halophyte species. Phenotypic variation among the accessions is observed under both control and salt stress conditions suggesting that future analysis of this variation could identify molecular determinants of enhanced salt tolerance within the population of accessions. The authors also show that all accessions accumulate the compatible osmolytes proline, glucose, fructose and sucrose, and that the content of these metabolites is correlated with salt tolerance. Metabolic profiling of the Yukon accession indicates both pre-adaptation to salt stress and a greater metabolic response to salt compared with *Arabidopsis*. To complement the metabolic profiling, the transcriptome response of the Yukon accession to salt stress is analysed using a 44k *Eutrema salsugineum* Agilent microarray platform developed by the Hincha group (Lee *et al.* 2013). The results indicate that the numbers of genes that respond to salt in the Yukon accession are similar to *Arabidopsis* but occur at a higher salt concentration. However, the identities of the salt-responsive genes are quite different between the two plant types suggesting species-specific transcriptome responses.

The next two papers in this issue examine salt tolerance mechanisms in halophyte species unrelated to *Arabidopsis*. Aymen *et al.* (2016) (pp. 607–619) address the response of the sabkha biotope halophyte, *Limonium delicatulum*, to high salinity. They demonstrate that for concentrations up to 200 mM NaCl, the plant positively responds to a saline growth medium in terms of growth thereby demonstrating its halophytic character. At higher salinities, however, growth is reduced below that of control plants. Photosynthetic parameters and pigments exhibit a similar optimum at 200 mM NaCl. *L. delicatulum* leaves accumulate large amounts of Na<sup>+</sup> and Cl<sup>−</sup> as salt in the growth medium increases, probably for osmotic adjustment. However, the plants also appear to excrete salt onto the surface of the leaves thus combining both tolerance and exclusion mechanisms in response to salt stress. As has been well documented in numerous plants, salt causes oxidative stress in *L. delicatulum* as evidenced by a rise in lipid peroxidation and H<sub>2</sub>O<sub>2</sub> levels. Antioxidant mechanisms are activated and the increase in Reactive Oxygen Species (ROS)-scavenging enzyme activities is highly correlated with salt level in the growth medium. On the other hand, there is a weak relationship between phenolic compounds (that in other species act as antioxidants), and salt tolerance.

Shelef *et al.* (2016) (pp. 620–631) analyse the fascinating phenomenon of positive root halotropism discovered by this group (Shelef *et al.* 2010) whereby roots of the Negev desert plant *Bassia indica* grow horizontally towards increasing saline concentrations in the soil. They hypothesise that development of *B. indica* roots is a trade-off between salt concentration and optimal nutrient supply. They show that while a higher root and shoot biomass is observed under saline conditions compared with fresh water, in split-root experiments, roots prefer fresh water-irrigated growth medium. Furthermore, roots do not favour a fertilised, saline soil over a fresh water non-fertilised soil, and grow better in fresh water, fertilised soil compared with a saline, non-fertilised environment. Thus, their results suggest that only in nutrient poor, saline soils characteristic of a desert habitat, will a positive effect of salt on root growth occur. Another interesting finding is a back-flow of water from the tap root to the tip of horizontal roots that could reduce soil salinity thereby facilitating nutrient uptake.

The section on salt-tolerant extremophytes closes with a study by Yang *et al.* (2016) (pp. 632–642) that focuses on improving the productivity of the facultative halophyte and seed crop, quinoa, by inoculation with halotolerant bacteria. After selection of two most salt-tolerant bacteria (*Enterobacter* sp. (MN17) and *Bacillus* sp. (MN54)), quinoa seeds are inoculated with these strains and grown in non-saline and saline soil conditions. The authors show that the bacteria ameliorate the salt-mediated reduction in shoot biomass and grain yield. This positive effect on growth parameters under saline conditions is correlated with improved photosynthesis and stomatal conductance (and reduced ABA levels), improved water relations and decreased leaf Na<sup>+</sup> content. Both bacterial strains exhibit ACC deaminase activity (that can decrease ethylene levels), exopolysaccharide production (that could bind Na<sup>+</sup> ions) and auxin production, all of which could aid in reducing the effects of salt stress on quinoa yields.

This paper thus demonstrates the feasibility of using biotic approaches to improve halophyte productivity.

### Desiccation-tolerant extremophytes

Resurrection plants are unique in that their vegetative tissues are tolerant of loss of up to 95% of their cellular water, are able remain in the desiccated state for prolonged periods (months to years) and yet resume full metabolic activity in existing tissues upon rehydration. Such vegetative desiccation tolerance (DT) is rare and has been reported to occur in only 135 angiosperm species to date (Gaff and Oliver 2013). DT is a complex multigenic and multifactorial phenotype and gaining a full understanding of this phenomenon requires, at best, an integrative systems biology approach to fully appreciate the adaptive responses to extreme water deficit. Such an approach would typically take into consideration changes in the transcriptome, proteome, metabolome and lipidome, contextualised by input from biochemical, biophysical and physiological studies. To date, this has not been fully achieved for any one species, although considerable information is available for at least 12 different resurrection plant species (reviewed in Farrant *et al.* 2012; Dinakar and Bartels 2013). Such studies have revealed common (presumably conserved) strategies among species, but yet with individual differences among them, which collectively ameliorate the stresses associated with extreme water deficit and ultimately enable a stable metabolically quiescent state in air dry tissues. This is nicely illustrated by the five papers on resurrection plant biology published in this special issue. Each paper provides novel species-nuanced insights into key mechanisms widely believed to be associated with DT, namely: protection of photosynthetic potential and maintenance of mechanical stabilisation (Karaschi *et al.* 2016; pp. 643–655); presence of robust antioxidant potential (Govender *et al.* 2016; pp. 669–683); the accumulation of sucrose (Zhang and Bartels 2016; pp. 684–694), Late Embryogenesis (LEA) proteins (Ataei *et al.* 2016; pp. 695–708) and seed longevity under extreme environmental conditions (Visscher *et al.* 2016; pp. 656–668).

Water plays multiple and various roles in supporting life, and loss thereof results in numerous stresses, the effects of these being exacerbated with progressive water loss (reviewed in Farrant *et al.* 2012). Among the first stresses experienced upon initial water loss are those associated with turgor loss (ultimately leading to cytorhesis) and photosynthetic ROS production (culminating in subcellular damage and loss of photosynthetic potential). Resurrection plants systematically minimise and prevent such damage, generally using in each instance, one of two strategies. In the case of minimising photosynthetic ROS production, the strategies of either homoiochlorophyll or poikilochlorophyll are adopted. Mechanical stabilisation is achieved by regulated cell wall folding and controlled reduction of cell volume and/or maintenance of cell volume by replacement of water in vacuoles with compatible solutes. In their paper entitled ‘*Tripogon loliiformis* elicits a rapid physiological and structural response to dehydration for desiccation tolerance’, Karaschi *et al.* (2016) show that this graminaceous species, native to Australia and New Guinea, utilises the strategies of homoiochlorophyll, cell wall folding

and vacuolar water replacement, the precise nature of implementation of these mechanisms being peculiar to graminaceous species such as *T. loliiformis*. The authors show that, like other resurrection plants, photosynthesis and carbon gain ceases below a relative water content (RWC) of 70%. This species retains up to 70% of its chlorophyll during drying, and like other homoiochlorophyllous types, uses leaf folding and pigment production (particularly anthocyanins) to shade and mask chlorophyll respectively, in order to minimise the formation of ROS associated particularly with the excitation of chlorophyll. Leaf folding is facilitated by water loss from bulliform cells, the pectin-rich elastic nature of their cell walls and those of the mesophyll cells, simultaneously enabling their mechanical stabilisation. In thicker walled bundle sheath cells, mechanical stabilisation appears to be achieved by water replacement in vacuoles. This paper provides an essential physiological characterisation of the responses of the graminaceous species *Tripogon loliiformis* to desiccation, and provides a basis for future molecular studies on this species.

ROS formation is an inevitable by-product of metabolism particularly that associated with photosynthesis and respiration. At low (non-toxic) concentrations, ROS have been shown to act as intracellular signalling molecules by altering the subcellular redox state, while excess formation of ROS inevitably leads to subcellular damage and ultimate loss of viability. Abiotic stresses, and water deficit in particular, result in considerable ROS formation so that maintenance of appropriate redox-balance becomes essential. This is for the most part achieved by the presence of efficient antioxidant systems. It is thus not surprising that resurrection plants have highly efficient antioxidant systems, which in turn have been proposed to be a valuable source of antioxidant potential for biotechnological applications both in plant stress tolerance as well as in the medicinal and cosmetic markets (Toldi *et al.* 2009; Gechev *et al.* 2014). Studies have shown that these comprise use of ‘housekeeping’ antioxidants (Illing *et al.* 2005), often presenting unusual characteristics at low plant water contents (Farrant *et al.* 2007, 2012), as well as antioxidants such as 1- and 2-cys-peroxiredoxins, glyoxylase I family proteins, metallothionein-like antioxidants, oxidoreductases, several members of the aldehyde- dehydrogenases, and various polyphenols that are not typically highly expressed during dehydration of desiccation-sensitive material (reviewed in Farrant *et al.* 2012, 2015; Dinakar and Bartels 2013). Here, Govender *et al.* (2016) report on a novel type II peroxiredoxin (XvPrx2) identified from the poikilochlorophyllous monocot resurrection plant *Xerophyta viscosa*, a species that has been relatively well characterised at the physiological and molecular levels (reviewed in Farrant *et al.* 2015), and the genome of which has recently been sequenced (Dias Costa 2016). The authors show that unlike most other type II peroxiredoxins present in angiosperms, XvPrx2 has only one of the two cysteine molecules typically present in the active site of this enzyme, the other being replaced by valine. Nevertheless, this antioxidant is effective in detoxification of H<sub>2</sub>O<sub>2</sub> and the authors propose a mechanism for how this atypical XvPrs2 might function under abiotic stresses.

The classical view of antioxidant systems is being reformed as it becomes clear that many primary and secondary metabolites are involved in free-radical scavenging (Keunen *et al.* 2013;

Noctor *et al.* 2014). Disaccharides, particularly sucrose, raffinose family oligosaccharides and sugar alcohols (e.g. galactinol) have been identified as being active in ROS-scavenging, being particularly effective in hydroxyl scavenging. Monosaccharides, on the other hand, while they have been evoked as regulators of sugar sensing and signalling, have been reported to exacerbate ROS production via Maillard type reactions, this becoming particularly prominent at low water contents (Vertucci and Farrant 1995). In their investigations into the role of D-glycero-D-ido-octulose (D-g-D-i-oct), the most abundant sugar in hydrated tissues of the well characterised resurrection plant *Craterostigma plantagineum*, Zhang and Bartels (2016) demonstrate the antioxidant potential of this monosaccharide. However, since this is realised only in relatively hydrated tissues, the authors propose that it serves as a natural (constitutive) antioxidant, responsible for protection against several abiotic stresses. The excessive amounts of D-g-D-i-oct in hydrated tissues of *C. plantagineum* have raised the question as to other roles it might play in facilitating DT. It has been demonstrated that it serves as the primary carbohydrate source for production of sucrose during dehydration when photosynthesis is no longer active (Bianchi *et al.* 1991; Norwood *et al.* 2000) thereby contributing towards one of the most ubiquitous and fundamental mechanisms associated with DT (reviewed in e.g. Hoekstra *et al.* 2001; Farrant *et al.* 2012; Dinakar and Bartels 2013). It has recently been proposed that another fundamental mechanism associated with DT is the ability to prevent senescence in all but older (and thus naturally more prone to senescence) tissues (Griffiths *et al.* 2014; Williams *et al.* 2015). Based on experiments performed on young (non-senescent) and mature (incipiently senescent) leaves of *C. plantagineum*, Zhang and Bartels (2016) propose that D-g-D-i-oct may be involved in the programmed regulation of senescence in these respective tissues. This study epitomises what is an emerging theme among resurrection plants: mechanisms believed to be fundamentally required for DT (such as presence of high levels of sucrose in desiccated tissues, controlled regulation of senescence and the necessity for robust but flexible antioxidant systems) can be variously achieved in different resurrection plant species.

Another ubiquitous feature associated with DT is the accumulation of LEA-like proteins within tissues at low water contents, this correlating with the late stages of seed development in orthodox seeds and during the mid to late stages of dehydration in vegetative tissues of resurrection plants and other anhydrobiotic organisms (reviewed in Cuming 1999; Illing *et al.* 2005; Tunnacliffe *et al.* 2010; Dinakar and Bartels 2013; Farrant *et al.* 2012; Dias Costa *et al.* 2016). However, to date this has largely been inferred from transcriptome studies and the exact role(s) of many LEA protein are still unknown. This is due to the fact that the proteins themselves are non-catalytic, intrinsically disordered proteins that are unfolded in aqueous solutions making it experimentally difficult to assign a structure and determine function. Given the fact that LEA proteins are present in the genomes of all species that produce orthodox seeds, the expression of such proteins in vegetative tissues, as is evident in resurrection plants, is presumably controlled by differences in their promoters. The Bartels group have published a series

of papers reporting on the presence and expression of the LEA-like protein they have termed 11–24 originally isolated from the resurrection plant *C. plantagineum* (Bartels 2005) but which they have subsequently shown to also be induced during dehydration of the related sister species *Lindernia brevidens*, which is DT and *Lindernia subracemosa*, which is desiccation-sensitive (van den Dries *et al.* 2011). The authors show that expression is considerably greater in the DT species than in *L. subracemosa* and in the article published here (Ataei *et al.* 2016), they go on to examine the promoters of these LEAs in attempt to explain these expression differences. Using site-directed mutagenesis, transient transformation assays, coupled with assessment of the role of ABA and mannitol in induction of the LEA-like 11–24 transcripts in each of these three species, the authors conclude that the dehydration-responsive element (DRE) is a key motif for induction of promoter activity. They attribute the variation in transcript levels observed among the three species to the number of ABRE's present in each species and propose that it is the localisation of the DRE element relative to these that determine transcription efficiency when plants are dehydrated. This paper indirectly confirms the notion that genes for DT are present in the genomes of DS species and suggests that their expression in vegetative tissues in response to dehydration can be induced by manipulation of promoter elements.

While vegetative desiccation tolerance is rare in angiosperms, it is a common occurrence in their seeds, with over 90% of angiosperms producing DT (or orthodox) seeds (Royal Botanic Gardens Kew 2008). Indeed, it has been shown that there is considerable similarity in the mechanisms instituted by resurrection plants and orthodox seeds and it has been proposed that DT in the former arose by 'retasking' seed genes in their vegetative tissues (Illing *et al.* 2005; Farrant and Moore 2011; Dias Costa *et al.* 2016). In the desiccated state, such seeds survive environmental extremes and this factor has served as a basis for long-term seed storage for conservation purposes. In most instances it has been shown that longevity of orthodox seeds is improved when dry seeds are cooled to sub-zero temperatures and thus the current international standards for long-term storage of such seeds requires that they be dried to 15% relative humidity, thus equating to an overall seed moisture content of between 3% and 7% (expressed on a dry mass basis), sealed in moisture-proof containers and stored at  $-18^{\circ}\text{C}$  or lower (FAO 2014). However, there is variation among species in their longevity when maintained under such (often also anoxic) conditions, which may be dictated *inter alia* by their physical and biochemical make up, and presence and depth of dormancy, all of which is likely to have been driven by the native environmental conditions in which the species evolved and exist. In keeping with the theme of this special issue, Visscher *et al.* (2016) provide a very elegant review of seed longevity under extreme environmental conditions such as high temperatures, anoxia and UV radiation. The authors propose that seeds from species that show best long-term survival of such conditions are good candidates for contributing towards solutions of food security in the face of the effects of global warming, as well as use in space travel. They thus also assess the effects of ultra-dry anoxic conditions, UVC irradiation and magnetic fields (all encountered in space) on longevity and germinability of seeds. The review points to gaps in our present knowledge in this field



and highlights areas where more research is required in order to gain a more comprehensive understanding of the underlying molecular and physiological mechanisms enabling tolerance to such extreme environmental conditions.

The papers comprising this special issue provide novel insights that individually and collectively add to the emerging bigger picture of the mechanisms whereby salt- and desiccation-tolerant extremophytes survive conditions that we might deem extreme now, but may become the norm in the future. What this special issue illustrates is the very real need to use extremophytes as models for an understanding of mechanisms associated with tolerance of extreme environmental stresses. Perusal of the greater body of plant science literature will reveal considerable work on effects of salinity and water deficit stress conducted on species that in fact have little tolerance to extremes of these stresses. While model species such as *Arabidopsis* (and increasingly other plant species with relatively small sequenced genomes that are easily transformed) have provided a platform for understanding many molecular processes, these species do not have the ability to withstand extreme conditions, and at best can inform us of what is absent, or not activated, in the face of environmental stress. Such discrepancies, in turn, can only be realised when compared with species that truly survive extreme environmental stresses. In this regard, the field of extremophyte biology is currently relatively new. As illustrated in this special issue, advances in high-throughput technology and genome sequencing of such species, accompanied by associated biochemical and physiological studies are increasingly enabling select extremophytes to themselves become 'Arabidopsis-like' model species, which we believe will have a major impact in future agri-technology.

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