# Molecular and genetic aspects of plant responses to osmotic stress

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# ABSTRACT

Drought, high salinity and freezing impose osmotic stress on plants. Plants respond to the stress in part by modulating gene expression, which eventually leads to the restoration of cellular homeostasis, detoxification of toxins and recovery of growth. The signal transduction pathways mediating these adaptations can be dissected by combining forward and reverse genetic approaches with molecular, biochemical and physiological studies. *Arabidopsis* is a useful genetic model system for this purpose and its relatives including the halophyte *Thellungiella halophila*, can serve as valuable complementary genetic model systems.

*Key-words*: Genetic analysis; reactive oxygen species; signal transduction.

#### INTRODUCTION

Water is required as a medium for biochemical activities by all known life forms. For plant cells, water-generated turgor pressure is also a driving force of cell expansion. However, vegetative growth of plants can only occur at a certain range of water status, which can be measured by the free energy state of water molecules – water potentials ( $\psi_w$ ). In a given cell,  $\psi_w$  mainly consists of pressure and osmotic potential. While maintaining a positive turgor pressure, plant cells usually adjust their osmotic potential to meet the requirement of the whole plant in balancing its water budget. Significant changes in water potentials in the environment can impose osmotic stress to plants, which disrupts normal cellular activities, or even causes plant death. Under natural conditions, high salinity and drought are the major causes of osmotic stress to plants. Here, we briefly analyse the molecular processes of plant responses to osmotic stress. Current methodology in dissecting stress signalling in plants is also discussed with particular reference to important and emerging genetic model systems.

# PLANT RESPONSES TO OSMOTIC STRESSES

Upon exposure to osmotic stress, plants exhibit a wide range of responses at the molecular, cellular and whole-

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plant levels (Greenway & Munns 1980; Zhu, Hasegawa & Bressan 1997; Yeo 1998; Bohnert, Su & Shen 1999; Hasegawa et al. 2000). These include, for example, morphological and developmental changes (e.g. life cycle, inhibition of shoot growth and enhancement of root growth), adjustment in ion transport (such as uptake, extrusion and sequestration of ions) and metabolic changes (e.g. carbon metabolism, the synthesis of compatible solutes). Some of these responses are trigged by the primary osmotic stress signals, whereas others may result from secondary stresses/ signals caused by the primary signals. These secondary signals can be phytohormones [e.g. abscisic acid (ABA), ethylene], reactive oxygen species and intracellular second messengers (e.g. phospholipids). Some of these secondary signals may not be confined to the primary stress sites such as the root, and their ability to move to other parts of the plant contributes to the co-ordination of whole-plant responses to the stress conditions. For example, under drought stress, root-derived ABA can ascend with transpirational flow to regulate stomatal aperture in leaves (Zhang & Davies 1991). Reactive oxygen species are also mobile in plants (Alvarez et al. 1998; Karpinski et al. 1999; Lopez-Huertas et al. 2000). In general, plant responses are of three kinds, i.e. maintenance of homeostasis, detoxification of harmful elements and recovery of growth.

#### Homeostasis

### Macromolecule homeostasis

Increased concentration of charged elements in the cytosol may change the hydration sphere of macromolecules and thus affects their conformation or charge interactions. Compatible osmolytes may be important for maintaining the conformation of macromolecules. Osmolytes were originally thought to function mainly in osmotic adjustment by lowering cellular osmotic potential to facilitate water absorption and restore intracellular salt concentrations. They are synthesized by virtually all organisms under hyperosmotic stress (Yancey *et al.* 1982). Common osmolytes include sugars, polyols and amino acids or their derivatives. These compounds appear to have little interference in the function of macromolecules, even at very high concentrations.

One osmolyte that has received much attention is proline. Accumulation of proline was reported in many plant species under diverse abiotic stress conditions (for review see Delauney & Verma 1993). The beneficial role of proline in plant stress tolerance as suggested by early correlative studies was recently confirmed by genetic as well as transgenic studies, which demonstrated that proline can increase the tolerance of plants to abiotic stress (e.g. Xin & Browse 1998; Nanjo et al. 1999; Hong et al. 2000; Ronde, Soreeth & Cress 2000). However, in transgenic studies, it seems that the magnitude of the increase in proline (or other osmolytes) in transgenic plants is too low to be significant for overall osmotic adjustment (Zhu 2001), despite the possibility that there might be higher levels in specific cell types or in subcellular compartments. Thus, the benefits of osmolytes in maintaining the natural status of macromolecules might outweigh their role in osmotic adjustment. This is probably due to, among others, the ability of osmolytes to scavenge reactive oxygen species (e.g. Smirnoff & Cumbes 1989; Hong et al. 2000), although the underlying mechanism is presently unclear.

Once damaged proteins are beyond repair, they have to be eliminated to prevent aggregation. In this case, cells initiate the degradation of the damaged proteins or the destruction of the entire cells (apoptosis); both of these processes have been observed for stressed plant cells (e.g. Katsuhara 1997). The major proteolytic system in eukaryotes is the ubiquitin-mediated degradation in the proteasome (Ciechanover, Otian & Schwartz 2000). Under drought stress, the expression of genes encoding ubiquitinrelated proteins and various proteases was found to be induced or enhanced (reviewed in Ingram & Bartel 1996), consistent with the requirement of protein degradation under stress conditions.

#### Ion homeostasis

Maintaining ion homeostasis is critical for plants to combat high salinity stress. Ion homeostasis under salt stress has thus received much attention both in plants and in yeast studies (Niu et al. 1995; Serrano et al. 1999). Processes important for ion homeostasis include cellular uptake, sequestration and export and long-distance transport. Previous gene regulation studies have suggested that various ATPases, water channel proteins and ion transporters are regulated by salt stress either at the mRNA or protein levels. The roles of various ion transporters in plant salt tolerance have been a focus point for a long time. Much progress has been made in molecular characterization of ion transporters and functional evaluation of their roles in stress tolerance. In higher plant cells, Na+ ions are extruded from the cells or compartmentalized in their vacuoles mainly by Na+/ H<sup>+</sup> antiporters. These antiporters make use of the pH gradient generated by P-type H+-ATPase (for plasma membrane-localized antiporters) or V-type H+-ATPases or H+pyrophosphatases (PPase) (for tonoplast antiporters). There are a number of genes encoding Na<sup>+</sup>/H<sup>+</sup> antiporters in the Arabidopsis genome. The first plant Na<sup>+</sup>/H<sup>+</sup> antiporter gene functionally characterized is AtNHX1, which encodes a tonoplast antiporter homologous to the yeast Na<sup>+</sup>/H<sup>+</sup> antiporter Nhx1 (Nass, Cunningham & Rao 1997). The role of AtNHX1 in plant salt tolerance was demonstrated by the work showing that its over-expression confers salt tolerance to transgenic Arabidopsis plants (Apse et al. 1999). This indicates that tonoplast Na<sup>+</sup>/H<sup>+</sup> antiporter activity may limit the capacity of glycophytic plants to compartmentalize Na<sup>+</sup>. By screening for Arabidopsis mutants that exhibit hypersensitivity to NaCl in root and shoot growth, several salt overly sensitive (SOS) loci were defined (Wu, Ding & Zhu 1996; Liu & Zhu 1997; Zhu, Liu & Xiong 1998). Among them, SOS1 encodes a plasma membrane-localized Na<sup>+</sup>/H<sup>+</sup> antiporter (Shi et al. 2000), which functions in excluding Na<sup>+</sup> from the cytoplasm at the expense of H<sup>+</sup> downhill movement from external solution into the cytosol. Loss-of-function mutation of SOS1 leads to the mutant seedlings unable to grow in medium containing as low as 50 mM NaCl, whereas wild-type plants can survive over 100 mM NaCl treatment. These studies demonstrate that ion homeostasis is key to salt tolerance, and its regulation may distinguish salt tolerance capacity between halophytes and glycophtyes.

#### Detoxification

In addition to imposing water stress and ionic stress, hyperosmolarity also generates secondary stresses, particularly, oxidative stress, which is caused by excessive reactive oxygen species (ROS). ROS include, for example, hydrogen peroxide, hydroxyl radicals and superoxide anions. ROS are usually generated by normal cellular activities such as photorespiration and  $\beta$ -oxidation of fatty acids, but their levels increase when plants are exposed to biotic or abiotic stress conditions. The capacity to scavenge ROS and to reduce their damaging effects on macromolecules such as proteins and DNA appears to represent an important stress-tolerance trait. For instance, an Arabidopsis mutant *pst1*, which exhibits increased tolerance to salt stress, was found to have an increased capacity to scavenge ROS (Tsugane et al. 1999). Elimination of ROS is mainly achieved by antioxidant compounds such as ascorbic acid, glutathione, thioredoxin and carotenoids, and by ROS scavenging enzymes (e.g. superoxide dismutase, glutathione peroxidase and catalase). Under drought stress conditions, the activity of the enzymes that participate in ROS scavenging increases and a higher scavenging activity may correlate with enhanced drought tolerance of the plants (Bowler, van Montague & Inze 1992). The expression of genes encoding many of these enzymes is also regulated by other stresses as well as by ABA (e.g. Guan & Scadalios 1998).

Superoxide dismutase (SOD) catalyses the conversion of superoxide anions to hydrogen peroxide and water. Several reports have shown that over-expression of superoxide dismutases leads to increased tolerance to abiotic stresses such as low temperature and water stress (reviewed in Bohnert & Sheveleva 1998). Under biotic stresses, one of the functions of ROS is to fortify cell wall to prevent pathogen penetration by increasing wall crosslinking. Seeds from

transgenic tobacco plants over-expressing a cell wall peroxidase gene are more tolerant to salt stress during germination, possibly due to an improved ability to retain water for germination as a result of physical modification of water permeability of the wall (Amaya et al. 1999). In tobacco plants, over-expression of glutathione S-transferase/glutathione peroxidase increases the tolerance to cold and salt stresses (Roxas et al. 1997). Another key enzyme, catalase, catalyses the conversion of H2O2 to oxygen and water. Plant catalases are mainly localized in peroxisomes and glyoxysomes where they remove  $H_2O_2$  that is generated during photorespiration and  $\beta$ -oxidation of fatty acids. Genes for catalase isoforms in plants are induced by abiotic stress as well as ABA (e.g. Guan, Zhao & Scadalios 2000). Upon ABA treatment, there is an increase in  $H_2O_2$  levels in maize cultured cells (e.g. Guan et al. 2000) and in Arabidopsis guard cells (Pei et al. 2000). Transgenic plants with reduced catalase activity showed increased sensitivity to salt stress (Willekens et al. 1997).

#### Recovery and maintenance of growth

Not all cellular activities are equally sensitive to salt stress. The ability to recover, sustain and enhance the activity of salt-sensitive physiological processes are a determinant of salt tolerance. When confronted with an acute osmotic stress, at the beginning, plant cells, like animal cells, usually arrest the progression of cell division. The wide adoption of mitogen activated protein kinase (MAPK) cascade as a signalling module for osmotic stress suggest that osmotic stress and cell cycle regulation might have common signalling characteristics. Previous studies have suggested that ABA inhibits cell division or DNA synthesis. Recently, it was found that ABA induces the expression of ICK1, a gene encoding a cyclin-dependent protein kinase inhibitor (Wang et al. 1998). Thus, one possibility for osmotic stress inhibition of growth may be through the production of ABA, which inhibits cell division by inducing ICK1. In yeast, the cell integrity pathway is activated by cell wall remodelling during vegetative growth and during pheromone-induced morphogenesis. This MAPK pathway can also be activated by osmotic stress (reviewed in Ruis & Schüller 1995). Cell integrity pathway additionally regulates gene expression during the G1/S phase transition in veast (Madden et al. 1997). Activation of this pathway by osmotic shock results in growth arrest at the G2 phase (Mizunuma et al. 1998) similar to that observed in murine kidney cells (Kültz & Burg 1998). However, osmotic stress may have different impacts on cell cycle progression depending on the severity and the duration of the stress. In fission yeast, it was shown that exposure to osmotic stress or nutrient limitation stimulates cells to enter mitosis (Shiozaki & Russell 1995). This speeds up cells to enter G1 where they may undergo conjugation and sporulation under the adverse conditions. Some components that connect this osmotic stress response to cell cycle control have been isolated in yeast (e.g. Humphrey & Enoch 1998).

# **OSMOTIC STRESS SIGNAL TRANSDUCTION**

#### Attributes of osmotic stress and the multitude of signal perception modes

At the beginning, water deficit from either drought or high salinity may have an ionic, osmotic, or even a mechanical impact on the cell. It is likely that all these different signals have their own cognate receptors. These receptors can either operate independently or co-operatively to initiate downstream signalling events. This multiplicity may underscore the complexity of the signalling network that is ascribed as 'crosstalk', redundancy or connectedness. Candidate receptors for ionic stress could be various ion transporters that host ion-binding sites on either the extracellular or intracellular side. Changes in receptor occupancy or receptor clustering under water stress may initiate the ionic stress pathways. Cell shrinkage from osmotic stress causes mechanical stimulation to the cell. In Escherichia coli, upon hypoosmotic stress, mechanosensitive channels (MscL), which are repressed under hyperosmolarity are rapidly activated and result in substantial solute/water efflux (Booth & Louis 2000; Blount & Moe 2000). Ion channels that sense mechanical changes are found in various organisms including plants. For example, plasma membrane mechanosensitive, non-selective cation channels in animal cells can be activated by membrane stretching caused by cell volume changes in response to osmotic stress (e.g. Christensen 1987; Strotmann et al. 2000). As plant cells possess a rigid cell wall, changes in cell volume in response to short-term hyperosmolarity may be not as significant as animal cells, yet the involvement of mechanosensitive signalling in osmotic adaptation is still likely as plant cell walls do respond to osmotic stress (e.g. Marshall et al. 1999). Additionally, alterations in turgor may generate a signal that could trigger conformational changes in membrane proteins and result in the initiation of a signalling cascade.

As the stress progresses, secondary signals such as second messengers, stress hormones and ROS may be produced and subsequently activate signalling pathways. These secondary signals can act as ligands and bind to cognate receptors to initiate signalling cascades. These receptors include, for example, the receptor-like protein kinases, twocomponent sensor kinases and G-protein associated receptors (Xiong & Zhu 2001).

# Acute signalling pathways

When cells are confronted with hyperosmolarity, the first consequence is the flux of water and ions across various membranes. Thus, maintenance of ion homeostasis may represent an acute response, both in terms of the short responsive time involved and its overall importance in combating the stress. In yeast cells, it is thought that the HOG1 MAPK pathway-mediated glycerol production may be a long-term adaptive strategy as it takes at least several hours for the accumulation of glycerol in these cells following exposure to hyperosmolarity. Similarly, the accumulation of osmolytes (e.g. proline) in plants is not instant, and often occurs when cell injury becomes evident (Delauney & Verma 1993). This suggests that other acute responses for the defence of the cell may exist. Studies with the yeast nhx1 mutant suggest that Nhx1-mediated ion homeostasis and cell volume regulation represent some aspects of these acute adaptive responses (Nass & Rao 1999). Previous studies with yeast also showed that under high salt stress, a calcineurin-dependent signalling cascade is activated and this results in the activation of the zinc finger transcription factor TCN1/CRZ1, which regulates the expression of a subset of ion transporters (i.e. PMC1, PMR1, PMR2A, FKS1) (Matheos et al. 1997). In Arabidopsis, a similar signalling pathway was uncovered through genetic analysis of the sos mutants. In the SOS pathway, salt stress elicits an intracellular Ca2+ transient, which activates the Ca2+ binding protein, SOS3 (Liu & Zhu 1998; Ishitani et al. 2000). SOS3 interacts with and activates the SOS2 protein kinase (Halfter, Ishitani & Zhu 2000; Liu et al. 2000) and the SOS2-SOS3 complex may regulate downstream effectors such as the plasma membrane-localized Na<sup>+</sup>/H<sup>+</sup> antiporter SOS1 and other ion transporters (Zhu 2000). The ion transporters then function directly in the restoration of ion homeostasis.

#### **ROS signalling pathways**

In plant cells, it is not very clear how environmental stress induces the accumulation of reactive oxygen species. In animal cells, a major pathway for  $H_2O_2$  generation is via NADPH oxidase and is triggered by growth factors (Rhee et al. 2000). H<sub>2</sub>O<sub>2</sub> is a potent activator of MAPK cascades in animal cells. H<sub>2</sub>O<sub>2</sub> activation of MAPK is probably through oxidation of the conserved cysteine residues in protein tyrosine phosphatases (PTP). A reduced PTP activity thus leads to a high level activation of receptor-tyrosine kinases and their downstream MAPK cascades in animal cells (for review, see Rhee et al. 2000). Because osmotic stress signalling also uses some of the MAPK modules, crosstalk between osmotic stress and oxidative stress signalling at these MAPK modules is likely. For example, in human epithelial cells, osmotic stress (NaCl)-regulated gene expression and production of interleukin-8 (IL-8) are mediated by the p38 MAPK pathway. It was shown that antioxidants blocked osmotic stress activation of p38 MAPK and the production of IL-8 (Loitsch et al. 2000).

Similarly, the yeast HOG1 pathway that mediates hyperosmolarity responses was also found to mediate oxidative stress signalling. Thus, mutants defective in the HOG1 pathways or the downstream zinc finger transcription factors Msn2/Msn4 are sensitive to many other stresses including oxidative stress, in addition to osmotic stress (for review, see Estruch 2000). The two-component sensor SLN1–SSK1 was shown to be involved in sensing H<sub>2</sub>O<sub>2</sub> and the oxidative agent diamide because the *sln1-ssk1* mutants are more sensitive to these compounds (Singh 2000). This raises a possibility that H<sub>2</sub>O<sub>2</sub> might be a direct signal for SLN1. Activation of the HOG1 pathway by  $H_2O_2$  might induce the catalase T gene *CTT1* and results in the breakdown of  $H_2O_2$ . To date, the mechanisms of hyperosmolarity perception by SLN1 are still unknown.

In plants, exogenous  $H_2O_2$  or ABA-induced  $H_2O_2$  activates  $Ca^{2+}$  channels in guard cells and mediates stomatal closure (Pei *et al.* 2000). This action of  $H_2O_2$  is also likely through the activation of a MAPK cascade. In *Arabidopsis*, it was shown that  $H_2O_2$  activates the MAP kinase kinase kinase ANP1 and the expression of downstream genes such as *GST6*, *HSP18*·2 and *GH3*. Interestingly, tobacco plants over-expressing the tobacco ANP1 ortholog, NPK1, exhibit an increased tolerance to heat shock, freezing and salt stress. However, the expression of the stress-responsive gene *RD29A* is not affected by either  $H_2O_2$  or the activated MAPK cascade (Kovtun *et al.* 2000), suggesting that the pathways for the regulation of DRE/CRT genes (Shinozaki & Yamaguchi-Shinozaki 2000) are activated through distinct mechanisms.

The expression of many genes is regulated by ROS. Transcription factors that bind to the cis elements in these gene promoters have been well studied in yeast. These are the Yap1 group b-Zip transcription factors in budding yeast and Pap1 in fission yeast. These AP-1 transcription factors are similar to the mammalian c-Jun protein. In fission yeast, Pap1 is activated by the MAPK Sty1 pathway, which regulates responses to osmotic stress, nutrient limitation, oxidative stress and UV irradiation. Moreover, the cytoplasmicnuclear partitioning of Pap1 (Toone et al. 1998) and Yap1 (Kuge, Jones & Nomoto 1997; Delaunay, Isnard & Toledano 2000) is regulated by ROS. These transcription factors have conserved cysteine residues that may act as sensors for the redox status of the cell (Delaunay et al. 2000; Estruch 2000). Upon activation by ROS, Yap1-related transcription factors activate genes that encode ROS scavenagers (e.g. superoxide dismutases and catalases) or antioxidant (e.g. glutathione, thioredoxins) synthases (Jamieson 1998). Surprisingly, although all these structural genes are found in plants, prototypic Yap1-like transcription factors do not appear to be present in the Arabidopsis genome.

#### Signalling for osmolyte synthesis

The obvious metabolic changes characterized by increased synthesis of osmolytes have attracted researchers to study the underlying mechanisms of hyperosmolarity signalling. The yeast HOG1 pathway thus emerged as the best studied osmolarity signalling cascade. The discovery of the HOG1 pathway inspired expectations of similar signalling networks in plants that activate the synthesis of osmolytes under osmotic stress conditions. The widely reported accumulation of proline in stressed plants makes proline a good candidate marker to study the signalling pathways leading to its accumulation (Hare, Cress & van Staden 1999). However, current work on this pathway has mostly been limited to the transcriptional or post-transcriptional regulation of several biosynthesis genes. For most glycophytes, osmolytes

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do not accumulate to a high level comparable to that of glycerol in yeast. In this regard, it is worth noting that the halophyte *Thellungiella halophila* (see below), which is capable of accumulating very high levels of proline, would be a good model system to use to dissect the signalling pathways that mediate osmolyte synthesis. The power of genetic analysis is being increasingly applied to stress signalling in plants. One mutant that shows increased tolerance to freezing was found to accumulate high concentrations of proline (Xin & Browse 1998). This work indicates that the signalling pathways leading to osmolyte synthesis is amenable to genetic analysis in plants.

## Protective or damage-repair pathways

The osmotic stress signalling pathways that have been more actively studied in plants are those that lead to the synthesis of a group of proteins with no clear biochemical function, and no significant sequence similarity to other well-characterized proteins. These proteins are highly hydrophilic, glycine-rich, and remain soluble even when boiled. They can be loosely grouped as LEA (late embryogenesis abundant)-like proteins.

LEA-like proteins are found to accumulate in vegetative tissues in all plant species in response to abiotic stress. LEA genes have been cloned from many plant species and are regulated by osmotic stress caused by drought, salinity and cold. Many LEA genes are regulated by ABA as well (Skriver & Mundy 1990; Ingram & Bartels 1996; Bray 1997; Zhu et al. 1997). Interestingly, LEA-like proteins are also found in bacteria (Close & Lammers 1993) and in yeast (Garay-Arroyo et al. 2000). The observation that LEA-like proteins likely exist in all organisms and most of them are induced by osmotic stress (Garay-Arroyo et al. 2000) suggests that this group of proteins may underlie a common adaptation strategy to osmotic stress. The yeast LEA-like GRE1 gene is up-regulated by osmotic, ionic, oxidative and heat stresses. GRE1 is negatively regulated by the protein kinase A (PKA) pathway, and positively regulated by the transcription factors Msn2 and Msn4 (Garay-Arroyo & Covarrubias 1999). However, disruption of GRE1 does not result in detectable phenotypic changes under any of the stress conditions (Garay-Arroyo & Covarrubias 1999). In contrast, E. coli rmf null mutant cells are more sensitive to osmotic stress (Garay-Arrovo et al. 2000). The RMF gene was previously described as a ribosome modulation factorencoding gene whose expression is induced during the transition from exponential phase to stationary phase of growth (Yamagishi et al. 1993). However, recent analysis showed that RMF is a typical LEA-like protein (Garay-Arroyo et al. 2000). LEA genes in diverse organisms may have similar functions. For example, a tomato LEA gene when expressed in yeast increases salt and freezing tolerance of the yeast cells (Imai et al. 1996). The function of LEA-like proteins has also been explored by over-expression studies in plants. Rice plants over-expressing a barley LEA gene, HVA1, show increased tolerance to cold and salt stress (Xu et al. 1996). Over-expression of transcription factors that regulate the LEA-like genes also significantly improves plant tolerance to various abiotic stresses (Jaglo-Ottosen *et al.* 1998; Kasuga *et al.* 1999).

The function of these stress proteins in plants and particularly their modes of action remain a mystery, although plenty of speculative suggestions have been put forward. A major hypothesis is that they act as chaperones to prevent misfolding or denaturation of proteins. One interesting analysis with the LEA group of proteins suggests that some of them have features of ribosomal proteins that interact with RNA (Garay-Arroyo *et al.* 2000), which is consistent with their over-representation of charged residues that facilitate nucleic acid binding. As mentioned above, the *E. coli* LEA-like RMF is a ribosomal protein. This alludes to the possibility that the COR/RD/LTI/KIN proteins, which share similar characteristics with LEA proteins, may have the ability to associate with ribosomes during stress.

ROS generated by osmotic stress may also damage DNA. Cellular responses to DNA damage include activation of stress signalling pathways, delay in cell cycle progression and the initiation of DNA damage repair. In yeast, the HOG1 pathway also activates DDR2 (DNA damageresponsive gene 2), which may be involved in DNA damage recognition and repair (Ruis & Schüller 1995). Similarly, the osmotic stress activated MAPK pathways in animals are responsive to other stresses such as UV and  $\gamma$  irradiation, cytokines, certain mitogens and oxidative stress. These pathways play a major role in apoptosis, cytokine production, transcriptional regulation and cytoskeletal reorganization (Obata, Brown & Yaffe 2000). They also activate GADD45 and GADD153, genes involved in DNA damagerepair (Kültz & Burg 1998). In Arabidopsis, the connection between genotoxic damage repair and stress sensitivity is suggested by the uvs66 mutant, which was identified as hypersensitive to UV-C and to the DNA-damaging agents methyl methane sulphonate and mitomycin. Interestingly, the uvs66 mutant is also sensitive to salt stress and ABA. Expression of the osmotic stress-responsive RAB18 gene is also altered in the mutant (Albinsky et al. 1999).

Traditionally, pathways that activate the expression of various stress-responsive genes are considered as signalling of the initial stresses such as drought, salinity and cold (Shinozaki & Yamaguchi-Shinozaki 2000). However, the possibility that these are activated by stress damage and represent actual pathways for damage repair has not been considered. In the case of activation of heat-shock protein synthesis, it is well recognized that damaged/denatured proteins are the signals that trigger the expression of HSP genes (Macario & Conway 2000). Similarly, evidence also suggests that the accumulation of proline may be a reflection of cell damage (Delauney & Verma 1993; Hare *et al.* 1999).

# GENETIC ANALYSIS OF OSMOTIC STRESS SIGNALLING NETWORK

Despite the accumulation of considerable information on gene expression under osmotic stress, which is continuing at

a greater pace due to the availability of microarray analysis of expression at a genome level, signal transduction pathways that lead to the activation of these genes are far from being understood. Obviously, to dissect the complex network of osmotic stress signalling requires the employment of a repertoire of research tools; among them, genetic analysis is indispensable.

# Genetic approaches to analysis of osmotic stress signalling

Conventional mutant screens using physiological criteria such as seed germination, root and seedling growth have been used to analyse plant abiotic stress responses (for review, see Xiong & Zhu 2001). For efficient genetic dissection of osmotic stress signalling networks in plants, the use of reporter genes under the control of stress-responsive promoters as a screening approach can be an excellent alternative to overcome the shortage of water stressspecific responses in plants (Xiong & Zhu 2001). Another promising approach is through RNA interference (RNAi) (Chuang & Meyerowitz 2000). Our recent work suggests that RNAi is very efficient in generating knock-out mutants in components of stress signalling pathways (J.-K. Zhu, unpublished).

Previously, we have used the firefly luciferase reporter gene (LUC) under control of the stress-responsive RD29A promoter as a molecular marker to screen for mutants that were defective in osmotic stress signalling. Hundreds of mutants were isolated in a moderate scale of screen (Ishitani et al. 1997; Xiong et al. 1999). Recently, dozens of these mutations have been cloned. Through these efforts, we have now learned several important messages regarding the application of this reporter gene approach. First, the reporter gene approach is very efficient in obtaining novel mutations. Second, mutants with a strong reporter gene phenotype many not necessarily have strong physiological phenotypes due to the redundancy or connectedness of signal pathways (Xiong & Zhu 2001). Third, although mutations in components of stress signal transduction pathways can be isolated, many mutated genes do not fit into our expected pathways. Rather, mutations in general cellular machinery that affects mRNA metabolism (general transcription, processing and nuclear export) and translation may also be recovered due to their effects on the reporter enzyme activity. Although some of the gene products related to general cellular machinery may participate directly in stress signalling, others may simply underlie general cellular lesions that are vulnerable to the particular stress administered during mutant screening.

#### Genetic model systems

One important prerequisite for genetic analysis of plant osmotic stress responses is the use of a genetically tractable model system. Traditionally, osmotic stress tolerance has been studied with extremophiles or with yeast. One model plant is the facultative halophyte, ice plant (*Mesembryan*- themum crystallinum). This plant can grow in the presence of 500 mM NaCl. Gene expression of ice plant under salt stress conditions has been scrutinized (Bohnert *et al.* 1988). Another model plant is the resurrection plant, *Craterostigma plantagineum* (Bockel, Salamini & Bartels 1998). An important problem with these plants is that it is extremely difficult to carry out genetic analysis.

With the complete sequencing of the *Arabidopsis* genome, *Arabidopsis* should find even more use as a model system for studying plant osmotic stress responses. Genetic analysis of salt stress signalling has demonstrated the promise offered by this model system, despite of its glycophytic nature (Zhu 2000). This is because the plants, whether glycophytes or halophytes, appear to have similar signalling and tolerance machinery. The difference between salt tolerance and sensitivity probably results from changes in the threshold of some regulatory switches or mutations in some key determinants. Thus, genetic analysis using the *Arabidopsis* model system will continue to contribute to building salt and water stress signalling networks applicable to all higher plants.

Once a framework has been set up, the application of knowledge from Arabidopsis to crop plants or, more specifically, the application of knowledge to the engineering of drought- or salt-tolerant crop plants also requires specific information from crop plants and from other plants that are more tolerant to osmotic stress than Arabidopsis. In this regard, genetic analysis of osmotic stress responses in halophytes should provide valuable information. One promising halophytic genetic model plant is Thellungiella halophila (Zhu 2001). This plant can complete its life cycle in the presence of more than 300 mM NaCl, compared with around 75 mM NaCl for Arabidopsis. Importantly, T. halophila has many traits that are similar to Arabidopsis, such as a small stature, short life cycle, self-pollination and a small genome. Thellungiella halophila can also be easily transformed via Agrobacterium-mediated flower-dipping transformation. One additional advantage is that at the cDNA level, homologous genes in this genome are over 90% identical in sequence to those in Arabidopsis (Zhu 2001). Therefore, genetic analysis of this halophytic plant can benefit from the availability of the Arabidopsis genome sequence.

#### **CONCLUDING REMARKS**

Although considerable information has been accumulated regarding plant responses to osmotic stress, one major deficiency is in the understanding of the signal transduction pathways that mediate these responses. In this post-*Arabidopsis* genomics era, the use of genetically tractable model plant systems and the application of forward and reverse genetic approaches (e.g. RNAi and T-DNA or transposon insertional knockouts) are expected to accelerate progress in our understanding of these signalling pathways. Elucidation of the signalling networks will pave the way for more rational engineering of stress-tolerant crop

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plants (e.g. Bartel & Nelson 1994; Winicov 1998; Cushman & Bohnert 2000).

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#### 138 L. Xiong & J.-K. Zhu

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