Engineering Salinity and Water-Stress Tolerance in Crop Plants: Getting Closer to the Field

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ABSTRACT

Abiotic stress is the primary cause of crop plant yield losses worldwide. Drought and salinity stress are the major environmental challenges faced by agriculture. Improving yield production and stability under stressful environments is needed to fulfil the food

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demand of the ever-growing world population. Numerous genes associated to plant response(s) to drought and salinity stress have been identified and characterized, in most cases, in the model plant *Arabidopsis*. However, while many of these genes are potential candidates for improving tolerance to abiotic stress, only a small proportion were transferred into crop plants. Further, transgenic crop plants overexpressing the genes of interest were, in most cases, tested under artificial conditions in the laboratory or controlled greenhouse. Thus, while many reports on drought and salinity tolerance in transgenic plants have been published, there is urgent need to test these traits under field conditions. In this chapter, we discuss recent advances in engineering drought and salinity tolerance in crop plants with emphasis on yield and the needs to close the gaps between the laboratory and the field conditions.

ABBREVIATIONS

ABA	abscisic acid
CAT	catalase
CDPK	calcium-dependent protein kinase
CIPK	calcineurin B-like protein-interacting protein kinase
CK	cytokinin
DREB	dehydration-responsive element binding protein
ERF	ethylene responsive factor
GB	glycine betaine
GST	glutathione S-transferase
IPT	isopentenyltransferase
JA	jasmonic acid
LEA	late embryogenesis abundant
MAPK	mitogen-activated protein kinase
MtlD	mannitol-1-phosphate dehydrogenase
NAM	no apical meristem
P5CS	D1-pyrroline-5-carboxylate synthetase
PEG	polyethylene glycol
PIP	plasma membrane intrinsic protein
RLK	receptor-like kinase
ROS	reactive oxygen species
RWC	relative water content
SOD	superoxide dismutase
SOS	salt overly sensitive
TE	transpiration efficiency
TIP	tonoplast intrinsic protein
TF	transcription factor
TPS	trehalose-6-phosphate synthase
OA	osmotic adjustment
WUE	water-use efficiency

I. INTRODUCTION

Crop plants are often grown under unfavourable environmental conditions that prevent the full expression of their genetic yield potential. The most frequently occurring abiotic stress conditions with adverse effects on crop yield are water, deficit or excess; ions, deficit or excess; temperature, low or high; and light, deficit or excess. The ever-increasing human population, concomitant with loss of agricultural land (due to urbanization processes) and diminishing water availability (associated with climate change) pose serious challenges to world agriculture (reviewed by Mittler and Blumwald, 2010). A significant increase (an estimated 50%) in grain yield of major crop plants such as rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.) and maize (*Zea mays* L.) is required to fulfil the food supply requirements for the projected population by 2050 (Godfray *et al*., 2010). The average production of major U.S. crops (corn, wheat, soybean, sorghum, oat, barley, potato and sugar beet) is only 21.6% of the highest yields attained under optimal conditions (Boyer, 1982). Diseases, pests and weed competition losses account for 4.1% and 2.6% yield reductions, respectively, with the remainder of the yield reduction (69.1%) attributed to unfavourable physicochemical (abiotic) environments induced by problematic soils and erratic climate patterns. Certainly, some of these losses are caused by inherently unfavourable environments and some by suboptimal management practices by farmers, often due to economic constraints or lack of training. Nevertheless, there is no doubt that a large fraction of potential crop productivity is lost to abiotic stress factors.

Plants respond to abiotic stresses at multiple levels such as molecular, cellular, tissue, anatomical, morphological and whole-plant physiological levels (Bartels and Sunkar, 2005; Bray, 1993, 1997; Chaves *et al*., 2003; Munns, 2002; Munns and Tester, 2008; Witcombe *et al*., 2008). The response to stress depends on the duration and severity of the event, as well as the age and developmental stage of the plant, which varies with the species and genotype level (Bray, 1997). For crop plants, tolerance to abiotic stresses is measured by yield loss rather than survival. Typically, early plant establishment (germination and seedling) and the reproductive stage are the most sensitive in determining yield under stress (Barnabas *et al*., 2008). However, a large segment of the research on abiotic stress in model systems (particularly *Arabidopsis*) in the past has focused primarily on the vegetative phase and strived to identify survival phenotypes. This has hindered our ability to readily translate the discoveries into improved yield in crop plants.

II. PLANT RESPONSES TO DROUGHT AND SALINITY STRESS

A. PLANTS RESPONSE TO WATER DEFICIT

Among the various abiotic stress conditions, water deficit is the most devastating factor (Araus *et al.*, 2008; Boyer, 1982). About one-third of the world's arable land suffers from chronically inadequate water availability for agriculture, and in virtually all agricultural regions, crop yields are periodically reduced by drought (Bruce *et al.*, 2002). While currently $\sim 80\%$ of the world's useable water resources are consumed by irrigated agriculture (Condon *et al.*, 2004), within a few decades, the expanding world population will require more water for domestic, municipal, industrial and environmental needs (Hamdy *et al.*, 2003). This trend is expected to accentuate due to global climatic change and increased aridity (Vorosmarty *et al.*, 2000). Thus, to meet the projected food demands, more crop per drop is required (Condon *et al.*, 2004).

B. PLANT RESPONSE TO SALINITY STRESS

Salinity (see definition of saline and sodic soils; Richards, 1954) is a major constraint on crop-plant productivity (reviewed by Apse and Blumwald, 2002; Flowers, 2004; Munns and Tester, 2008; Witcombe *et al.*, 2008). More than 800 million hectares of land throughout the world are salt affected, which accounts for 6% of the world total land area (Munns and Tester, 2008). In most cases, salinity results from natural causes (salt accumulation over long periods of time). In addition, a significant portion of the cultivated agricultural land is becoming saline due to deforestation or excess irrigation and fertilization (Shannon, 1997). Current estimates indicate that 20% of the roughly 230 million hectares of irrigated land is affected by salinity. Given that a third of the food production comes from irrigated agriculture, salinity is becoming a serious problem for crop-plant productivity.

C. PLANT ADAPTATIONS TO ABIOTIC STRESS

Plant resistance to stress conditions may arise from escape, avoidance or tolerance strategies (Levitt, 1972). *Escape* relies on successful completion of reproduction before the onset of severe stress (i.e. developmental plasticity), achieved by early flowering and/or short growth duration (Mooney *et al.*, 1987). *Avoidance* involves the prevention or decreasing the impact of the stress on the plant, such as minimizing water loss and maximizing water

uptake (Chaves *et al*., 2003) or exclusion of salt ions, a feature observed in halophytes (Munns and Tester, 2008). *Tolerance* relies on the inherent ability of the plant to sustain growth (likely at a reduced rate) even when the conditions are unfavourable for the maintenance of basic plant processes. This strategy involves coordination of physiological and biochemical alterations at the cellular and molecular levels, such as osmotic adjustment (Morgan, 1984) and the sequestration of ion in the plants, in the vacuole or leaf sheath and/or older leaves (Mimura *et al*., 2003). In most cases, plants subjected to stress conditions combine a suite of responses, exhibiting a number of physiological and biochemical responses at the molecular, cellular and whole-plant level (Bohnert *et al*., 1995; Bray, 1993, 1997; Chaves *et al*., 2003).

D. NEW TECHNOLOGIES TO STUDY PLANT RESPONSE TO ABIOTIC STRESS

New technologies are providing opportunities to address the challenging problem of maintaining high-yield crop production under stressful and changing climates. The information provided by high-resolution transcript profiling, the identification of large-scale specific protein networks and their association with the plant responses to environmental perturbations are allowing the application of a systems-level approach to uncover the bases of plant responses to environmental changes. Model plants, such as *Arabidopsis thaliana*, *Brachypodium distachyon* and *Medicago truncatula*, have been and will continue to offer insights into the genetic and biochemical basis of abiotic stress adaptations (Bohnert *et al*., 2006; Hirayama and Shinozaki, 2010). Further, the identification of stress-related genes and pathways has been facilitated by introducing new tools and resources developed in these model plants. Numerous genes related to plant response to drought and salinity stress have been identified and characterized (Ashraf, 2010; Pardo, 2010; Shinozaki and Yamaguchi-Shinozaki, 2007; Umezawa *et al*., 2006). Many of the genes so identified are considered as potential candidates for improving tolerance to abiotic stress. In the majority of cases, these genes are overexpressed in the target plant(s), whether with a strong constitutive promoter or a stress-responsive promoter. Early generations (T_1–T_3) are screened for responses to stresses to assess the efficacy of the construct. However, the vast majority of these studies were conducted under laboratory conditions (i.e. dehydration) in the vegetative phase (i.e. seedling, or plate assays) using artificial stress (e.g. PEG, mannitol), with very high concentration (i.e. osmotic shock) and for short periods (i.e. hours). Moreover, most of these studies showed stress tolerance and/or survival, but not the effects of the different stress conditions on plant productivity (Parry *et al*., 2005).

Under rain-fed drought prone agriculture, water stress at the reproductive stage is the most prevalent problem as in most rain-fed ecosystems, the crop season's rains diminish towards flowering and harvest time (Blum, 2009). Thus, more emphasis should be given to the study of the response of crop plants to abiotic stress at the reproductive stage and under field conditions.

III. ENGINEERING OF DROUGHT AND SALINITY-TOLERANT CROP PLANTS

Plant responses to abiotic stress affect all aspects of plant physiology and metabolism, leading to severe yield losses. Thus, tolerance mechanisms depend on the prevention or alleviation of cellular damage, the re-establishment of homeostatic conditions and the resumption of growth. Discovering and understanding the molecular/genetic basis of these tolerance components have been the focus of crop biotechnology in the past 2 decades. Despite these enormous research efforts, the role of very few genes in enhancing abiotic stress tolerance has been demonstrated under field conditions. However, this is expected to change primarily because research is increasingly focused on high yields under stress rather than plant survival. Other factors include better facilities for testing the transgenic materials and the increasing acceptance of genetically engineered plants. Genetic engineering of candidate genes for abiotic stress was found to be successful in model plants growing under controlled conditions and provided insights on the role of these genes in key physiological and biochemical processes (reviewed by Pardo, 2010; Umezawa *et al*., 2006; Vinocur and Altman, 2005). In this chapter, we have focused on efforts towards the improvement of drought and salinity stresses tolerance in crop plants with emphasis on field trials.

A. GENES INVOLVED IN OSMOREGULATION

The biosynthesis and accumulation of compatible solutes in is an adaptive response of plants to both drought and salinity stress (Munns, 2002). Compatible solutes are non-toxic small molecules which do not interfere with normal cellular metabolism. A variety of substances have been identified in plants as compatible solutes, including sugars (trehalose, fructan), sugar alcohols (galactinol, trehalose and mannitol), amino acids (proline) and amines (glycine betaine, GB). There are many examples in the literature of increasing compatible solute synthesis as a strategy to improve tolerance to abiotic stress. In most cases, tolerance to either water or salinity stress has been reported as comparisons of plant recovery from treatments of rapid

drying or high salinity. Survival, protection of photosynthetic activity, degree of lipid peroxidation and membrane leakage are common parameters for assessing the effects of increased synthesis of compatible solutes. In rare cases, evaluations are made over longer term growth, but even so, effects on yield are rarely reported, and we are aware of no reports of field performance under both normal and stress conditions of transgenic plants engineered to produce increased amounts of compatible solutes. In this section, we highlight some of the promising candidate technological approaches that remain to be substantiated with field testing for yield performance.

1. Proline

The accumulation of proline in response to osmotic stress has been reported in many plant species (Delauney and Verma, 1993). Proline is believed to act as a store of carbon and nitrogen, as a scavenger of reactive oxygen species (ROS), a molecular chaperone and even as a signal for other adaptive responses to abiotic and biotic stresses (Verbruggen and Hermans, 2008). Transformation of chickpea (*Cicer arietinum*) with the osmoregulatory gene *P5CSF129A* (under 35S promoter) encoding the mutagenized Δ^1-pyrroline-5-carboxylate synthetase (*P5CS*) for the overproduction of proline showed significantly higher proline accumulation. However, the transgenic plants resulted only in a modest increase in transpiration efficiency (TE), suggesting that enhanced proline had little bearing on the components of yield in chickpea (Bhatnagar-Mathur *et al*., 2009). Wheat plants overexpressing *P5CS* (under the control of a stress-induced promoter complex-AIPC) showed accumulation of proline, which resulted in improved tolerance to water deficit (Vendruscolo *et al*., 2007). Likewise, transgenic rice overexpressing *P5CS* showed significantly higher tolerance to salinity and water stress produced in terms of faster growth of shoots and roots (Su and Wu, 2004). Rice plants overexpressing the *ZFP252* gene, resulted in increased amount of free proline and soluble sugars, elevated the expression of stress defence genes and enhanced tolerance to salt and drought stresses (Xu *et al*., 2008). Soybean plants expressing Δ^1-pyrroline-5-carboxylate reductase (*P5CR*) under control of an inducible heat shock promoter were found in greenhouse trials to accumulate proline without deleterious effects and to retain higher relative water content (RWC), and higher glucose and fructose levels than the antisense and control plants (de Ronde *et al*., 2004). Field trials have been conducted in South Africa with apparent yield advantages for the proline accumulating soybean transgenic plants under reduced watering conditions and heat stress (ARC Research Highlights, 2006). However, these results have yet to appear in a scientific peer-reviewed publication.

2. Mannitol

Mannitol is accumulated as a compatible solute in many plants and organisms of other kingdoms, although its accumulation in celery is often cited, perhaps because in celery up to half of fixed CO_2 is converted to mannitol (Stoop *et al.*, 1996). The overexpression of *mannitol-1-phosphate dehydrogenase* (the *Escherichia coli* locus *mtlD*) resulted in the accumulation of a small amount of mannitol and also in the improved tolerance to salinity and drought in *Arabidopsis* (Thomas *et al.*, 1995) and tobacco (Karakas *et al.*, 1997). In wheat, where mannitol is normally not synthesized, constitutive expression of the m*tlD* (under the control of the *ZmUbi-1* promoter) improved growth and tolerance to water stress and salinity, although growth in the absence of stress was accompanied with sterility, stunted growth and leaf curling at levels of mannitol higher than 0.7 μmol/gFW (Abebe *et al.*, 2003). As with other compatible solutes discussed above, the concentration of mannitol in the transgenic plants that showed better response to water and salinity stress at the whole-plant level was too small to be osmotically relevant. Rather, the ameliorative effect of mannitol was likely to be exerted through the scavenging of hydroxyl radicals and stabilization of macromolecular structures (see Abebe *et al.*, 2003, and references therein).

3. Glycine betaine

GB, a fully *N*-methyl-substituted derivative of glycine, accumulates in the chloroplasts and plastids of many species such as Poaceae, Amaranthaceae, Asteraceae, Malvaceae and Chenopodiaceae, in response to drought and salinity. In some species, GB accumulates to concentrations that would contribute to cellular osmotic pressure (Munns and Tester, 2008), but in most cases, plants accumulate less than this amount. At lower concentrations, GB stabilizes the quaternary structures of enzymes and complex proteins and protects the photosynthetic machinery via ROS scavenging (Chen and Murata, 2008). Transgenic maize expressing the *betA* locus of *E. coli*, encoding choline dehydrogenase, showed more GB accumulation under drought and salinity in the field (Quan *et al.*, 2004). Under drought stress, imposed at the reproductive stage, transgenic maize lines that showed the highest amounts of GB accumulation (between 5.4 and 5.7 μmol/gFW) also had a 10–23% higher yield than wild-type plants under the same treatment (Quan *et al.*, 2004). Quantitative data describing yields in the field in the absence of stress were not reported. Cotton plants (*Gossypium hirsutum* L.) expressing *betA* were also described as more drought tolerant (Lv *et al.*, 2007). Under water-stress conditions, the transgenic cotton lines had higher RWC, OA, increased photosynthesis, reduced ion leakage and lower lipid membrane peroxidation than wild-type plants. As with the transgenic maize

(Quan *et al*., 2004), GB levels in the transgenic cotton were up to threefold greater than that measured in the wild-type controls. Yield was tested in pots in the greenhouse and one line showed a reduced loss of yield on water-stress treatment at anthesis. Recently, *betA* was transformed (under control of a maize ubiquitin promoter) into bread wheat and resulted in improved salt tolerance (He *et al*., 2010). Under 200 mM NaCl treatment, the transgenic wheat seedling (five-leaf stage) had higher levels of GB and chlorophyll, lower Na^+/K^+ ratios and solute potential, and less cell membrane damage. Further, in a field experiment under saline conditions (0.42–0.47% NaCl w/w), the transgenic plants dramatically outyielded the wild-type control plants (He *et al*., 2010).

A *CMO* gene (*AhCMO*), cloned from *Atriplex hortensis*, was introduced into cotton, showing enhance resistance to salinity stress (Zhang *et al*., 2009). GB levels in the leaves of the transgenic cotton plants were on the high end of the range of GB reported in transgenic plants (43 μmol/gFW). While yield in the absence of stress was approximately 10% lower in the transgenic lines, these were T_3 generation materials that were being compared to untransformed controls. At least one backcross to the wild type would be useful to make comparisons with wild type and to minimize tissue culture effects in the transgenic lines. Seed cotton yields of the transgenic lines were 20–30% higher than wild type in three seasons of field trials on what was reported as saline soil (Zhang *et al*., 2009); however, no description of the salinity level was provided in the publication. Transgenic potato (*Solanum tuberosum* L.) plants, developed via the introduction of the bacterial choline oxidase (*codA*) gene, expressed under the control of an oxidative stress-inducible *SWPA2* promoter and directed to the chloroplast with the addition of a transit peptide at the N-terminus, showed enhanced tolerance to NaCl and drought stress at the whole-plant level (Ahmad *et al*., 2008). While not yet tested under field conditions, greenhouse testing with transgenic potato plants having relatively low levels of GB (0.9–1.4 μmol/gFW) showed greater dry weight accumulation after recovery from 150 mM NaCl treatment and water withholding stress treatments. Recently, wheat plants overexpressing a *BADH* gene, encoding betaine aldehyde dehydrogenase (BADH), were shown to be more tolerant to drought and heat, by improving the photosynthesis capacity of flag leaves (Wang *et al*., 2010).

4. Trehalose

Trehalose (α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside) is a nonreducing disaccharide composed of two molecules of glucose that functions as a compatible solute in the stabilization of biological structures under abiotic stress in bacteria, fungi and invertebrates (Goddijn and van Dun, 1999).

Trehalose is not thought to accumulate to detectable levels in most plants, with exception of the desiccation-tolerant ''resurrection plants''. However, there is thought to be a signalling role for trehalose at least in part through its inhibition of *SNF-1*-related kinase (*SnRK1*), which results in an up-regulation of biosynthetic reactions supporting photosynthesis and starch synthesis, among others (reviewed by Iturriaga *et al*., 2009). Transgenic tomatoes (*Solanum lycopersicum*) overexpressing the yeast trehalose-6-phosphate synthase (*TPS1*) gene (under control of 35S promoter) showed higher tolerance to salt, drought and oxidative stresses (Cortina and Culiáñez-Macià, 2005). The transgenic plants exhibited pleiotropic changes such as thick shoots, rigid dark-green leaves, erected branches and an aberrant root development and higher chlorophyll and starch content compared to wild-type plants. The alteration of soluble carbohydrate content suggests that the stress tolerance phenotype in trehalose genetically engineered plants could be partly due to modulation of sugar sensing and carbohydrate metabolism (Fernandez *et al*., 2010). In rice, the overexpression of a synthetic fusion of *E. coli* trehalose biosynthetic genes (*otsA* and *otsB*), under the control of tissue-specific (rbcS) and rice stress-dependent promoter ABA-inducible), resulted in sustained plant growth, less photo-oxidative damage and more favourable mineral balance under salt and drought stress conditions. The transgenic rice plants accumulate up to 3–10 times more trehalose than the wild-type plants (Garg *et al*., 2002). A similar fusion construct was made with the constitutive promoter maize ubiquitin, and used to transform rice (Jang *et al*., 2003). Incredibly, the transgenic rice accumulated up to 1000 μg/g FW trehalose, which was attributed to the increased efficiency of the fusion protein over two separate enzymes (Jang *et al*., 2003). Even more surprising was the absence of abnormal developmental and morphological phenotypes, given the high level of trehalose and the occurrence of such deleterious phenotypes in Arabidopsis, potato and tobacco (Goddijn and van Dun, 1999). Jang *et al*. (2003) suggested that the fusion protein would reduce the amount of the trehalose-6-phosphate intermediate, which is the metabolite responsible for signalling cytosolic carbon status and regulation of chloroplastic starch synthesis (reviewed by Paul *et al*., 2008). However, constitutive expression of such fusion proteins in potato (Jang *et al*., 2003) and alfalfa (Suarez *et al*., 2009) results in a range of stunted plant growth phenotypes. It may be the case that sensitivity to trehalose and the synthetic pathway intermediates are different for monocots and dicots. The use of inducible promoters has been an approach that appears to circumvent the deleterious effects of trehalose synthesis and accumulation in alfalfa (Suarez *et al*., 2009). A fusion of yeast trehalose biosynthetic genes, *TPS1* and *TPS2*, was driven either by the constitutive strong promoter *35S* or by the drought-inducible

promoter *rd29A*. Stunting of growth in the absence of stress was apparent for the alfalfa plants harbouring the constitutive expression of the fusion gene, but was not apparent for plants with the inducible construct. Both rice and alfalfa were tested in controlled growth conditions for tolerance to water and salinity stresses and were found to outperform the wild-type controls (Jang *et al*., 2003; Suarez *et al*., 2009). Though promising as tools for the application to abiotic stress tolerance in agriculture, we are not aware of field trials or testing of this technology as yet.

5. Osmotin genes

Osmotin is a stress-responsive multifunctional 24-kDa protein with roles in plant response to fungal pathogens and osmotic tolerance. Overexpression of a heterologous osmotin-like protein (under control of 35S) in potato (*S. tuberosum*) improved tolerance to salinity stress (Evers *et al*., 1999). The tobacco osmotin gene (driven by the 35S promoter) was transformed into tomato and was reported to enhance tolerance to salt and drought stresses (Goel *et al*., 2010). Estimation of several physiological traits such as RWC, chlorophyll, leaf proline, leaf expansion and plant height was observed in transgenic lines as compared to the wild-type plants. Yield of potted plants grown in the greenhouse showed a dramatic advantage for the transgenic osmotin tomatoes after recovery from 150 mM NaCl treatment for 3 weeks. Strawberry (Fragaria × ananassa Duch) plants overexpressing osmotin gene of *Nicotiana tabacum* (driven by the 35S promoter) showed increased accumulation of proline and higher chlorophyll content compared with wild-type plants (Husaini and Abdin, 2008). Under salinity stress conditions, transgenic plants perform better than the wild-type control plants; however, under normal conditions, growth rate was slower.

B. GENES FOR MITIGATING OXIDATIVE DAMAGE

Another physiological and biochemical cellular component common to a suite of abiotic stresses including drought and salt stress is oxidative stress. Oxidative stress involves the generation of ROS during stress. The most common ROS are hydrogen peroxide (H_2O_2), superoxide, the hydroxyl radical and singlet oxygen. Under normal conditions, ROS are continuously produced through cellular metabolism and plant cells are well equipped with antioxidants and scavenging enzymes to keep their levels low (Jaspers and Kangasjärvi, 2010). Under stress conditions, increased ROS production results from an increased production of superoxide due to reduced CO_2 availability and the over reduction of the photosynthetic electron transport chain. Increased photorespiration also generates more H_2O_2, which, if not

adequately balanced by scavenging molecules and enzymes, can lead to further generation of ROS via lipid peroxidation. Oxidative damage is believed to be a consequence of inadequate ROS scavenging, which might be mitigated by the inducible or constitutive overexpression of enzymes that can reduce ROS under stress.

McKersie *et al.* (1996) reported that alfalfa constitutively expressing a tobacco *MnSOD* directed at either chloroplasts or mitochondria had improved survival and yield over 3 years of field trials, relative to the untransformed control plants. Increased SOD activity in the transgenic plants was accompanied by increased photosynthetic efficiency (F_v/F_m) and shoot regrowth during water-deficit stress treatments in controlled growth conditions. A wheat mitochondrial *MnSOD*, regulated by either constitutive (35S) or the stress-inducible (COR78) promoter, was used to transform canola (Gusta *et al.*, 2009). In both constitutive and stress-inducible *MnSOD* transgenic canola plants, SOD activity was increased by 25–45% over that in control plants, and survival and recovery from water withholding was greater. Field experiments showed that the *MnSOD* transgenic canola had superior germination and emergence, as well as earlier time to flowering; yield testing is to occur in future trials using these transgenic plants (Gusta *et al.*, 2009).

Improving the antioxidant capacity in plants has also been accomplished indirectly, with the overexpression of proteins involved in signalling upstream of ROS scavenging. Recently, a rice gene coding for a receptor-like kinase (RLK) was reported to improve the drought and salt tolerance (DST) of transgenic plants overexpressing the RLK (*OsSIK1*) (Ouyang *et al.*, 2010). The transgenic plants had higher activity of peroxidases, SOD and catalase (CAT) during stress, as well as reduced stomatal density. The improved tolerance to osmotic stress treatments (using very high concentrations of NaCl or water withholding) of the transgenic plants may be attributed to reduced stomatal density as much as to the increased antioxidant activity (Ouyang *et al.*, 2010). What cannot be determined from the data provided by Ouyang *et al.* (2010) is whether the changes in antioxidant activity are dependent on the changes in stomatal density, or vice versa, or if the two are independent. Overexpression of the *Arabidopsis* gene *GF14λ*, encoding a 14-3-3 protein that interacts with proteins involved in numerous metabolic processes, including antioxidant activity, demonstrated a "stay-green" phenotype and improved tolerance to moderate water stress in cotton (Yan *et al.*, 2004).

CAT is one of the major endogenous enzyme antioxidants. It catalyses H_2O_2 decomposition and is up-regulated at the transcriptional level upon exposure to high salinity stress. In cyanobacteria, introduction of a *CAT* gene of *E. coli*, *kat*E, was found to reduce ROS production under salt stresses

and confer salt tolerance (Kaku *et al*., 2000). Transgenic rice plants' constitutive overexpression of the *kat*E gene showed improved growth under salinity stress (Nagamiya *et al*., 2007). Plants were evaluated at the vegetative and reproductive stages for salt tolerance. T_1 seedlings were soaked in 0, 50, 100, 150, 200, 250, 300, 400, 500 or 600 mM NaCl and surviving rate (green tissue) was recorded. In addition, flowering T_1 transgenic lines grown under normal conditions were soaked in 250 mM NaCl solution for 14 days. The transgenic rice seedlings showed improved growth under high salinity (250 mM), and were able to form flower and produce seeds in the presence of 100 mM NaCl. CAT activity in the transgenic rice plants was 1.5- to 2.5-fold higher than in nontransgenic rice plants.

Pyramiding of ROS-scavenging genes may provide more effective tolerance of oxidative stress resulting from drought or salinity. Two genes (from *Suaeda salsa*) coding *GST* (glutathione *S*-transferase, EC 2.5.1.18) and *CAT* (EC 1.11.1.6) were transformed under the control of a constitutive promoter into rice plants. Transgenic rice seedlings showed a marked enhanced tolerance to salinity and oxidative stresses (Zhao and Zhang, 2006). Expression of three antioxidant enzymes, copper zinc superoxide dismutase (CuZnSOD), ascorbate peroxidase (*APX*) and dehydroascorbate (*DHA*) reductase (*DHAR*), in tobacco chloroplasts resulted in a higher tolerance to oxidative stress induced by salinity stress (Lee *et al*., 2007). These studies suggested that the simultaneous expression of multiple antioxidant enzymes could be more effective than the expression of single genes for developing transgenic plants with enhanced tolerance to abiotic stresses.

ROS, and H_2O_2 in particular, also play a role in the signalling pathways involved in the adaptation to the stress response (Miller and Mittler, 2006). Samis *et al*. (2002) combined the mitochondrial and chloroplastic SOD expression by crossing the transgenic alfalfa plants that had shown superior field performance in earlier trials (McKersie *et al*., 1996). The plants carrying both constructs had higher SOD activity than either of the sibling controls that carried only one of the *MnSOD* transgenes, but biomass production in the field of the plants carrying both genes was reduced, relative to the single gene siblings (Samis *et al*., 2002). The authors suggested that there might be an optimum level of SOD activity, above which processes such a H_2O_2 signalling might be impaired. The use of inducible promoters for driving the expression of antioxidant enzymes is also being tested as an alternative to constitutive expression. In rice, transformation of chloroplast-targeted manganese superoxide dismutase isolated from pea (*MnSOD*) under the control of an oxidative stress-inducible *SWPA*2 promoter resulted the improvement of indicators of oxidative stress tolerance in T_1 plants tested in the greenhouse (Wang *et al*., 2005a).

C. GENES FOR IONIC BALANCE

In most saline soils, Na^+ and Cl^- are the predominant ions in the soil solution. At sufficiently high concentrations, both ions contribute to an unfavourable osmotic gradient between the soil solution and the plant roots. Both ions also cause ion-specific toxicity when accumulated in salt-sensitive plants. And while it is clear that the exclusion of Na^+ or Cl^-, or both, is correlated with improved salinity tolerance in some species (and the accumulation of both with others), the state knowledge of Na^+ transport mechanisms is more advanced than that for Cl^- transport (Teakle and Tyerman, 2010).

1. Decreasing Na^+ uptake

In both glycophytes and halophytes, the net uptake of sodium into the roots is the sum of sodium influx and efflux. The negative electrical membrane potential difference at the plasma membrane of root cells (− 140 mV) favours the passive transport of sodium into root cells, and especially so when sodium concentrations increase in the soil solution. The entry of sodium into root cells is mediated by uniporter or ion channel-type transporters, like *HKT*, *LCT1* and *NSCC* (reviewed in Plett and Moller, 2010). The reduction of Na^+ uptake might be accomplished by decreasing the number or activity of these transporters in the roots. Reduction of *TaHKT2;1* expression in wheat by antisense suppression resulted in lower net sodium uptake of transgenic roots and higher fresh weight of plants grown under salinity stress in controlled growth conditions (Laurie *et al.*, 2002). Similarly, *Arabidopsis* T-DNA knockout mutants of *AtCNGC3*, a cyclic nucleotide gated channel which catalyses Na^+ uptake, had lower net influx of Na^+ and were more tolerant to salinity at germination (Gobert *et al.*, 2006).

The efflux of sodium from the roots is an active process, which is presumed to be mediated by plasma membrane Na^+/H^+ antiporters. These secondary transporters use the energy of the proton gradient across the plasma membrane to drive the active efflux of sodium from the cytosol to the apoplast. The Na^+/H^+ antiporter, *SOS1* (identified in a mutant screen as salt overly sensitive 1), is the only Na^+ efflux protein at the plasma membrane of plants characterized so far. The overexpression of *AtSOS1*, a plasma membrane-bound Na^+/H^+ antiporter, improved the ability of the *Arabidopsis* transgenic plants to grow in the presence of high NaCl concentrations (Shi *et al.*, 2003). And the rice orthologue, *OsSOS1*, is able to complement the *Arabidopsis sos1* mutant (Martinez-Atienza *et al.*, 2007). The *SOD2* (*Sodium2*) gene was identified in yeast, *Schizosaccharomyces pombe*, as a Na^+/H^+ antiporter on the plasma membrane involved in salt tolerance. Transformation of rice with

the *SOD2* gene (under 35S promoter) resulted in accumulation of more K^+, Ca^{2+}, Mg^{2+} and less Na^+ in the shoots compared with wild type (Zhao *et al.*, 2006b). The transgenic rice plants were able to maintain higher photosynthesis level and root proton exportation capacity, whereas reduced ROS generation. Although yield data were not reported, the trials were conducted outdoors, which is the closest to field level study of a crop plant for this approach in the literature.

2. Decreasing root to shoot translocation of Na^+

The accumulation of sodium in shoots occurs via the translocation of sodium from the roots along the transpirational stream. The removal of sodium from the xylem, which reduces the rate of sodium transfer to the shoot tissue, has been shown to be mediated by members of the *HKT* gene family (reviewed in Plett and Moller, 2010). *AtHKT1;1* in *Arabidopsis*, *OsHKT1;5* in rice, and *HKT1;4* in wheat are all critical in reducing Na^+ shoot concentrations by transporting Na^+ from the xylem into the root stele (reviewed in Hauser and Horie, 2010). One strategy for improving salinity tolerance is to increase the expression of such genes to further reduce sodium concentrations in the xylem (Plett *et al.*, 2010). The overexpression of *AtHKT1;1* under the control of the constitutive promoter CaMV35S leads to increased salt sensitivity, presumably because Na^+ fluxes are increased in inappropriate cells and tissues (Moller *et al.*, 2009). However, when expressed under the control of a promoter directing expression in root epidermal and cortical cells, both in rice and in *Arabidopsis*, HKT1;1 overexpression causes an increase in root cortical sodium, a decrease in shoot sodium and a higher accumulation of fresh weight during the course of the experiment (Plett *et al.*, 2010).

3. Sequestering Na^+

The accumulation of Na^+ ions into vacuoles through the operation of a vacuolar Na^+/H^+ antiporter provided an efficient strategy to avert the deleterious effect of Na^+ in the cytosol and maintain osmotic balance by using Na^+ (and Cl^-) accumulated in the vacuole to drive water into the cells (Apse *et al.*, 1999; Apse and Blumwald, 2002). Transgenic plants overexpressing an *Arabidopsis* vacuolar Na^+/H^+ antiporter, *AtNHX1*, exhibited improved salt tolerance in *Brassica napus* (Zhang *et al.*, 2001), tomato (Zhang and Blumwald, 2001), cotton (He *et al.*, 2005), wheat (Xue *et al.*, 2004), beet (Yang *et al.*, 2005) and tall fescue (Zhao *et al.*, 2007). The transformation of an orthologue gene (*AgNHX1*) from halophytic plant *Atriplex gmelini* into rice improved salt tolerance of the transgenic rice (Ohta *et al.*, 2002). Maize plants overexpressing rice *OsNHX1* gene accumulated more biomass, under 200 mM NaCl in greenhouse (Chen *et al.*, 2007). Moreover, under field trail

conditions, the transgenic maize plants produced higher grain yields than the wild-type plants. Transformation of another Na^+/H^+ antiporter family member, *AtNHX3* (from *Arabidopsis*), in sugar beet (*Beta vulgaris* L.) resulted in increased salt accumulation in leaves, but not in the storage roots, with enhanced constituent soluble sugar contents under salt stress condition (Liu *et al*., 2008).

The introduction of genes associated with the maintenance of ion homeostasis in halotolerant plant into crop plants confirmed salinity tolerance. The yeast gene *HAL1* was introduced into tomato (Gisbert *et al*., 2000), watermelon (*Citrullus lanatus* (Thunb.); Ellul *et al*., 2003) and melon (*Cucumis melo* L.; Bordas *et al*., 1997), which confirmed higher level of salt tolerance, with higher cellular K^+ to Na^+ ratio under salt stress. Likewise, the introduction of the yeast *HAL2* gene into tomato resulted in improved root growth under NaCl conditions, contributing to improved salt tolerance (Arrillaga *et al*., 1998). Overexpression of *HAL3* (from *S. cerevisiae*) homologue *NtHAL3* in tobacco increased proline biosynthesis and the enhancement of salt and osmotic tolerance in cultured tobacco cells (Yonamine *et al*., 2004).

The electrochemical gradient of protons across the vacuolar membrane is generated by the activity of the vacuolar H^+-translocating enzymes, H^+-ATPase and H^+-pyrophosphatase. Increasing vacuolar H^+ pumping might be required to provide the additional driving force for vacuolar accumulation via sodium/proton antiporters. A gene coding for a vacuolar H^+-pyrophosphatase proton pump (*AVP1*) from *Arabidopsis* was overexpressed in tomato (Park *et al*., 2005), cotton (Pasapula *et al*., 2011) and rice (Zhao *et al*., 2006a) and induced improved growth during drought and salt stress. Interestingly, the overexpressed *AVP1* resulted in a more robust root system which could possibly improve the plants ability to absorb more water from the soil (Pasapula *et al*., 2011).

D. REGULATORY AND SIGNALLING GENES

1. DREB/CBF

Dehydration-responsive element (DRE)/C-repeat (CRT) was identified in *Arabidopsis*, a *cis*-acting element regulating gene expression in response to dehydration (drought, salinity and cold stress; Baker *et al*., 1994; Yamaguchi-Shinozaki and Shinozaki, 1994). Several DRE-binding proteins (DREB)/CRT-binding factor (CBF) were isolated and identified as key players in dehydration (drought, salinity and cold stress) responsive gene expression (Yamaguchi-Shinozaki and Shinozaki, 1994). Using transgenic approaches, the DREB/CRF signalling pathway is one of the most studied in

numerous plant species. The overexpression of these genes activated the expression of many downstream genes with the DRE elements in their promoters, and the resulting transgenic plants showed improved stress tolerance (Agarwal *et al*., 2006). In *Arabidopsis*, two classes of *DREBs* were isolated: *DREB1* expression was found to be highly up-regulated during cold stress, and *DREB2* expression was responsive to drought and salinity.

Transgenic rice lines overexpressing *OsDREB1A* and *OsDREB1B* under the control of a constitutive ubiquitin promoter showed more tolerance to drought and salinity conditions (in term of survival rate); however, under normal conditions, the transgenic lines showed reduced growth (Ito *et al*., 2006). In this experiment, rice seedlings (17–19 days) that were grown in very small pots under continuous light were exposed to high salinity (250 mM NaCl, 3 days) or drought (withholding water for 9 days), followed by re-watering. While drought associated traits (as proline) were measured, no data on yield were reported. Further, the transgenic rice plants overexpressing *OsDREB1* or *DREB1* showed growth retardation under normal growth conditions (Ito *et al*., 2006). Constitutive (35S promoter) overexpression of *AtDREB1A* in transgenic rice resulted in increased tolerance to drought (Oh *et al*., 2005). Transgenic plants were grown in small pots for 4 weeks and exposed to 4 days of drought followed by re-watering. Survival rate was measured. In contrast to previously reported reduction in growth, in this experiment, neither growth inhibition nor visible phenotypic alterations were noted, despite constitutive expression of *DREB* gene. Overexpression of two other *OsDREB* genes, *OsDREB1G* and *OsDREB2B*, also showed significantly improved survival rate under water-deficit stress in rice seedling (Chen *et al*., 2008).

Overexpression of *DREB1A/CBF3*, driven by the stress-inducible *RD29A* promoter in bread wheat, improved drought tolerance in greenhouse (Pellegrineschi *et al*., 2004). Small seedlings (six leaf stage) grown in pots (5×5 cm) of T_2 plants were exposed to 10–12 days of withholding water and re-watering. Survival rate was used to measure tolerance, but no yield was reported. Transformation of *AtDREB1A* into peanut (*Arachis hypogaea* L.) was reported to improve TE under water-limited conditions (Bhatnagar-Mathur *et al*., 2007). T_3 plants were grown in pots and water stress was applied after 28 days. Interestingly, most transgenic events had higher TE than the wild type under well-watered conditions, and one event showed 40% improvement than wild-type plants under water stress. While P_{35S}*::DREB1A* plants exhibited stunted growth even under control conditions, the transgenic P_{rd29A}*::DREB1A* peanut plants did not show any growth retardation (Bhatnagar-Mathur *et al*., 2007). In contrast, transgenic potato expressing the same P_{rd29A}*::DREB1A* gene showed growth retardation (Behnam *et al*.,

2006). Overexpression of a soybean DREB orthologue, *GmDREB1*, in alfalfa (*Medicago sativa* L.) plants under the control of *Arabidopsis Rd29A* promoter was tested in greenhouse pot experiment (Jin *et al*., 2010). Four-week-old plants were watered with NaCl solution (0, 100, 200, 300 and 400 mM) for 60 days at 5-day intervals. The transgenic lines showed improved tolerance to salinity in terms of survival as compared with wild-type plants; however, no biomass production data were reported.

Tomato plants overexpressing the *AtDREB1B/CBF1* under constitutive 35S promoter showed a higher level of proline, as compared with the wild-type plants grown under normal or water-deficit conditions (Hsieh *et al*., 2002). T_1 plants, grown in controlled greenhouse conditions, were exposed to water deficit (after 3 months) for 3 weeks and survival rate was calculated. However, severely reduced growth was found in the transgenic tomato plants. Further, the transgenic tomato plants showed a decrease in fruit, seed number, and fresh weight as compared with wild-type plants under normal conditions (Hsieh *et al*., 2002).

HARDY (*HRD*), a gene encoding AP2/ethylene response factor (ERF)-like transcription factor (TF) that belongs to the BREB/CRB family, was identified as a gain-of-function mutation in *Arabidopsis* (Karaba *et al*., 2007). The *hrd* mutant showed abnormally dense root system, increased mesophyll cell layer and enhanced tolerance to drought and salinity (Karaba *et al*., 2007). Overexpressing of the *HRD* gene in rice resulted in increased water-use efficiency (WUE) in controlled greenhouse conditions. Rice plants of T_3 generation lines were grown in pots under 100% and 70% field capacity. Under control conditions, the transgenic lines showed no growth reduction, an increase in leaf biomass and an increase in bundle sheath cells. The *HRD* expression in rice caused significant increases of instantaneous and whole-plant WUE in well-watered and drought conditions, with a very remarkable increase of 100% in absence of drought and a consistent 50% increase under drought stress (Karaba *et al*., 2007). The efficiency of this approach still needs to be tested for yield under greenhouse and field conditions.

2. Protein kinase

Several studies have suggested that many protein kinases are involved in drought resistance, among them, members of the calcium-dependent protein kinase (CDPK), calcineurin B-like protein-interacting protein kinase (CIPK) and mitogen-activated protein kinase (MAPK) families. Ca^{2+} cytosolic levels increase rapidly in plant cells in response to environmental stress, including drought and salinity (Sanders *et al*., 1999). This Ca^{2+} influx is likely to be mediated by a combination of protein phosphorylation/dephosphorylation cascades involving members of the CDPK family. In rice, overexpression of

OsCDPK7 (under the control of the 35S promoter) resulted in increased seedling recovery rate after a salt treatment (Saijo *et al.*, 2000). T_1 seedlings (10 days) old treated with 150/200 mM NaCl and transferred again to a normal nutrient solution. The transgenic plants showed normal development and yield. It was suggested that *OsCDPK7* underwent post-translational regulation, since the presence of *OsCDPK7* was not sufficient to induce expression of stress-associated target genes. Overexpression of three *CIPK* genes (*OsCIPK03*, *OsCIPK12* and *OsCIPK15*) enhanced tolerance to cold, drought and salt stress, respectively, in transgenic rice (Xiang *et al.*, 2007). Overexpression of a MAPK family gene *OsMAPK5a* in rice leads to increased *OsMAPK5a* kinase activity and enhanced tolerance to drought and salt stresses (Xiong and Yang, 2003). Overexpression of another rice MAPK family, *OsMAPK44*, resulted in increased tolerance to salt stress (Jeong *et al.*, 2006). Recently, overexpression in rice of *DSM1* (*drought-hypersensitive mutant1*), a putative MAPK kinase kinase (*MAPKKK*) gene, increased the tolerance of the seedlings to dehydration stress (Ning *et al.*, 2010). It was suggested that *DSM1* might be a novel MAPKKK functioning as an early signalling component in regulating mechanisms of ROS scavenging in rice

Expression of a *MAPKKK* gene was shown to activate an oxidative signal cascade and led to the tolerance to environmental stress in transgenic tobacco. The catalytic domain of *Nicotiana* protein kinase 1 (*NPK1*) activated a bypass of BCK1-mediated signal transduction pathway in yeast, which was found to be conserved among different organisms (Banno *et al.*, 1993). *NPK1* was reported to be upstream of oxidative pathways inducing expression of heat shock proteins and GST (Kovtun *et al.*, 2000). Constitutive overexpression of the tobacco MAPKKK in maize enhanced the drought tolerance of the transgenic plants (Shou *et al.*, 2004). Under drought conditions, the transgenic plants maintained significantly higher photosynthesis rates and kernel weight as compared with wild-type plants. However, the effect of *NPK1* on yield components was less apparent.

3. Nuclear factor Y-B subunit

In *Arabidopsis*, *AtNF-YB1*, a nuclear factor Y (NF-Y complex), was found to mediate transcriptional control through CCAAT DNA elements and confer tolerance to abiotic stress when constitutively expressed in *Arabidopsis* (Nelson *et al.*, 2007). NF-Y is a conserved heterotrimeric complex consisting of NF-YA (*HAP2*), NF-YB (*HAP3*) and NF-YC (*HAP5*) subunits (Mantovani, 1999). An orthologous *NF-YB* gene was found in maize with similar response to drought. Transgenic maize lines constitutively overexpressing *ZmNF-YB2* showed improved drought tolerance under field conditions (Nelson *et al.*, 2007). Under water-limited conditions, transgenic plants

show tolerance to drought based on grain yield and on the responses of a number of stress-related parameters, including chlorophyll content, stomatal conductance, leaf temperature, reduced wilting and maintenance of photosynthesis.

4. NAC proteins

Several NAC [*NAM* (No Apical Meristem), *ATAF1-2* and *CUC2* (cup-shaped cotyledon)] domain proteins, which are one of the largest plant TF families (Riechmann *et al.*, 2000), have been reported to be associated with abiotic stresses. Of the 140 putative rice *NAC* genes, the expression of 40 *NAC* genes increased with drought or salinity stress (Fang *et al.*, 2008). Twenty of these genes were induced at least twofold with stress treatment and a majority of these form the group III clade of *NAC* genes, called SNAC or the stress-responsive NACs (Fang *et al.*, 2008). The overexpression of a stress-responsive gene *SNAC1* (*STRESS-RESPONSIVE NAC 1*) in rice significantly enhanced the drought tolerance (22–34% increase in seed setting) of the transgenic plants under severe water-stress conditions at the reproductive stage in the field (Hu *et al.*, 2006). Biomass accumulation at the vegetative stage was improved in rice plants overexpressing *SNAC1* under both salinity and drought stress (Hu *et al.*, 2006). The phenotype was partially attributed to increased stomatal closure and ABA sensitivity in the transgenic plants (Hu *et al.*, 2006). Overexpression of *OsNAC45* in rice improved tolerance to drought and salt treatments as discussed in more detail in Section 5 (LEA gene expression). Recently, the overexpression of *OsNAC10* in rice, under the control of the constitutive promoter *GOS2* and the root-specific promoter *RCc3*, improved tolerance to drought and salinity of the transgenic plants at the vegetative stage. However, only the root-specific overexpression of *OsNAC10* (*PRCc3*::*OsNAC10*) significantly enhanced drought tolerance at the reproductive stage, increasing grain yield (25–42%) in the field under drought conditions (Jeong *et al.*, 2010). The yield advantage in the *PRCc3::OsNAC10* plants was attributed to the larger root diameter in these plants, which were approximately 20% larger than both the wild type and *PGOS2::OsNAC10* plants (Jeong *et al.*, 2010).

5. Increasing LEA gene expression

Late embryogenesis abundant (LEA) proteins are low-molecular weight proteins that, in molar excess, and synergistically with trehalose, prevent protein aggregation during desiccation or water stress (Goyal *et al.*, 2005). The overexpression of *OsLEA3-1* under the control of a strong constitutive

promoters (35S and Actin1) and a stress-inducible promoter (*HVA1*-like promoter isolated from the upland rice IRAT109) in a drought-sensitive Japonica (lowland) rice resulted in improved drought tolerance (Xiao *et al.*, 2007). Transgenic rice plants with 35S and *HVA1*-like promoters displayed improved yields when grown in PVC pipes and under field conditions without yield penalties. The improved yield under drought conditions was primarily due to improved spikelet fertility under stress (Xiao *et al.*, 2007). Spring wheat lines expressing the barley *HVA1* gene (under the control of the ubiquitin promoter) tested across multiple years and locations in dry land cultivation yielded better than the untransformed controls (Bahieldin *et al.*, 2005). In an earlier study, wheat lines were taken to the T_4 generation and compared to newly developed lines using the same construct (Sivamani *et al.*, 2000). Yields of the transgenic *HVA1* lines were not significantly different than the wild-type and non-transformed control lines under irrigated conditions; however, under dry land conditions, the *HVA1* lines produced 7–35% more yield. The yield under water stress was correlated with the amount of *HVA1* protein detected in leaf extracts of the transgenic lines (Bahieldin *et al.*, 2005).

Increasing LEA gene expression under stress, and presumably LEA protein abundance, has also been accomplished indirectly, with the overexpression of *NAC* genes. LEA gene expression under stress may account for improved tolerance to drought and/or salinity stress in plants overexpressing *OsNAC5* and *OsNAC6* (Takasaki *et al.*, 2010), and *OsNAC45* (Zheng *et al.*, 2009). The overexpression of the stress-responsive proteins *OsNAC5* and *OsNAC6* resulted in enhanced stress tolerance by up-regulating the expression of stress-inducible rice genes such as *OsLEA3*, although the effects of these proteins on plant growth were different. However, the tolerance of the *UBIpro::OsNAC5* transgenic rice plant to salinity was measured in 2-week-old transgenic plants that were grown in 250 mM NaCl for 3 days and then grown for 30 days under normal conditions (i.e. survival rate), and no yield data were presented. The overexpression of *OsNAC45* leads to increased *LEA3* and *PM1* gene expression Zheng *et al.* (2009). Preliminary assays of the response to drought stress showed that young seedlings overexpressing *OsNAC45* had improved survival rates, relative to wild-type controls, 10 days after recovery from a 9.5-h period of root drying (Zheng *et al.*, 2009). Although these hydroponic assays on T_2 generation transgenics are not sufficient to assess the response of the transgenic plants to drought under field conditions, the increased expression of *LEA3*, taken together with the results of Xiao *et al.* (2007), provides an incentive to take later generations of these transgenic rice plants to field testing.

6. Aquaporins

Aquaporins are intrinsic membrane proteins that mediate the transport of water, small neutral solutes and CO_2 (Tyerman *et al.*, 2002). The regulatory role of aquaporins in cellular water transport had been demonstrated (Knepper, 1994). The stress-induced expression of the aquaporin, RWC3, a member of the plasma membrane intrinsic protein 1 (*PIP1*) subfamily, resulted in improved water status of lowland rice (Lian *et al.*, 2004). Four-week-old plants grown hydroponically in nutrient solution were exposed to a osmotic shock treatment of 20% polyethylene glycol (PEG) 6000 for 10 h (Lian *et al.*, 2004). However, transgenic tobacco plants constitutively expressing the *Arabidopsis* plasma membrane aquaporin *PIP1b* displayed enhanced growth vigour under well-watered conditions, but the transgenic plants wilted rapidly during water stress (Aharon *et al.*, 2003). A comparison between the results obtained by overexpressing PIP-type aquaporins in tobacco and rice is difficult. In addition to the difference between the constitutive (tobacco) and stress-inducible (rice) expression, two different treatments (osmotic shock vs. gradual dehydration) were applied. Further, transgenic rice plants constitutively overexpressing a barley *HvPIP2;1* (a plasma membrane aquaporin) showed more sensitivity (reduction in growth rate) to salinity stress (Katsuhara *et al.*, 2003). T_2 rice plants were grown hydroponically and exposed to 100 mM NaCl after 4 weeks. Although the growth of transgenic rice plants was similar to that of control plants under normal conditions, the growth of the transgenic plants was greatly inhibited and eventually withered and died under a salinity treatment (Katsuhara *et al.*, 2003).

Recently, tomato plants' constitutive overexpressing of atonoplast *SlTIP2;2* showed increased cell water permeability and whole-plant transpiration (Sade *et al.*, 2009). The expression of *SlTIP2;2* resulted in increased transpiration under normal growth conditions, limited transpiration reduction under drought and salt stresses and also accelerate transpiration recovery after stress Two field experiments of F_1 hybrids of transgenic MicroTom and M82 plants were conducted in commercial net-house. Salinity was applied by irrigation with saline water (80–200 mM NaCl) and in parallel, the same F_1 hybrids were grown under well-watered and water-limited conditions. Transgenic plants showed significant increases in fruit yield, harvest index and plant mass relative to the control under both normal and water-stress conditions (Sade *et al.*, 2009). It was postulated that overexpression of the *SlTIP2;2* could bypass the stress-induced down-regulation of the endogenous *aquaporins* genes of the tonoplast and thus prevent the slowdown of tonoplast osmotic water permeability (Sade *et al.*, 2009).

7. Hormonal homeostasis and abiotic stress

Hormones play a major role in stress signalling. One of the fast responses of plants to soil water stress is the accumulation of ABA in the roots (Thompson *et al*., 2007), which is transported through the xylem to the shoot (Wilkinson and Davies, 2010) causing stomatal closure reducing water loss via transpiration (Schroeder *et al*., 2001) and eventually restricting cellular growth. ABA can also be synthesized in leaf cells and transported through the plant (Wilkinson and Davies, 2010). In *Arabidopsis*, a large number of genes associated with ABA metabolic pathway have been characterized, and genes coding ABA receptors and downstream signal relays have been recently reviewed (Cutler *et al*., 2010; Huang *et al*., 2008). However, in crop plants, only one gene involved in ABA metabolism (*LOS5/ABA3*, a key enzyme in the last step of ABA biosynthesis) has been manipulated in rice with enhanced drought tolerance (Xiao *et al*., 2009). *LOS5* gene was overexpressed under the control of constitutive or drought-inducible promoters and tested in the field. Plants were grown under normal conditions for 1 month and then water was stopped during the initiation of panicle development. The improved yield of the transgenic lines under field conditions was a result of a significant increase in the spikelet fertility (Xiao *et al*., 2009). While many reports on the development of transgenic plants with improved tolerance to drought or salinity by manipulating the expression of stress-related genes in laboratory or greenhouse conditions are available, only few studies were tested under natural field condition. In tomato, the constitutive overexpression of *LeNCED1* (drought-inducible and a rate-limiting enzyme for ABA biosynthesis) resulted in increased ABA accumulation (Thompson *et al*., 2007). Plants were grown to a four- to five-leaf stage in a controlled environment cabinet in 500-mL free-draining pots and exposed to drought treatment. The constant elevation in ABA level resulted in physiological and morphological changes in the transgenic plants. Under well-watered conditions, plants showed reduction in assimilation rates, leaf flooding and chlorosis, but under water-deficit conditions, these effects were insufficient to reduce biomass production, presumably because of counteracting positive effects on leaf expansion through improvements in water status, turgor and antagonism of epinastic growth (Thompson *et al*., 2007).

Cytokinins (CKs) have been found linked to a variety of abiotic stresses (Hare *et al*., 1997). In *Arabidopsis*, examination of public microarray expression data revealed many genes encoding proteins associated with CK signalling pathways that were differentially affected by various abiotic stresses (reviewed by Argueso *et al*., 2009). CK is an antagonist to ABA, and the exposure of plants to drought results in decreased levels of CK. Elevated CK levels could promote survival under water-stress conditions, inhibit leaf

senescence and increased levels of proline (Alvarez *et al.*, 2008). The manipulation of endogenous CK levels was effective in delaying senescence (Gan and Amasino, 1997). Isopentenyltransferase (*IPT*, mediating the rate-limiting step in CK biosynthesis) has been overexpressed in several plant species. However, drought tolerance varied with the type of promoter used to drive *IPT* expression (Ma, 2008). Transgenic tobacco (*N. tabacum*) expressing the *IPT* gene under control of a drought-induced promoter (*SARK*, senescence-associated receptor kinase) resulted in increased drought tolerance, photosynthetic capacity and yield (Rivero *et al.*, 2007, 2009, 2010). Recently, we have showed that transgenic rice plants expressing *PSARK::IPT* resulted in enhanced drought tolerance and superior yields (Peleg *et al.*, 2011). Transgenic Cassava (*Manihot esculenta* Crantz), expressing *IPT* under control of a senescence-induced promoter, *SAG12*, were tested for drought tolerance under field conditions (Zhang *et al.*, 2011). The transgenic cassava plants displayed higher tolerance to drought due to the inhibition of stress-induced leaf abscission and fast recovery from stress. Creeping bentgrass (*Agrostis stolonifera*) expressing P_{SAG12}*::IPT* was tested hydroponically using osmotic stress induced by different PEG concentrations (Merewitz *et al.*, 2011). The transgenic plants were able to maintain higher osmotic adjustment, chlorophyll content, WUE and greater root viability under osmotic stress compared with the wild-type plants (Merewitz *et al.*, 2011). However, these results should be taken with caution since the use of PEG to stimulate osmotic stress is artificial, and did not represent the multidimensional response of plants to water deficit under natural conditions.

Jasmonic acid (JA) is involved in plant development and the defence response. Transgenic rice plants overexpressing the *Arabidopsis* JA carboxyl methyltransferase gene (*AtJMT*) under the control of the *Ubi1* promoter showed increased JA levels in panicles (Kim *et al.*, 2009). Plants were grown in the greenhouse and were subjected to 2 weeks of drought before panicle initiation. The *PUbi1::AtJMT* plants resulted in significantly grain yield reduction, due to a lower numbers of spikelets and lower filling rates than wild-type plants (Kim *et al.*, 2009).

Rice plants overexpressing the ERF, *AP37*, under the control of the constitutive promoter *OsCc1*, displayed increased tolerance to drought and high salinity at the vegetative stage (Oh *et al.*, 2009). More importantly, when these transgenic lines were tested in the field, the *POsCc1::AP37* plants showed increased grain yield over controls under severe drought conditions, while no significant differences were noted under well-watered conditions (Oh *et al.*, 2009). Overexpression in rice of another *ERF* gene, a protein *TSRF1* that binds to the GCC box, showed enhanced osmotic and drought tolerance in seedlings (Quan *et al.*, 2010). T_2 rice seedlings (10 days old) were exposed

osmotic shock (20% PEG for 3 days) or withholding water for 6 days followed by recovery under control conditions. Under normal conditions the transgenic *TSRF1* plants did not show any differences in growth or development. In another experiment, 2-week-old seedlings overexpressing *TERF1* (a tomato ERF protein) were exposed to drought by withholding water for 9 days, or salinity by immersing in 200 mM NaCl. The transgenic plants showed improved survival rate after exposure to drought or salinity (Gao *et al.*, 2008). Further study is needed to test the efficiency of this strategy under field experiment and more critical growth phases (i.e. reproductive stage).

Plant hormone crosstalk and the regulation of various hormone-regulated biosynthetic pathways (see Nemhauser *et al.*, 2006) during water stress play important roles in abiotic stress adaptation. The homeostatic regulation of phytohormones could play significant roles in the regulation of source/sink relationships and its manipulation could provide a significant avenue for the development of abiotic stress tolerance in plants.

8. The regulation of the stomatal response to stress

Reducing transpiration rates without affecting CO_2 assimilation would result in increase WUE and may contribute to improve yields. It was postulated recently that reductions in stomata density and stomatal aperture can reduce transpirational water loss while maintaining sufficient CO_2 uptake to sustain biomass and yield under water-deficit conditions (Yoo *et al.*, 2009). There are a handful of examples where the modification of a single gene resulted in reduced stomatal aperture and stomatal density, and consequently increasing WUE (reviewed in Yoo *et al.*, 2009). These modifications also resulted in improved plant resistance to water-deficit stresses like salinity and drought. Some of these modifications have been tested in crop plants and in some cases, under field conditions. *ERA1* is a negative regulator of the ABA response in *Arabidopsis*, and was found in a screen for hypersensitivity of seed germination to ABA (Cutler *et al.*, 1996). *era1* rosettes were slower to wilt under severe water deficit, owing to the smaller stomatal aperture in the mutant plants (Pei *et al.*, 1998). The *ERA1* locus is the beta subunit of farnesyltransferase, which adds a farnesyl group to proteins containing a CaaX motif (Andrews *et al.*, 2010). In *era1* plants, and to a lesser degree in plants expressing a constitutive *AtFTB* (farnesyltransferase B) hairpin construct, growth and development are impaired, owing to the loss (or reduction) of function of FTB in other aspects of plant development, including meristem organization (Bonetta *et al.*, 2000), among others. An agriculturally relevant application FTB down-regulation was accomplished by the use of a stress-inducible promoter, rd29. While early seedling development was impaired in canola plants expressing *Prd29::antiFTB*, yields of the field

grown transgenic plants were no different that wild-type controls under sufficient water conditions (Wang *et al.*, 2005b). Down-regulation of FTB in canola provided improved yield relative to wild-type controls under mild and moderate water-deficit stress conditions in the field (Wang *et al.*, 2005b). The concept of reducing stomatal aperture and transpiration during drought stress was further refined and confirmed by Wang *et al.* (2009) by using the *hydroxypyruvate reductase* (*HPR1*) promoter to drive the expression of an RNAi construct directed against the farnesyltransferase A (*FTA*) subunit. The *HPR1* promoter is up-regulated by drought stress and is preferentially expressed in the shoot tissues. P_{HPR1}::*antiFTA* transgenic canola seedlings were not impaired in early shoot and root growth, as was the case with P_{rd29}::*antiFTB* seedlings, and P_{HPR1}::*antiFTA* plants had no yield drag relative to wild-type controls under water-sufficient conditions in the field (Wang *et al.*, 2009). Under water-deficit conditions, experienced primarily during flowering and pod filling, P_{HPR1}::*antiFTA* plants yielded 14–16% greater seed than wild-type controls, which experienced yield losses of 20% (Wang *et al.*, 2009). Whether this technology can be applied to crops other than canola is yet to be reported. However, the successful application of *SNAC1* overexpression to improving rice yields under drought and salinity stress, by increasing stomatal closure without decreasing CO_2 assimilation, shows the concept viability.

Loss of function of the zinc finger protein *DST* resulted in reduced stomatal aperture and stomatal density, and increased drought and salt tolerance in rice (Huang *et al.*, 2009). While field testing has not been reported for the *dst* plants, under controlled growth conditions, they retained a higher RWC under soil drying conditions and recovered more rapidly on re-watering than the wild-type control plants (Huang *et al.*, 2009). *DST* negatively regulates the expression of hydrogen peroxide scavenging enzymes in guard cells, which balances the ROS signalling for stomatal closure that is propagated through the ABA signal. Therefore, in the *dst* mutant, the ROS signal was less attenuated and stomatal apertures remained smaller than in the wild type. While CO_2 assimilation was not measured, Huang *et al.* (2009) reported that seed yields were not reduced in the *dst* mutant. Genetic modifications, where stomatal aperture and stomatal density reduce water loss under stress, but do not reduce CO_2 assimilation in the absence of stress, are attractive targets for engineering abiotic stress tolerance.

9. Other transcription factors

Although multiple TFs have been well characterized in various plant species, transcriptional reprogramming under drought and stress is not fully understood. Overexpression of the *AtMYB2* gene (from *Arabidopsis*) in rice under the control of an ABA-inducible promoter conferred salt stress tolerance to

the transgenic plants, with higher biomass and decreased ions leakage (Malik and Wu, 2005). Overexpression of *OsWRKY11* (encoding a TF comprising a WRKY domain), under the control of a *HSP101 promoter*, conferred heat and drought tolerance at the seedling stage (slower leaf wilting and higher survival rate of green parts of plants; Wu *et al*., 2009).

Recently, it was shown that the constitutive overexpression of two members of a family of bacterial RNA chaperones, *CspA* (from *E. coli*) and *CspB* (from *Bacillus subtilus*), conferred abiotic stress tolerance to transgenic *Arabidopsis*, rice and maize (Castiglioni *et al*., 2008). The transgenic maize plants showed yield benefits of up to 15% (0.75 t/ha) as compared to the non-transgenic controls, under water-stressed environment. Importantly, the observed yield improvements in water-limited field trials were not associated with a yield penalty in non-stressed (high-yielding) environments (Castiglioni *et al*., 2008). These results suggested that chaperones molecules may be good candidates for abiotic stress enhancement in crop plants.

IV. CONCLUSIONS AND PERSPECTIVES

Developing drought and salinity tolerance crop plants using conventional plant breeding methods had limited success during the past century. New technologies are providing opportunities to address the challenging problem of maintaining high-yield crop production under stressful environmental conditions and changing climates. The information provided by high-resolution transcript profiling, the identification of large-scale specific protein networks and their association with the plant responses to environmental perturbations are allowing the application of a systems-level approach to uncover the bases of plant responses to environmental changes. The application of an integrated approach is of paramount importance because the crops of the future are likely to be stacked with multiple traits (water deficit, nitrogen use efficiency, pathogen challenges, etc.). However, a review of the different transgenic crops produced so far revealed very limited success in producing drought- and salinity-tolerant cultivars through genetic transformation. Most transgenic plants developed with improved tolerance based on the performance of transgenic lines under controlled conditions in growth room or greenhouse, while only few lines were tested under field conditions (Flowers, 2004).

Numerous genes related to plant response to abiotic stress have been identified and characterized (Araus *et al*., 2008; Wang *et al*., 2005b). However, the vast majority of these studies were conducted on the model species such as *Arabidopsis* and tobacco under laboratory conditions (reviewed by Ashraf and Akram, 2009; Pardo, 2010; Umezawa *et al*., 2006). While for

crops, the reproductive stage in the most critical stage for productivity, in the majority of studies cited here, stress tolerance has been assessed at the initial growth stages, that is, germination and seedling stage, using survival rate as the main trait to represent tolerance to stress. In many of these experiments, artificial extreme conditions were applied (i.e. high salinity, osmotic shock, etc.). Under field conditions, plants have to cope with multiple stresses (as water deficit and heat) for longer periods. Hence, more emphasis should be given to the study of the responses of crop plants to a combination of environmental stresses at the reproductive stage and under field conditions.

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