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Narrowing Down the Targets: Towards Successful Genetic Engineering of Drought-Tolerant Crops

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ABSTRACT Drought is the most important environmental stress affecting agriculture worldwide. Exploiting yield potential and maintaining yield stability of crops in water-limited environments are urgent tasks that must be undertaken in order to guarantee food supply for the increasing world population. Tremendous efforts have been devoted to identifying key regulators in plant drought response through genetic, molecular, and biochemical studies using, in most cases, the model species *Arabidopsis thaliana*. However, only a small portion of these regulators have been explored as potential candidate genes for their application in the improvement of drought tolerance in crops. Based on biological functions, these genes can be classified into the following three categories: (1) stress-responsive transcriptional regulation (e.g. *DREB1*, *AREB*, *NF-YB*); (2) post-transcriptional RNA or protein modifications such as phosphorylation/dephosphorylation (e.g. *SnRK2*, *ABI1*) and farnesylation (e.g. *ERA1*); and (3) osomoprotectant metabolism or molecular chaperones (e.g. C*spB*). While continuing down the path to discovery of new target genes, serious efforts are also focused on fine-tuning the expression of the known candidate genes for stress tolerance in specific temporal and spatial patterns to avoid negative effects in plant growth and development. These efforts are starting to bear fruit by showing yield improvements in several crops under a variety of water-deprivation conditions. As most such evaluations have been performed under controlled growth environments, a gap still remains between early success in the laboratory and the application of these techniques to the elite cultivars of staple crops in the field. Nevertheless, significant progress has been made in the identification of signaling pathways and master regulators for drought tolerance. The knowledge acquired will facilitate the genetic engineering of single or multiple targets and quantitative trait loci in key crops to create commercial-grade cultivars with high-yielding potential under both optimal and suboptimal conditions.

Key words: Abiotic stress; drought tolerance; gene expression; genetic engineering; crop yield potential; field trials.

INTRODUCTION

One of the major challenges facing agriculture today is the global water shortage caused by the increasing world population and worldwide climate change. The Intergovernmental Panel on Climate Change (IPCC) has concluded that elevated greenhouse gas concentrations are likely to lead to the general drying of the subtropics by the end of this century, creating widespread drought stress in agriculture (IPCC, 2007). This global water scarcity threatens sustainable crop farming, as agricultural activities account for about 75% of global water consumption, and, in particular, irrigation represents over 90% of water used in many developing countries (UNEP, 2009). Stresses such as drought and salinity affect the productivity of most field crops to variable degrees, depending on the onset time, duration, and intensity of the stress. Rice (*Oryza sativa*), one of the most important food crops in the world, is very sensitive to drought stress because of its limited adaptation to water-deficit conditions. It is predicted that the depletion of ground water resources and rising soil salinity are pushing some rice-cropping systems away from traditional paddy land to aerobic fields (Delmer, 2005; Bernier et al., 2008). Maize (*Zea mays*), another staple crop, is also very sensitive to water-deficit stress (Boyer and Westgate, 2004), as its pollination and embryo development during and post flowering are greatly affected by soil water supply (Grant et al., 1989; Bolaños and Edmeades, 1996).

Plants, sessile in their environments, have evolved to adapt to environmental stresses via morphological and physiological changes. These include the efficiency with which the plant draws water from the surrounding soil, the water-retaining

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capacity within plant tissues or cells, control of water loss from transpiration through stomatal pores, and developmental adaptations to avoid seasonal water shortage during flowering (Zhu, 2002; Shinozaki et al., 2003; Umezawa et al., 2006; Neumann, 2008). At the molecular level, plants utilize multiple chains of signaling molecules (e.g. abscisic acid; ABA), which regulate different sets of stress-responsive genes to initiate the synthesis of various classes of proteins, including transcription factors (TFs), enzymes, and molecular chaperones (Valliyodan and Nguyen, 2006). These proteins function accordingly to enhance plants' tolerance via processes such as vegetative growth attenuation, osmoprotectant accumulation, and transpiration reduction. Hundreds of genes and their related signaling pathways that control the key processes of plants' responses to abiotic stresses have been identified (Umezawa et al., 2006; Yamaguchi-Shinozaki and Shinozaki, 2006; Tran et al., 2007; Wan et al., 2009). In general, the primary response of plants to water deficit is the inhibition of shoot growth, allowing cellular essential solutes to be diverted from growth requirements to stress-related functions. However, since this growth arrest decreases plant size and hence limits yield potential, the development of crop varieties able to maintain near-normal growth under moderate water stress is critical for yield stability.

Traditional breeding for yield stability under water-deficit conditions is labor-intensive and time-consuming, primarily because of the complicated genetics that control yield and the difficulty in controlling moisture levels in the field. Furthermore, the long-time practice of breeding for higher yield under well watered conditions has narrowed the genetic spectrum of current cultivars with respect to their ability to tolerate drought stress. Genetic engineering is thus being intensively explored to enhance plants' tolerance to abiotic stresses, and many engineered plants have manifested improved stress-resistance phenotypes (Bartels and Sunkar, 2005; Vinocur and Altman, 2005; Umezawa et al., 2006; Pennisi, 2008; Wan et al., 2009). Although such genetic engineering has been successfully applied to some crop species, such as rice, canola (*Brassica napus*), and maize, the majority of published results were based on the analysis of transgenic *Arabidopsis*, and stress tolerance was often evaluated in potted soil with rapid stress imposition under controlled growth conditions.

Many excellent reviews regarding plant drought response and resistance focus on either particular molecules (e.g. ABA, Wasilewska et al., 2008; aquaporin, Johansson et al., 2000), *cis*-acting regulatory elements (Yamaguchi-Shinozaki and Shinozaki, 2005), long-distance signals (Schachtman and Goodger, 2008), signaling pathways (Xiong et al., 2002; Chinnusamy et al., 2004; Yamaguchi-Shinozaki and Shinozaki, 2006), crop engineering (Zhang et al., 2004a; Umezawa et al., 2006; Valliyodan and Nguyen, 2006), particular crop species (e.g. maize, Bruce et al., 2002; rice, Leung, 2008; canola, Wan et al., 2009), soybean (*Glycine max*, Manavalan et al., 2009), or offer an overall retrospective (Ingram and Bartels, 1996; Bartels and Sunkar, 2005; Maggio et al., 2006; Bressan et al., 2009; Charron and Quatrano, 2009). This review, on the other hand, focuses mainly on the recent studies of genes that are involved in molecular or biochemical processes affecting drought tolerance and that have been used successfully in the genetic engineering of staple crop species such as rice, maize, wheat (*Triticum aestivum*), soybean, and canola for improvement of drought tolerance (summarized in Tables 1–3 and Figure 1). Particular attention is given to those gene candidates that were assessed in a crop species under field conditions for successful yield protection against the stress. Hyperosmotic, high salinity, and cold stress responses are discussed minimally, regardless of their close cross-talk and interaction with drought-resistance responses (Seki, 2002).

TRANSCRIPTIONAL REGULATION OF DROUGHT STRESS RESPONSES

Environmental stresses on plants often induce the expression of numerous transcriptional regulators, which, in turn, up-regulate a series of downstream genes for self-protection or stress adaptation. Typical *de novo* synthesized regulatory proteins include TFs (Yamaguchi-Shinozaki and Shinozaki, 2006). In *Arabidopsis*, over 1700 genes encoding TFs in more than 50 families have been identified (Xiong et al., 2005; Palaniswamy et al., 2006; Riaño-Pachón et al., 2007; Guo et al., 2008), and some of them have been implicated in plants' response to drought (Bartels and Sunkar, 2005; Umezawa et al., 2006). In particular, TFs from basic leucine zipper (bZIP) (e.g. ABA responsive element binding protein/ABRE binding factor (AREB/ABF)), AP2/EREBP (e.g. DRE binding protein/CRT binding factor (DREB/CBF)), NAM (no apical meristem), ATAF1-2, CUC2 (cup-shaped cotyledon) (NAC) (e.g. stress-responsive NAC (SNAC)), CCAAT-binding (e.g. nuclear factor Y (NF-Y)), and zinc-finger (e.g. C2H2 zinc finger protein (ZFP)) families have been characterized in detail with regard to their roles in the regulation of drought stress responses (Zhu, 2002; Shinozaki et al., 2003), and the ectopic expression of several of these TFs resulted in improved crop tolerance to drought (Hu et al., 2006; Nelson et al., 2007; Xiao et al., 2009; Table 1).

Drought and ABA-Inducible AREB/ABF

ABA responsive element binding proteins/factors (AREB/ABF) belong to a family of basic leucine zipper (bZIP) plant TFs known to function in ABA signaling during dehydration and seed maturation. In the *Arabidopsis* genome, 75 AREB/ABF homologs have been identified (Jakoby et al., 2002). In response to ABA, an activated AREB/ABF binds to the conserved regulatory *cis*-element sequence (ACGTGT/GC) called the ABA-responsive element (*ABRE*, Mundy et al., 1990) to trigger gene expression. *ABRE* is found in the promoters of the genes that are induced by ABA (Baker et al., 1994; Yamaguchi-Shinozaki and Shinozaki, 2005, 2006).

Overexpression of *AREB1/ABF2*, *ABF3*, or *AREB2/ABF4* in *Arabidopsis* resulted in enhanced ABA response, greater guard cell closure, and reduced transpiration (Kang et al., 2002). The transgenic plants appeared to be tolerant to drought (Fujita

Table 1. Genes Studied in Transcriptional Regulation of Drought Response and Tolerance in Crops.

TF family	Gene category	Gene name	Promoter	Crop	Evaluation	Physiological change	Tolerance	References
bZIP	AREB/ABF	*AtABF3*	ZmUbi1	rice	GH	less leaf rolling, wilting; higher Fv/Fm	drought	Oh et al., 2005
NAC	SNAC	*AtSNAC1* *SNAC2* *OsNAC6*	35S; ZmUbi; OsNAC6	rice	GH; field	ABA-hypersensitive; stomatal closure; oxidative stress protection	drought; salt	Rabbani et al., 2003; Hu et al., 2006; Nakashima et al., 2007
AP2/ERF	DREB1/CBF; DREB2	*OsDREB1A; OsDREB1B; OsDREB1F; AtDREB1A; AtDREB1B*	35S; ZmUbi; RD29A; OsHVA22P; OsActin1	rice; wheat; peanut; tomato	GH; field	fast stomatal closure; high proline; high spikelet fertility & yield	drought; salt; cold	Hsieh et al., 2002; Pellegrineschi et al., 2004; Oh et al., 2005; Ito et al., 2006; Bhatnagar-Mathur et al., 2007; Wang et al., 2008b; Xiao et al., 2009
	HARDY	*AtHARDY*	35S	rice	GH	high WUE; low transpiration; high photosynthesis	drought	Karaba et al., 2007
TFIIIA-type Zinc finger	ZFP252; DST	*OsZFP252; DST*	35S; LOF	rice	GH	proline & sugar accumulation; better H_2O_2 homeostasis	drought; salt	Xu et al., 2008; Huang et al., 2009
EAR Zinc finger	Zat10/STZ	*AtZat10*	OsActin1; OsHVA22P	rice	GH; field	high spikelet fertility; high yield	drought	Xiao et al., 2009
NF-Y (A, B, C)	NF-YB	*AtNF-YB1; ZmNF-YB2*	35S; OsActin1	maize	GH; field	high photosynthesis; polysaccharide metabolism	drought	Nelson et al., 2007
WRKY Zinc finger	WRKY	*OsWRKY11*	HSP101	rice	GH	less leaf wilting; slow water loss	drought; heat	Wu et al., 2009

Table 2. Genes Studied in Post-Translational Regulation of Drought Tolerance in Crops.

Functional group	Gene category	Gene name	Promoter	Crop	Evaluation	Physiological change	Tolerance	Reference
Farnesylation	FTA/FTB	*AtERA1; BnFTA*	RD29A; HPR	canola	GH, field	stomatal closure	drought	Wang et al., 2005; Wang et al., 2009
	CDPK	*OsCDPK7*	35S	rice	GH	LEA gene expression	drought; salt; cold	Saijo et al., 2000
Protein Phosphorylation	SnRK3	*OsCIPK03; OsCIPK12; OsCIPK15*	ZmUbi	rice	GH	higher proline and sugar accumulation; less leaf rolling	drought; salt; cold	Xiang et al., 2007
	MAPK cascade	*NPK1*	35S; OsHVA22P	maize; rice	GH; field	high photosynthesis; high spikelet fertility; high yield	drought	Shou et al., 2004; Xiao et al., 2009

et al., 2005), cold, heat, and oxidative stresses (Kim et al., 2004). Rice seedlings overexpressing *Arabidopsis ABF3* showed less leaf rolling, wilting, and higher Fv/Fm values after withholding water for 4 d (Oh et al., 2005). However, this ABF3-mediated rice drought tolerance was outperformed by DREB1A/CBF3-mediated stress tolerance (discussed in the ABA-independent DREB/CBF section, below) when the two transgenic events were compared side by side (Oh et al., 2005).

Stress and ABA-Inducible NAC (SNAC)

NAC (NAM, ATAF1-2, CUC2) is a plant-specific TF family consisting of about 100 members that contain a highly conserved DNA-binding NAC domain (Palaniswamy et al., 2006; Guo et al., 2008). Three *Arabidopsis NAC* genes (*ANAC019*, *ANAC055*, and *ANAC072*) were isolated based on their binding to the *NACRS* (NAC recognition sequence, CATGTG) element in the promoter of the early responsive to dehydration 1 (*ERD1*)

Table 3. Metabolites and Osmoprotectants Studied in Drought Tolerance in Crops.

Functional Group	Gene category	Gene name	Promoter	Crop	Evaluation	Physiological change	Tolerance	Reference
ABA biosynthesis	LOS5/ABA3	*AtLOS5*	OsActin1; OsHVA22P	rice	field	high spikelet fertility; high yield	drought	Xiao et al., 2009
	NCED	*LeNCED*	Super Promoter; rbcS3C	tomato	GH	high WUE;	N/A	Thompson et al., 2000; Tung et al., 2008
Lipid metabolism	PtdIns-PLC2	*BnPtdIns-PLC2*	35S	canola	GH	early flowering; small stomatal aperture; low transpiration rate	drought	Georges et al., 2009
	PI-PLC1	*ZmPLC1*	ZmUbi	maize	GH	more solutes; less membrane damage; high yield	drought	Wang et al., 2008a
Heat/cold-shock protein	sHSP	*OsHSP17.7*	35S	rice	GH	protein protection	drought	Sato and Yokoya 2008
	HSP70	*soyBiP D*	35S	soybean	GH	stomatal closure; delayed leaf senescence	drought	Valente et al., 2009
	CSP	CspA (*E. Coli*); CspB *(B. Subtilis)*	35S; OsActin1	rice; maize	GH; field	high photosynthesis; increased yield	drought; heat; cold	Castiglioni et al., 2008
late embryogenesis abundant protein	LEA	barley *HVA1*	OsActin1; Ubi1	rice; wheat	GH; field	high WUE; high RWC; membrane protection	drought; salt	Xu et al., 1996; Sivamani et al., 2000; Rohila et al., 2002
	LEA	*OsLEA-3*	OsLEA-3; 35S; OsActin1	rice	GH; field	higher spikelet fertility; higher grain yield	drought	Xiao et al., 2007
Poly(ADP-ribose) polymerase	PARP	*hpAtPARP1;* *hpAtPARP2*	35S	canola; maize	GH; field	less ROS; low mitochondrial respiration; ABA signaling	drought; heat; high light	de Block et al., 2005; Vanderauwera et al., 2007
Proline	P5CS P5CR (AtP5C1)	*VaP5CS* (mothbean); *AtP5CR*	AIPC (Hva22+ OsActin1); IHSP	rice; soybean	GH	proline accumulation; less wilting	drought; salt; heat	Zhu et al., 1998; de Ronde et al., 2004a; de Ronde et al., 2004b
Glycinebetaine (GB)	betA	*betA (E. Coli)*	35S	maize; cotton	GH	better reproductive development; high RWC; membrane protection	drought; chilling	Quan et al., 2004; Lv et al., 2007

Table 3. Continued

Functional Group	Gene category	Gene name	Promoter	Crop	Evaluation	Physiological change	Tolerance	Reference
Sugar	trehalose	*TPS*; *TPP* (*E. coli*; yeast)	35S; AIPC; ZmUbi1	rice; tomato	GH	more trehalose; less leaf rolling	drought; salt; cold; oxidation	Garg et al., 2002; Jang et al., 2003; Cortina et al., 2005
	mannitol	*mtlD* (*E. coli*)	ZmUbi1	wheat	GH	mannitol accumulation; increase in plant biomass and height	drought; salt	Abebe et al., 2003
Polyamine	spermine spermidine	Oat *adc* jimsonweed *adc*	35S ZmUbi1	rice	GH	spermine/spermidine accumulation; more chlorophyll; less leaf rolling, wilting	drought	Capell et al., 1998, 2004

gene (Tran et al., 2004), suggesting that at least some of the NAC TFs play an important role in plant response to dehydration and drought. These three *NAC* genes are inducible by drought, high salinity, and ABA. In rice, 140 putative *NAC* or *NAC*-like genes are predicted according to sequence analysis, of which 40 are likely to be responsive to drought and/or salt stresses (Kikuchi et al., 2000; Fang et al., 2008). Several rice *NAC*s such as *SNAC1* (Hu et al., 2006), *SNAC2* (Hu et al., 2008), *OsNAC6* (Nakashima et al., 2007), *ONAC045* (Zheng et al., 2009), and soybean *GmNACs* (Tran et al., 2009) are induced by drought, salt, cold, and ABA; canola *BnNAC5-1* is slightly induced in seedlings by dehydration (Hegedus et al., 2003).

SNAC1 is also specifically induced in guard cells by drought. *SNAC1*-overexpressing rice showed significant reduction in stomatal aperture (Hu et al., 2006). In moderate drought or severe drought (sheltered) field conditions, *SNAC1*-transgenic plants exhibited 17–22% higher spikelet fertility and 22–34% higher seed setting rate than non-transgenic plants, whereas in well irrigated field conditions, transgenic plants performed similarly to wild-type plants. The enhanced drought resistance appeared to be partially due to the reduction in stomatal aperture as a result of increased ABA sensitivity in the guard cells (Hu et al., 2006). In contrast, *SNAC2*-overexpressing plants did not demonstrate any improvement with respect to drought tolerance but showed improved tolerance to cold, salinity, and osmotic stresses (Hu et al., 2008). Transgenic rice seedlings expressing *OsNAC6* under the control of the maize ubiquitin (*ZmUbi*) promoter had 42–57% higher recovery rate after the hydroponically grown seedlings were removed from their growth medium and exposed to air for 12 h. However, the *OsNAC6*-transgenic rice suffered from growth retardation with yield penalty (Nakashima et al., 2007). In order to circumvent these problems, the ubiquitin promoter was replaced with the endogenous stress-inducible *OsNAC6* promoter (Rabbani et al., 2003). The resulting transgenic rice seedlings showed increased salt tolerance without any growth and yield penalty (Nakashima et al., 2007); however, no data were given regarding the dehydration tolerance of these transgenic rice seedlings.

ABA-Independent DREB/CBF

Unlike AREB/ABF and SNAC described above, some TFs are responsive to dehydration but not to ABA, and are thus called ABA-independent dehydration-responsive TFs. These TFs typically bind to a specific and conserved *cis*-element (A/GCCGAC) in the promoters of target genes to activate their expression. The signature *cis*-element is termed the dehydration-responsive element (*DRE*), first discovered in the promoter of the *Arabidopsis RD29A* gene (Yamaguchi-Shinozaki and Shinozaki, 1994). Interestingly, *DRE* was simultaneously characterized as a C-repeat (*CRT*) element in the promoters of several cold-responsive genes (Baker et al., 1994). The *DRE*-binding (DREB, Liu et al., 1998) or *CRT*-binding factors (CBF, Stockinger et al., 1997) were then isolated and found to be the major

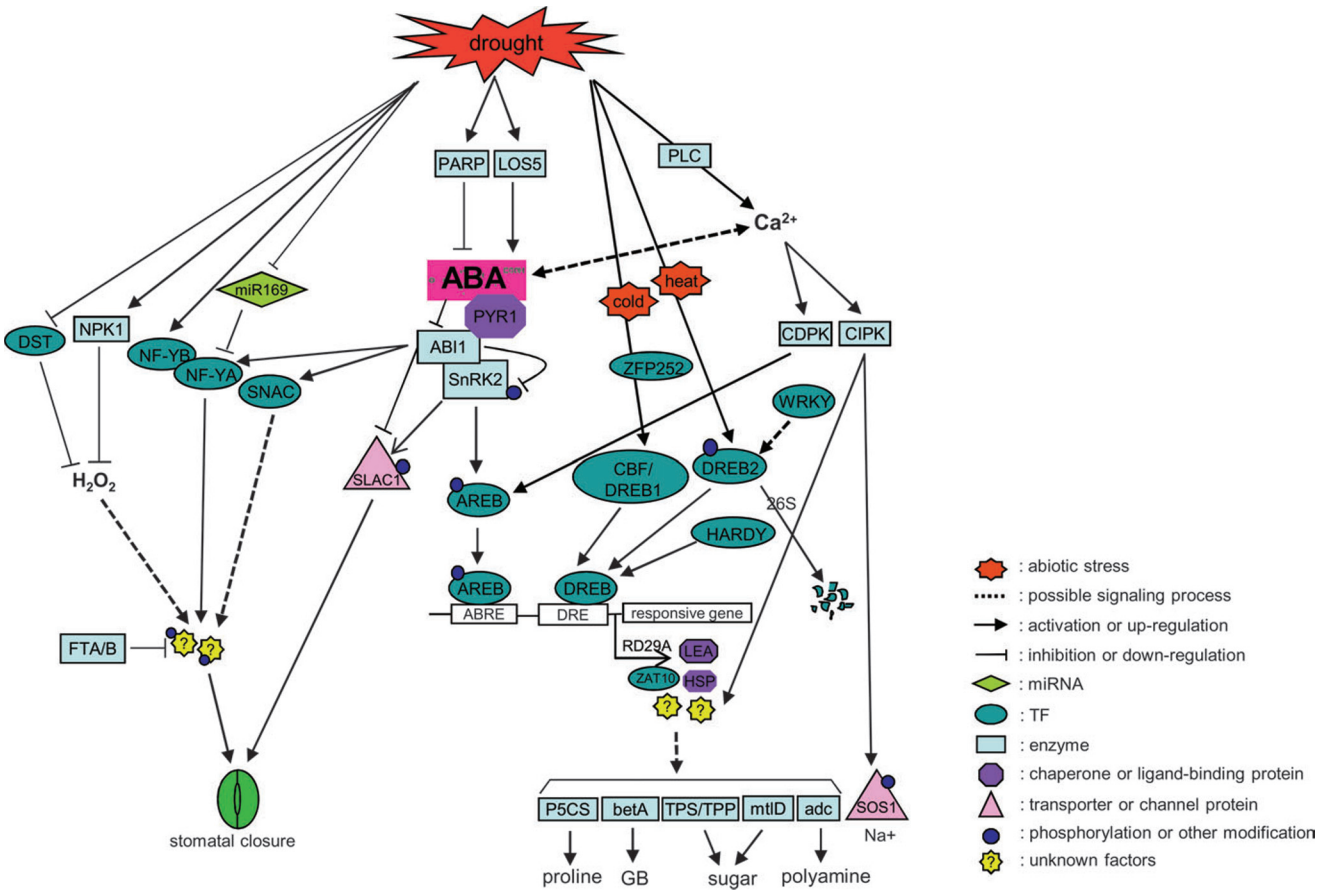

Figure 1. Schematic Presentation of the Signaling Relationships among the Genes Described in this Review.

Drought induces the accumulation of ABA, which is affected by PARP and LOS5. ABA is perceived by the ABI1/PYR1 receptor and induces downstream TFs such as NF-YA, SNAC, and AREBs through, in some cases, SnRK2 kinase activity. SnRK2 also physically interacts with and activates the S-type anion channel protein SLAC1 to promote stomatal closure. Drought also induces the expression of CBF/DREB1, DREB2, ZFP252, NF-YB, and ZAT10 independently of ABA. DREBs and AREBs regulate, through the interaction with DRE and ABRE *cis*-elements, the expression of a series of downstream genes to produce osmoprotectants such as LEA, HSP, proline, GB, sugars, and polyamines, whereas farnesyltransferase α-/β-subunit (FTA/B), SNAC, NF-YA5, DST, and NPK1 are involved in the regulation of stomatal movements via, in the cases of DST and NPK1, H_2O_2 signaling. Drought- or ABA-induced Ca^{2+} fluxes are affected by PLC and perceived by CDPK and CIPK for downstream drought-tolerance responses.

players in driving dehydration- or cold-induced gene expression (Yamaguchi-Shinozaki and Shinozaki, 1994, 2005, 2006; Stockinger et al., 1997; Gong et al., 2008). All DREB/CBFs belong to the plant-specific AP2/ERF TF superfamily, which includes 147 members (Nakano et al., 2006). Besides their involvement in regulation of dehydration and cold responses, these TFs play a wide range of roles in seed, leaf, and flower development (Riechmann and Meyerowitz, 1998; Fujimoto et al., 2000; Lin et al., 2008).

Two classes of DREBs (i.e. DREB1 and DREB2) were isolated from *Arabidopsis* using yeast one-hybrid screening for proteins that bind to *DRE* elements (Stockinger et al., 1997; Liu et al., 1998). *DREB1* expression is strongly up-regulated by cold whereas *DREB2* is more responsive to drought, salt (Liu et al., 1998), and heat stresses (Sakuma et al., 2006b). *DREB1s* and *CBFs* correspond to identical loci in the *Arabidopsis* genome; for instance, DREB1A is identical to CBF3; DREB1B to CBF1; DREB1C to CBF2; and DREB1D to CBF4. DREB1/CBF TFs belong to Group III of the DREB/CBF subfamily (Nakano et al., 2006), which are expressed in response to various stresses in *Arabidopsis*, rice, wheat, barley, corn, soybean, and canola (Qin et al., 2004). To date, the DREB1/CBF signaling pathway has been one of the most explored for improving crop stress tolerance (Stockinger et al., 1997; Liu et al., 1998; Nakashima et al., 2009). When rice *OsDREB1A* (Dubouzet et al., 2003) or corn *ZmDREB1A* (Qin et al., 2004) were constitutively overexpressed in *Arabidopsis*, the downstream target genes regulated by the *Arabidopsis DREB1* (e.g. *RD29A*) were induced, resulting in desiccation tolerance under 15% humidity (Qin et al., 2004) as well as salt and cold tolerance (Dubouzet et al., 2003). This suggests that the DREB1/CBF pathway and the function of DREB1 proteins are conserved in both dicot and monocot species.

To employ the DREB1 regulon for the purpose of improving rice drought stress tolerance, *OsDREB1A*, *OsDREB1B*,

AtDREB1A, *AtDREB1B*, and *AtDREB1C* were ectopically overexpressed in rice under the control of either the constitutive 35S, *ZmUbi*, *OsActin1*, or the drought-inducible *OsHVA22p* promoter (Oh et al., 2005; Ito et al., 2006; Xiao et al., 2009). Among the transformants that overexpressed either of these proteins, the majority of the transgenic plants could survive the treatment of a 9-d water withholding while all wild-type plants died from water deprivation in pot trials (Ito et al., 2006). In rain-free and water-stressed fields, *AtDREB1A*/*CBF3* driven by the *OsHVA22p* promoter significantly improved rice spikelet fertility (42% higher) and yield per plant (11% higher), whereas *AtDREB1A*/*CBF3* driven by the constitutive *OsActin1* promoter had only marginal effects (Xiao et al., 2009). It is worthwhile to note that the field drought treatment was quite severe, which resulted in a yield loss in wild-type rice plants of 82%. Under normal field conditions, however, the spikelet fertility and grain yield per plant were comparable between the wild-type control and the *AtDREB1A*/*CBF3*-transgenic rice (Xiao et al., 2009), indicating that no growth defect was derived from *DREB1A* transgene. This result is in agreement with an earlier report that showed the normal growth under greenhouse conditions of *AtDREB1A*-transgenic rice under the control of the *ZmUbi* promoter (Oh et al., 2005) but differs from the observation that all *ZmUbi*-driven *DREB1*-transgenic rice plants suffer from growth retardation (Ito et al., 2006). This phenotypic discrepancy is possibly due to the different rice cultivars used in these reports. Growth retardation was also observed in *35S:AtDREB1B*-transgenic tomato plants, which, similarly to rice, were more resistant to water-deficit stress by showing less plant wilting and leaf curling than wild-type controls after a 21-d water withdrawal in the same pot (Hsieh et al., 2002). Unlike *OsDREB1A* and *OsDREB1B* (Ito et al., 2006), *OsDREB1F* caused no negative growth effects when overexpressed in transgenic rice or *Arabidopsis* (Wang et al., 2008b) but resulted in enhanced tolerance to drought, salt, and cold stresses in laboratory tests. By activating the expression of both ABA-dependent and ABA-independent downstream genes, *OsDREB1F*-transgenic rice seedlings grown in liquid medium showed less leaf coil and withering after a 5-h air dry; and transgenic *Arabidopsis* grown in pots survived an 8-d water withdrawal while controls failed (Wang et al., 2008b). Since OsDREB1F is also induced by ABA application but does not directly bind to the *ABRE* element, OsDREB1F might be a molecular bridge between ABA-independent DREB and ABA-dependent AREB pathways. Moreover, ICE1, inducer of CBF/DREB1 expression 1, a TF upstream of DREB1, was recently characterized as regulator of stomatal cell differentiation in *Arabidopsis* (Kanaoka et al., 2008). Therefore, it could be another link between the DREB1/CBF regulon and stomatal function/formation.

One of the main drawbacks for DREB1/CBF application for the enhancement of drought tolerance is the growth defects caused by their constitutive overexpression. In order to minimize this side effect, *AtDREB1A*, driven by the osmotic stress-inducible *RD29A* promoter, was transformed into *Arabidopsis* (Kasuga et al., 1999), bread wheat (*Triticum aestivum*, Pellegrineschi et al., 2004), tall fescue (*Festuca arundinacea* Schreb., Zhao et al., 2007b), and peanut (*Arachis hypogaea*, Bhatnagar-Mathur et al., 2007). In these cases, the transgenic plants demonstrated substantial resistance to water stress in comparison with the non-transgenic controls without displaying obvious defects in growth. Transgenic wheat seedlings in pots developed leaf wilting and bleaching symptoms 5 d later than controls after water withdrawal (Pellegrineschi et al., 2004). Transgenic peanut plants had relatively higher transpiration efficiencies in both well watered and water-limited conditions, likely explained by lower stomatal conductance (Bhatnagar-Mathur et al., 2007), while tall fescue had higher proline contents (Zhao et al., 2007b).

HARDY, another Group III member of the DREB/CBF subfamily (Nakano et al., 2006), is not induced by drought but is normally expressed in inflorescence tissues of *Arabidopsis*. When constitutively overexpressed in *Arabidopsis*, HARDY caused pleiotropic effects on vegetative growth, forming small, thick green leaves and an abnormally dense root system (Karaba et al., 2007). In rice, ectopically expressed *HARDY* improved the plants' water use efficiency (WUE) in greenhouse tests (Karaba et al., 2007). The transgenic rice did not show any reduction in growth and seed yield, but had dark green leaves with more bundle sheath cells and a significant increase in leaf canopy size and number of tillers. The significant increase (50%) in rice WUE was explained by lower transpiration and higher photosynthetic carbon assimilation under both drought and well watered conditions (Karaba et al., 2007). HARDY and DREB1/CBF may share some common steps in stress signal transduction, since both of them induce the expression of a subset of genes known to respond to water deprivation and osmotic stress.

Arabidopsis DREB2A, a Group IV member of the DREB/CBF subfamily (Liu et al., 1998; Nakano et al., 2006), only weakly induced downstream genes when overexpressed, and had little effect on stress tolerance enhancement because of its possible post-translational modification or quick degradation by the 26S proteasome (Qin et al., 2008). However, as the DREB2A protein can be made constitutively active (DREB2A-CA) by specific residue deletion (Sakuma et al., 2006a), rendering DREB2A constitutively active or blocking DREB2 degradation through ubiquitination could be possibilities for the application of DREB2 in enhancing crop drought tolerance. Indeed, Sakuma et al. (2006a) showed that overexpression of DREB2A-CA significantly enhanced the drought tolerance of *Arabidopsis* but, as has been the case with the constitutive expression of other *DREBs*, this was also associated with growth retardation. On the other hand, stress-inducible or constitutive ectopic expression of maize *ZmDREB2A* (Qin et al., 2007) and soybean *GmDREB2* (Chen et al., 2007) in *Arabidopsis* resulted in the improved tolerance to drought, heat, and salt stress of transgenic plants without growth defects, suggesting also that maize *ZmDREB2A* and soybean *GmDREB2* may not require modification to be active. It has yet to be determined, however, whether

the overexpression of *ZmDREB2A* and *GmDREB2* would have similar effects on maize or soybean plants, respectively. Importantly, DREB2A regulates the expression of not only dehydration-inducible genes, but also heat-shock-related genes (Sakuma et al., 2006b). Therefore, the active form of DREB2A could be applicable to crop engineering for both drought and heat tolerance.

Zinc Finger Protein (ZFP) TFs

There are about 134 C2H2-type ZFPs in the *Arabidopsis* genome, and they play a critical role in many cellular functions through DNA or RNA binding and protein–protein interactions (Ciftci-Yilmaz and Mittler, 2008). The strong induction of some *Arabidopsis* C2H2 ZFPs by dehydration, salinity, cold, and ABA treatments suggests that these ZFPs may also be actively involved in the stress response by acting as transcriptional activators or repressors (Sakamoto et al., 2000, 2004; Sugano et al., 2003).

Recent reports on rice ZFPs, such as OsZFP252 (Xu et al., 2008), DST (drought and salt tolerance, Huang et al., 2009), and ZAT10 (Xiao et al., 2009), have shed some light on the possible roles of rice ZFP TFs in the regulation of drought responses. Overexpression of *OsZFP252*, a C2H2-type ZFP, enhanced the tolerance of rice seedlings to drought by showing 74–79% higher survival rate after a 14-d water withdrawal (Xu et al., 2008). The tolerance was correlated with the induced expression of *OsDREB1A*, suggesting that *OsZFP252* might be an upstream regulator of *OsDREB1A*, and with a higher accumulation of free proline and soluble sugars. On the other hand, DST, another C2H2-type ZFP, was shown to function as a negative regulator in stomatal closing via the direct modulation of H_2O_2 homeostasis in guard cells (Huang et al., 2009). The rice *DST* knockdown mutant (*dst*) acquired a significant improvement in drought and salt tolerance without any yield penalty. In addition, an *EAR* (ERF-associated amphiphilic repression) C2H2 ZFP, ZAT10 (Xiao et al., 2009), and a cytoplasmic (non-TF) ZFP, OsiSAP8 (Kanneganti and Gupta, 2008), were reported to affect rice drought tolerance. *ZAT10* is induced by DREB1A/CBF3 (Maruyama et al., 2004), and its overexpression in rice conferred drought tolerance and, importantly, 17–36% yield increase under rain-free field conditions (Xiao et al., 2009). *OsiSAP8* is induced by multiple abiotic stresses (dehydration, salinity, heat, cold, and ABA treatment), and its overexpression in rice resulted in 50% yield penalty even under normal water conditions, but these plants survived water withdrawal for 23 d, while the non-transgenic plants did not (Kanneganti and Gupta, 2008). As the C2H2 zinc finger TFs appear to function in association with either the DREB1 pathway or H_2O_2-mediated stomatal response, they may be candidates that could be tailored for crop engineering for stress tolerance.

Nuclear Factor (NF-Y)

NF-Ys are ubiquitous CCAAT-binding TFs composed of A, B, and C subunits. The *Arabidopsis* genome encodes for 10, 13, and 13 A, B, and C subunits, respectively (Gusmaroli et al., 2002), whereas there is only a single gene for each subunit in mammals and yeast. This suggests that plants may have evolved additional functions for the NF-Y heterotrimeric complexes. Using a functional genomics strategy, a B subunit of the *Arabidopsis* NF-Y complex (named AtNF-YB1, Nelson et al., 2007) and, subsequently, an A subunit named AtNF-YA5 (Li et al. 2008) were isolated in searches for TFs able to improve drought tolerance (Nelson et al., 2007) or for small RNAs involved in stress responses (Li et al., 2008), respectively. In the latter case, the *AtNF-YA5* gene in the *Arabidopsis* genome partially overlaps with its neighbor gene in reverse orientation, resulting in the production of natural antisense transcript (nat)-derived siRNA (nat-siRNA, Borsani et al., 2005). AtNF-YB1 and AtNF-YA5 were both able to increase drought tolerance in *Arabidopsis* when constitutively overexpressed. Whereas AtNF-YB1 regulates genes not known to respond to ABA or to the DREB/CBF regulon, AtNF-YA5 is induced by both drought and ABA and is prominently expressed in the vascular system and in guard cells, where it functions to reduce stomatal aperture. AtNF-YA5 regulates a number of known stress-responsive genes. Interestingly, AtNY-YA5 is post-transcriptionally regulated by microRNA 169 (miR169), suggesting that there might be a complex mechanism controlling the functions of AtNF-YA5 (Li et al., 2008).

When *ZmNF-YB2*, the maize ortholog of *AtNF-YB1*, was constitutively expressed in an elite maize inbred line, the transgenic lines showed improved drought tolerance compared to wild-type plants under water-stressed conditions in the field, suggesting the existence of a common regulator for stress response in maize and *Arabidopsis*. Under such conditions, the transgenic maize had less leaf rolling, a higher photosynthetic rate, cooler leaf temperature, and higher stomatal conductance. Although earlier flowering (1–3 d) and slightly compressed internodes were observed in *ZmNF-YB2* transgenic plants under normal water supply, an increase of ~50% in yield from a best-performing line in relatively severe drought conditions highlights the potential of the application of this gene target for genetic engineering in suboptimal, drought-prone maize production systems (Nelson et al., 2007).

WRKY TFs

TFs containing conserved WRKY domain (WRKY) TFs (more than 70 in *Arabidopsis*) are best known for their involvement in the regulation of plant growth, development, and in response to biotic stresses (Ulker and Somssich, 2004). While many studies on WRKYs focused on disease-induced responses, relatively little is known about their roles in abiotic response. One exception is *OsWRKY11*, which, when driven by the heat-inducible *heat shock protein* (*HSP*)*101* promoter, rendered transgenic rice significantly heat and drought-tolerant compared to controls, as indicated by slower leaf wilting in potted soil (Wu et al., 2009). It is very interesting to note that the overexpression of soybean *GmWRKY54* in *Arabidopsis* (Zhou et al., 2008) significantly induced the expression of *DREB2A*, which is known to have dual functions in both drought and heat tolerance (Sakuma

et al., 2006b). Unfortunately, the tolerance of *GmWRKY54*-transgenic *Arabidopsis* to drought, salt, and cold but not to heat was evaluated by the authors (Zhou et al., 2008). Transgenic plants showed significantly enhanced tolerance to drought and salt, but only slightly improved tolerance to freezing.

Challenges with Transcriptional Manipulation for the Purpose of Enhancing Drought Tolerance

Using TF regulons to improve tolerance of crops to abiotic stresses is a promising strategy because of the ability of a TF to regulate an entire set of genes in a stress-response pathway. Of all the TFs discussed above, only SNAC1 (Hu et al., 2006), NF-YB1 (Nelson et al., 2007), and DREB1A/CBF3 (Xiao et al., 2009) were confirmed as being able to enhance drought tolerance in crops under field conditions. The effects of other TFs were only assessed in potted soil under greenhouse conditions or by air-drying over a rather short period of time. In addition, the physiological assessments were often carried out with transgenic seedlings rather than with flowering plants, which are most sensitive to water deprivation with respect to yield output. According to the literature published so far, the manipulation of DREB regulons appears to be quite effective for improving plants' tolerance to drought at their seedling stage. Their value for increasing grain yield in crops in the field has only been demonstrated in rice under rather severe drought conditions (Xiao et al., 2009); therefore, further evaluation of this promising gene target is required to determine its impact on economically important crops at the flowering stage under suboptimal field conditions.

Constitutive overexpression of some stress-responsive TFs such as DREBs (controlled by the 35S promoter) frequently caused unwanted side effects, such as growth retardation and yield penalty (Kasuga et al., 1999; Hsieh et al., 2002; Zhang et al., 2004a). In order to minimize the negative effects, *DREB1A* was expressed under the control of the osmotic stress-inducible *RD29A* promoter (Kasuga et al., 1999, 2004). One of the unique features of the *RD29A:DREB1A* expression cassette is the forward feedback between the *RD29A* promoter and the DREB1A protein. Under drought stress conditions, the drought-induced and *de novo* synthesized DREB1A binds to the *DRE cis*-element of the *RD29A* promoter, which drives the expression of DREB1A for rapid production of this TF. Caution should be taken, however, when using an *Arabidopsis*-inducible promoter in crops. For example, it was reported that the *Arabidopsis RD29A* promoter functions in rice root but, unexpectedly, not in leaves (Ito et al., 2006). In the case of *OsNAC6* expression, the endogenous *OsNAC6* promoter was used in order to minimize growth defects in transgenic rice plants (Nakashima et al., 2007), although the resulting level of *OsNAC6* transcripts would be expected to be only double the normal level.

So far, most candidate genes for drought tolerance were first unearthed from studies in the *Arabidopsis* system. Although most stress-response and signaling pathways are thought to be ubiquitously present throughout the plant kingdom, it will be crucial to examine the involvement and contribution of additional and related gene candidates from crop species in drought tolerance.

POST-TRANSLATIONAL PROTEIN MODIFICATIONS IN DROUGHT RESPONSES

Protein Farnesylation

Farnesylation is a post-translational protein modification that adds a farnesyl group to a target protein. Farnesylated proteins function to regulate many different growth and developmental processes (Nambara and McCourt, 1999; Galichet and Gruissem, 2003). The plant farnesyltransferase consists of an α- (FTA) and a β- (FTB) subunit. Loss of function of *FTB* in *Arabidopsis* resulted in mutants that display an enhanced response to ABA (*era1*, Cutler et al., 1996). ERA1, a negative regulator of the ABA response, is involved in the regulation of stomatal aperture (Pei et al., 1998), suggesting a potential role for protein farnesylation in drought response.

The effectiveness of down-regulation of *FTB* using antisense technology (Wang et al., 2005) and *FTA* via RNAi techniques (Wang et al., 2009) for improvement of drought tolerance and yield protection has been demonstrated in transgenic canola. As the *era1* mutants exhibit pleiotropic developmental abnormalities, the suppression of endogenous *FTA* or *FTB* was fine-tuned to be dependent upon water availability by using the drought stress-inducible *RD29A*, or the shoot-specific peroxisomal hydroxypyruvate reductase promoters. Under drought stress, transgenic canola showed reduced stomatal conductance and transpiration, likely as a result of being hypersensitive to ABA. Field trials over the course of three consecutive years indicated that under moderate drought stress at flowering stage, transgenic canola had yields significantly higher than controls, while with adequate water supplies, their yields were equal (Wang et al., 2005, 2009).

Protein Phosphorylation

Some TFs such as AREB1 need to be phosphorylated by protein kinases in order to become active (Furihata et al., 2006). Several protein kinases, especially a subset of 604 *Arabidopsis* receptor-like kinases, have been implicated in osmotic stress responses based on their transcriptional responses to different environmental stimuli (Boudsocq and Laurière, 2005; Chae et al., 2009). Mitogen-activated protein kinases (MAPKs) are often the focus of studies in plant response to environmental stimuli because of the well known yeast HOG1 (high-osmolarity glycerol) MAPK cascade in hyperosmotic signaling. Other protein kinases related to osmotic adaptation include calcium-dependent protein kinases (CDPKs), CBL (calcineurin B-like) interacting protein kinase (CIPK/sucrose non-fermenting protein (SNF1)-related kinase 2 (SnRK3) and SNF1-related kinase 2 (SnRK2).

MAPK cascades, consisting of three interlinked protein kinases (MAPKKK, MAPKK, and MAPK), function in many signal transduction pathways, including those activated by dehydration, cold, and high salt conditions (Nakagami et al., 2005). Based on the results of comparative sequence analysis, about 90 genes have been identified and assigned to the *Arabidopsis* MAPK cascades, and multiple homologs or orthologs have been reported in crop species such as maize and rice (Ning et al., 2008). The overexpression of a tobacco (*Nicotiana tobacum*) MAPKKK (designated *Nicotiana* protein kinase 1 (*NPK1*) activated an oxidative stress-response signaling cascade and led to enhanced tolerance to freezing, heat, and salt stress in transgenic tobacco (no drought test was performed, Kovtun et al., 2000). When the kinase domain of NPK1 was constitutively expressed in maize, the overexpression lines showed enhanced drought tolerance and maintained significantly higher photosynthetic rates under greenhouse conditions (Shou et al., 2004). The drought-stressed transgenic plants produced kernels comparable to those under well watered conditions. The mechanism behind this drought tolerance is likely that the active NPK1 switches on the oxidative stress-responsive signaling cascade at the onset of drought stress, thus protecting the photosynthetic machinery from oxidative damage. Overexpression of *NPK1* had no negative effect on maize growth. In rice, four *NPK1*-like genes (*OsNPKLs*) are strongly induced by drought and salt stress, and are genetically co-localized with a drought resistance quantitative trait loci (QTL) on chromosome 1 (Ning et al., 2008). *NPK1*-transgenic rice under control of the drought-inducible *OsHVA22P* promoter also showed significantly increased spikelet fertility (23% higher) and grain production (28% higher) under rain-free field conditions, while its constitutive expression under *OsActin1* promoter had a less significant effect with spikelet fertility of only 1% higher and grain yield of 11% higher (Xiao et al., 2009).

In plants, CDPKs are important sensors of environmentally induced Ca^{2+} fluxes. The majority of Ca^{2+}-stimulated protein phosphorylation is performed by CDPKs (Ludwig et al., 2004). Rice *OsCDPK7* is induced by salt and cold in roots and shoots. When *OsCDPK7* was constitutively overexpressed in transgenic rice, the extent of the tolerance of rice seedlings to drought, salt, or cold stresses correlated well with the expression level of the transgene (Saijo et al., 2000), with 50% fewer transgenic seedlings showing wilting after a 3-d water withdrawal when compared with non-transgenic controls. Three rice late embryogenesis abundant (*LEA*) genes were found to be induced in the overexpression lines. It was proposed that OsCDPK7 is likely kept in an inactive form under normal conditions, but is then quickly activated by Ca^{2+} once stress occurs. As a consequence, no negative effects on plant growth were observed as a result of *OsCDPK7*'s overexpression.

Another group of Ca^{2+}-sensing protein kinases involved in stress signaling are CIPKs. CIPKs are activated by Ca^{2+} through interaction with Ca^{2+}-binding CBL proteins during the ABA response (Hirayama and Shinozaki, 2007). Activated CIPKs transduce Ca^{2+} signals by phosphorylating downstream protein components such as the Na^{+} transporter SOS1 (Quintero et al., 2002). *Arabidopsis* has at least 10 CBLs and 25 CIPKs, which provide a high level of diversity and flexibility with respect to CBL–CIPK interactions. The CIPK-mediated signaling pathways are also found in rice (Kim et al., 2003) and maize (Zhao et al., 2009). Three rice *CIPKs* (*OsCIPK03*, *OsCIPK12*, and *OsCIPK15*) and a maize *ZmCIPK16* are all responsive to osmotic stress (Xiang et al., 2007; Zhao et al., 2009). Interestingly, when overexpressed in rice seedlings, *OsCIPK12* enhanced tolerance to drought, *OsCIPK15* to salt, and *OsCIPK03* to cold stress (Xiang et al., 2007). This suggests that each *OsCIPK* may have a distinctive role in the response to different stresses. The observed drought tolerance conferred by *OsCIPK12* expression was observed to be mediated by a significant increase in the proline and soluble sugar content after the onset of drought. As a result, the transgenic plants displayed less leaf rolling and a 33–57% higher growth recovery rate after a week-long water withdrawal and re-watering. No negative growth effects were observed in plants overexpressing the *OsCIPK* genes. Since CIPK's kinase activity also depends on its interaction with CBLs, the manipulation of CIPK alone may not always result in drought enhancement, as expected. However, constitutively active forms of CIPKs generated by mutations in the catalytic kinase domains or by deletion of the auto-inhibitory FISL motif in the kinases (Gong et al., 2002; Pandey et al., 2008) may prove useful for the application of these kinases in crop genetic engineering.

Unlike CDPKs and CIPKs, the activity of plant SnRK2 (also called SRK2 for the *Arabidopsis* ortholog) does not require direct Ca^{2+} binding, but can be induced by ABA or osmotic stress. SnRK2s belong to a relatively small plant-specific kinase family of about 10 members. SnRK2.6/SRK2E/OST (open stomata) in *Arabidopsis* was originally characterized as an ABA-activated protein kinase involved in the regulation of stomatal movement (Li and Assmann, 1996; Mustilli et al., 2002). ABA is known to induce stomatal closure by activating specific anion channels, such as the slow-type anion channel (SLAC1). It has recently been demonstrated that SnRK2.6 physically interacts with SLAC1 (Lee et al., 2009; Geiger et al., 2009). Furthermore, the interaction leads to SLAC1 activation through phosphorylation (Lee et al., 2009). Conversely, SLAC1 activity appears to be negatively regulated by selected members of the protein phosphatase 2C (PP2C) family such as ABA-insensitive (ABI)1 via dephosphorylation (Lee et al., 2009; Geiger et al., 2009). Therefore, the movement of guard cells might be controlled through the actions of activation/inactivation by a specific pair of kinase–phosphatase (Figure 1). It would be interesting to see whether activation of SLAC1 constitutively or conditionally would lead to drought stress tolerance.

SnRK2 also phosphorylates a motif in the Constant (C) subdomains in bZIP TFs including AREB1, AREB2, and ABI5 to make them active for transcription (Furihata et al., 2006). SRK2E (SnRK2.6) was identified as a key regulator of stomatal closure in *Arabidopsis* leaves (Mustilli et al., 2002), while SRK2C (SnRK2.8), abundant in roots tips, mediates drought stress

signaling in roots. When overexpressed in *Arabidopsis*, SRK2C (SnRK2.8) improved drought tolerance by up-regulating stress-responsive genes such as *DREB1* (Umezawa et al., 2004). Since the signal transduction pathways of stress-activated SnRK2s are highly conserved in higher plants, the manipulation of SnRK2 expression in *Arabidopsis* could be developed into a valuable tool for enhancing drought stress tolerance in important crops.

Two types of proteins are critical to the process of ABA perception: pyrabactin resistance (PYR)1/PYL and protein phosphatase 2C (e.g. ABI1 and ABI2) (Park et al., 2009). ABI1, a negative regulator of the ABA response, is repressed upon ABA binding to ABI1 and PYR1. Inactivation of multiple ABI1 functional homologs was shown to enhance ABA sensitivity and reduce water consumption in *Arabidopsis* (Saez et al., 2006). Certainly, the ABA receptor proteins are of immediate interest as targets for improvement of drought tolerance in crops. However, due to the high redundancy of these receptors in each species as well as in their interacting components downstream in the ABA signaling pathways, a crop-specific, precise engineering approach would be required in order to utilize these components to achieve satisfactory effects in protecting crop productivity against stresses.

Protein Poly(ADP-ribosyl)ation

Many abiotic stresses cause the accumulation of reactive oxygen species (ROS) in plant cells, which can, among other things, cause damage to genetic material and subsequently interfere with cellular energy homeostasis through the activation of Poly(ADP-ribose) polymerase (PARP, Amor et al., 1998; Doucet-Chabeaud et al., 2001), which catalyzes the synthesis of long branching poly(ADP-ribose) polymers covalently attached to proteins by consuming NAD+ pools (Bakondi et al., 2002). Reducing the activity of PARP, thus limiting its consumption of NAD+, could conceivably reduce the impact of environmental stresses on cellular energy homeostasis, resulting in the enhanced ability of the plants to tolerate stress. Both *Arabidopsis* and canola with reduced PARP activity following transformation with a PARP hairpin (hpPARP) construct were more resistant to various abiotic stresses such as drought, heat, and high light without accompanying growth defects (de Block et al., 2005). This stress tolerance was initially attributed to the maintenance of energy homeostasis due to reduced NAD+ consumption (de Block et al., 2005); however, another explanation for stress tolerance conferred by PARP deficiency is the apparent induction of specific ABA signaling pathways (Vanderauwera et al., 2007). PARP-deficient plants had elevated ABA levels and an over-representation of transcripts of ABA-responsive genes such as *ABF3* and *RD29A* in addition to transcripts for genes involved in starch metabolism and flavonoid biosynthesis. Field trials with canola and corn hpPARP-transgenic plants showed a significant difference in yield (20–40% increase for best lines) under drought (Vanderauwera et al., 2007). Since PARP is able to modify some chromatin-associated proteins and function in transcriptional regulation, it will be of great interest to dissect the chromosomal control of ABA-related gene expression by PARP activity.

Challenges with Post-Translational Manipulation for the Purpose of Enhancing Drought Tolerance

The majority of known regulatory genes (e.g. TFs and protein kinases) were originally isolated and characterized as important stress regulators based on their transcriptional induction by various stresses. However, as discussed, many proteins undergo some form of post-translational modification in order to be biologically active *in vivo* (e.g. DREB2A, Sakuma et al., 2006a; AREB1/ABF2, Furihata et al., 2006; CIPK, Gong et al., 2002; see Table 2). The expression of such genes may be constitutive in terms of transcript accumulation as revealed by transcript profiling, so the challenge in this regard is to identify components whose activities are controlled by post-translational modification or intracellular translocation rather than by transcription during stress signaling. Direct manipulation of the active form of a regulator could bypass the control from its upstream modifier, and thus may be a better approach for genetic engineering for drought-resistant plants. For instance, modifying the phosphorylation site of the bZIP TF TRAB1 (Kagaya et al., 2002) or AREB1/ABF2 (Furihata et al., 2006) to enhance protein activity resulted in the induction of many ABA-responsive genes without exogenous ABA application.

The successful application of manipulating genes involved in protein modification was well demonstrated in drought-tolerant canola suppressing protein farnesylation (Wang et al., 2005, 2009) and rice with elevated levels of CDPK or CIPK (Saijo et al., 2000; Xiang et al., 2007). Many regulatory proteins and enzymes can be switched on and off by phosphorylation and dephosphorylation so as to control a wide range of cellular processes or signal relays. However, the *in vivo* substrates of many stress-responsive protein kinases remain unknown because of the low substrate specificity of kinases in *in vitro* activity assays. Therefore, one of the great challenges ahead is the identification of the *in vivo* substrates of these protein kinases or other post-translational modifiers in order to be able to genetically fine-tune the effectors for stress tolerance.

METABOLITES AND OSMOPROTECTANTS IN DROUGHT RESPONSE

ABA Metabolism

One of the quick responses of plants to drought stress is the accumulation of ABA in plant cells (Hsiao, 1973), which triggers ABA-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006) and stomatal closure to reduce transpirational water loss (Schroeder et al., 2001). ABA *de novo* biosynthesis occurs in leaves, stems, and roots of almost all plant species. The initial four catalytic steps of ABA biosynthesis, which

involves the conversion of β-carotene to xanthoxin, primarily take place in plastids (e.g. chloroplasts in the leaf) via ABA1/low expression of osmotically responsive gene 6 (LOS6), ABA4, and 9-*cis*-epoxycarotenoid dioxygenase (NCED) enzymatic activities, while the last two steps occur in the cytoplasm to produce bioactive ABA via ABA2 and ABA3/LOS5 (Wasilewska et al., 2008; Wan et al., 2009). In the cytoplasm, ABA is readily perceived by the recently identified ABA receptors (PYR1/ABI1, Park et al., 2009), which then relay the signal to the AREB TFs to regulate the ABA-dependent expression of numerous downstream target genes for drought adaptation. ABA can also be directly converted from its inactive glucose ester conjugate storage form in vacuoles to bioactive ABA via β-glucosidase (AtBG1, Lee et al., 2006) and *vice versa*. After rehydration or drought release, ABA is catabolized to phaseic acid or dehydrophaseic acid by Cyp707A-mediated hydroxylation to maintain its homeostasis (Kushiro et al., 2004).

Although the genes for the metabolism of ABA in *Arabidopsis* have all been characterized, and those for ABA perception and the downstream signal relay are being described at rapid pace, the manipulation of only one gene (*LOS5/ABA3*) has been reported so far to have an effect on crop (rice) drought tolerance enhancement (Xiao et al., 2009). LOS5/ABA3 is a key enzyme working in the last step of ABA biosynthesis, and its loss-of-function mutant *los5* had reduced tolerance to drought, salt, and cold stresses (Xiong et al., 2001). The constitutive or drought-inducible overexpression of *LOS5* in rice significantly increased the spikelet fertility and yield of transgenic plants under field conditions (Xiao et al., 2009). NCED is drought-inducible and a rate-limiting enzyme for ABA biosynthesis. Thus, it may be the best candidate for gene manipulation to regulate ABA homeostasis in plants. However, although overexpression of *LeNCED1* in tomato (*Solanum lycopersicum* L.) did increase ABA accumulation and WUE in transgenic tomato, it had no effect on dehydration tolerance (Thompson et al., 2007) while causing severe growth defects such as stunted plants and increased seed dormancy (Thompson et al., 2000; Tung et al., 2008).

Phospholipid Signaling

Membrane-component phospholipids and their metabolites are important second messengers in plant development and in response to environmental stimuli (Testerink and Munnik, 2005; Xue et al., 2009). Phosphatidylinositol-specific phospholipase C2 (PtdIns-PLC2) hydrolyzes PtdIns-4,5-biphosphate (PtdIns(4,5)P2) to produce Inositol 1,4,5,-triphosphate (Ins(1,4,5)P3), which acts as a second messenger for Ca^{2+} release from internal storages. The Ca^{2+} oscillation mediated by PtdIns(4,5)P2 and Ins(1,4,5)P3 is crucial for guard cell movement as well as for other plant growth processes. In addition, phospholipase D (PLD) hydrolyses phospholipids at the terminal phosphodiester bond to generate phosphatidic acid (PA), which interacts with and represses the activity of ABI1, resulting in enhanced ABA response and stomatal closure (Zhang et al., 2004b). PA is likely the functional link between the phospholipid and ABA signaling pathways with respect to stomatal movement and drought response.

Arabidopsis PLC (Tasma et al., 2008) and *PLD* (Katagiri et al., 2001) genes are both induced by dehydration and involved in drought tolerance (Sang et al., 2001; Hong et al., 2008). Maize overexpressing *ZmPLC1* (Wang et al., 2008a) exhibited a higher cellular solute content and increased rate of photosynthesis, whereas canola overexpressing *BnPtdIns-PL2* (Georges et al., 2009) exhibited a lower rate of stomatal transpiration and a smaller stomatal aperture. Transgenic maize were more productive, as indicated by showing 14% higher kernel weight per ear under drought, while transgenic canola were able to survive progressive water deprivation for 24 d, under which conditions the control plants died.

Heat or Cold Shock Protein (HSP or CSP) Chaperones

Drought, salt, and high temperature can cause the misfolding and dysfunction of many RNAs and proteins. Some stress-induced genes encode proteins to protect the conformation of other proteins, RNAs, or cell structures. These include numerous HSPs and CSPs, which are required not only for quick adaptation to temperature changes, but also for rapid recovery after heat or cold release. Small HSPs (sHSPs) represent the major family of HSPs induced by heat in plants. It has been observed that the exposure of rice seedlings to high temperature (42°C for 24 h) resulted in a significant increase in drought tolerance, and rice seedlings overexpressing rice *sHSP17.7* also had enhanced tolerance to drought stress, suggesting that the observed drought-tolerance acquisition after heat shock was associated with the accumulation of sHSP proteins (Sato and Yokoya, 2008). Endoplasmic reticulum (ER) luminal binding protein (BiP), a member of the HSP70 family of protein chaperones, is induced by abiotic stresses and overexpression of *soyBiPD* conferred drought resistance to soybean plants and resulted in a delay in drought-induced leaf senescence through an unknown mechanism (Valente et al., 2009).

CSPs, which rapidly accumulate in some bacteria under low-temperature stress, function as RNA chaperones to stimulate growth following stress acclimation. Bacterial CspA and CspB, when expressed in *Arabidopsis*, rice, and maize, conferred enhanced tolerance to drought, cold, and heat by protecting and improving vegetative growth, photosynthesis, and reproductive development (Castiglioni et al., 2008). Multiple-location, multiple-year, and multiple-hybrid field trials with *CspA* or *CspB*-expressing maize showed yield increases of 11–21% for maize plants under limited water supply during or after vegetative-to-reproductive transition. No yield drag was observed for *CspB*-transgenic maize under normal water supply; however, the significant effect of CspB on corn yield protection was achieved only under severe drought that often caused about 50% overall yield losses when compared with yields from unstressed transgenic plants (Castiglioni et al., 2008). Nevertheless, commercial grade hybrid corn modified with this technology is on its way to the marketplace, intended for

sub-optimal corn farming systems, and the regulatory approval for *CspB* transgenic corn is underway (Edmeades, 2008).

Late Embryogenesis Abundant (LEA) Proteins

LEA proteins accumulate in embryos during seed desiccation and are also induced in vegetative tissues by dehydration, cold, salt, and ABA treatment. LEAs are extremely hydrophilic and are involved in adaptive responses to hyperosmotic conditions through the maintenance of protein or membrane structure, sequestration of ions, binding of water, and their operation as molecular chaperones (Bray, 1997). ABA-inducible HVA1, a group 3 LEA protein from barley, was reported to sequester ions (e.g. Na^+) during cellular dehydration (Hong et al., 1988). The idea of overexpressing *LEA* genes for drought tolerance has been tested. These include rice expressing barley *HVA1* (Xu et al., 1996; Rohila et al., 2002; Babu et al., 2004) or rice *OsLEA3-1* (Xiao et al., 2007); spring wheat (*Triticum aestivum*) expressing barley *HVA1* (Sivamani et al., 2000); and Chinese cabbage (*Brassica chinensis*) expressing a canola *LEA* (Park et al., 2005). The transgenic wheat had significantly higher dry mass accumulation and WUE under moderate water-deficit conditions in greenhouse tests (Sivamani et al., 2000); however, no consistent results were obtained in multiple-season/location field trials (Bahieldin et al., 2005). The *HVA1*-transgenic rice and *LEA*-transgenic Chinese cabbage exhibited improved tolerance at the vegetative stage to water deprivation and to salt stress in potted soil, as indicated by higher growth rates and faster recovery from the stresses (Xu et al., 1996; Park et al., 2005). The dehydration and salt tolerance likely resulted from better cell membrane protection, as indicated by low membrane electrolyte leakage, but was not likely due to osmotic adjustment (the ratio of leaf osmotic potential before and after dehydration stress), which was found to be quite similar among transgenic and control plants (Rohila et al., 2002; Babu et al., 2004). Under rain-free field drought conditions, the transgenic rice expressing *OsLEA3-1* under either its own drought-inducible promoter or the 35S promoter had significantly higher spikelet fertility and grain yield (~45%) than wild-type (Xiao et al., 2007). Even more promisingly, no significant yield penalty was detected between the transgenic and wild-type plants under normal field conditions. However, the significant yield protection attributed to *OsLEA3-1* expression was observed only under severe stress that still led to about 45% yield loss when compared with wild-type plants under normal field condition (Xiao et al., 2007).

Amino Acids (Proline)

Drought resistance could also be improved by increasing in plant cells the accumulation of metabolites that function as adaptive osmolytes or antioxidants. These metabolites function via either osmotic adjustment (OA) to increase water retention or as osmoprotectants to stabilize other molecules under stress conditions.

Proline is one of the most common compatible solutes (small hydrophilic organic molecules that accumulate to high concentrations) in water-stressed plants and in many other organisms (Delauney and Verma, 1993). The accumulation of proline in dehydrated plants is caused by both the promotion of its biosynthesis and by the prevention of its degradation. Water stress-induced overexpression of 1-pyrroline-5-carboxylate synthase (P5CS), the rate-limiting enzyme for proline biosynthesis in rice (Zhu et al., 1998), or 1-pyrroline-5-carboxylate reductase (P5CR) in soybean (de Ronde et al., 2004a, 2004b) increases proline content by about 2.5-fold after drought induction, resulting in increased relative water content (RWC) and better growth under water-deficit conditions, whereas the suppression of P5CR in soybean resulted in an increased sensitivity to osmotic, drought, and heat stresses (de Ronde et al., 2000). Other evidence suggests a role for proline in drought adaptation through the DREB1 pathway. For example, tall fescue and *Arabidopsis* overexpressing *AtDREB1A*/*CBF3* (Gilmour et al., 2000; Zhao et al., 2007b), tomato overexpressing *AtDREB1B*/*CBF1* (Hsieh et al., 2002), and rice overexpressing *OsDREB1* or *AtDREB1* (Ito et al., 2006) have been shown to accumulate higher levels of proline than wild-type plants under both normal and water-deficit conditions. Correspondingly, the transgenic plants were more resistant to water-deficit stress than the wild-type controls.

Glycine Betaine (GB)

Plants from several families (e.g. *Gramineae*, *Chenopodiaceae*, *Compositae*) produce GB in response to salt or water stress. GB accumulates in the chloroplasts and plastids of these and many other halotolerant plants, presumably to stabilize photosynthetic and translational machineries via ROS scavenging and protein protection (Chen and Murata, 2008). When applied exogenously, GB translocates actively to all parts of plants, whereas GB produced *in vivo* accumulates five times more in flowers and siliques than in leaves of transgenic *Arabidopsis*, indicating its potential role in protection in reproductive machinery (Sulpice et al., 2003).

Genetic engineering to increase GB biosynthesis (by dehydrogenation or oxidation of choline) had only a marginal effect on GB levels (Bartels and Sunkar, 2005), and tolerance enhancement by this method has been more successful in salt and chilling stresses than in drought stress (Chen and Murata, 2008). Maize plants transformed with *betA*, which encodes a choline dehydrogenase, accumulated more GB than controls when exposed to drought stress in the field (Quan et al., 2004). After a 3-week drought stress, reproductive development of transgenic plants was less inhibited, as evidenced by a 10–23% higher grain yield per plant than that of wild-type. *betA*-transgenic cotton were tolerant to drought stress, as they displayed a higher RWC, increased photosynthesis, better OA, a lower percentage of ion leakage, and less lipid membrane peroxidation than the wild-type controls (Lv et al., 2007).

A novel GB biosynthesis pathway via glycine was reported recently in halophilic microorganisms (Nyyssola et al., 2000;

Waditee et al., 2003). *Arabidopsis* expressing genes from this pathway accumulated more GB than plants expressing the choline monooxygenase gene from the conventional choline-mediated pathway (Waditee et al., 2005). Whether commercially important crops can be engineered to take advantage of this novel pathway remains to be tested.

Sugars (Trehalose, Mannitol)

Sugar osmolytes include simple sugars (e.g. glucose), sugar alcohols (e.g. mannitol), and complex sugars (e.g. trehalose). Trehalose is synthesized in response to dehydration stress in various organisms, such as insects and lower plants, to stabilize dehydrated proteins, lipid membranes, and biological structures. But, among higher plants, most species, except for highly desiccation-tolerant resurrection plants, accumulate only trace amounts of trehalose. To increase the biosynthesis of trehalose in rice (Garg et al., 2002; Jang et al., 2003) and tomato (Cortina et al., 2005), bacterial and yeast trehalose-6-phosphate synthase (TPS) and phosphatase (TPP), key enzymes in trehalose biosynthesis, were engineered into the plant genomes under the control of either tissue-specific, stress-responsive, or constitutive promoters. Transgenic rice plants could accumulate two to nine times more trehalose than non-transgenic controls (Garg et al., 2002) or even more when TPS and TPP were expressed as bifunctional fusion proteins in rice (Jang et al., 2003). After 8 or 12 d of water withholding, non-transgenic rice exhibited leaf rolling and wilting, while transgenic rice showed vigorous shoot growth with considerably fewer visual stress symptoms (Garg et al., 2002; Jang et al., 2003). In addition, transgenic rice exhibited less photooxidative damage under salt and low-temperature stresses.

Wheat does not naturally synthesize mannitol; however, expressing the *E. coli mtlD* gene caused the accumulation of mannitol in transgenic wheat cells (Abebe et al., 2003). A high accumulation of mannitol generally causes severe abnormalities such as sterility and stunted growth; however, a moderate accumulation of mannitol was shown to improve the tolerance of transgenic wheat to water and salt stresses by facilitating increased biomass and plant height under stressed conditions compared to wild-type (Abebe et al., 2003).

Polyamines

Polyamines are small nitrogenous compounds involved in plant responses to various stresses. The physiological significance of polyamines in stress response is not yet thoroughly understood (Groppa and Benavides, 2008). In plants, polyamines (spermidine and spermine) are derived from arginine via enzymes such as arginine decarboxylase (adc) and S-adenosylmethionine decarboxylase (samdc). Transgenic rice expressing oat *adc* (Capell et al., 1998) or jimsonweed (*Datura stramonium*) *adc* had a significant increase in putrescine, a precursor of spermidine and spermine (Capell et al., 2004). The increase surpassed the threshold required to trigger the conversion of putrescine to spermidine and spermine; however, only 1.5-fold increases of each compound were observed in transgenic rice subjected to drought stress, suggesting a tight control of polyamine homeostasis in plants. Nevertheless, *adc*-transgenic plants showed no chlorophyll loss after 8 d of drought stress (Capell et al., 1998) and developed no stress symptoms, such as rolling leaves or wilting, as controls did under drought (Capell et al., 2004). Since the spermidine and spermine content in rice was not correlated with mRNA levels of *adc* but was with that of *samdc*, the latter, which actually catalyzes the formation of the polyamines from putrescine precursors, should be the primary candidate for plant engineering. An alternative explanation for the low polyamine accumulation in transgenic rice is that the over-accumulation of polyamines is toxic to plants (Capell et al., 1998), in which case constitutive overexpression of *adc* or *samdc* should be avoided for genetic engineering.

CHALLENGES WITH THE MANIPULATION OF METABOLITES AND OSMOPROTECTANTS FOR THE PURPOSE OF ENHANCING DROUGHT TOLERANCE

There have been very few successful genetic engineering examples to improve plant tolerance against dehydration stress by increasing cellular content of compatible solutes (see Table 3). This is likely due to two main reasons: (1) the transgene-induced increase in compatible solutes, in most cases, represented only a small portion of the total OA of plant cells under osmotic stress; and (2) the induced increase often resulted in impaired plant growth, even in the absence of stress (Holmström et al., 1996; Abebe et al., 2003; Karim et al., 2007). Osmolyte-mediated OA responsible for maintaining cell turgor, when it occurs in the root or root tips, could allow continued root development into deeper soil and thus give plants access to a deep water reservoir (Serraj and Sinclair, 2002; Sinclair et al., 2004). In contrast, OA-mediated cell turgor maintenance in leaves may prolong stomatal opening and thus lead to more water transpiration during drought stress. Taking into account the growth retardation of plants overproducing osmolytes, investigations that seek to improve crop performance by enhancing OA need to focus on the root or root tip by using tissue-specific expression systems.

The functional mechanisms of some compatible solutes in drought tolerance enhancement are, however, still unclear or controversial. Experiments expressing tomato *P5CS* in yeast cells, for example, showed that yeast growth is inversely correlated with proline accumulation under osmotic stress conditions (Maggio et al., 2002), suggesting that proline or other osmolytes may not act as osmoprotectants.

The successful application of *betA* gene expression (GB biosynthesis) in drought-tolerance enhancement of maize and cotton propelled the search for the mechanism behind GB function. GB, like plant hormones, can be exogenously applied to, quickly taken up by, and actively spread to all parts of the plant. Moreover, GB has an *in vivo* concentration in the millimolar range, which is the effective concentration range of most plant hormones. As such, it was postulated that, like phytohormones,

GB's effects might be manifested through the induction of specific downstream genes. However, no drought-related genes have been confirmed so far to be directly induced by GB.

In the case of sugar solutes, it was proposed that the primary effect of trehalose is not actually as a compatible solute (Garg et al., 2002). Rather, increased trehalose accumulation correlates with higher soluble carbohydrate levels and an elevated capacity for photosynthesis under both stress and non-stress conditions. This suggests that the role of trehalose in stress tolerance is actually through the modulation of sugar sensing and carbohydrate metabolism. In transgenic wheat that accumulated mannitol, it was found that the concentration of mannitol was too low to function as an osmolyte, but may have functioned as a ROS scavenger (Abebe et al., 2003). It should also be noted that the high level of osmolyte accumulation in plants necessary for drought tolerance improvement is biologically expensive; therefore, the effect on plant biomass production or on yield should be evaluated.

Single genes are more amenable to manipulation but less sustainable under various field conditions. To compensate for built-in feedback control mechanisms or to avoid the over-accumulation of intermediate products, it is sometimes required that multiple genes be manipulated. For instance, stress-tolerant *TPS*-expressing tobacco plants had a higher trehalose content but also presented with developmental irregularities such as stunted growth and altered leaf shape (Holmström et al., 1996; Karim et al., 2007), possibly due to the over-accumulation of trehalose-6-phosphate (T6P, Schluepmann et al., 2004). To avoid growth defects while maintaining drought tolerance, three strategies were successfully applied: (1) co-expression of *TPS* and *TPP* to reduce the over-accumulation of harmful T6P intermediates; (2) stress-induced expression of TPS using an appropriate promoter; and (3) the targeting of TPS to chloroplasts where it functions naturally (Karim et al., 2007). In addition, a fusion-gene strategy was successfully applied in rice to allow the overexpression of two trehalose biosynthesis genes (*otsA* and *otsB*), requiring only a single transformation event and resulting in a higher net catalytic efficiency for trehalose formation (Garg et al., 2002).

Nevertheless, very encouraging results for enhanced drought tolerance of commercial crops is coming from, for instance, the overexpression of some molecular chaperones in maize or rice (Castiglioni et al., 2008). Constitutive overexpression of ubiquitous RNA chaperones (e.g. CSP) of bacterial origin made transgenic plants more tolerant to multiple stresses, but caused no negative pleiotropic effects, suggesting that the small chaperone molecules might be good candidates for drought tolerance enhancement.

CONCLUSION

This review focused on the recent progress in genetic manipulation of important crops for the enhancement of drought tolerance, which is a very complex plant trait. It should be borne in mind that tolerance to a given stress such as drought should ultimately be evaluated in association with other major stresses such as high temperature, salt, and cold because of the cross-talk between these stress-response pathways. The majority of the genes in the literature reported to play a role in drought tolerance also, to some extent, confer some degree of tolerance to salt and cold stresses. However, crops tolerant to drought as well as heat will be in immediate demand for a world that is generally hotter and drier as a result of global climate change (Battisti and Naylor, 2009). Since our knowledge about stress signaling networks is still limited, the challenge in the near future will be to identify the signaling elements missing in our current models of pathways and increasing our understanding of the cross-talk between pathways.

Outside of the scope of this review due to the absence of literature available on the subject as it pertains to drought tolerance in crop species are non-coding regulatory RNAs. Regulatory RNAs including siRNAs and miRNAs have been emerging as important regulators in abiotic stress response and tolerance (Borsani et al., 2005; Zhao et al., 2007a; Li et al., 2008). It is very likely that regulatory proteins work in concert with regulatory RNAs to fine-tune the temporal and spatial expression of genes in stress response (Li et al., 2008; Alexandre et al., 2009).

The identification of commercial grade transgenes that enhance crop performance under both drought and optimal conditions is a lengthy, tedious, and expensive process. Nevertheless, the successful genetic engineering of canola (Wang et al., 2005, 2009), rice (Hu et al., 2006; Xiao et al., 2007, 2009), and maize (Nelson et al., 2007; Castiglioni et al., 2008) for improved drought tolerance as reviewed herein confirms that the approach is feasible (Tables 1–3 and Figure 1). However, among the reports with promising drought-tolerance enhancement under field conditions, there were only a few showing significant net crop yield benefit (Wang et al., 2005, 2009), while the majority of the reports showing a yield benefit are based on experiments in which the drought conditions were too severe and yields were usually too low to be acceptable for agricultural practice, especially in developed countries. So far, none of the reported technologies has been tested in actual farmers' fields, although some commercial plans have been proposed for within the next couple of years by some biotech companies. Ultimately, with the use of the powerful molecular and genetic tools available, such as stress-metabolite profiling (Urano et al., 2009), functional genomics, and proteomics, more and more key regulators of plant stress responses will be identified and utilized as target genes or molecular markers for crop genetic engineering or marker assistant selection to create cultivars with yield stability in drought-prone environments.

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