Evaluation of Drought-Tolerance Strategies in Cotton

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Research made possible through the use of the wealth of genomic information and genetic resources available for model plant species such as *Arabidopsis thaliana* has led to a substantial increase in our knowledge about how plants respond to stressful environmental conditions. This leap in understanding provides the basis for many new strategies for optimization of stress tolerance in crop plants through both traditional and transgenic approaches. Although, to date, most of these strategies have been tested only in model plants, evaluation of some of them in important crop species, including cotton, is now underway. Application of novel stress-tolerance technologies will require extensive physiological and agronomic evaluation of plants that express these genes to determine their ultimate applicability to crop systems. A broad-based evaluation strategy for candidate genes will be necessary to test their value under a wide variety of field conditions. In cotton, this will include testing the effects of candidate genes on germination, seedling survival and stand establishment, vegetative growth and the transition to flowering, flowering period, boll maturation, and fiber development and quality. Though experiments in model species are valuable, experience shows that they do not guarantee similar results in a heterologous crop species. Our preliminary work with transgenic cotton plants that express transcription factors, such as ABF3 and CBF3, show that optimization of expression patterns using different promoters or other regulatory strategies will be necessary. The recent identification of complex post-transcriptional and post-translational regulatory mechanisms for stress-responsive gene expression reinforces the need for direct testing of new technologies in crop species. These mechanisms are able to fine-tune the expression of stress-responsive genes by ensuring their suppression under non-stressful conditions and attenuating stress responses in the face of prolonged stress exposure. However, since these mechanisms can be sequence-specific, we anticipate that, in some cases, different

results will be achieved in plants depending on whether endogenous or heterologous coding sequences are used.

The translational genomic approach, in which the depth of knowledge gleaned from a model system is systematically translated to economically important crop plants that are less amenable for basic research, is a powerful strategy for crop improvement. Although it may not yet be possible to accurately predict the outcome of any particular transgenic experiment, the ability to narrow the group of candidate genes based on experimental data from model species makes it possible to approach these experiments in a much more rational way. As our understanding of the complex regulatory networks that control stress-responsive gene expression develops and the roles of these regulatory mechanisms in plant productivity are revealed, it is likely that substantial increases in crop yield under suboptimal conditions can be realized.

Abiotic Stresses

Water deficit, salinity, and temperature extremes are the primary factors that limit crop productivity, accounting for more than a 50% reduction in yields worldwide (Boyer, 1982). Approximately 22% of agricultural land is saline (FAO, 2004) and drought-affected areas are expanding and this trend is expected to accelerate (Burke *et al.,* 2006). Growth of the human population combined with increasing prosperity in developing countries and a decrease in arable land are creating greater demands for food, fiber, biomaterials, and sustainable agriculture (Ragauskas *et al.*, 2006). A large proportion of available fresh water is consumed by agriculture and drought is a perennial environmental constraint, affecting approximately 25% of all crops worldwide at enormous cost. For example, dry-land cotton crops are decimated by drought on a regular basis and estimates of the value of cotton varieties with increased photosynthetic and water-use efficiencies, enhanced flowering, and improved seed qualities exceed $1 billion/yr in the United States, and $5 billion/yr globally (Wilkins *et al.*, 2000). The task of identifying gene functions and developing effective strategies to use these functions for crop improvement is daunting and a more complete understanding of these mechanisms is needed to achieve the promises of plant biotechnology.

Drought induces a wide range of plant responses, including stomatal closure, changes in gene expression, accumulation of abscisic acid (ABA), production of osmotically active compounds, and the synthesis of protective proteins that scavenge oxygen radicals or act as molecular chaperones (Wang *et al.*, 2003). These responses are controlled by molecular networks that activate stress-responsive mechanisms to re-establish homeostasis and protect and repair damaged cellular components (Ramachandra-Reddy, 2004). Abiotic-stress responses are genetically complex and, thus, difficult to manipulate. Early experimental strategies for engineering abiotic-stress tolerance in plants relied largely on the expression of genes that encode protective molecules such as dehydrins and antioxidant enzymes or on enzymes involved in the synthesis of functional and structural metabolites (for examples, see Roxas *et al.*, 2000; Kornyeyev *et al.*, 2001; Payton *et al.*, 2001; Park *et al.*, 2005). More recently, strategies to use genes that are involved in signaling and regulatory pathways have shown great promise (Umezawa *et al.*, 2006).

Development of transgenic strategies by the introduction of selected genes provides a focused approach for the improvement of abiotic-stress tolerance in plants. Genetic engineering allows the transfer of genes from any source, including non-plant species and permits the expression of the introduced genes to be controlled both temporally and spatially. This capability can be critical if expression of a given gene is needed only at a defined developmental stage, in a certain organ or tissue, or in response to specific environmental conditions. Although promoters that are constitutively expressed at high levels are still widely used, they are not appropriate for all transgenes. This is especially true for stress-responsive genes, which often have serious deleterious effects when constitutively expressed. Therefore, transgenic modification provides a wide variety of options for the development of novel strategies for crop improvement.

In addition to the technology used to generate transgenic plants that express their introduced genes in an appropriate way, it is also important to evaluate these transgenic plants such as to determine the effects of the introduced gene on stress-tolerance characteristics. In many cases, transgenes have been tested only in model-system plants such as arabidopsis [*Arabidopsis thaliana* (L.) Heynh.] or tobacco (*Nicotiana tabacum* L.). While these "proof-of-concept" experiments can give important clues about the potential usefulness of specific genes in plants such as cotton, much of the published work depends on artificial environments that are unlike those faced by crops under field conditions. In addition, physiological characterization often does not extend beyond evaluation of growth or survival under severe conditions. Therefore, rigorous physiological evaluation of the tolerance of transgenic crop plants to abiotic stresses and the effects of specific transgenes on agronomic traits such as yield and quality are needed. In the paragraphs below, I briefly summarize recent progress in the understanding of stress-responsive regulatory pathways in plants and the evaluation of these mechanisms for their potential utility in the improvement of abiotic-stress tolerance, with emphasis on drought, salinity and temperature extremes. I also outline, where possible, the published and preliminary experiments in which transgenic modification of stress tolerance in cotton has been attempted.

Plant Stress Responses

Much has been learned about the role of specific stress-protective genes in determining stress-tolerance phenotypes through experiments in transgenic plants. Many of the transgenic plant lines produced show detectable increases in tolerance to specific or, sometimes, multiple stresses under laboratory conditions. However, in only a few cases have the effects of these genes been tested in the field. Although promising, the levels of stress tolerance provided from the transfer of a single gene encoding a specific stress-protective protein may not reach the levels necessary to justify incorporation into a commercial variety. Doubts about whether the resulting improvement in stress tolerance is of sufficient magnitude to provide an appreciable improvement in the performance of a certain crop under field conditions make it difficult to justify the huge financial investment necessary to bring a transgenic variety to market. Thus, efforts have more recently focused on functional evaluation of genes that play crucial roles in the regulation of native stress responses in plants. These experiments provide important new understanding of the complex regula-

tory networks that plant cells use to sense and respond to stressful conditions and provide additional opportunities for the use of transgenic strategies to alter plant-stress responses that may provide stress-tolerance traits that are sufficiently robust to justify commercial investment.

Transcription Factors

Genetic dissection of plant signal transduction has provided an important framework for the development of a more complete understanding of the complex signal-transduction pathways that regulate plant responses to abiotic stress. Due to amenability of analysis, genetic characterization of plant-stress responses has focused primarily on arabidopsis. These efforts have identified diverse classes of transcription factors that are associated with stress-responsive gene expression, including the MYC/MYB, basic leucine zipper domain (bZIP), homeodomain leucine zipper (HD-Zip), nuclear factor Y (NF-Y), ABI3/VP1, WRKY, and various zinc-finger protein families.

Considerable overlap exists between the signal-transduction events that occur during exposure to cold and drought stress (for reviews, see Shinozaki *et al.*, 2003; Zhang *et al.*, 2004). Some of these processes are regulated by abscisic acid (ABA), whereas others appear to be ABA independent. For detailed reviews of the regulation of gene expression by ABA, see Finkelstein *et al.*, (2002); Himmelbach *et al.*, (2003); Kuhn and Schroeder (2003). ABA-response elements (ABREs) are located upstream of many ABA-responsive genes. These *cis*-acting elements interact with a class of bZIP transcription factors known as ABFs. Promoters with optimal ABA responsiveness often contain a second *cis*-acting element (Shen and Ho, 1995) that is similar to a C-repeat/dehydration-responsive element (CRT/DRE). Thus, ABRE-binding bZIP proteins and CRT/DRE-binding AP2 factors (CBF/DREB1) may interact to control gene expression in response to ABA, osmotic stress and cold temperatures (Narusaka *et al.*, 2003) (Figure 1).

Constitutive expression of the ABA-dependent bZIP transcription factors ABF3 or ABF4 in arabidopsis resulted in up-regulated expression of several ABA/stress-responsive genes including *RD29B, RA18*, *ABI1* and *ABI2*, leading to enhanced drought tolerance (Kagaya *et al.*, 2002; Kang *et al.*, 2002). Constitutive over-expression of ABF2 in arabidopsis also resulted in other ABA-associated phenotypes, including hypersensitivity to ABA and sugar and stunted growth (Kang *et al.,* 2002). However, expression of the arabidopsis *ABF3* gene in rice and lettuce under control of the constitutive *Ubiquitin 1* promoter from maize resulted in enhanced drought tolerance without negative effects on plant growth and development (Oh *et al.,* 2005; Vanjildorj *et al.,* 2005).

Based on these successful examples, transgenic cotton plants with increased expression of arabidopsis ABF3 were created in our laboratory (L. Aleman, H. Abdel-Mageed, R.D. Allen, unpublished data). Our preliminary observations indicate that transgenic cotton plants that constitutively express ABF3 under control of the CaMV 35S promoter exhibit enhanced expression of ABA-responsive genes under non-inductive conditions and show enhanced survival under severe water deficit (Figure 2). However, these plants also exhibit deleterious side-effects including reduced vegetative growth and delayed flowering. The relatively severe negative effects associated with constitutive expression of

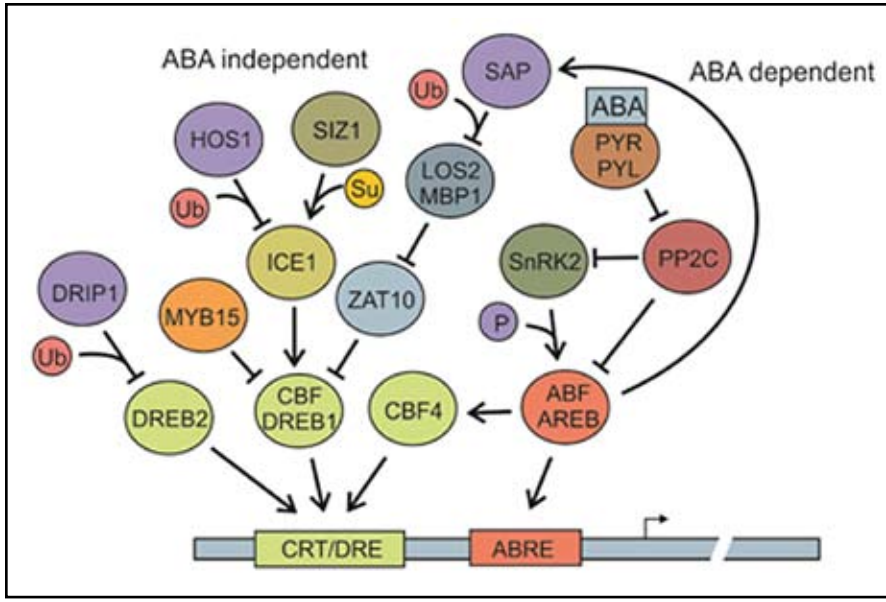

Figure 1. Simplified model for the transcriptional and post-transcriptional regulatory mechanisms that mediate stress-responsive gene expression in plant cells. Upstream regulatory sequences of many stress-responsive genes carry *cis*-acting sequences that bind stress-regulated transcription factors. In this model, ABA-dependent activation is mediated via ABF/AREB transcription factors, whereas ABA-independent responses are controlled by CBF/DREB1 factors. Under normal conditions, transcription of *CBF* genes is suppressed by MYB15 and the zinc-finger transcriptional regulator ZAT10. Levels of the transcriptional activator protein ICE1 remain low under these conditions due to HOS1-dependent ubiquitination and degradation. Stress exposure leads to stabilization of ICE1 thorough SIZ1-dependent sumoylation.[1] In contrast, activation of the ABA-responsive ABF/AREP transcription factors is mediated primarily through a protein phosphorylation/dephosphorylation cascade. Under normal conditions, ABFs are maintained in the inactive non-phosphorylated state. Binding of ABA to the ABA receptor (PYR/PYL) inactivates PP2C protein phosphatases that prevent auto-phosphorylation of SnRK2 protein kinases. Activation of SnRK2s leads to phosphorylation of ABF. In this hypothetical example, crosstalk between these ABA-dependent and ABA-independent pathways can occur through the ABA-responsive expression of genes that encode CBF4 and SAP ubiquitin ligases. SAPs may target the LOS2 MBP-1-like transcriptional repressor. Expression of stress-protective genes by the CBF/DREB1 and ABF/AREB signaling pathways is controlled by a complex web of transcriptional and post-transcriptional regulatory mechanisms. Each of these regulatory steps provides an opportunity for the development of novel strategies for the genetic optimization of stress tolerance in plants.

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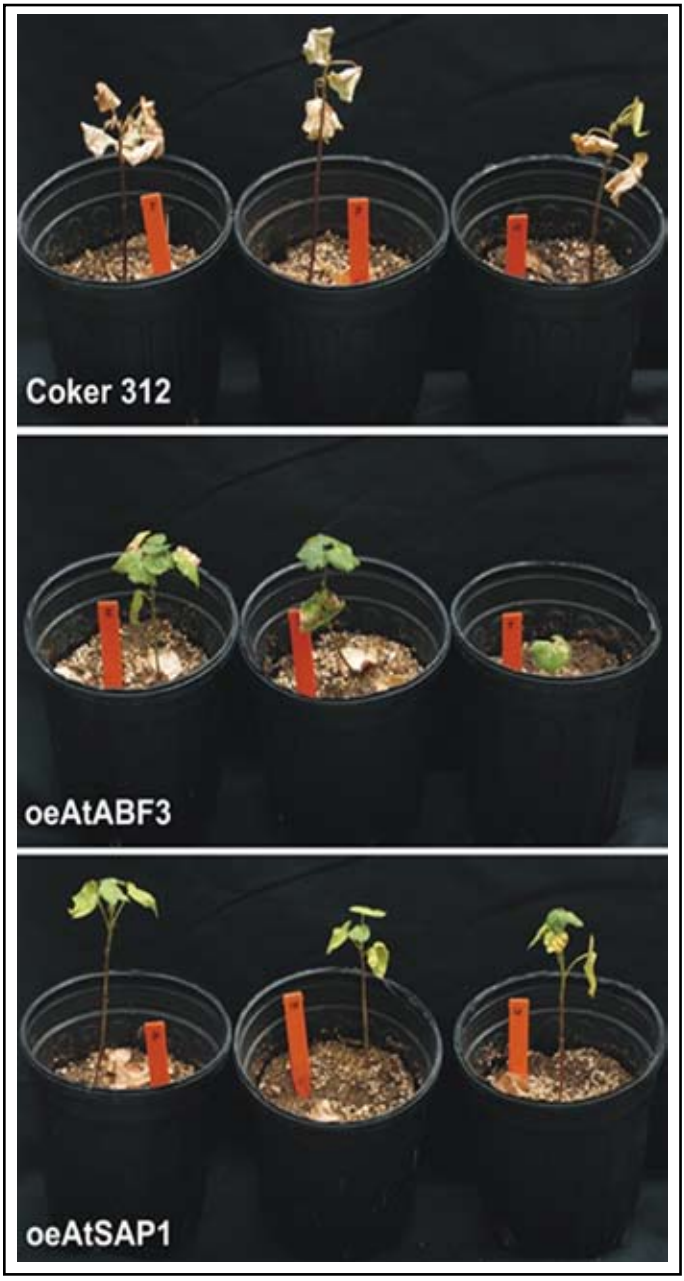

Figure 2. Comparison of water-deficit survival of greenhouse-grown wild-type cotton plants (Coker 312) and transgenic cotton plants that constitutively express either AtABF3 or AtSAP1. Plants were grown with ample moisture to the 5th leaf stage then water was withheld until the wild-type plants reached terminal wilt. The plants were re-watered one day before they were photographed. Wild-type plants were dead after this severe water-deficit exposure whereas plants of both transgenic genotypes survived and recovered from the stress treatment.

ABF3 in cotton led us to conclude that it responds too strongly to this foreign transcription factor. Therefore, development of transgenic plants using native *ABF* genes or in which *ABF3* expression is controlled by stress-responsive promoters may be required if this gene is to be used to improve stress tolerance without negative impacts on yield and other properties. Cotton plants that express these stress-responsive *ABF3* transgenes are now being tested.

Cold acclimation in arabidopsis involves the cold-responsive expression of a large number of genes, many of which are regulated by the *CBF/DREB1* regulon (Thomashow, 1998). *CBF/DREB1* genes in arabidopsis are expressed at low levels under normal growth conditions, but their expression increases within minutes after exposure to cold or drought stress. This gene family includes *CBF1*, *CBF2* and *CBF3* genes (Gilmour *et al.*, 1998; Medina *et al.*, 1999; Jaglo *et al.*, 2001), also known as *DREB1B*, *DREB1C* and *DREB1A*, respectively (Liu *et al.*, 1998). These genes encode transcriptional activators that bind to the conserved CRT/DRE DNA elements located in the promoters of certain cold-responsive genes (Baker *et al.*, 1994; Yamaguchi-Shinozaki *et al.*, 1994; Stockinger *et al.*, 1997; Gilmour *et al.*, 1998). Ectopic expression of these transcription factors in transgenic plants led to elevated freezing tolerance without prior cold treatment (Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Gilmour *et al.*, 2000). Microarray analyses of arabidopsis genes during cold acclimation indicated that the expression of about 500 genes was either up- or down-regulated in response to low temperature (Fowler and Thomashow 2002; Vogel *et al.*, 2005). However, only about 15% of these genes were also responsive to CBF/DREB1 expression and could, therefore, be assigned to the CBF/DREB1 regulon. Therefore, although many of the genes that are most strongly induced in response to low temperature are regulated by CBF/DREB1, it is apparent that cold acclimation involves several low-temperature-responsive regulatory pathways.

Transgenic arabidopsis plants that constitutively over-express CBF1/DREB1B exhibited increased tolerance to freezing without negative side-effects on growth or development (Jaglo-Ottosen *et al.*, 1998). Expression of cold-responsive genes was shown to be activated in these plants at non-inducing temperatures (Jaglo *et al.*, 2001). Constitutive overexpression of CBF1/DREB1B in tomato plants also led to improved tolerance of chilling, drought and salt-stress, but these plants were stunted with reductions in fruit set and seed production (Hsieh *et al.*, 2002). Over-expression of CBF3/DREB1A in transgenic arabidopsis also leads to enhanced expression of target genes including *COR15a*, *RD29*, *KIN1*, *COR6.6*, and *COR47/RD17* (Liu *et al.*, 1998; Kasuga *et al.*, 1999; Maruyama *et al.*, 2004) under non-inducing conditions and these plants showed enhanced tolerance of freezing, drought and salt-stress. Constitutive expression of CBF3/DREB1A in transgenic rice plants resulted in increased tolerance to drought-, salt-, and cold-stress and these plants grew and developed normally (Oh *et al.*, 2005). Subsequently, the over-expression of CBF3/DREB1A was shown to improve the drought- and low-temperature-stress tolerance in tobacco, wheat and groundnut (Kasuga *et al.*, 2004; Pellegrineschi *et al.*, 2004; Bhatnagar-Mathur *et al.*, 2008), but the stress-inducible *RD29A* promoter was used to minimize the negative effects of CBF/DREB1 over-expression in these species.

Attempts to develop cotton plants that constitutively express CBF3/DREB1A under control of the CaMV 35S promoter in our laboratory failed. Regeneration proceeded normally through somatic embryogenesis, but the plantlets would not grow and most failed to develop roots (Y. Sun, J. Lee and R.D. Allen, unpublished data). Use of the stress-responsive *RD29A* promoter to drive stress-responsive CBF3/DREB1A expression allowed regeneration of plants that were able to thrive but were severely stunted and virtually all were sterile (J. Lee, Y. Sun and R.D. Allen, unpublished data). Development of transgenic cotton plants that contain a CBF3/DREB1A transgene under a different stress-responsive promoter that does not contain a CRT/DRE element has been carried out and these plants grow normally and are fertile. Initial characterization of the stress-tolerance characteristics of these plants indicates that they exhibit increased tolerance of water-deficit stress.

Protein Kinases

Protein kinases represent another type of regulatory protein that has been used to improve stress tolerance in plants. Protein kinases initiate phosphorylation cascades that control downstream regulatory factors leading to altered stress-responsive gene expression and tolerance of abiotic stress. An advantage of engineering signaling factors is that they can control the signal output involved in different aspects of homeostasis or damage prevention under abiotic stress (Verslues *et al.*, 2006). One of these genes is the tobacco *NPK1* kinase and its arabidopsis ortholog *ANP1*, which activates a mitogen-activated protein kinase (MPK3 and MPK6) signaling cascade that leads to enhanced tolerance of multiple environmental stresses in tobacco and maize (Kovtun *et al.*, 2000; Shou *et al.*, 2004). *NPK1/ANP1* acts upstream of the oxidative-stress-response pathway and can induce expression of HSPs, APX, GST and other stress-responsive gene products. These proteins protect the photosynthetic machinery from damage during drought, thereby improving agronomic traits such as yield (Shou *et al.*, 2004). Other kinases interact with proteins directly to confer a stress response. For example, SOS2 (salt overly sensitive 2) directly regulates the Na^+/H^+ antiporter SOS1 (Shi *et al.*, 2000) that is known to be an important determinant of salt tolerance because of its role in ion homeostasis (Apse *et al.*, 1999; 2003; Gaxiola *et al.*, 1999). Although SOS2 is known to interact with the myristolyated calcium-binding protein, SOS3, in a calcium-dependent manner, SOS2 appears to limit plant salt tolerance (Guo *et al.*, 2004) and studies have shown that SOS2 is sufficient for activation of SOS1 and for increasing salt tolerance *in planta*. Recently developed cotton plants that express *AtANP1* and *At*SOS2 under control of the CaMV 35S promoter are now being tested for increased stress tolerance (Y. Sun, J. Lee, and R.D. Allen, unpublished data).

Ubiquitin and SUMO Ligases

Post-translational modification of proteins via the attachment of a variety of small polypeptides such as ubiquitin and small ubiquitin-like modifiers (SUMOs) is an important regulatory mechanism in eukaryotic cells, including those of plants (Melchior, 2000; Hay,

2001; Pickart, 2001; Vierstra, 2003; Gill, 2004; Kerscher *et al.*, 2006). Ubiquitin ligases, which catalyze the attachment of ubiquitin to target proteins and thereby regulate their stability and/or activity, are involved in a wide range of regulatory pathways including those of virtually all phytohormones. Several of these are involved in stress-signaling pathways; for example, transcriptional regulation of *CBF3/DREB1A* is modulated by post-translational modification of transcription factors while the stability of DREB2A is directly regulated by ubiquitination (see below). Furthermore, ring-finger proteins such as SDIR and XERICO affect ABA signaling and biosynthesis in arabidopsis (Ko *et al.*, 2006; Zhang *et al.*, 2007b), and a family of stress-associated proteins (SAPs) that contain A20- and AN1-like zinc-finger motifs are also involved in regulating stress responses (Mukhopadhyay *et al.*, 2004).

Conjugation of the SUMO peptide to a target motif of a protein substrate is known as sumoylation (Bernier-Villamor *et al.*, 2002; Melchior *et al.*, 2003; Schmidt and Müller, 2003; Johnson, 2004). Like ubiquitination, SUMO conjugation occurs in a series of biochemical steps mediated by E1-activating, E2-conjugating, and E3-ligating enzymes (Chosed *et al.*, 2006*)*. Whereas ubiquitin is primarily responsible for the degradation of cellular proteins, ligation of SUMO to target proteins can interfere with ubiquitination, alter protein–protein interactions and subcellular localization, and modify transcription-factor activity (Hochstrasser, 2000; 2001; Gill, 2003; Girdwood *et al.*, 2004; Johnson, 2004; Watts, 2004). Sumoylation has also been associated with the regulation of a wide range of cellular processes in eukaryotes including innate immunity, cell-cycle progression, heat adaptation, DNA repair, nucleocytoplasmic trafficking, subnuclear targeting, and transcriptional regulation (Mao *et al.*, 2000; Saitoh and Hinchey, 2000; Freiman and Tjian 2003; Bohren *et al.*, 2004; Dohmen 2004; Johnson 2004; Gill, 2005; Hay, 2005; Shuai and Liu, 2005; Zhao and Blobel 2005; Hietakangas *et al.*, 2006). In plants, levels of SUMO conjugates increase in response to a range of stresses, and sumoylation correlates with elevated expression of ABA- and stress-responsive genes (Kurepa *et al.*, 2003; Lois *et al.*, 2003; Miura *et al.*, 2005; 2007; Yoo *et al.*, 2006). An arabidopsis SUMO E3 ligase, SIZ1 regulates the expression of genes involved in controlling plant responses to phosphate starvation and cold stress (Miura *et al.,* 2005; 2007) and characterization of a *siz1* knockout mutant showed that loss of SIZ1 alters the expression of a set of genes in response to water deficit (Catala *et al.*, 2007). Recently, Miura *et al.* (2009) presented evidence that SIZ1 negatively regulates ABA signaling through sumoylation of ABI5. These authors demonstrate an epistatic genetic interaction between *SIZ1* and *ABI5*, and show that K391 in ABI5 is essential for SUMO1 conjugation. SUMO proteases encoded by the overly tolerant to salt 1 (*OTS1*) and *OTS2* genes, regulate salt-stress responses in arabidopsis. Double mutants are sensitive to salt and they accumulate higher levels of SUMO-conjugated proteins than wild-type plants under both normal and salt-stress conditions (Conti *et al.*, 2008). Over-expression of OTS1 in transgenic arabidopsis plants led to reduced levels of sumoylated proteins and increased salt tolerance compared to wild-type plants.

Ubiquitination and sumoylation can cooperate in the regulation of transcription factors involved in stress-responsive gene expression. For example, transcriptional regulation of members of the *CBF/DREB1* gene family, which are transiently induced by low temperature (Gilmour *et al.*, 1998; Liu *et al.*, 1998; Medina *et al.*, 1999) is controlled both at transcriptional and at post-transcriptional levels (Figure 1). Transcription of these genes is activated in response to low temperature by a constitutively expressed transcription factor, ICE1 (for inducer of CBF/DREB1 expression 1). ICE1 is a MYC-like basic helix-loop-helix transcription factor that binds to canonical MYC *cis*-elements (CANNTG) in the *CBF3/DREB1A* promoter. This interaction induces expression of CBF/DREB1, which leads to induction of the CBF/DREB1 regulon (Chinnusamy *et al.*, 2003). Negative transcriptional regulation of *CBF/DREB1* expression is mediated by MYB15, which binds to *CBF/DREB1* promoter elements and represses expression of *CBF/DREB1* genes and, thus, the CBF/DREB1 regulon (Agarwal *et al.,* 2006). While transcription of the *ICE1* gene is constitutive, the stability of the ICE1 protein is regulated by the RING-type E3 ubiquitin ligase HOS1 (Lee *et al.*, 2001), which ubiquitinates ICE1, targeting it to the 26S proteasome for degradation (Dong *et al.*, 2006). Since ubiquitination of ICE1 by HOS1 is induced by cold, HOS1 appears to be involved in the attenuation of plant responses to low temperatures. The activity of ICE1 is positively regulated via sumoylation by the SUMO ligase SIZ1 under cold conditions (Miura *et al.*, 2007). ICE1 sumoylation interferes with HOS1-dependent ubiquitination and suppresses expression of *MYB15*. Thus, under non-inducing conditions, transcription of *CBF/DREB1* genes is repressed by MYB15 and the accumulation of ICE1 is inhibited by HOS1-mediated ubiquitination. In response to stressful conditions, sumoylation of ICE1 by SIZ1 blocks its ubiquitination, leading to stabilization and/or activation. The accumulation of active ICE1 leads to the repression of *MYB15* and the transcriptional activation of *CBF/DREB1*.

Unlike the transcriptional regulation of the *CBF1/DREB1* gene family, regulation of DREB2A is primarily post-transcriptional. Over-expressed *DREB2A* in transgenic plants failed to confer an altered phenotype and had no apparent effect on the expression of stress-responsive genes. However, deletion of a small region in the central part of the *DREB2A*-coding sequence produced a constitutively active form called DREB2A-CA that, when expressed in arabidopsis, resulted in dwarfed growth and increased stress tolerance (Sakuma *et al.*, 2006). A recent search for protein factors that interact with the negative regulatory domain of DREB2A resulted in the identification of a unique RING-finger domain-containing E3 ubiquitin ligase named DREB2A interacting protein 1 (DRIP1) (Qin *et al.*, 2008). DRIP1 catalyzes the ubiquitination of DREB2A *in vitro* and mediates the stability of DREB2A *in planta*. Although E3 ligase genes such as HOS1 are expressed in response to stress and are thought to attenuate stress responses, DRIP1 is constitutively expressed and may be responsible for suppression of DREB2A expression under non-stressful conditions.

While the RING-finger E3 ubiquitin ligases HOS1 and DRIP1 negatively regulate plant-stress responses, other members of the RING-finger-domain protein family positively regulate stress responses. For example, over-expression of the RING-finger domain protein SDIR1 leads to increased drought-stress tolerance and enhanced expression of

several ABA-responsive genes (Zhang *et al.,* 2007b) while over-expression of another RING-finger domain protein, XERICO, leads to increased ABA biosynthesis (Ko *et al.,* 2006). Although the cellular targets of these ubiquitin ligases have not been specifically identified, it is likely that they are transcription factors or other regulatory molecules that function to down-regulate stress responses.

A family of stress-responsive genes has been identified that encode proteins containing conserved A20-like and AN1-like zinc-finger motifs at their N- and C-terminal domains, respectively. Expression of the *OsiSAP1* gene from rice is induced in response to a variety of environmental stresses including cold, salt, drought, anoxia, wounding and heavy metals, and over-expression of this gene in transgenic tobacco plants conferred increased tolerance to abiotic stress (Mukhopadhyay *et al.*, 2004). Similarly, overexpression of OsiSAP8 in both transgenic tobacco and rice conferred increased tolerance of salt, drought and cold stress, compared to unstressed transgenic plants, without a yield penalty (Kanneganti and Gupta, 2008). Vij and Tyagi (2006) identified 18 genes encoding putative SAP1-like proteins in the rice genome and 14 genes of this type were identified in arabidopsis. Like OsiSAP1, several of the rice *SAP* genes were found to be stress responsive (Vij and Tyagi, 2006) and analysis of public microarray data indicated that several of the *AtSAP* transcripts are strongly induced by ABA and a range of abiotic-stress treatments.

Transgenic arabidopsis plants that ectopically express the *AtSAP5* gene under control of the CaMV 35S promoter, as well as arabidopsis plants with antisense-suppressed *AtSAP5* expression and T-DNA knock-out mutants, were developed and characterized in our laboratory (Kang *et al.*, 2011). The growth and development of these plants were indistinguishable from those of wild-type plants under normal, non-stressful conditions. However, when these plant were grown under chilling temperatures or exposed to water deficit, phenotypic differences became apparent. In general, AtSAP5 knockout and knock-down plants were more stress sensitive than wild-type plants under chilling and osmotic stress, whereas transgenic arabidopsis plants that over-express *AtSAP1* showed substantial increases in stress tolerance under water-deficit conditions.

Development of transgenic cotton plants that ectopically express AtSAP5 has been completed in our laboratory and several independent transgenic lines that express this transgene were regenerated (Hozain *et al.*, 2012). When compared with wild-type plants, AtSAP5-expressing cotton plants showed increased survival under water deficit in greenhouse experiments (Figure 2). No differences were seen between AtSAP5 cotton plants and wild-type plants when they were grown under irrigated conditions in small-scale field trials. However, differences in stress tolerance were readily apparent in dry-land plots. When grown under water-deficit conditions, AtSAP5-expressing plants showed increased vegetative growth and reduced wilting and chlorosis relative to non-transformed plants. While bolls of wild-type cotton plants tended to open prematurely under these conditions, bolls of AtSAP5-expressing plants remained closed. Premature boll opening under drought stress results in production of immature, poor-quality cotton fibers, whereas fiber produced by AtSAP5 expressing plants was of higher quality. Thorough physiological evaluation and quantitative analysis of yield and fiber-quality parameters from these plants is now underway.

MicroRNAs

MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) represent an additional mode of post-transcriptional regulation in eukaryotic cells. These small non-coding RNAs can silence gene expression by targeting specific mRNAs for degradation or by repressing their translation (Bartel, 2004; Baulcombe, 2004; Jones-Rhoads *et al.*, 2006; Mallory and Vaucheret 2006). Although the function of miRNAs in developmental processes has been extensively studied (for recent reviews see Kidner and Martienssen, 2005; Mallory and Bouché, 2008), the roles of these molecules in the regulation of plant responses to abiotic stresses is now beginning to emerge (Sunkar *et al.*, 2007).

Jones-Rhoades and Bartel (2004) identified arabidopsis miRNAs that were predicted to target genes involved in abiotic-stress responses. For example, miR395, which is induced by sulfate starvation, targets transcripts for ATP sulfurylases and a sulfate transporter (Allen *et al.*, 2004; Jones-Rhoades and Bartel, 2004). Sunkar and Zhu (2004) identified several stress-responsive miRNAs, including miR393, which was strongly up-regulated by cold, dehydration, salinity, and ABA treatments. miR393 mediates cleavage of transcripts from several closely related F-box auxin-receptor genes, including transport inhibitor response 1 (*TIR1*), which, targets AUX/IAA proteins for ubiquitination (Vierstra, 2003). Thus, miR393-mediated inhibition of TIR1 down-regulates auxin signaling under abiotic-stress conditions. Accumulation of miR159 is induced by ABA via the seed-specific ABA-dependent transcription factor ABI3 (Reyes and Chua, 2007). miR159 targets transcripts for the MYB transcription factors MYB33 and MYB101 that are positive regulators of ABA signaling. Thus, miR159 may act to attenuate ABA responses in plants. Genes for two closely related Cu-Zn superoxide dismutase genes (*CSD1* and *CSD2*) are transcribed under normal growth conditions, but their mRNAs do not accumulate due to miR398-directed cleavage. miR398 is transcriptionally down-regulated in response to oxidative stress to allow increased accumulation and translation of *CSD1* and *CSD2* transcripts (Sunkar *et al.,* 2006), whereas induction of miR398 by sucrose-repressed expression of these mRNAs (Dugas and Bartel, 2008). Furthermore, plants that express a transgene for a mutant *CSD2* that is resistant to miR398-mediated cleavage accumulate higher levels of CSD2 transcripts and these plants showed improved tolerance to oxidative stress when compared to transgenic plants that express the miR398-susceptible CSD2 gene (Sunkar *et al.*, 2006). The recent identification of conserved microRNAs in cotton (Zhang *et al.*, 2007a) shows that opportunities exist to use this important post-transcriptional regulatory system in future efforts to optimize abiotic-stress tolerance in cotton.

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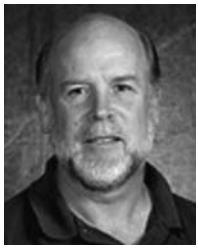

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