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Review

# Achievements and Challenges in Understanding Plant Abiotic Stress Responses and Tolerance

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**Intensive research over the last decade has gradually unraveled the mechanisms that underlie how plants react to environmental adversity. Genes involved in many of the essential steps of the stress response have been identified and characterized. In particular, the recent discovery of ABA receptors, progress in understanding the transcriptional and post-transcriptional regulation of stress-responsive gene expression, and studies on hormone interactions under stress have facilitated addressing the molecular basis of how plant cells respond to abiotic stress. Here, we summarize recent research progress on these issues, especially focusing on progress related to the essential and classically important signaling pathways and genes. Despite this wealth of achievements, many challenges remain not only for the further elucidation of stress response mechanisms but also for evaluation of the natural genetic variations and associating them with specific gene functions. Finally, the proper application of this knowledge to benefit humans and agriculture is another important issue that lies ahead. Collaborative wisdom and efforts are needed to confront these challenges.**

**Keywords:** ABA perception • Abiotic stress • Gene expression • Signal transduction • Tolerance improvement.

**Abbreviations:** ABAR, ABA-binding protein; ABC transporter, ATP-binding cassette transporter; ABF, ABA responsive factor; ABI, ABA Insensitive; AIP2, ABI3-interacting protein 2; AP2/ERF, APETALA2/ethylene-responsive factor; AREB, ABRE-binding protein; ABRE, ABA-responsive element; AHK, Arabidopsis histidine kinase; bHLH, basic helix–loop–helix; bZIP, basic domain leucine zipper; CAMTA, calmodulin-binding transcription activator; CBF/DREB1, C-repeat-binding factor/dehydration-responsive element-binding protein 1; CBL, calcineurin B-like; CHLH, H subunit of Mg-chelatase; CIPKs, CBL-interacting protein kinases; CK, cytokinin; CPK, calcium-dependent protein kinase; CRT/DRE, C-repeat-binding factor/dehydration responsive element; DPBF, Dc3 promoter binding factor; DREB2A-CA, constitutively active form of DREB2A; DRIP, DREB2A-interacting protein; E1, ubiquitin-activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligase; EEL, enhanced Em level; ERD1, early response to dehydration 1; ERE, ethylene-responsive element; ESL1, ERD six-like 1; GCR2, G protein-coupled receptor 2; GID, gibberellin-insensitive dwarf; GTG1/GTG2, GPCR-type G protein 1/2; GUN5, genomes uncoupled 5; GWAS, genome-wide association studies; HOS, high expression of osmotically responsive gene; HSFA3, heat shock transcription factor A3; ICE, inducer of CBF expression; ICEr, ICE region; IPT, isopentenyltransferase; KEG, keep on going; NAC, NAM, ATAF and CUC; NF-Y, nuclear factor Y; OST1, open stomata 1; PIF, phytochrome-interacting factor; PP2C, protein phosphatase 2C; PYL, PYR1-like; PYR, pyrabactin resistance; RAP, related to APETALA; RCAR, regulatory component of ABA receptor; ROS, reactive oxygen species; SA, salicylic acid; SIZ1, SAP and Miz1; SARK, senescence-associated receptor protein kinase; SLAC1, slow anion channel-associated 1; SNAC, stress-responsive NAC; SNP, single nuclear polymorphism; SnRK2, sucrose non-fermenting 1-related protein kinase 2; SOS, salt overly sensitive; SUMO, small ubiquitin-related modifier; TFs, transcription factors; ZFHD, zinc-finger homeodomain.

## Introduction

As sessile organisms, plants have evolved sophisticated mechanisms to adapt to environmental changes and challenges. The mechanism underlying the environmental stress response in plants is probably more advanced and prominent than in animals. Moreover, the question of how plant cells react to various environmental stresses is one of the most attractive topics not only to plant biologists but also to agronomists, because abiotic

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stress is a particular threat to crop productivity. It is estimated that abiotic stress such as drought, salinity and extreme temperatures, which usually cause primary crop losses worldwide, lead to an average yield loss of >50% for most major crop plants (Boyer 1982, Bray et al. 2000). Furthermore, world food production needs to be doubled by the year 2050 to meet the ever-growing demands of the population (Tilman et al. 2002). For these reasons, understanding the mechanisms underlying plant abiotic stress responses and the generation of stress-tolerant plants has received much attention in recent years.

However, because of the complexity of stress tolerance traits, conventional approaches are less effective at directly connecting tolerance traits to the determinant genes that play key roles in the stress response. Owing to recent progress in functional genomics, genes involved in many of the essential steps of the stress response have been identified and characterized. In particular, the discovery of ABA receptors, progress in understanding the transcriptional and post-transcriptional regulation of stress-responsive gene expression, and studies on hormone interactions under stress have facilitated addressing the molecular basis of how plant cells respond to abiotic stress. Importantly, the physiological functions of a number of genes have been investigated in transgenic model plants and some crops, and an approach for utilizing useful genes for crop genetic improvement by gene transfer has been proposed. This review describes recent progress towards understanding plant abiotic stress responses, primarily focusing on ABA receptor identification, stress-responsive gene expression regulation by transcription factors (TFs), signal transduction mediated by protein modification and the roles of phytohormones in plant stress responses and development. The roles of small RNAs and RNA-directed DNA modifications in this regard have been extensively reviewed elsewhere (Sunkar and Zhu 2004, Sunkar et al. 2007, Chinnusamy and Zhu 2009).

## Recent breakthroughs in ABA receptor identification

The phytohormone ABA modulates many important plant development processes, such as the inhibition of germination, maintenance of seed dormancy, regulation of growth, fruit abscission and stomatal closure (Finkelstein et al. 2002, Parent et al. 2009, Raghavendra et al. 2010). In addition, ABA serves as an endogenous messenger in abiotic stress responses in plants; therefore, it is called a 'stress hormone'. Physiological experiments have shown that under abiotic stress, especially drought and salinity, plants accumulate high levels of ABA accompanied by major gene expression changes. The perception, signaling and transportation of ABA are some of the most central issues in plant science (Fujii et al. 2009, Ma et al. 2009, Park et al. 2009, Umezawa et al. 2009, Kang et al. 2010, Kuromori et al. 2010). The sites of ABA perception have intrigued plant biologists for many years, and the issue has reached some resolution with the recent identification of several ABA receptors.

In 2009, two independent research groups from the USA and Germany reported in the same issue of *Science* that they had identified a small protein family that binds ABA and interacts with ABA Insensitive 1 and 2 (ABI1 and ABI2), two type 2C protein phosphatases (PP2Cs). These genes are negative regulators of ABA signaling (Ma et al. 2009, Park et al. 2009). Although the strategies they used were different, both groups found the same group of proteins to be ABA receptors and to function at the apex of a negatively regulated ABA pathway that is controlled by ABI1 and ABI2. This small protein family was named PYR1/PYLs/RCARs (pyrabactin resistance 1/Pyr-likes/regulatory component of ABA receptors; subsequently referred to as PYRs) by both groups. PYR1/PYLs were identified by genetic screening for mutants resistant to pyrabactin, an ABA analog (Park et al. 2009). The quadruple mutant *pyr1-pyl1pyl2pyl4* was found to be insensitive to ABA in terms of germination, root growth, ABA-activated sucrose non-fermenting 1-related protein kinase 2 (SnRK2) activity and ABA-responsive gene expression. Further research revealed that PYR1/PYLs interact with PP2C in an ABA-dependent manner. Furthermore, in the presence of PYR1/PYLs, ABA is a saturable inhibitor of phosphatase activity. All of the evidence suggests that PYR1/PYLs meets the criteria expected of an ABA sensor, revealing how the ABA signal is perceived and transduced to regulate downstream gene expression. RCARs were identified in a yeast two-hybrid screen with ABI1 and ABI2 (Ma et al. 2009). They were found to be able to bind ABA and inactivate PP2C activity in vitro, further explaining how the ABA signal is perceived and how the activity of the negative regulator is inhibited in the presence of ABA.

Soon after the identification of the PYR proteins, five groups independently determined their protein structures by X-ray crystallographic analysis (Melcher et al. 2009, Miyazono et al. 2009, Nishimura et al. 2009, Santiago et al. 2009, Yin et al. 2009). In a common paradigm, in the absence of ABA, the receptor contains a ligand-binding pocket surrounded by two open-lid loops. This pocket closes in the presence of ABA through an ABA-induced conformational change. Thus, the ligand (ABA) is locked in the pocket by the lid. Moreover, a docking site is created on the closed lid where a conserved tryptophan in PP2C can bind, further locking the closed conformation of the receptor. On the other hand, the active site of PP2C is covered by the lid of the closed receptor, which inactivates the phosphatase activity of PP2C. This structural research has unraveled how the receptor binds to ABA and changes its conformation to inhibit PP2C activity.

Further work connecting PP2C to its downstream effector in ABA signaling, the SnRK2 kinase, was conducted by the research groups of Shinozaki and Zhu, who reconstructed ABA signaling in vitro by combining all of the key components, including the ligand (ABA), the receptor PYR1, PP2C (such as ABI1 and/or ABI2), SnRK2, basic domain leucine zipper (bZIP) TFs and an ABA-responsive promoter-driven reporter gene

(Fujii et al. 2009, Umezawa et al. 2009). Their research beautifully reconstituted ABA signaling in vitro and found that without ABA, the negative regulator PP2C dephosphorylated the SnRK2 kinases and blocked the signal (**Fig. 1A**). With ABA, the ABA-bound receptor inactivated PP2C, which caused the SnRK2 kinases (SnRK2.2/2D, 2.3/2I and 2.6/2E/OST1) to be in a phosphorylated state, allowing them to activate bZIP group TFs, such as ABA-responsive element-binding protein 1/ABA responsive factor 2 (AREB1/ABF2) by protein phosphorylation. Once activated, AREB1/ABF2 promoted downstream ABA-responsive gene transcription. In this way, plant cells generate a series of physiological reactions to ABA (**Fig. 1B**).

The discovery of PYRs as ABA receptors not only facilitates the elucidation of regulation of ABA-responsive gene expression, but it also sheds light on ion channel control in guard cells. ABA signals and drought stress can induce stomatal closure, which is mediated by the turgidity of two bordering guard cells. SnRK2E/OST1 (open stomata 1) directly interacts with and phosphorylates SLAC1 (slow anion channel-associated 1), thereby activating the channel, which mediates the efflux of

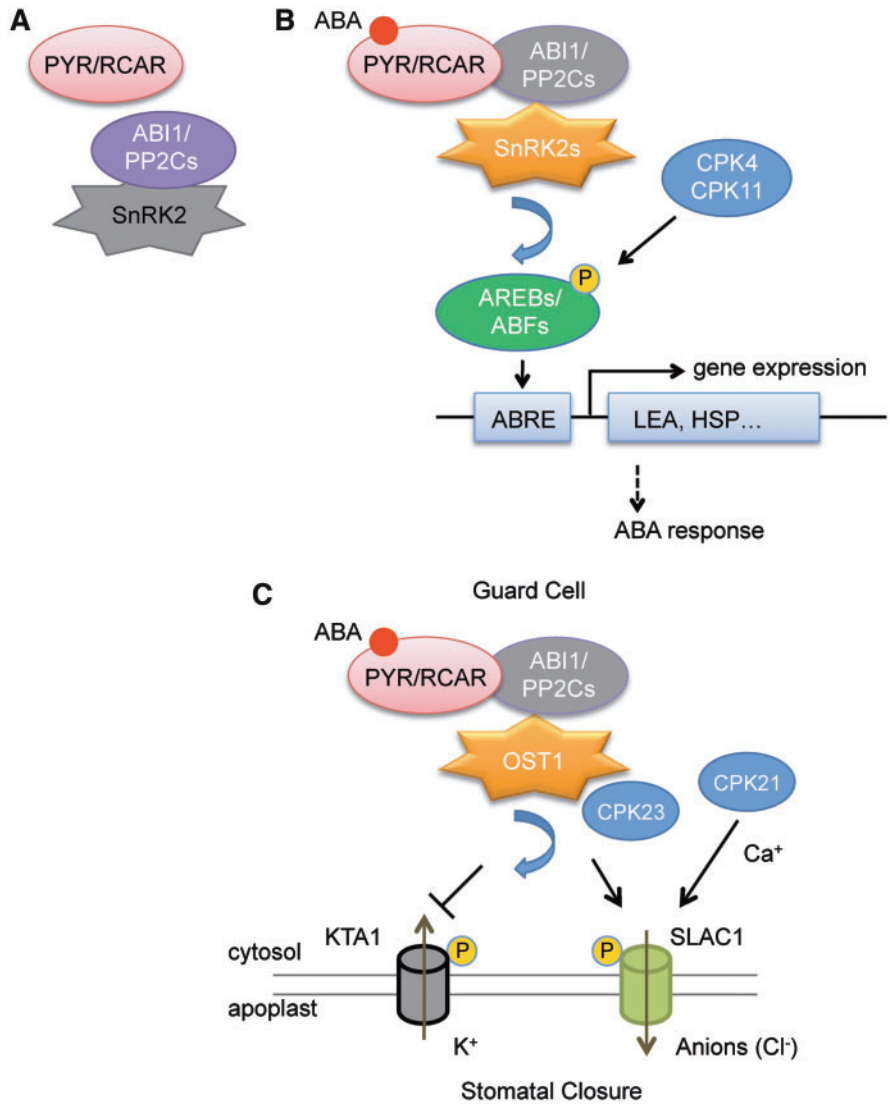


**Fig. 1** A model of ABA signal reception and transduction through the PYR/RCAR ABA receptor and the PP2C and SnRK2 kinases. (A) In the absence of ABA, PP2Cs (such as ABI1) dephosphorylate the SnRK2 kinases and keep them inactive. (B) In the nuclei, when ABA is present, PYR/RCAR receptors bind ABA and specifically interact with PP2Cs to inhibit their phosphatase activity, which causes the SnRK2 kinases to become active. Subsequently, the SnRK2s phosphorylate the AREB/ABF transcription factors, which directly bind ABREs (ABA-responsive elements) located in the promoters of the ABA-responsive genes. (C) In guard cells, when PP2C activity is inhibited by ABA-bound PYR/RCAR, OST1, the main SnRK2 kinase in guard cells, becomes active. Activated OST1 phosphorylates the potassium channel KTA1, which allows it to import $K^+$. In contrast, OST1 phosphorylates the anion channel SLAC1 to promote $Cl^-$ efflux. CPK21 also interacts with and phosphorylates SLAC1; this is dependent on $Ca^{2+}$ signaling. Consequently, stomatal closure occurs due to the turgor and ionic changes of the two guard cells.

osmoregulatory anions ($Cl^-$ and $malate^{2-}$) from guard cells during stomatal closure (Negi et al. 2008). In contrast, the phosphatase activity of ABI1 prevents SLAC1 activation (Geiger et al. 2009, Lee et al. 2009). KAT1 is one of the best characterized $K^+$ channels in Arabidopsis, and an inward-rectifying $K^+$ channel has been suggested to have a key role in stomatal opening. SnRK2E/OST1 can phosphorylate the C-terminal region of KAT1 and reduce its $K^+$ transport uptake activity (Sato et al. 2009). Thus, the previous proposal that OST1 is a central positive regulator of stomatal closure in the $Ca^{2+}$-independent pathway is addressed, and the pathway turns out to be unexpectedly short and simple. In addition to the OST1-mediated calcium-independent pathway, there is also a calcium-dependent ABA pathway that controls ion channels in stomatal closure. Calcium-dependent protein kinases 21 and 23 (CPK21 and CPK23) are able to interact with SLAC1 (Geiger et al. 2010). Therefore, OST1 and CPK21/23 are two positive regulators of ABA-induced stomatal closure that act by controlling the activity of SLAC1, a major anion channel located on the membranes of guard cells, both $Ca^{2+}$ independently and dependently (**Fig. 1C**).

In addition to the PYR proteins, three other types of proteins are reported to be ABA receptors, including chloroplast protein ABA-binding protein/H subunit of Mg-chelatase/genomes uncoupled 5 (ABAR/CHLH/GUN5) (Shen et al. 2006, Wu et al. 2009), plasma membrane-localized G protein-coupled receptor 2 (GCR2) (Liu et al. 2007) and GPCR-type G protein 1/2 (GTG1/GTG2) (Pandey et al. 2009). The ability of these proteins to bind ABA is controversial, and the molecular mechanisms through which they transduce the ABA signal to initiate downstream gene expression are unclear. Subsequent studies on the affinity of ABAR for ABA and its interaction with the WRKY TF (which was named due to the conserved amino acid sequence WRKYGQK at its N-terminal end) have provided more evidence for its role in ABA signaling (Wu et al. 2009, Shang et al. 2010), but its physiological functions in chloroplasts with respect to ABA responses and metabolism remain to be elucidated.

In addition to ABA receptor identification, two ABA transporter genes have been separately reported by two independent groups, and both genes belong to the ATP-binding cassette (ABC) transporter gene family. AtABCG25 is an ABA exporter and AtABCG40 is an importer responsible for ABA transport in plants (Kang et al. 2010, Kuromori et al. 2010). Overall, the collection of research published in 2009 represents a breakthrough in understanding ABA signal perception and transduction. All of these studies elegantly explained and defined a minimal set of core components that constitute a major ABA signaling pathway. In addition to the newly identified ABA receptor, the functions of the formerly characterized ABA negative regulators, ABI1 and ABI2, have been reviewed. From the perception of the signal to downstream responsive gene expression, every step has been molecularly clarified (also reviewed by Umezawa et al. 2010). At this time, PYRs are undisputedly accepted as the ABA receptors in plants.

## Transcription factors play central roles in stress-responsive gene expression

Since the end of the last century, the functions of TFs in abiotic stress responses have received much research attention. Owing to technical developments in detecting differential gene expression, multiple stress-inducible genes have been simultaneously identified. In these types of studies, a similar expression pattern is predicted to reflect control by a common *cis*-acting promoter element. Moreover, a specific TF that recognizes the element may activate gene expression in response to stress stimuli. This type of model has been proven by the establishement of the C-repeat-binding factor/dehydration responsive element-binding protein 1 (CBF/DREB1) regulon. CBF/DREB1 proteins belong to a small group of the AP2/ERF super TF family, which is unique to plant species. This group of proteins recognizes the DRE/CRT *cis*-acting element (core motif: G/ACCGAC) located in the promoters of many cold- and drought-inducible genes (Stockinger et al. 1997, Liu et al. 1998). Most of these genes are transcriptionally stress inducible and transactivate downstream gene expression under stress. More than 40 genes have been identified as the downstream targets of a single CBF3/DREB1A protein. All of these genes meet the criteria in that their promoter contains a C-repeat/dehydration responsive element (DRE/CRT) sequence, they are cold stress inducible and they are up-regulated by ectopic expression of CBF3/DREB1A (Fowler and Thomashow 2002, Maruyama et al. 2004, Vogel et al. 2005). Therefore, this set of genes is called the CBF/DREB1 regulon. Extensive transcriptional reprogramming and reconfiguration upon cold or freezing stress were found to be attributable to central regulation by CBF/DREB1 TFs, which are considered to be the master regulators of the low temperature response in plants (reviewed by Hua 2009, Nakashima et al. 2009b). Moreover, CBF4/DREB1D, DREB1E/DDF2 and DREB1F/DDF1 are weakly induced by osmotic stress, indicating an additional function of CBF/DREB1 proteins in other abiotic stress responses (Haake et al. 2002, Magome et al. 2004). Comparative genomics studies on the functions of CBF/DREB1 orthologs in other species have supported the regulatory functions of these genes (Jaglo et al. 2001, Gao et al. 2002, Dubouzet et al. 2003, Shen et al. 2003, Qin et al. 2004). Molecular manipulation of the CBF/DREB1 genes through bioengineering is a promising means of improving plant stress tolerance (Celebi-Toprak et al. 2005, Oh et al. 2005, Behnam et al. 2006, Ito et al. 2006).

Because of the importance of CBF/DREB1 regulators in plant low temperature responses, it is critical to understand how the expression of these genes is activated by stress. The control of *CBF/DREB1* gene expression by various transcriptional regulators is summarized in **Fig. 2**. Two conserved sequences have been identified to contribute to the cold induction of *CBF/DREB1* genes. They are inducer of *CBF* expression region 1 and 2 (ICEr1 and ICEr2), which were similar to boxes IV and VI named by another group (Zarka et al. 2003, Shinwari et al. 1998). A major positive regulator (ICE1) was isolated from a

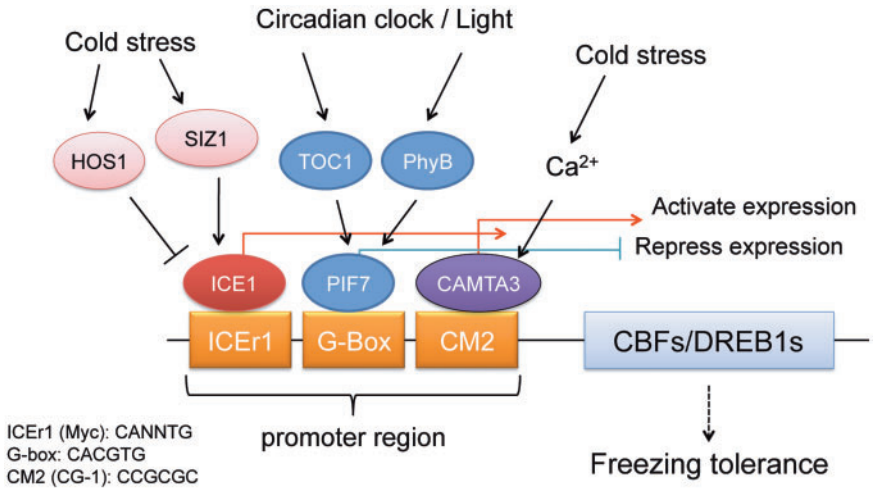


**Fig. 2** Transcriptional control of the cold-inducible expression of the *CBF/DREB1* genes is coordinated by various TFs. ICEr1, G-box and ICEr2 are *cis*-acting elements located upstream of CBF genes, and ICE1, PIF7 and CAMTA3 bind to these elements, respectively (Chinnusamy et al. 2003, Doherty et al. 2009, Kidokoro et al. 2009). ICE1, a MYC-like bHLH TF, binds to ICEr1 (a MYC-like sequence) and activates CBF3/DREB1A gene expression under cold stress. HOS1 is a ubiquitin E3 ligase, and SIZ1 is a SUMO E3 ligase, and both modify ICE1 to regulate its protein abundance and activity in response to cold stress. PIF7 is a helix–loop–helix type TF that binds to the G-box element and functions as a transcriptional repressor of CBF1/DREB1B and CBF2/DREB1C under circadian and light control. PIF7 also interacts with TOC1 and PhyB, which may modulate the activity of PIF7. CAMTA3 is a calmodulin-binding TF that binds the CM2 sequence in the promoter of CBF2/DREB1C and functions as an activator that promotes gene expression.

forward genetic screen for reduced expression of a reporter gene driven by the *CBF3* promoter. ICE1 is a basic helix–loop–helix (bHLH) TF, and it binds to multiple Myc DNA sequences present in the *CBF3* promoter. The *ice1* mutation blocks *CBF3* expression and reduces the expression of its downstream genes under cold stress, whereas overexpressing *ICE1* enhances the expression of the CBF regulon and improves plant freezing tolerance (Chinnusamy et al. 2003). *ICE1* is constitutively expressed in plant cells, suggesting that a protein modification is required to activate the protein upon stress, which will be discussed in the following section. Another upstream regulator of *CBF3* is MYB15, which binds a Myb sequence in the *CBF3* promoter and negatively affects *CBF3* gene expression. *MYB15* gain of function results in the reduced expression of *CBF* genes in response to cold stress, whereas *MYB15* loss of function leads to an increase in this response (Agarwal et al. 2006). Recently, a group of calmodulin-binding transcriptional activators (CAMTAs) have been reported to bind to the *CBF2* promoter and positively regulate its expression (Doherty et al. 2009). The CAMTA binding sequence is a CG-1 element, and it is located near ICEr2 and the box VI region in the *CBF2* promoter. Double *camta1 camta3* mutants are impaired in freezing tolerance. It is worth noting that the phytochrome-interacting factor PIF7, a bHLH TF, was isolated from a yeast one-hybrid screen and binds the G-box sequence located in box V in the *CBF2/DREB1C* promoter. In addition to its cold-inducible expression, the expression of the CBF/DREB1 gene is also regulated by the circadian clock (Harmer et al. 2000, Maruyama et al. 2004). Box V lies between boxes IV and VI, and it is responsible for the circadian expression of *DREB1C*. PIF7 functions as a transcriptional repressor of *CBF/DREB1* expression under circadian control (Kidokoro et al. 2009).

Another subgroup of the AP2/ERF TF family includes the DREB2 proteins, which were isolated together with DREB1 in a yeast one-hybrid screen (Liu et al. 1998). There are eight genes in this subgroup in the Arabidopsis genome (Sakuma et al. 2002). Unlike the DREB1 genes, DREB2A expression is highly inducible by high salinity and drought stress rather than cold. It has been proposed that DREB2A protein requires post-transcriptional modification to be stable and active (to be discussed later). Overproduction of a constitutively active form of DREB2A (DREB2A-CA) protein in plants causes an increase in the expression of a number of genes responsive to water deficit stress, and enhanced drought stress tolerance in the transgenic plants (Sakuma et al. 2006a). Moreover, in transgenic plants that overexpress DREB2A-CA, some heat stress-inducible genes are also transcriptionally activated, and the basal thermotolerance is enhanced in the plants (Sakuma et al. 2006b). Further work has demonstrated that heat shock transcription factor 3 (AtHSFA3) is a direct target gene of DREB2A protein. The promoter of *AtHSFA3* contains two copies of the DRE sequence recognized by DREB2A, and DREB2A is sufficient to activate *AtHSFA3* gene expression even at a normal temperature (Schramm et al. 2008, Yoshida et al. 2008). Thus, DREB2A has dual functions in modulating the expression of different sets of downstream genes under both heat and water deficit stress. Three different sets of downstream genes are known to be DREB2A targets. They are drought, drought and heat, and heat inducible (Sakuma et al. 2006b). Moreover, overexpressing *DREB2C* in plants significantly enhances their thermotolerance

(Lim et al. 2007), further indicating the role of DREB2-type TFs in plant heat stress responses. Additionally, DREB2A and DREB2C interact with AREB/ABF proteins, which may function cooperatively to activate the transcription of ABA-responsive genes (Lee et al. 2010).

Recently, two DREB A-6 subgroup genes, *RAP2.4* and *RAP2.4B*, were found to be able to regulate multiple aquaporin genes, indicating roles for them in maintaining water homeostasis under water deficit stress (Rae et al. 2011). Several aquaporin genes are both up-regulated in *RAP2.4* and *RAP2.4B* overexpressors and down-regulated in the *rab2.4 rab2.4b* double mutant. An analysis of direct binding to the promoter regions of these aquaporin genes by RAP2.4 and RAP2.4B and of their DNA-binding specificity would be of interest.

Under stress conditions, monosaccharides, disaccharides, trisaccharides and sugar alcohols accumulate in plants, and the gene expression levels of some of the relevant enzymes also increase accordingly. Engineering biosynthetic pathways for the production of osmolytes, such as galactinol, fructans, trehalose, ononitol, proline and glycine betaine, to modulate osmotic pressure has been shown to be an effective means of enhancing plant abiotic stress tolerance. Both cold and drought stress can cause water deficit stress in plant cells. How plants adjust their metabolic level properly and differentially is being intensively investigated. A comparative metabolic study of DREB1A- and DREB2A-overexpressing plants has revealed that the metabolic configuration of DREB1A overexpressors resembles that of plants subjected to freezing stress; likewise, DREB2A overexpressors share similarities with drought-exposed plants (Maruyama et al. 2009). Several types of metabolites, such as myo-inositol, sucrose, galactinol and raffinose, are known to accumulate specifically in both cold-treated plants and DRE1A overexpressors. However, argininosuccinate, fumarate and malic acid are only elevated in dehydrated plants and DREB2A overexpressors (Maruyama et al. 2009). These metabolites probably help plants to maintain better osmotic regulation to retain water and reduce transpiration under different stresses. Recently, a facilitated diffusion transporter for sugars, encoded by the *ESL1* (*ERD six-like 1*) gene, was reported in Arabidopsis (Yamada et al. 2010). *ESL1* is highly inducible by drought and salinity stress, and it is mainly expressed in the pericycle and xylem parenchyma cells. ESL1–green fluorescent protein (GFP) localizes to the tonoplast membrane. The majority of hexoses are stored in tonoplasts in plant cells. It has been proposed that ESL1 functions in the efflux of hexoses from the tonoplast to the cytoplasm under osmotic stress to regulate osmotic pressure (Yamada et al. 2010).

Water deficit stress induces ABA synthesis and accumulation in plant cells, and a host of ABA-responsive genes have been identified. Most of these genes contain a conserved, ABA-responsive, *cis*-acting element named ABRE (ABA-responsive element, PyACGTGG/TC) in their promoters. This sequence is specifically recognized by AREBs, a group of bZIP TFs (reviewed by Yamaguchi-Shinozaki and Shinozaki 2005, Yamaguchi-Shinozaki and Shinozaki 2006, Nakashima et al. 2009b). Nine AREB homologs have been identified in the Arabidopsis genome: AREB1/ABF2, AREB2/ABF4, AREB3/DPBF3, ABF1, ABF3/DPBF5, ABI5/DPBF1, EEL/DPBF4, DPBF2 and AT5G42910. The expression levels of *AREB1/ABF2*, *AREB2/ABF4* and *ABF3/DPBF5* are clearly induced in vegetative tissues by ABA, drought and high salinity (Fujita et al. 2005). Yoshida et al. (2010) generated *areb1areb2abf3* triple mutants and discovered that these genes were functionally redundant for ABA responses. AREB1, AREB2 and ABF3 could form homo- and heterodimers in nuclei. The triple mutant was impaired in ABA-responsive gene expression under ABA treatment and drought stress, and it was less tolerant to drought stress than the wild type. ABA-reduced root growth was relieved in this mutant. Collectively, these results indicate that these three TFs are master regulators of ABA-responsive gene expression in plants. Protein phosphorylation is required for this group of TFs to achieve their full activity. SnRK2s are the direct upstream activators of AREB proteins in ABA signaling (Fujita et al. 2009). The function of this TF class has been also tested in transgenic rice. Overexpression of the ABF3 gene resulted in improved drought stress tolerance, and some up-regulated genes have been identified (Oh et al. 2005).

NAC (NAM, ATAF1, 2 and CUC2) and zinc-finger homeodomain (ZFHD) TF genes have also been found to impart stress signals by binding to the early response to dehydration 1 (ERD1) gene promoter (Tran et al. 2004, Tran et al. 2007a). Inspired by the research in Arabidopsis, researchers are also investigating NAC family TF gene functions in rice. *OsNAC6* is highly inducible by both ABA and salinity stress, and overexpressing the *OsNAC6* gene enhances plant tolerance to drought, salt and blast disease (Nakashima et al. 2007). OsNAC5 is another stress-inducible NAC TF in rice, and transgenic rice overexpressing *OsNAC5* exhibited enhanced salinity stress tolerance with no obvious constraints on plant growth under favorable conditions (Takasaki et al. 2010). Hu et al. (2006, 2008) reported that two other NAC TF genes, SNAC1 and SNAC2, enhance plant resistance to drought and salinity. SNAC1 transgenics have been tested in the field under severe water deficit conditions, and they were found to have an approximately 30% higher seed setting than wild-type plants.

In addition to the aforementioned TF families, the nuclear factor Y (NF-Y), Cys2/His2-type zinc-finger, MYC and MYB families have also been shown to function in various biotic stress conditions (Abe et al. 2003, Sakamoto et al. 2004, Villalobos et al. 2004, Davletova et al. 2005, Ciftci-Yilmaz et al. 2007, Nelson et al. 2007). The idea that TFs play central roles in regulating stress-responsive gene expression has been accepted by plant biologists.

## Signal transduction through protein modification

The discovery of the regulatory role of TFs in stress-inducible gene expression revealed how the similar expression pattern of

a set of genes is controlled. Recently, more exciting research has focused on how a stimulus signal is transmitted from one molecule to another, from the cytosol to the nuclei. Most signal transduction occurs through protein modifications, such as phosphorylation, ubiquitination and sumoylation. Reversible post-translational modifications play roles in these processes. Historically, the best studied protein modification is phosphorylation; however, other types of modifications have recently become more prominent, such as ubiquitination and sumoylation. Here, we focus on how these types of protein modifications regulate the activity of key TFs in plant abiotic stress responses.

Phosphorylation cascades regulated by protein kinases and phosphatases impart stress signals and modulate target protein activity. Recently, SnRK1, SnRK2 and SnRK3 have received much attention regarding stress responses. Two Arabidopsis SnRK1-type kinases (KIN10/SnRK1.1 and KIN11/SnRK1.2) function to link energy balance and stress responses to regulate growth and development (Baena-Gonzalez et al. 2007). In the Arabidopsis genome, there are 10 SnRK2-type kinases, and, except for SnRK2J/SnRK2.9, all of them are activated by osmotic stress (Boudsocq et al. 2004). Additionally, SnRK2D, 2E and 2I are responsive to ABA (Yoshida et al. 2002). The identification of the new ABA receptor PYL1 and its molecular partner ABI1 directly clarified the functions of SnRK2D, 2E and 2I in ABA-activated responses, as previously mentioned. In addition to the SnRK2-type kinases, two calcium-dependent protein kinases, CPK4 and CPK11, have been identified as positive regulators of ABF1 and ABF4 in response to ABA (Zhu et al. 2007). SnRK2C/SnRK2.8 and SnRK2F/SnRK2.7 were recently shown to play roles in regulating drought-responsive gene expression (Mizoguchi et al. 2010). Twenty-five Arabidopsis SnRKs belong to the SnRK3 group, which are also referred to as calcineurin B-like (CBL)-interacting protein kinases (CIPKs) (Hrabak et al. 2003, Luan et al. 2009). CBL proteins, which have 10 members in Arabidopsis, are $Ca^{2+}$ sensors in higher plants. CBL1 is highly inducible by cold, ABA and osmotic stress. Overexpressing CBL1 results in enhanced salt and drought stress tolerance; however, freezing tolerance has been reported to be impaired (Cheong et al. 2003). Another stress-responsive CBL gene is CBL9. The loss-of-function mutant *cbl9* is hypersensitive to ABA, which may be due to increased accumulation of ABA and elevated ABA signaling (Pandey et al. 2004). The interaction between CBLs and CIPKs is dependent on $Ca^{2+}$ signaling in plant cells, and the specificity of each CBL for each CIPK has been determined in a yeast system (Kim et al. 2000). Two of the most extensively studied interacting pairs are SOS3/CBL4 and SOS2/CIPK24. Mutations in *Salt Overly Sensitive* (*SOS*) genes render Arabidopsis plants hypersensitive to NaCl stress. Moreover, CIPK23/LKS1 (low-$K^+$-sensitive 1) activates the $K^+$ transporter AKT1 by protein phosphorylation, and CIPK23 directly interacts with and is activated by CBL1 and CBL9. It has been suggested that low potassium stress may trigger cytosolic $Ca2^+$ signaling and activate the calcium sensors CBL1 and CBL9 (Xu et al. 2006).

The correct regulation of gene expression is a vitally important process in plant cells because controlling when and where a gene is active is essential for normal growth and stimulus responses. The presence and abundance of each signaling factor and TF must be exactly correct. Protein ubiquitination plays key roles in this regard. In this type of protein modification, a highly conserved 76 amino acid protein, ubiquitin, is attached to the target protein as either single or multiple molecules, and, thereafter, the ubiquitin-labeled protein is recognized and degraded by the 26S proteasome. In this process, the ubiquitin molecule is first activated by the ubiquitin-activating enzyme (E1) in an ATP-dependent manner, and then activated ubiquitin is transferred to the ubiquitin-conjugating enzyme (E2), which forms an E2–ubiquitin intermediate. A ubiquitin ligase (E3) finally facilitates the attachment of ubiquitin to substrate proteins. E3 proteins confer most of the substrate specificity in this proteolysis pathway (reviewed by Hare et al. 2003, Vierstra et al. 2003, Moon et al. 2004). More than 5% of the predicted genes in the Arabidopsis genome are involved in this process. There are two isoforms of E1 proteins, and 41 genes contain a ubiquitin-conjugating domain (UBC) for E2 proteins; however, there are approximately 1,300 genes predicted to function as E3 ligases (Kraft et al. 2005, Stone et al. 2005). In addition to the major proteolytic function of ubiquitination, this modification participates in protein activation, histone modification and protein translocation. One of the most important E3 ligases in regulating plant response to freezing stress is high expression of osmotically responsive gene 1 (HOS1), which mediates ICE1 degradation and negatively regulates plant stress responses (Dong et al. 2006). HOS1 is a RING finger E3 ligase that is normally expressed in the cytoplasm, but, upon cold stress, it translocates into the nucleus, where it interacts with and degrades ICE1 to attenuate the latter's effects on cold-responsive gene expression. It is worth mentioning that ICE1 is also modified by small ubiquitin-related modifier (SUMO) via SIZ1 (SAP and Miz1), which competes with the function of HOS1 (**Fig. 2**). The sumoylation of ICE1 is thought to activate ICE1 protein activity and play a positive role in the plant cold response because the *siz1* mutant is sensitive to freezing stress. The K393R residue in the ICE1 protein is the target of SIZ1-mediated protein sumoylation, and $ICE1^{K393R}$ transgenic plants are more sensitive to stress (Miura et al. 2007). The HOS1–SIZ1 system appears to fine-tune the CBF/DREB1 regulon. Cold stress activates ICE1 through SIZ1-mediated protein sumoylation to up-regulate cold-responsive gene expression. At the same time, HOS1 inhibits extra ICE1 activity to attenuate the potentially deleterious effects brought on by the stress. The major remaining question is how cold stress regulates the activities of HOS1 and SIZ1 in this apex of cold stress response to ensure that the plant cell achieves an immediate and correct response.

DREB2A protein undergoes 26S proteasome-mediated proteolysis under favorable growing conditions. A negative regulatory domain has been identified within this protein, and it directly follows the AP2 DNA-binding domain. Removal of this domain enhances the protein's stability, and

overexpressing this modified DREB2A protein confers both drought and heat stress tolerance to the transgenic plants (Sakuma et al. 2006a, Sakuma et al. 2006b). DRIP1 and DRIP2 are two ubiquitin E3 ligases that contain C3HC4-type RING domains and interact with the DREB2A protein. In DRIP1 overexpressors, the expression of many DREB2A target genes is delayed in response to dehydration stress. However, in *drip1-1* and *drip2-1* single and double mutants, these gene expression responses are stronger than in the wild type, and the DREB2A protein is more stable. This finding suggests that DRIP1 and DRIP2 function as negative regulators of the plant drought response by mediating the proteolysis of DREB2A. DRIP1 and DRIP2 have been proposed to degrade the leaky expression of DREB2A under normal conditions to minimize the negative effects of this protein on plant growth (Qin et al. 2008). This research reveals another example of the delicate modulation of the abundance of a key TF through protein modification (**Fig. 3**).

ABI5 is a bZIP TF that is essential for ABA-dependent post-germinative growth arrest, and it is highly inducible by either ABA treatment or stress. It was recently reported that ABI5 is phosphorylated by SnRK2D/E/I kinases. Loss of this modification in *snrk2dei* mutants results in a loss of dormancy and an extreme sensitivity of seeds to desiccation (Nakashima et al. 2009a). The ubiquitin E3 ligase KEG (keep on going) is a negative regulator of ABA signaling, and it mediates ABI5 degradation during post-germinative growth. A loss-of-function *keg* mutant undergoes growth arrest immediately after germination because of a large accumulation of ABI5 protein (Stone et al. 2006, Liu and Stone 2010). Moreover, ABA promotes KEG self-ubiquitination and degradation, thus leading to the accumulation of ABI5 protein. Miura et al. (2009) discovered that ABI5 protein undergoes sumoylation to protect it from proteolysis, and the K391 residue in ABI5 is the target site of SIZ1-mediated sumoylation. ABI3 is another TF modified by ubiquitination, and ABI3-interacting protein 2 (AIP2) mediates the degradation functions of ABI3 by acting as a ubiquitin E3 ligase. The mutation of AIP2 leads to high ABI3 protein levels after germination, whereas in AIP2 overexpressors the opposite has been observed (Zhang et al. 2005).

## Hormonal homeostasis and interactions in plant stress responses

More information has indicated that homeostasis and cross-talk converging on phytohormones are implicit in plant stress responses and signaling. As previously mentioned, ABA is a key hormone in this adaptation, especially under water stress conditions. In addition to ABA, gibberellin, cytokinin (CK), ethylene and even auxin have recently been found to play roles in abiotic stress responses. Gibberellin is generally regarded as a growth-promoting compound that positively regulates processes such as seed germination, leaf expansion, stem and root elongation, flowering time and fruit development. Research on gibberellin signaling has revealed that GID1 (gibberellin-insensitive dwarf 1) is a soluble gibberellin receptor in plant cells that binds gibberellin and promotes ubiquitination-mediated DELLA protein destruction (Ueguchi-Tanaka et al. 2005). DELLA protein is a repressor of plant growth, and its degradation relieves this repressive effect, which results in a gibberellin response in plants. A sublethal level of salt stress

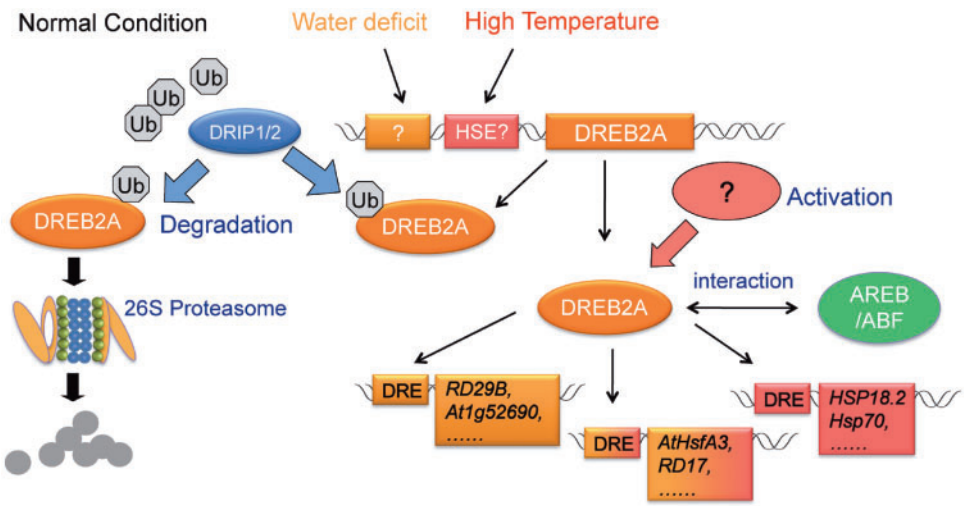


**Fig. 3** A model of the dual functions of the DREB2A TF under drought and high temperature stress. DREB2A gene expression is gradually and transiently induced by both water deficit and high temperature stress. DRIP1 and 2 are RING-type ubiquitin E3 ligases that mediate DREB2A protein ubiquitination and degradation under favorable growth conditions. Under stress, the active form of the DREB2A protein can regulate three types of gene expression, i.e. drought-inducible, drought- and heat-inducible, and heat-inducible genes, by binding to DRE sequences in their promoters. Thus, overexpressing the active form of the DREB2A protein enhances plant tolerance to both drought and heat stress. The DREB2A and DREB2C proteins also interact with AREBs/ABFs to mediate cross-talk between ABA-independent and -dependent gene expression.

usually slows plant leaf expansion and growth and reduces biomass. Moreover, gibberellin levels in salt stress-treated plants are lower than those in unstressed plants. Thus, the restriction of growth by salt stress is at least partly because of this reduced gibberellin content. Further research has revealed that a quadruple DELLA mutant lacking GAI, RGA, RGL1 and RGL2 is more sensitive to salt stress than the wild type. Thus, it has been proposed that stress reduces gibberellin levels and leads to the accumulation of DELLA proteins, which suppresses plant growth and confers tolerance to stress (Achard et al. 2006). DELLA proteins are also able to promote the expression of genes encoding reactive oxygen species (ROS)-detoxifying enzymes to reduce ROS levels in cells after biotic or abiotic attack, thereby delaying cell death and enhancing tolerance (Achard et al. 2008b). Moreover, CBF1 can stimulate the expression of the *GA 2-oxidase* gene, which encodes an enzyme that decreases bioactive gibberellin levels. Reduced gibberellin content leads to DELLA protein stabilization, so that CBF1 overexpressors are dwarves and delayed in growth. In addition, these mutants reveal that DELLA contributes to CBF1-induced cold acclimation and freezing tolerance (Achard et al. 2008a). More evidence that gibberellin is involved in plant abiotic stress responses is based on the fact that the exogenous application of gibberellin to plant seeds reverses the salt stress, oxidative stress and heat stress inhibition of germination and seedling establishment. A gibberellin-responsive gene from beechnut, *FsGASA4*, was recently discovered to function positively under abiotic stress. Overexpressing this gene in Arabidopsis enhances abiotic stress tolerance, which is accomplished by an increased level of salicylic acid (SA) (Alonso-Ramirez et al. 2009).

CKs are known to be able to induce cell division, promote lateral bud initiation, promote leaf expansion and delay senescence. Under stress conditions, CK levels decline, accompanied by an increase in ABA levels, which leads to stomatal closure and ABA-dependent gene expression. These changes promote leaf senescence and abscission, which results in a small canopy and reduced water loss; thus, resources are redirected to ensure survival. Rivero et al. (2007) generated transgenic tobacco carrying an *Agrobacterium tumefaciens Isopentenyltransferase* gene (*IPT*), which encodes a key enzyme for CK biosynthesis. The promoter of a senescence-associated receptor protein kinase (SARK) gene, from *Phaseolus vulgaris*, was constructed in front of the *IPT* gene. These transgenic plants ($P_{SARK}$::*IPT*) are more tolerant of drought-induced leaf senescence, which results in a remarkable level of water stress tolerance. Under mild water stress conditions, the transgenic plants display minimal yield loss compared with the wild type. Further characterization revealed no differences in stomatal apertures between the transgenic and wild-type plants under either optimal or restricted water conditions. Moreover, a greater reduction in photosynthesis occurs in wild-type plants under water stress compared with the transgenic plants. Although the photosynthetic apparatus is degraded in wild-type plants under low photosynthesis conditions, it is not affected in the transgenic plants. The CK-mediated occurrence of photorespiration, which may protect photosynthetic processes, has been proposed to contribute to the stress tolerance in the transgenic plants (Rivero et al. 2009, Rivero et al. 2010). Manipulating IPT gene expression in plants is one more exciting means of enhancing drought stress tolerance by gene transfer. Recent research on CK signaling has suggested that this hormone is indeed involved in plant abiotic stress responses. Double mutations in two CK receptor genes, AHK2 and AHK3 (Arabidopsis histidine kinase 2 and 3), greatly enhance plant osmotic stress tolerance. Under lethal (200 mM) NaCl conditions, the survival rate of the mutant is significantly higher than the survival rate in the wild type. Similarly, a higher survival rate was observed when the plants were compared under severe water stress, indicating that these two CK receptors play negative roles in plant stress responses (Tran et al. 2007b). Jeon et al. (2010) found that *ahk2 ahk3* double mutants are more resistant to freezing stress than wild-type plants. Research on another Arabidopsis histidine kinase, AHK1, demonstrated that it is a positive regulator that functions in plant osmotic stress responses. AHK1 gain of function confers drought stress tolerance, whereas *AHK1* gene loss of function, through either knock-down or knock-out, impairs plant stress tolerance (Tran et al. 2007b, Wohlbach et al. 2008). CK-mediated plant stress tolerance has been attributed to both ABA-dependent and ABA-independent responses because alterations in expression of both ABA-dependent and ABA-independent genes are observed in transcriptional comparisons. Research on plants with modified CK biosynthesis will probably provide further indications of the functions of CK in plant abiotic responses.

Research on auxin, ethylene and SA has revealed that they also are involved in plant abiotic stress. In the auxin pathway, many NAC TFs are responsive to auxin, ABA and abiotic stress. For example, although AtNAC2 is highly inducible by salt stress, it is also highly inducible by auxin, ABA and ethylene. However, the salt-inducible expression of AtNAC2 is suppressed in both ethylene-insensitive and auxin-insensitive mutants, but it is not affected in an ABA-insensitive mutant (He et al. 2005). This finding indicates that the AtNAC2-mediated salt response requires both auxin and ethylene signaling, and AtNAC2 is a TF that probably incorporates environmental and endogenous hormone stimuli (He et al. 2005). Recently, it was found that under heat stress, endogenous auxin levels are specifically suppressed in developing anthers, which may be due to the repression of *YUCCA*, an auxin biosynthesis gene. The application of auxin can completely reverse the male sterility caused by high temperature stress in barley and Arabidopsis, which suggests a role for auxin in maintaining pollen fertility under heat stress (Sakata et al. 2010). Many ethylene-responsive TFs belonging to the AP2/ERF superfamily are inducible by both abiotic stress and ethylene. Sun et al. (2008) reported that TINY, a DERB-like TF, can bind the DRE and ERE (ethylene-responsive element) with similar affinities and activate reporter genes driven by them. When *TINY* expression is induced, both DRE-regulated and ERE-regulated genes are up-regulated. Thus, TINY is

considered to be a signaling molecule that connects biotic and abiotic responses (Sun et al. 2008).

## Future challenges

As described above, great leaps towards understanding plant abiotic stress responses have occurred in the last decade. However, many challenges still lie ahead, both in basic research and in field applications. For example, whether additional ABA receptors exist remains to be determined. Although GTG1/GTG2 proteins have been reported to be membrane-bound ABA receptors, their biological roles in ABA downstream signal transduction remain unknown. In addition to ABA perception, the processes of ABA biosynthesis, transportation, storage and turnover in response to abiotic stress are not fully understood. Studies on TFs have revealed their complexities and pleiotropic functions in both plant stress responses and development (Kanaoka et al. 2008). Key components underlying stress responses and development remain to be elucidated. Furthermore, the cross-talk and interactions between ABA and other phytohormone signals need to be better addressed.

With the rapid development of genomic technology and robust statistical analysis methods, there is growing interest in using association mapping strategies to identify genes underlying quantitative or complex traits of particular agricultural or evolutionary importance. Using genomic sequence and single nuclear polymorphism (SNP) information, association mapping based on linkage disequilibrium analysis of a naturally varying population can resolve complex traits down to the sequence and genome-wide levels (Zhu et al. 2008). Genome-wide association studies (GWAS) have proven to be a good complementary tool to the traditional biparental crossing and mapping strategy. GWAS of 107 Arabidopsis phenotypes using 191 different genotypes have provided candidate genes for further studies (Atwell et al. 2010). Han and his colleagues sequenced 517 rice landrace genomes with 1-fold coverage and conducted GWAS for 14 agronomic traits, including drought resistance (Huang et al. 2010).

Another great challenge is how to apply the knowledge obtained to improve the environmental stress tolerance of crops, a potentially significant benefit of this research. Classical genetics suggests that plant abiotic stress tolerance is controlled by multiple loci, each contributing a minor effect (minor genes); therefore, the manipulation of a master regulatory gene through biotechnology is considered to be more efficient than conventional breeding strategies in which it is difficult to break negative linkage for many loci at one time. Nevertheless, large gaps remain between basic research and the production of stress-tolerant crops. First, the standard assays, including screening criteria and methods, are different between Arabidopsis and crop species. Proper assessments of environmental stress tolerance for crops need to be established according to the actual requirements of agricultural production. Some pioneering work has been performed in producing stress-tolerant plants and testing them in the field, such as the transfer of *ZmNF-YB2*, an NF-Y gene, to transgenic maize, and the use of *SNAC1*, a rice NAC-type TF, to improve drought tolerance (Hu et al. 2006, Nelson et al. 2007). This type of research is valuable not only because it applies the genes to agriculturally important crops but also because it evaluates the improved traits under actual productive field conditions. Secondly, yield potential is the primary concern of breeders. However, several conflicts exist between high yield and stress tolerance. For example, a smaller canopy and lower seed set are beneficial for maintaining survival under adverse conditions. Stomatal closure to reduce water loss inhibits photosynthetic efficiency and carbon assimilation. Under laboratory conditions, plants with higher survival rates are considered to be tolerant, which does not always suit the requirements of breeding or the desires of breeders. To make it possible to combine stress tolerance with high yield potential while avoiding the negative effects of a stress gene on plant growth under favorable conditions, strategies that spatially and temporally restrict transgene expression via tissue-specific and stress-inducible promoters are used (Sakuma et al. 2006a, Nakashima et al. 2007). Alternatively, several traits, such as a strong root system, high water use efficiency and flexible osmotic adjustment, could be integrated with yield potential. To achieve the combination of high yield and stress tolerance in one variety depends on our understanding of how the individual traits develop and how they interact with each other. Thirdly, intimate collaborations among plant molecular biologists, physiologists and breeders are required. Previous work regarding gene identification and functional characterization has been performed by molecular biologists; photosynthetic damage, stomatal aperture movement and osmotic adjustments in stress responses are topics for specialists in plant physiology; and tolerant resource collection and evaluation are performed by breeders. Differences in their research strategies and their primary concerns cause them to work independently. However, the transformation of upstream knowledge into an end-product will require their cooperation.

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