Development of Stress-tolerant Crop Plants

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ABSTRACT Adverse environmental conditions such as drought, high salt and cold/freezing are major factors that reduces crop productivity worldwide. According to a survey, 50-80% of the maximum potential yield is lost by these "environmental or abiotic stresses", which is approximately ten times higher than the loss by biotic stresses. Thus, improving stress-tolerance of crop plants is an important way to improve agricultural productivity. In order to develop such stress-tolerant crop plants, we set out to identify key stress signaling components that can be used to develop commercially viable crop varieties with enhanced stress tolerance. Our primary focus so far has been on the identification of transcription factors that regulate stress responsive gene expression, especially those involved in ABA-mediated stress response. Be sessile, plants have the unique capability to adapt themselves to the abiotic stresses. This adaptive capability is largely dependent on the plant hormone abscisic acid (ABA), whose level increases under various stress conditions, triggering adaptive response. Central to the response is ABA-regulated gene expression, which ultimately leads to physiological changes at the whole plant level. Thus, once identified, it would be possible to enhance stress tolerance of crop plants by manipulating the expression of the factors that mediate ABA-dependent stress response. Here, we present our work on the isolation and functional characterization of the transcription factors.

Key words: Abscisic acid, basic leucine zipper protein, drought tolerance, environmental stress, gene regulation, stress response

Introduction

Abscisic acid (ABA) is one of the major plant hormones that plays an important role during plant growth and development (Zeevaart and Creelman 1988; Leung and Giraudat 1998). The hormone controls several physiological processes during seed development and germination. During vegetative growth, ABA mediates adaptive responses to various abiotic stresses (i.e., drought, high salt and cold/freezing). The responses include stomatal closure and expression of a large number of stress-inducible genes. These and other ABA-dependent stress responses are critical to plant survival and productivity. Hence, ABA biosynthetic mutants are prone to wilting and cannot grow well even under normal, unstressed conditions.

Central to the ABA-mediated stress response is the ABA-regulated gene expression, which eventually leads to physiological changes at the whole plant level. From numerous promoter analyses, several *cis*-elements known as ABA responsive elements (ABREs) have been identified (Giraudat et al. 1994; Busk and Pages 1998). Among them, those sharing a (C/T)ACGTGGC consensus sequence (G-ABRE) are found to be most ubiquitous in ABA and/or stress-regulated genes. The elements, typified by the Em1a element (GGACACGTGGC) of wheat *Em* gene (Guiltinan et al. 1990), contains the ACGT core sequence and can be considered a subset of a larger group of *cis*-elements known as "G-box" (CACGTG) (Giuliano et al. 1988; Menkens et al. 1995). Another group of ABREs, known as "coupling element", "*hex3*" or "motif III" (C-ABRE) (Busk and Pages 1998), shares the CGCGTG core sequence. Based on their interaction with these two types of ABREs, a number of putative *trans*-acting factors have been isolated (Busk and Pages 1998). Also, their homologs and numerous other G-box binding factors, all belonging to the bZIP class proteins (Landschulz et al. 1988), are able to interact

with the ABREs *in vitro* (Foster et al. 1994). However, several observations suggest that hitherto unidentified factors are involved in ABA-regulated gene expression during stress response, especially in vegetative tissues. ABA-induction of rice *rab16A* and *Arabidopsis rd29B* genes requires *de novo* protein synthesis (Yamaguchi-Shinozaki and Shinozaki 1994; Nakagawa et al. 1996), suggesting the involvement of ABA-inducible factors. Such ABA-inducible DNA-binding protein(s) has been identified in a tobacco leaf nuclear extract by *in vitro* binding study (Chung 1996). Furthermore, it has been firmly established by genetic studies that different ABA signaling pathways operate in seeds and in vegetative tissues, respectively. However, none of the source materials used in the previous protein-DNA interaction clonings were ABA- or stress-treated young plant tissues, and thus, inducible factors that may be critical for the ABA-mediated stress response during vegetative growth phase may have been missed in the previous attempts. As a first step toward the elucidation of the ABA/stress responsive signal transduction pathways, we set out to isolate such transcription factors. Here, we report a novel subfamily of *Arabidopsis* bZIP proteins that are involved in the ABA/stress signal transduction.

Results

Isolation of ABRE-binding protein factors

We employed a modified yeast one-hybrid system (Kim et al. 1997; Kim and Thomas 1998) in order to isolate ABRE-binding factor(s) using the prototypical ABRE, Em1a element (GGACACGTGGCG). A cDNA expression library representing 2 $\times 10^7$ cfu was constructed in a yeast expression vector pYESTrp2, using a mixture of equal amounts of mRNAs isolated from ABA- and salt-treated *Arabidopsis* plants. From a screen of 4 million yeast transformants, ca. 40 His^+ blue colonies were obtained, among which 19 isolates were characterized further. Analysis of the cDNA inserts of the positive clones indicated that they could be divided into 4 different groups according to their restriction patterns.

Sequence analysis of the representative clones, here referred to as ABFs (ABRE-binding factors), revealed that they have basic regions near their C-termini (Figure 1). The region immediately downstream of it contains 4 heptad repeats of leucine, indicating that ABFs are bZIP proteins. The basic regions of ABF1 and ABF3 are identical to each other, and those of ABF2 and ABF4 are also identical. The two, shared basic regions are the same except that one of the lysine residues of ABF1 and ABF3 is replaced by arginine in ABF 2 and ABF4. The analysis shows that a family of bZIP proteins with conserved basic regions interacts with the ABRE.

ABFs

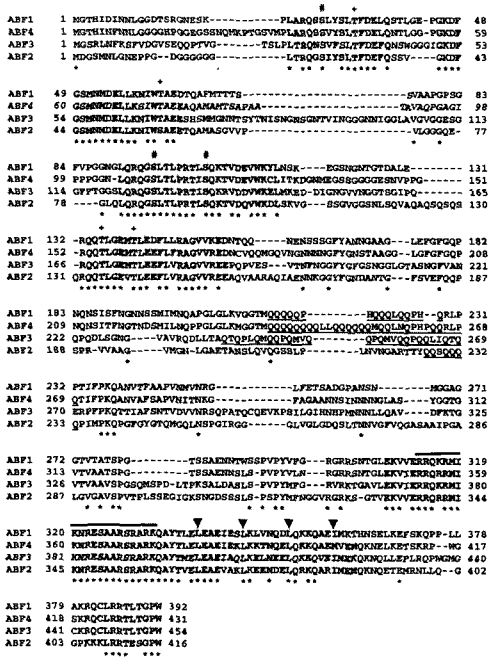

Figure 1. Deduced amino acid sequences of ABFs. Deduced amino acid sequences of ABFs are aligned together. The basic region and the leucine repeats are shown by a thick line and arrowheads, respectively. Conserved regions are highlighted and glutamine-rich regions are underlined. #, CaMK II sites. +, CK II sites. *, conserved amino acids. GenBank Accession Numbers: AF093544 (ABF1), AF093545 (ABF2), AF093546 (ABF3), AF093547 (ABF4).

Binding site preference of ABFs

We performed *in vitro* binding assay to confirm the binding of ABFs to ABREs. The result (data not shown) showed that, unlike other plant bZIP proteins, ABFs can interact with both G- and C-ABREs, although mutual competition assay showed that they have higher affinity to the G-ABRE. In order to investigate ABF binding sites further, we performed a random binding site selection assay (RBSA) (Pollock and Treisman 1990), using the recombinant ABF1. The selected sequences are presented in Figure 2A. The sequences could be divided into 4 groups (groups IA, IAA, IB, and II) according to their consensus sequences. The group I sequences contain an ACGT element, while the group II sequences contain the C-ABRE core. The most frequently selected sequences are those sharing a strong G-ABRE, CACGTGGC: gACACGTGGC (group IA) or CCACGTGGC (group IB).

Expression of ABFs is ABA-inducible

ABA-inducibility of ABF expression was investigated by RNA gel blot analysis. As shown in Figure 2B, expression of all four ABFs increased when treated with exogenous ABA, indicating that they are ABA-inducible. We also examined the effect of various environmental stresses on the expression of ABFs. The results (Figure 2B) showed that expression of ABF1 was induced by cold treatment, but not by other stress treatments. ABF2 and ABF3, on the other hand, were not induced by cold, but by high salt treatment. ABF4 expression was induced by all three treatments, although induction level after cold treatment was relatively low. Expression of ABFs is, thus, inducible also by various environmental stresses and their induction patterns are different from each other, suggesting that they function in different stress signaling pathways.

ABFs can transactivate an ABRE-containing reporter gene in yeast

Our result so far demonstrated that ABF1, and probably other ABFs also, can bind to various ABREs and that their expression is both ABA- and stress-dependent. Thus, ABFs have a potential to activate a large number of ABA/stress responsive genes, if they have transactivation capability. Therefore we investigated whether ABFs can activate an ABRE-containing reporter gene. Coding regions of ABFs were cloned into a yeast expression vector and the constructs were individually introduced into a yeast strain that harboured a G-ABRE-containing *lac*Z reporter gene. Subsequently, reporter enzyme activity was measured.

With the ABF1 construct, β-galactosidase activity was 6 times higher than that obtained with the control construct (Figure 2C). Thus, ABF1 could transactivate the reporter gene and the activation was ABRE-dependent. With the ABF2 construct, reporter enzyme activity two times higher than the background activity was detected, indicating that the factor also can transactivate the reporter gene. Likewise, ABF3 and 4 could transactivate the reporter gene. The activation level of ABF3 was higher than the ABF1's, while ABF4 showed weaker activation. The result of our transactivation assay demonstrates that ABFs can activate an ABRE-containing gene in yeast.

In vivo function of ABFs

We determined the *in vivo* functions of ABF3 and ABF4, employing an overexpression approach. ABF3 and ABF4 were constitutively expressed in *Arabidopsis* under the control of a strong *35S* promoter, then ABA-associated phenotypes such as

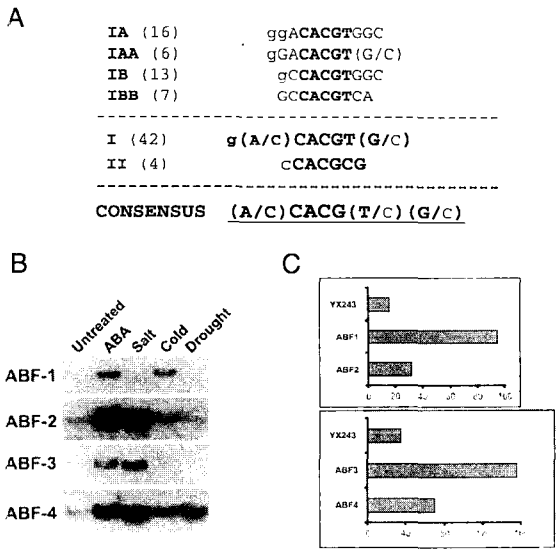

Figure 2. Binding site preference, expression pattern and transcriptional activity of ABFs. A, The consensus sequences of selected sites are shown with the number of selected sequences in the parentheses; B, ABA- and stress-inducibility of ABF expression were examined by RNA gel blot analysis; C, Transactivational function of ABFs was tested by using a yeast system. ABFs were expressed in yeast that harboured an ABRE-containing *lacZ* reporter gene. The β-galactosidase activity was then assayed and indicated as Miller units. For each construct, 5 different transformants were assayed in duplicates. YX243, control vector without any inserts.

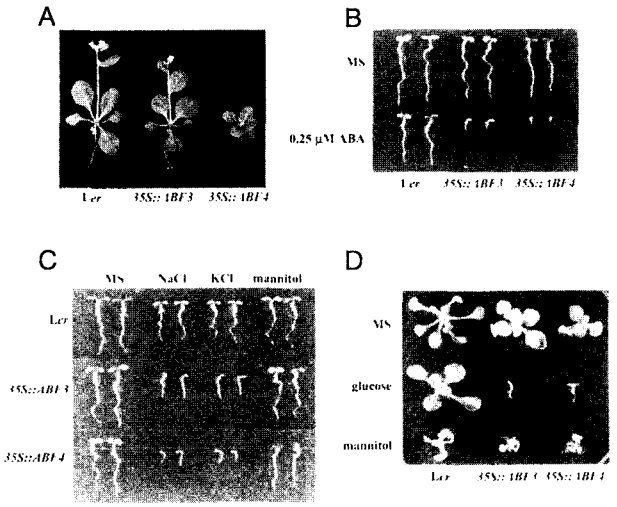

Figure 3. Phenotypes of *35S::ABF* transgenic lines. A, Growth on soil. *35S::ABF3* and 35S::ABF4 transgenic plants were grown for 3 weeks on soil; B, ABA sensitivity of *35S::ABF3* and *35S::ABF4* plants. Seeds were germinated on the medium containing 0.25 mM ABA for 3 days and representative plants are shown; C, Salt sensitivity of *35S::ABF3* and *35S::ABF4* plants. Seeds were germinated for 4 days on media containing 100 mM of NaCl , KCl or mannitol, and representative seedlings are shown; D, Sugar sensitivity of *35S::ABF3* and *35S::ABF4* plants. Seeds were germinated and grown for 14 days on the regular growth medium or the same medium supplemented with 3% glucose or mannitol, and representative plants are shown.

germination/growth inhibition, ABA sensitivity, stress response and interaction with other signaling pathways were scored.

The *ABF* transgenic lines exhibited growth retardation. Compared with wild type plants, *ABF3* transgenic lines exhibited slightly delayed germination and mild growth retardation in the aerial parts in the absence of ABA (Figure 3A). *ABF4* transgenic lines, on the other hand, germinated normally, but their growth was severely retarded; petioles were shorter, leaves were smaller, flowering was delayed and plants were shorter (Figure 3A).

The *35S::ABF* transgenic lines were hypersensitive to ABA. Germination was more severely inhibited by ABA than wild type plants, and the growth of newly germinated seedlings was also more sensitive to ABA (Figure 3B). The ABA hypersensitivity was observed also at later growth stages, e.g., primary root elongation of seedlings was more sensitive to ABA (data not shown). Thus, both germination and post-germination growth of the *35S::ABF* plants were hypersensitive to ABA.

High concentrations of salts inhibit germination of *Arabidopsis* (Werner and Finkelstein 1995; Leon-Kloosterziel et al. 1996; Quesada et al. 2000). Several studies showed that ABA plays a role in the inhibition process. Although the reverse is not true, all ABA deficient and ABA insensitive mutants exhibit salt-insensitivity during germination. This is probably because ABA, whose level rises under high salt conditions, promotes the inhibition process. Since *ABF3* and *ABF4* expression is salt-inducible, we asked whether the germination inhibition process was affected in the *35S::ABF* transgenic lines. Under our experimental conditions, germination of wild type plants was not significantly affected by NaCl at $\leq$ 100 mM although growth after germination was inhibited somewhat (Figure 3C). In contrast, germination and seedling growth (cotyledon greening/expansion and true leaf development) of *35S::ABF3* and *35S::ABF4* plants were severely inhibited by 100 mM NaCl. In a parallel experiment, the transgenic lines responded to KCl in a similar way to NaCl, but their response to mannitol was normal. Thus, *35S::ABF3* and *35S::ABF4* plants were hypersensitive to salt, and the hypersensitivity appeared to be ionic rather than osmotic in nature.

High concentrations of sugars inhibit the development of young seedlings (Jang et al. 1997). Recently, ABA has been shown to play an essential role in the sugar signal transduction (Arenas-Huertero et al. 2000; Huijser et al. 2000; Laby et al. 2000). For example, ABA deficient mutant, *aba2*, is allelic to the sugar insensitive mutant, *sis4*, and the glucose or sugar insensitive mutants, *gin6*, *sis5* and *sun6* are allelic to *abi4* mutant. Also, other *aba* mutants and, to some degree, *abi5* mutants are insensitive to glucose. Thus, ABF overexpression might have affected sugar sensitivity if ABF3 and ABF4 mediate ABA-dependent sugar signaling. We addressed this by examining their response to glucose, which exerts more severe growth inhibition than other sugars. Wild type seedlings showed defects in aerial part growth at glucose concentrations above 4% (data not shown). With *35S::ABF3* and *35S::ABF4* plants, a complete arrest of the aerial part growth was observed at 3% glucose, at which wild type plants developed fully (Figure 3D). Thus, *35S::ABF3* and *35S::ABF4* plants were hypersensitive to glucose. The enhanced response of the *35S::ABF* plants was not observed with the same concentration of mannitol, which inhibited the growth of both wild type and transgenic lines significantly but similarly. The results show that the hypersensitivity is glucose-specific rather than osmotic.

One of the key ABA-controlled processes is the stomatal closure under water deficit condition, which minimizes water loss through transpiration. To address whether ABF3 or ABF4 overexpression affected water stress response, we examined the drought tolerance of the *35S::ABF* plants. Wild type plants withered completely when withdrawn from water for 11 days and only 16% of them survived to maturity when re-watered afterwards (Figure 4A). *35S::ABF3* plants, however, were not affected noticeably and all survived the treatment to set seeds. Similarly, *35S::ABF4* plants also exhibited higher survival rates under water deficit conditions; all of them survived a 12-day drought treatment, whereas 33% of the wild type plants survived to set seeds (Figure 4B). Thus, the *35S::ABF* transgenic plants were more tolerant to the drought conditions than wild type plants. In accordance with the results,

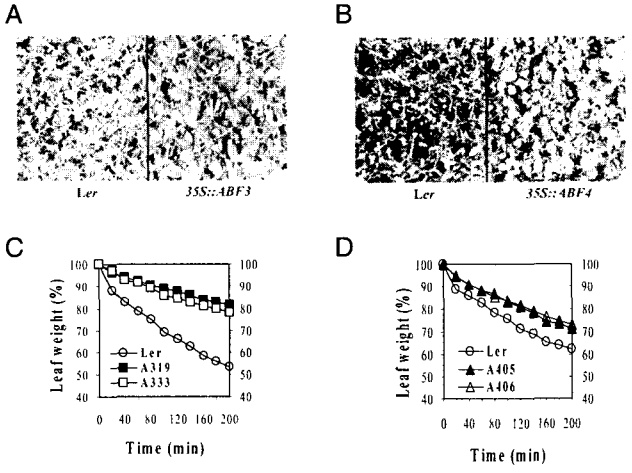

Figure 4. Drought tolerance of *35S::ABF* plants. A, Drought tolerance of *35S::ABF3* plants; B, Drought tolerance of *35S::ABF4* plants. Transgenic and wild type plants (n=100 each) were grown on soil for two weeks, withheld from water for 11 (ABF3) or 12 (ABF4) days, and then re-watered. The pictures were taken 3 days after the re-watering; C and D, Transpiration rates of *35S::ABF3* and *35S::ABF4* transgenic plants, respectively. Leaves of similar developmental stages were excised and weighed at various times after the detachment.

35S::ABF plants exhibited reduced transpiration. When measured by the fresh weight loss of detached rosette leaves, transpiration rates of the *35S::ABF* transgenic lines were less than half (ABF3) or approximately 70% (ABF4) of the wild type plants' (Figure 4, C and D). Thus, constitutive expression of ABF3 or ABF4 resulted in reduced transpiration and enhanced drought tolerance.

Discussion

Numerous studies, both genetic and biochemical, show that ABA mediates stress response in vegetative tissues, although not all stress responses are ABA-dependent (Leung and Giraudat 1998). Central to the response is the ABA-regulation of gene expression through the G- or C-ABREs. Thus, identifying relevant transcription factors is critical for the delineation of ABA signal transduction cascades. Many studies showed that ABA plays an essential role also in seed development, and several seed-specific ABA signaling components (*ABI3*, *ABI4*, and *ABI5*) have been identified by genetic screens (Leung and Giraudat 1998). *ABI3* and *ABI4* encode transcription factors (Giraudat et al. 1992; Finkelstein et al. 1998), whose binding sites and immediate target genes are not known. Recently, *ABI5* has been shown to encode a bZIP factor that belongs to a seed-specific subfamily of ABF-related factors (Finkelstein and Lynch 2000; Lopez-Molina and Chua, 2000; Lopez-Molina et al. 2001). Also, transcription factors mediating ABA-independent cold and drought responses have been reported (Jaglo-Ottosen et al. 1998; Liu et al. 1998). However, those transcription factors involved in stress-responsive ABA signaling have not been reported previously.

In a search for such transcription factors, we isolated a family of G-ABRE-binding proteins from *Arabidopsis* plants. The factors, referred to as ABFs, are ABA/stress-inducible bZIP class transcription factors with shared basic regions. Our *in vitro* binding assay showed that the most preferred binding site of ABF1 *in vitro* can be represented as CACGTGGC (Figure 2A). The element, first identified as EmBP-1 recognition site (Guiltinan et al. 1990), is highly conserved among ABA/stress inducible promoters and strongly affects ABA-inducibility *in vivo*. In addition, ABF1 could interact with C-ABREs. Together with its ABA/stress-inducibility and transactivation capability, the broad binding specificity suggests that ABF1, and probably other ABFs as well, can potentially activate a large number of ABA/stress responsive genes.

Our *in vivo* data indicate that ABF3 and ABF4 indeed function in stress responsive ABA signaling. First, their overexpression resulted in ABA hypersensitivity. Second, their overexpression enhanced salt and glucose sensitivities at the germination/young seedling stages, which are further indications that they are involved in ABA signaling. Third, it reduced transpiration rate with concomitant enhancement of drought tolerance, suggesting that ABF3 and ABF4 regulate stomatal movement and/or guard cell ABA signaling. In addition, expression of many ABA/stress responsive genes whose products are known to participate in various stress responses was up- or down-regulated in ABF-overexpressing lines (data not shown). Together, these observations point to the ABF3 and ABF4's involvement in stress responsive ABA signaling.

The adaptive role of ABA in stress response has been documented extensively. Thus, it would be possible to improve stress tolerance of plants by genetically engineering ABA signaling components. Our results show that ABF3 and ABF4 participates in ABA/stress signaling and that their overexpression indeed results in enhanced drought tolerance of Arabidopsis. It appears that ABFs are excellent targets for genetic engineering to develop drought and, perhaps, other environmental stress tolerant crop plants.

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