



Expression and Characterization of Salt Stress Induced ABF Transcription Factors from French Bean (*Phaseolus Vulgaris*)

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Abstract

ABA responsive element binding factor (ABF) plays an important role in stress responses via regulating the expression of stress-responsive genes. ABF is a bZIP transcription factor and functions in positive modulation of abiotic stress tolerance. It may be an important candidate gene for molecular breeding of stress-tolerant plants. In this study, four ABF coding genes were isolated from French bean (*Phaseolus vulgaris*) under salt stress (400mM NaCl) conditions. All the four PvABFs exhibit complete open reading frame of 1347 bp, encoding a 448 amino acid peptide, and shared high sequence identities with ABFs from other plants. PvABF was sub-cellular targeted to the nucleus, exhibited transactivation activity and could bind to ABF, supporting its role as a transcription factor. Expression levels of PvABFs were induced by treatments with salt stress conditions.

Key Words: ABF; Absciscic acid; French bean; Transcription factor

Introduction

Plants are exposed to many types of environmental constraints during their life cycle. These limitations result in both general and specific effects on plant growth and development. For example, drought retards the growth due to decline in photosynthesis and non-availability of nutrients. Similarly, salinity leads to physiological dryness. Chilling and freezing temperatures can also cause osmotic stress. Plants respond to these conditions with an array of morphological, physiological, biochemical and molecular changes, which enable plants to survive and reproduce. Plants under these conditions, an assortment of genes and proteins with diverse functions are induced or repressed. These proteins could be categorized into two groups. The first group includes functional proteins namely late embryogenesis abundant (LEA) proteins, molecular chaperones, key enzymes for osmolyte biosynthesis like proline, sugar and sugar alcohols, betaines, detoxification enzymes, and membrane transporters which are directly associated with protection of plants from ill effects of abiotic stress. The second group is comprised of proteins that are regulatory in nature and further regulate signal transduction and stress-responsive gene expression. These include various transcription factors, protein kinases, enzymes involved in phospholipid metabolism, and other signaling molecules such as calmodulin-binding protein and 14-3-3 protein.

During the evolutionary process, plants have developed some complex molecular mechanisms probably for their survival under the extreme environmental constraints. In this way, another category of genes encoding regulatory proteins have emerged. Such genes play important roles in survival of plants under stress situation by serving as master regulator of sets of downstream stress-responsive genes.

Thus, expression of many genes responsive to abiotic stresses can be regulated and coordinated by manipulating a single regulatory gene for management of crops under stress conditions. Among the regulatory proteins, transcription factors (TFs) have a central role in activating defense gene expression (Chen and Zhu 2004; Xu et al. 2008). The TFs interact with *cis*-acting elements present in the promoter region of various stress-responsive genes and thus activate cascades or whole network of genes that act together in enhancing tolerance towards multiple stresses at a time. This property of TFs makes them an attractive category of genes for manipulation of abiotic stress tolerance. Analyzing and elucidating the function of these genes is very critical for further understanding of the molecular mechanisms governing plant abiotic stress tolerance, and this may help in genetic manipulation of crops for enhanced stress tolerance. Absciscic acid (ABA) is one of the major plant hormones that play an important role during plant growth and development. The hormone controls several physiological processes during seed development and germination. ABA mediates responses to various adverse environmental conditions such as drought, high salt, and cold/freezing (Shinozaki, et al 1996; Thomashow, 1998).

The ABA-mediated adaptive responses to environmental stresses include stomatal closure and expression of a large number of genes involved in stress tolerance. These and other ABA-mediated stress responses are critical to plant survival and productivity, although ABRE binding factors have been inducible by ABA (Kusano, et al; 1995). Several observations suggest that hitherto undefined factors are involved in ABA-regulated gene expression during stress response, especially in vegetative tissues. Furthermore, it has been well established by genetic studies that different ABA signaling pathways operating in seeds and vegetative tissues, (Ono, et al, 1996), and tissue-specific ABRE binding activities have been demonstrated (Pla, et al. 1993). In rice, ABA positively or negatively regulates the transcripts of some WRKYs (Xie, et al., 2005). None of the source materials used in the previous protein-DNA interaction cloning, however, 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 so far. In this study, we are interested in ABA-regulated gene expression during salt stress response and set out to isolate the transcription factors. Here, we report the expression of ABFs induced by salt stress in French bean, and the key role mediated during hormonal pathway.

Materials and Methods

Plant materials and stress treatments

Seedlings of French bean (*Phaseolus vulgaris* S-9) were grown hydroponically at 25 °C for 06 days and were subjected for salt stress. Seedlings were transferred into solutions containing 400 mM NaCl and the tissue was collected after 48h, and stored at -80 °C for further analysis.

Identification of French bean ABF gene

Based on the homology of the *ABF* family genes among different species, we designed degenerate primers that bordered the C3 conservative region and the bZIP domain using the Arabidopsis genes as a template. Four central fragments of the *ABF* coding regions were amplified using degenerate primers and sequenced (Additional Table 1).

Quantitative RT-PCR and RNA gel blot Analysis

Real-time RT-PCR was performed with oligonucleotides that were specific for the 3' non-translated regions of the different *ABF* genes. Each amplified product was cloned and sequenced to verify the specificity of the primers; the actin gene was used as control. The qRT-PCR was performed according to manufactures instructions (Takara Bio Sciences Japan) using gene specific primers and a SYBR-Green PCR Master kit. The expression levels were measured three times for each cDNA sample. The Delta-delta-Ct method was used to quantify the fold change in *ABF* gene expression during stress exposure with respect to expression levels under the control conditions. The primers that were used in the real-time RT PCR are presented in (additional Table 1). Expression analysis and Northern blot hybridization were conducted using high sensitive RNA Northern blot assay kit (Signosis, USA). 30 µg total RNA of each sample was electrophoresed on 15% polyacrylamide gel and transferred to membrane. Antisense RNA biotin labeled in the 5' end (Invitrogen) was used for hybridization probes. The SYBR Green® II stained (Biotech). rRNA bands in the polyacrylamide gel are used as a loading control.

Electrophoretic Mobility Shift Assay (EMSA)

Recombinant and fusion GST-PvABF2/AREB1 plasmids were transferred to the *E. coli* BL21 cell line. Under 16 °C and 0.2 mM isopropyl β-D-1-thiogalacto-pyranoside (IPTG) for overnight incubation, the recombinant proteins were induced and purified by Nickel NT column. The EMSA was performed essentially carried according to (SYBR® Green EMSA kit Invitrogen USA).

Results

Characterization of ABF transcription factors: expression analysis

Using degenerate primers, four open reading fragments of the putative *ABF* genes were amplified by PCR. The newly identified genes were named *PvABF1-4* according to their most similar Arabidopsis counterparts. The similarities of the deduced amino acid sequences of French bean ABF1, ABF2, ABF3 and ABF4 to their Arabidopsis homologs were 71, 77, 85 and 88%, respectively. All of the identified isoforms share the bZIP domain with four leucine repeats as well as 3 N-terminal (C1, C2 and C3) and one C-terminal (C4) conserved domains. Similarly to those from Arabidopsis, the ABFs from French bean contain invariable Ser/Thr residues in their conserved domains. These residues are the putative targets for Ser/Thr kinases. Moreover, the C4 conserved domain contains the canonical mode II 14-3-3 interaction motif (RRTL/SGP), shown to be involved in the interaction with 14-3-3 proteins in barley, Arabidopsis and rice.

ABF gene expression in French bean plants, exposed to stress.

To determine whether the expression of *ABFs*; we performed measurements at different time points over 24 after stress treatment. A slight increase in the expression of *ABF2* (10%) was detected in the shoots immediate and 30% at 8 h after the treatment, which may be due to water loss. The expression of *ABF2* and *ABF3* genes was strongly induced by salinity. The induction of the *ABF4* gene was moderate. The expression of *ABF1* was not significantly affected by the stress (data not shown). In the shoot, *ABF2* showed the strongest induction. Generally, ABA stress resulted in a rapid induction of the *ABF* genes in the shoot with peaks between 2 and 8 h after the onset of stress, whereas the expression returned to the near basal level after 24 h. As ABFs are known for their great importance in ABA-mediated stomatal closure, such a quick expression change in the *ABF* genes might play a crucial role in plant adaptation by reducing transpiration levels (Figure 1). Gene expression changes were further evident with northern assays with gene specific probes and showed ABF2 and ABF3 were significantly up-regulated under salt stress conditions, however the levels of ABF1 and ABF4 were not affected.

In Vitro Binding Activity of ABFs

In-vitro DNA binding assays of ABFs were performed by EMSA using recombinant ABFs (1-4) and PCR amplified products of promoter elements respectively. The results were found to be all ABFs showed the excellent binding characteristic in concentration dependent manner. However, in-vitro binding efficiency was found to be highest (gel retardation complex) for stress induced ABF2 and ABF3 (Figure 3).

Discussion

High salinity or drought results in increase in plant ABA levels, which in turn affect the expression of a vast number of stress-related genes. In the salt- and ABA-mediated regulation of gene expression, ABF transcription factors play a pivotal role (Agarwal, et al., 2010). In our research, we identified and characterized four members of the *ABF* family in French bean. In addition to the bZIP domain, the identified proteins contain four highly conserved regions that are almost invariable among French bean and Arabidopsis. The ABF transcription factors of the test plant share up to 88% identity in the amino acid sequence with their Arabidopsis counterparts with the differences in amino acid sequence observed

mainly in inter-domain regions. The highest diversity was observed for *PvABF1*, whose *Arabidopsis* homolog was reported to be induced by ABA and cold but not by salt and drought (Choi, et al., 2000). Although the identity between *PvABF1* and *AtABF1* was not high, the expression pattern of *PvABF1* (data not shown) was similar to that observed for *AtABF1*, which is salt-insensitive (Choi et al., 2000). ABF2, ABF3 and ABF4 were characterized as the master transcription factors that regulate the salt and ABA-induced ABRE-dependent gene expression based on the observed phenotype of the *abf2 abf3 abf4* triple mutant plants (Agarwal et al., 2010; Yoshida et al 2010).

The quantitative RT-PCR analysis revealed some clear differences in the salt-induced *PvABF* expression patterns in the shoot. Of the ABF genes, ABF2 and ABF3 had the strongest response. In addition to the evidence in favor of ABF redundancy, data concerning the different expression patterns of ABFs as reported here and in a number of other studies indicate that there are both similarities and specificities in their functions under various conditions (Choi et al., 2000; Yoshida et al., 2010). In addition to the above, the short-term up-regulation of ABF genes in response to salt stress. Similar results also observed, in the expression rates of the *Arabidopsis* ABF genes after 18-24 h of stress treatment (Uno, et al., 2000; Fujita et al., 2005). The unregulated ABF transcription factors may involve in post-translational regulation, phosphorylation and proteasome-mediated degradation (Sirichandra, et al., 2010). Although members of the SNRK2 protein kinase family have been shown to phosphorylate and activate ABF transcription factors, the precise mechanism of activation remains unclear (Kobayashi et al., 2005 and Fujita et al., 2009). Interaction with members of the 14-3-3 protein family was shown to form another level of control over the activity of a number of ABF transcription factors (Schoonheim et al., 2009). Recently, it has become clear that ABA-induced phosphorylation, 14-3-3 interaction and the prevention of degradation of ABFs are related events that control the amount of available ABF protein and thereby the associated transcriptional regulation. There might be a complex network of ABA responsive transcription factors involved in stress and ABA response. The expression pattern of ABFs implies that they are involved in the regulation of ABA-responsive genes whose expression requires protein synthesis. Depending on the requirement of *de novo* protein synthesis, ABA-responsive gene activation events can be divided into two classes: one involving protein synthesis and the other independent of protein synthesis. Expression of rice *rab16A*, *Arabidopsis rd29B* and rice *Osem* genes, for example, requires protein synthesis, whereas ABA induction of the wheat *Em* gene does not. Our result (*i.e.* ABA inducible of ABF expression) thus, suggests that ABFs are likely to regulate the expression of the former class of genes. Each ABF may function in different ABA-dependent stress signaling pathways. ABF1 expression is induced by cold, ABF 2 and ABF3 by high salt, and ABF4 by cold, high salt and drought. The simplest interpretation of the result would be that ABF1 is involved in cold signal transduction, whereas ABF2 and ABF3 function in osmotic stress signaling. ABF4, on the other hand, appears to participate in multiple stress responses. In addition, ABFs differ in their ABA induction patterns. Expression of ABF1 was induced rather slowly and the accumulation of its RNA was transient, whereas induction of other ABFs appeared faster, and their RNA levels remained relatively stable once a plateau was reached. The multiplicity of ABA-dependent stress-signaling pathways has been demonstrated in *Arabidopsis* by genetic analysis (Choi, et al., 2000). Previous studies found that ABF2/ABF2/AREB11 is an important mediator in glucose signaling and drought stress tolerance in vegetative tissues (Kim et al., 2004; Fujita et al., 2005). Over-expression of an activated form of ABF2 increased the expression of some stress-inducible genes and greatly enhanced drought tolerance in *Arabidopsis* (Fujita et al., 2005). However, ABF2 over-expressing plants did not change ABA-mediated stomatal closure, which suggests that the increased drought tolerance was attributed mainly to the elevated expression of downstream genes, such as LEA-type genes (Fujita et al., 2005). *In-vitro* binding assay showed that the most preferred binding site of ABF1 is represented as CACGTGGC. The element first identified as EmBP-1 recognition site is highly conserved among ABA/stress-inducible promoters and strongly affects ABA expression *in-vivo* (Choi, et al., 2010). Together with the fact that ABF1 is ABA/stress-inducible and has transcriptional activity, this suggests that ABF1 can potentially activate a large number of ABA/stress-responsive genes. Also, ABF1 can bind to other ABREs including the C/ABREs, further supporting the broad spectrum of potential ABF1 target genes. The affinity to C/ABREs, however, was relatively low. It cannot be ruled out, therefore, that factors other than ABFs interact with C/ABREs with higher affinity.

Conclusion

ABA-dependent stress-responsive gene expression is critical to plant growth and productivity. Here, we reported a family of transcription factors that interact with cis-regulatory elements mediating this process. Although their specific roles in plants remain to be determined, our data presented here suggest that they are likely to be involved in various ABA-mediated stress responses. They can bind to ABREs highly conserved among stress-responsive promoters. They can trans-activate ABRE reporter gene, their expression is induced by ABA and by various environmental stresses.

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Figures and Legends

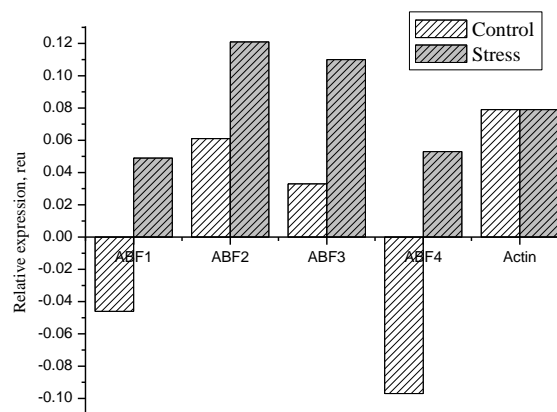


Figure-1: Levels of ABF Transcription factors induced under salt stress conditions in French bean; Sixth day old seedlings were treated with 400 mM NaCl. The shoots were harvested at 0, 2, 8 and 24 h after the plants were exposed to stress. Total RNA was isolated, converted to cDNA and subjected to quantitative real-time RT-PCR. The relative expression levels (relative units, reu) were normalized using the level of *Actin* gene expression and calculated with respect to the gene expression of the control plants.

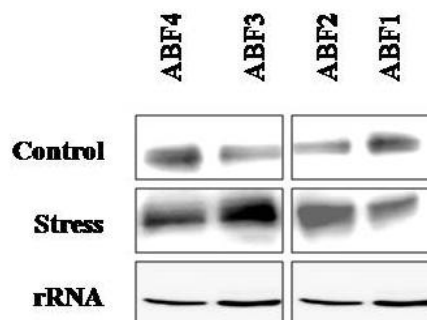


Figure-2: Northern blot analysis of salt-stress induced ABF genes from French bean. Each sample contains 20 µg of total RNA from stress and control seedlings respectively. rRNA was used as loading control as shown at the bottom of hybridization set.

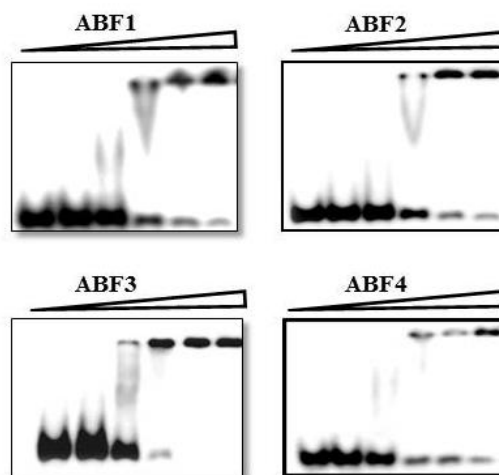


Figure-3: Recognition of the G/CREB promoter by ABF genes. Sequence specific binding French bean ABF1-4 recombinant proteins were studied by EMSA. For each binding assay were studied with concentration gradient of each recombinant proteins and biotin labeled DNA probe were used.

Additional File

Table 1: Primers used in ABF expression analysis

Gene	Primer Name	Sequence 5'-3'
Central fragments of ABF genes (degenerated primers)	ABF1-4fw	AAAGATTTYGGGTCDATGAAYATGG
	ABF1-4 rv	ATCATYCTYYTTTGCCTYCTCTC
PvABF 1	ABF1-RT fw	GTGTATATGAGGCTGATGATGG
	ABF1-RT rv	GAACTGGAAGTGGAGATGACC
PvABF 2	ABF2-RT fw	CTTAGGTGTTTAGTTTGAAGTCTG
	ABF2-RT rv	GATCCCGTGTCTGTATAACGC
PvABF 3	ABF3-RT fw	TGTACGTGACTTAGGCAGGTG
	ABF3-RT rv	ATCACGATCCGTTACATCAGG
PvABF 4	ABF4-RT fw	GAGATAGAGCACCTGTACAGG
	ABF4-RT rv	AACCTAGACTAATGAAGCAAGG