Functional characterization of the ABF gene family in upland cotton (*Gossypium hirsutum* L.)

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**Highlight**

The *Gossypium hirsutum* ABF homeologs are differentially expressed in response to abiotic stress, and their ectopic expression in *Arabidopsis* can confer increased water deficit tolerance.

**Abstract**

The AREB/ABF bZIP transcription factors play a pivotal role in abscisic acid-dependent abiotic stress-responsive gene expression. Despite the perennial damage and reduced productivity that result from water-deficit and unpredictable early season temperature fluctuations, these critical genes have not been previously examined in upland cotton (*Gossypium hirsutum*). Here, we report the isolation of the *G. hirsutum* ABF homologs, characterization of their expression patterns in response to abiotic stress treatments, and examination of their functions through heterologous ectopic expression in *Arabidopsis*.

As expected for an allotetraploid, *G. hirsutum* ABF homologs are present in the genome as homeologous pairs. These genes are differentially expressed, both among the homologs and within the homeologous pairs, in response to exogenous abscisic acid (ABA) application, dehydration, and chilling temperatures. Furthermore, heterologous ectopic expression of many of the *G. hirsutum* ABF genes in *Arabidopsis* conferred increased tolerance to water deficit and osmotic stress, as well as cold tolerance, in a gene specific manner. These results indicate the *G. hirsutum* ABF homologs are functional in *Arabidopsis* and, as in other species, are likely to play an essential role in the abiotic stress response.

**Key words:** abiotic stress, abscisic acid, AREB/ABF, cold tolerance, cotton, drought tolerance, tetraploid

**Abbreviations:** ABA, ABF, ABRE, AREB
Introduction

Plants experience damage and reduced productivity as a result of exposure to stressful environmental conditions including water deficit and temperature extremes (Boyer, 1982; Bray et al., 2000; Wang et al., 2003). The pernicious effects of abiotic stress are especially problematic in agricultural settings, where economic viability is dependent on predictable, high yields. Cultivated upland cotton (Gossypium hirsutum L.) is particularly susceptible to these acute effects, as it is grown mainly in arid or semi-arid regions where rainfall is limited and early and late season temperatures can fluctuate widely. These conditions make rain-fed production difficult and risky, therefore, more often than not, irrigation is required to consistently produce profitable yields. With the depletion of groundwater resources, the diversion of surface water to other uses, and increasingly extreme and unpredictable temperature fluctuations, the need to understand the mechanisms used by plants, including cotton, to acclimate to stressful conditions and the development of strategies to optimize these systems in order to produce varieties that can remain productive with less water, has become a priority.

Plants have robust abiotic stress responsive networks that include myriad differentially regulated genes (Wang et al., 2003; Bartels and Sunkar, 2005; Yoshida et al., 2014). Among these, the abscisic acid (ABA)-responsive element binding proteins/ABRE-binding factors (AREB/ABFs) have been identified as essential regulators of the osmotic stress response (Yamaguchi-Shinozaki and Shinozaki 2006; Fujita et al., 2013; Yoshida et al., 2015). The expression of many of the members of this small sub-family of transcription factors is induced in response to ABA and various other abiotic stressors, and in turn, they modulate the expression of downstream target genes that ultimately result in the up-regulation of abiotic stress-protective factors including membrane and protein stabilizing molecules, antioxidants, and the accumulation of osmocompatible solutes (Wang et al., 2003; Reddy et al., 2004).

As ABA-dependent, bZIP transcription factors, the AREB/ABFs interact with the conserved cis-acting ABA-responsive element (ABRE: PyACGTGG/TC) found in the 5’ flanking regions of many ABA responsive genes (Choi et al., 2000; Kang et al., 2002; Fujii et al., 2009; Yoshida et al., 2010; Yoshida et al., 2014). Nine AREB/ABF family members have been identified in Arabidopsis. Of these, three are induced by osmotic stress: AREB1/ABF2, AREB2/ABF4, and ABF3; a fourth, ABF1, has been shown to be a functional homolog (Yoshida et al., 2015). These Arabidopsis AREB/ABF paralogs contain the basic region and the leucine repeats characteristic of the bZIP domain, and five conserved Ser/Thr
kinase phosphorylation sites (RXXS/T), that are phosphorylated by SnRK2 protein kinases. Although many of these Arabidopsis AREB/ABF genes are induced by similar abiotic stressors, and their target genes overlap, each exhibits unique temporal and spatial expression patterns (Choi et al., 2000; Fujita et al., 2005; Fujii et al., 2009; Fujita et al., 2013; Yoshida et al., 2015).

Ectopic over-expression of this subset of genes from the AREB/ABF family in Arabidopsis has been shown to confer increased tolerance to various abiotic stressors, as a result of their positive regulation of ABA signaling (Kim et al., 2004; Fujita et al., 2005; Chinnusamy et al., 2006; Novillo et al., 2007; Fujii et al., 2009; Yoshida et al., 2010; Medina et al., 2011). These studies show that these AREB/ABF transcription factors play an essential role in the response to abiotic stress, and thus, have been extensively examined in model species. Although the endogenous ectopic expression of these Arabidopsis AREB/ABF genes confers increased abiotic stress tolerance, these improvements are often also accompanied by slower vegetative growth and delayed reproduction.

Despite the economic importance of cotton as the world's primary source of natural fiber, accounting for 40% of all textile fibers produced, the ABF gene family has not been fully characterized in G. hirsutum, the most commonly cultivated cotton species (Wendel and Cronn, 2003; Meyer et al., 2007; Osakwe, 2009). This is likely due, at least in part, to the allotetraploid nature of the G. hirsutum genome (Wendel & Cronn 2003; Chaudhary et al., 2009). Here, we characterize the G. hirsutum AREB/ABF homologs (hereinafter GhABFs), including their expression in response to various abiotic stressors and their ability to confer improved abiotic stress tolerance when ectopically expressed in Arabidopsis. Furthermore, to address the putative tradeoff between improved stress tolerance and developmental delay, multiple independent transgenic lines, with various levels of ectopic expression, are evaluated for each GhABF transgenic gene construct to ascertain if there is an acceptable balance of positive and negative functional effects.

Materials and methods

Gossypium ABF homolog isolation and phylogenetic analysis

Gossypium arboreum, G. hirsutum, and G. raimondii coding sequences were isolated as previously described (Kerr et al. 2017). In brief, a BLAST query of the NCBI EST database was performed using publicly available Arabidopsis AREB/ABF gene coding sequences for similar sequences from G.
hirsutum to identify ESTs representing putative homologs. To recover full-length coding sequences, total RNA from G. hirsutum (c.v Coker 312) was extracted using the Spectrum Plant Total RNA kit (Sigma) and consecutive rounds of RACE-PCR were used to derive the 5' and 3' ends of the target transcripts using the SMARTer RACE cDNA amplification kit (Clontech). G. arboreum and G. raimondii ABF coding sequences were derived in a similar fashion for those sequences not found in the NCBI database. Populus trichocarpa orthologs with the highest homology to Arabidopsis ABF1, AREB1/ABF2, ABF3, and AREB2/ABF4, as identified by Ji et al. (2013) and Brassica napus ABF/ABF orthologs were obtained via BLAST queries of the NCBI database. Coding sequences were imported into MEGA6.06-mac (Tamura et al., 2013), aligned with ClustalW, and used to generate a maximum likelihood tree; bootstrapped 250 times. Aligned amino acid sequences were imported into Jalview 2.9.Ob2 (Waterhouse et al., 2009) for annotation.

qRT-PCR analysis

Arabidopsis thaliana Columbia (Col-0) seeds, sown on solid medium containing half strength MS and 1% sucrose, were placed in the dark for 24 h at 4°C, then transferred to a growth chamber at 24°C with a 15 h light/9 h dark cycle for three weeks. Samples for basal expression level determination were taken prior to stress treatments. To measure expression levels in response to exogenous ABA, plants were sprayed to saturation with a 100 µM ABA solution and sampled 0.5 h, 1 h, and 2 h after application. To measure the dehydration response, plants were removed from the media, keeping the roots intact, and sampled after 1.5 h, 3 h, and 6 h. To measure the response to chilling temperatures, plants were transferred to 4°C, and sampled after 1 h, 2 h, and 4 h. G. hirsutum (Coker 312) plants were grown in soil, in 1 L pots, for six to eight weeks under long-day conditions (15 h light/9 h dark) at 30°C. Following pre-treatment sampling for the determination of basal expression, the plants were subjected to the following treatments. Plants were sprayed to saturation with a 500 µM ABA solution, and sampled after 0.5 h, 1 h, and 2 h. Water was withheld, and dehydration stress treatment samples were taken after 48 h (before visible wilting), 72 h (moderate wilting), and 78 h (severe wilting). Chilling temperature treatment samples were taken after 1 h, 2 h, and 4 h exposure to 4°C. Arabidopsis RNA was extracted using the RNeasy Mini kit (Qiagen). G. hirsutum RNA was extracted using the Spectrum Plant Total RNA kit (Sigma). All RNA concentrations were quantified via Nanodrop, normalized to 100 ng/µL, and cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad). All qRT-PCR reactions were performed using the iTAQ Universal SYBR Green Supermix.
(Bio-Rad) in 10 µL reactions. Standard curves were derived from pGWB12 plasmid constructs (described in the following section) containing the Arabidopsis AREB/ABF or G. hirsutum ABF homolog coding sequences.

**Generation of transgenic Arabidopsis lines**

The coding sequences of the G. hirsutum ABF genes were amplified in accordance with the pENTR Directional TOPO Cloning kit (Invitrogen). Half-reactions were used for TOPO cloning, then transformed into One Shot Chemoically Competent *Escherichia coli* (Invitrogen). Plasmids were purified using the QIAprep Spin Miniprep kit (Qiagen). LR recombination (Invitrogen) was used to transfer the target sequences to the pGWB12 expression vector (provided by T. Nakagawa, Research Institute of Molecular Genetics, Shimane University, Matsue, Japan), then transformed into Library Efficiency DH5-α *E. coli* (Invitrogen). Purified plasmid was transformed into *Agrobacterium tumefaciens* C58, the culture was incubated at 30°C with shaking for 3 h, then plated to solidified LB supplemented with 10 µg mL⁻¹ gentamicin, 50 µg mL⁻¹ kanamycin, and 50 µg mL⁻¹ rifampicin. Colonies positive for the insert were cultured for 48 h in 25 mL liquid LB supplemented with 10 µg mL⁻¹ gentamicin, 50 µg mL⁻¹ kanamycin, and 50 µg mL⁻¹ rifampicin at 30°C with shaking, then transferred to 250 mL LB for 24 h. Cells were pelleted, then resuspended in a 400 mL 5% sucrose, 0.01% Silwet L-77 solution. Flowering Arabidopsis plants were dipped for 20 s with agitation, then placed under cover in the dark for 24 hours before being transferred to growth conditions at 24 °C with a 15 hour light/9 hour dark cycle (Clough and Bent, 1998). Harvested seeds were surface sterilized in 30% chlorine bleach and plated on solidified ½ MS media containing 1% sucrose and 50 µg mL⁻¹ kanamycin. Independent transformed lines were transferred to soil, verified via PCR, and expression levels were measured using qRT-PCR (as above) for a minimum of ten lines. Three lines, the first representing a relatively low level of ectopic expression, the second representing the highest level of ectopic expression of the lines quantified, and the third, representing an approximate average expression level of the low and high expressing lines (hereinafter “medial”), were selected for further examination.

**Immunoprecipitation**

Immunoprecipitation assays were performed as previously described (Chen *et al.*, 2013) with the following modifications. Total protein from eight-day-old 35S::FLAG-GhABF expressing transgenic...
Arabidopsis seedlings was extracted in immunoprecipitation (IP) buffer (50 mM Tris-HCl (pH 8.1), 150 mM NaCl, 1% NP-40 (v/v), 1 mM EDTA, 5% glycerol, 1mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (1:100)). The protein extracts (1 mg) were precleared by incubation with Protein A/G beads (Santa Cruz) for 2 h at 4°C, and immunoprecipitated using 20 µl of Anti-FLAG Affinity Gel (Sigma) at 4°C for 1 h. Beads were washed three times with IP buffer for 20 min each at 4°C. The precipitated proteins were eluted using 2x SDS sample buffer. Eluted samples were subjected to Western blot analysis using an Anti-FLAG Alkaline Phosphatase antibody (Sigma). Each experiment was replicated three times.

Transgenic Arabidopsis development and abiotic stress tolerance evaluation

To determine the effects of ectopic expression of the G. hirsutum ABF homologs in Arabidopsis on the reproductive transition, three to four T3 generation transgenic plants were grown in soil in 15 ml pots alongside wild-type (WT) Arabidopsis. The reproductive transition, defined by the initiation of bolting, was monitored for each transgenic line as compared to WT. To measure differential survival following dehydration, homozygous T3 and WT seeds were surface sterilized in 30% bleach, plated on ½ MS, 1% sucrose solid medium, placed in the dark for 24 hours at 4°C, then transferred to a growth chamber at 24°C with a 15 h light/9 h dark cycle for 3 weeks. An average of ten plants from three plates for each transgenic line and WT were removed from the media and transferred to petri dishes lined with glass beads to dehydrate. Plants were re-watered, in 30 min intervals, after a minimum of 4 h dehydration, to a maximum of 6.5 h. Survival was recorded following a 48 h recovery period. Electrolyte leakage, as the result of low water potential-induced damage, was measured as described by Verslues and Bray (2004) and van der Weele et al. (2000), with minor modifications. Briefly, three-week-old seedlings were transferred to PEG-infused plates of increasingly negative water potentials (-0.25, -0.50, -0.75, and -1.25 MPa) for 24 h, rinsed in a mannitol solution of the same water potential, and placed in 5 mL deionized water for 1 h. Conductivity was measured, the samples were autoclaved, and conductivity was measured again. Relative electrolyte leakage was calculated by dividing initial conductivity by conductivity following autoclaving. Each genotype and treatment was replicated three times. To measure differential survival following exposure to freezing temperatures, T3 seeds were sown on soil-filled petri dishes. An average of ten 4 week-old plants per plate were then transferred to a growth chamber at -7°C. After a minimum of 3 h at -7°C, plates were returned to the growth chamber at approximately 24°C, at 30 min intervals, to a maximum 5.5 h. Survival was recorded following a 48 h
recovery period. Electrolyte leakage as the result of freezing damage was measured as described by Guo et al. (2002) and Ristic and Ashworth (1993), with minor modifications. Briefly, leaves of 4 week old plants were excised and placed in tubes containing 5 mL deionized water then transferred to a water bath at 1°C. The temperature was decreased at a rate of 1.5°C h\(^{-1}\) and samples were removed at -2, -5, -8, and -11°C, and placed on ice overnight. Following the measurement of initial conductivity, the samples were autoclaved, conductivity was measured again, and relative electrolyte leakage was calculated. Each genotype and temperature was replicated three times.

Results

Isolation and phylogenetic analysis of GhABF homologs

The allotetraploid *G. hirsutum* genome is a result of a polyploidy event between A and D genome *Gossypium* diploid species (Wendel & Cronn, 2003; Chaudhary et al., 2009). Therefore, we expected that the target *G. hirsutum ABF* orthologs would occur in the *G. hirsutum* genome as highly similar, albeit distinct, homeologous gene pairs. To confirm this hypothesis, we isolated the coding sequences and portions of the promoter regions of multiple putative *ABF* homologs from *G. hirsutum* and the diploid *Gossypium* species, *G. arboreum* (A genome) and *G. raimondii* (D genome), and aligned them with the *Arabidopsis AREB/ABF* orthologs (Supplementary Fig. S1). Eight putative polypeptides encoding *GhABF* orthologs (four homeologous pairs) were derived that contained the conserved basic region and leucine repeats requisite of the bZIP domain, and the five putative Ser/Thr phosphorylation sites characteristic of the *Arabidopsis* AREB/ABFs (Furihata et al., 2006; Fujii et al., 2009).

To confirm the homology of these putative *Gossypium* orthologs, we constructed a maximum likelihood phylogenetic tree (Supplementary Fig. S2) including the isolated *G. arboreum, G. hirsutum,* and *G. raimondii ABF* coding sequences, their *Arabidopsis* and *B. napus* orthologs (Rosid II), and the *P. trichocarpa AREB/ABF* homologs (Rosid I; Ji et al., 2013). Significant support was found for the homology of the eight isolated *GhABF* sequences. Each of the Brassicaceae family *AREB/ABF* sequences resolved in a one-to-one fashion, as did the *G. hirsutum* homeologous pairs with their corresponding A or D genome diploid *Gossypium* progenitor. However, no one-to-one *AREB/ABF* gene relationship was found between the Malvaceae (*Gossypium*) and Brassicaceae families, or between the Rosid I and Rosid II clades. The coding sequences of the *Gossypium ABF* homeologous pairs *ABF1*, *ABF3*, and *ABF4* were found to be more closely related to each other than to the *AREB/ABF* orthologs
from any of the other genera examined, and also more closely related to two of the four *AREB/ABF*
m homologs from *P. trichocarpa*, rather than the examined species from the Rosid II clade, of which the
genus *Gossypium* is a member. Furthermore, the *Gossypium ABF2* orthologs resolved with the
remaining two Rosid I clade homologs, and were more similar to the *Arabidopsis ABF1, ABF3*, and
*AREB2/ABF4* and *Gossypium ABF1, ABF3, and ABF4* homologs than to the corresponding
*Arabidopsis AREB1/ABF2* homolog. Since no clear one-to-one phylogenetic orthologous relationship
was found between the Malvaceae (*Gossypium*) and Brassicaceae species examined, we opted to label
the isolated *GhABF* homologs based on a combination of their phylogenetic relationships and
similarities to the *Arabidopsis AREB/ABFs* in their expression patterns in response to abiotic stress (as
described in the following section).

**The AtAREB/ABFs and GhABFs are differentially expressed in response to abiotic stress**
The *AREB/ABFs* have been widely reported to be differentially expressed in response to various abiotic
stressors in several plant species (Choi *et al.*, 2000; Fujita *et al.*, 2005; Orellana *et al.*, 2012; Li *et al*.,
2014; Yoshida *et al.*, 2015). Therefore, we used qRT-PCR to measure the expression patterns of the
*Arabidopsis AREB/ABF* and *GhABF* homologs in response to exogenous ABA application, water
deficit, and cold temperature stress (Figs. 1 and 2). Analyses of the *Arabidopsis* homologs was carried
out to provide baseline expression level data to which the *GhABF* expression levels could be compared.
Absolute quantification methods were used to measure transcript copy number so that expression
changes between the different genes could be compared directly. Relative quantification was also
performed to confirm that our results were consistent with previously published data (Choi *et al.*, 2000;
Kim *et al.*, 2004; Fujita *et al.*, 2005; Oh *et al.*, 2005; Yoshida *et al.*, 2015). We found basal expression
levels of the *Arabidopsis AREB/ABFs* ranged from an average low of 10 transcripts per ng total RNA
for *AtABF1*, to 21 and 27 copies for *AtAREB1/ABF2*, and *AtABF3* and *AtAREB2/ABF4*, respectively
(Fig. 1A,C,E). Similar low levels of basal expression were measured for the *GhABF* homologs, ranging
from an average of 2 copies per ng total RNA for *GhABF1D*, to an average of 18 copies for *GhABF2A*
and *GhABF4D* (Fig. 2).

As previously reported, we found the *Arabidopsis AREB/ABF* homologs were differentially expressed
in response to exogenous ABA and abiotic stress treatments. While expression of each *AtAREB/ABF*
gene was induced, at least to some degree, in response to exogenous ABA application, the magnitude of
increase differed substantially. *AtABF1* expression doubled and *AtAREB1/ABF2* expression tripled relative to basal levels, while the expression of *AtABF3* increased 6 fold and *AtAREB2/ABF4* increased 7 fold (Fig. 1A,B). Similarly, all *Arabidopsis AREB/ABF* genes were induced in response to water deficit, though *AtABF1* and *AtAREB1/ABF2* transcript levels increased only slightly, while the *AtAREB2/ABF4* transcript level increased steadily to 20 times its basal level over the 6 h sampling period, and the *AtABF3* level increased quickly after 3 h to ultimately reach a level 75 fold greater than the basal level after 6 h (Fig. 1C,D). Though the *AREB/ABFs* genes are primarily associated with the response to drought via the ABA-dependent pathway (Lee *et al.*, 2010; Fujita *et al.*, 2013; Yoshida *et al.*, 2014), we also examined their expression in response to low temperature stress. The expression of *AtABF1* and *AtAREB2/ABF4* did not change in response to chilling, however, the *AtAREB1/ABF2* transcript level increased gradually to 3 times its basal expression over 4 h at 4 °C, and *AtABF3* expression rose quickly to 6 six times its basal level after 1 h at 4 °C, then declined after the 2 h time point (Fig. 1E,F).

The expression of each *GhABF* homeolog was induced in response to at least one stress treatment, though the magnitude of induction varied widely between treatments (Fig. 2), and no consistent bias in expression of the A or D genome was observed. While expression of each of the eight *GhABF* homologs increased in response to exogenous ABA application, induction of *GhABF3A* was by far the strongest, rising to a level 30 times its basal expression over the course of the 2 h assay. Expression of *GhABF3D* and both *GhABF4* homeologs increased more gradually in response to ABA, reaching levels 8 to 10 fold above basal levels, while expression of the *GhABF2* homeologs increased by about 5 fold, and the *GhABF1* homeologs increased only by about 2 to 3 fold during the 2 h assay (Fig. 2A-D). In response to water deficit stress, again, *GhABF3A* expression showed the largest increase in transcript copy number. In addition, the increase in expression of the *GhABF3* homeologs in response to water deficit treatment began earlier than the other *GhABF* genes, becoming apparent after 48 h, as compared to 72 h for the *GhABF1, GhABF2*, and *GhABF4* homeologous gene pairs (Fig. 2E-H). Furthermore, both ABA- and drought-induced expression of *GhABF3A* was considerably stronger than that of *GhABF3D*, illustrating differential expression among these homeologous pairs.

Again, while the AREB/ABF bZIP transcription factors are not generally associated with temperature stress, we found the expression of *AtABF2* and *AtABF3*, and at least one member of each *GhABF*
homeologous gene pair, was induced during exposure to low temperature, although the magnitude of change exhibited by most of these GhABF homologs was far less than in the exogenous ABA application or water deficit treatments (Fig. 2I-L). Transcript levels of most of the GhABF genes induced by low temperature reached a maximum after 1 h at 4 °C, then leveled off or dropped back to near basal levels over the duration of the treatment. GhABF1A, which showed a relatively weak response to ABA or water deficit stress, was the most strongly induced GhABF homolog in response to low temperature, increasing from single digit levels to more than 100 copies per ng total RNA within 1 h, before returning to near basal levels after 4 h. Expression of GhABF4D also increased considerably in response to chilling stress, and like GhABF1A, expression returned to near basal levels after 4 h.

**Generation of GhABF expressing transgenic Arabidopsis lines**

In order to characterize the functions of the individual GhABF homeologs and test the impact of their ectopic expression on development and abiotic stress tolerance, we generated independent transgenic Arabidopsis lines that ectopically express each of the eight isolated GhABF genes, under the control of the constitutive CaMV 35S promoter. The ectopic expression levels of a minimum of ten independent Arabidopsis lines for each gene construct were quantified, and three lines for each were selected for phenotypic examination. These transgenic lines were selected as per the following criteria: 1) the line with the lowest measurable ectopic expression level, 2) the line with the highest measured ectopic expression level, and 3) a line with an ectopic expression level approximating the midpoint between the high and low expressing lines for each gene construct (Table 1). Each of these three selected lines, from each of the eight GhABF gene constructs, were subsequently evaluated, in parallel, for differences in growth and development, and their ability to tolerate drought and low temperature stress.

Although the same binary vector and CaMV35S promoter were used in the generation of all gene constructs, we found substantial differences in the levels of constitutive ectopic expression among the independent transgenic Arabidopsis lines. Wide variation in the range of event-specific expression was seen between the transgenic lines expressing the individual GhABF orthologs and, in some cases, between the lines expressing the A or D genome-derived homeologs (Table 1). For example, the high-expressing lines containing the transgenes that encode the GhABF2 A and D genome homeologs had similar levels of expression, averaging 455 transcripts per ng total RNA, while the GhABF2A-expressing lines showed little event-specific variability, with less than a 2-fold difference detected.
between the highest and lowest expressing lines, in contrast to the difference between highest and lowest expressing GhABF2D lines, which was nearly 16-fold. Greater event-specific variation in expression was seen in the GhABF3 homeolog expressing lines, with the selected GhABF3A lines ranging from a low of 175 transcripts per ng total RNA to a high of 6383 transcripts per ng total RNA, a 36-fold difference, while the overall expression difference among the GhABF3D lines was approximately 1/10th the level of the GhABF3A lines, ranging from 17 to 770 transcripts per ng total RNA, a 45-fold difference from lowest to highest. Even more substantial differences in expression between the paired homeologs was seen among the GhABF4 lines, with the expression of the GhABF4A lines ranging from 224 to 1563 transcripts per ng RNA (a 7-fold difference), while expression levels in the GhABF4D lines were far lower, ranging from 22 to 63 transcripts per ng RNA, a difference of only about 3-fold. Thus, in addition to the expected event-specific variation in transgene expression that is typically attributed to position effects associated with the insertion site, substantial gene-specific differences in mRNA accumulation are also apparent.

**GhABF protein expression is largely independent of transcript level**

To better understand the patterns of ectopic G. hirsutum ABF expression in Arabidopsis and determine the effects of ABA on ABF accumulation (Chen et al., 2013) we examined FLAG-GhABF fusion protein accumulation in the selected GhABF D genome expressing transgenic Arabidopsis lines with or without ABA treatment (Fig. 3). Ectopic GhABF protein expression was not detected in crude protein extracts from any of our transgenic lines by Western blot analysis but specific bands were detectable after enrichment by immunoprecipitation. Unlike the wide variation in transcript expression levels, relatively little variation in G. hirsutum ABF protein accumulation was seen between the low, median, and high transcript expressing GhABF2D or GhABF4D transgenic Arabidopsis lines without ABA treatment and these levels did not change in response to ABA. In contrast, GhABF3D protein levels were nearly undetectable in immunoprecipitated samples taken from plants without ABA treatment but, after ABA treatment, the protein accumulated to substantially higher levels and clear differences were seen between the low, median, and high expressing lines. Thus, it appears that the steady state levels of the GhABF proteins in plants that express GhABF2D and GhABF4D are relatively stable and largely independent of transcript levels or ABA treatment. On the other hand, accumulation of GhABF3D appears to be under ABA-dependent post-transcriptional regulation.
Ectopic GhABF expression can delay the reproductive transition

Previous studies have shown that endogenous ectopic expression of Arabidopsis AREB/ABFs delays growth and the reproductive transition (Kang et al., 2002; Kim et al., 2004; Fujita et al., 2005). To determine if ectopic GhABF gene expression in Arabidopsis affects development, selected transgenic lines were grown alongside wild type and monitored for differences in the reproductive transition, defined by the initiation of bolting (Fig. 4). None of the GhABF1A or GhABF1D expressing lines examined differed significantly from wild type plants; however, the majority of the GhABF2, GhABF3, and GhABF4 transgenic Arabidopsis lines exhibited significant delays in reproductive transition (Fig. 4B). Except for the GhABF1 expressing lines, the reproductive transition delay was most severe the lines that express the highest ectopic levels of the GhABF transcripts, indicating a relationship between expression level and reproductive delay. For example, while GhABF2D lines showed some line to line variation in mRNA expression, the level of GhABF2D protein was relatively stable and this is reflected in a limited range of developmental delay phenotypes. Likewise, expression of GhABF4D mRNA was low but protein accumulation in these lines was relatively high and stable, which corresponds with the strong developmental delay in all three lines. On the other hand, GhABF3D lines showed strong variation in expression at the mRNA level and, following ABA treatment, at the protein level. Thus, not unexpectedly, the severity of developmental delay in GhABF expressing Arabidopsis plants appears to correlate more closely with GhABF transgene expression at the protein level than at the mRNA level.

Ectopic GhABF expression can improve tolerance to water deficit and osmotic stress

To determine if ectopic GhABF expression in Arabidopsis confers improved water deficit tolerance, we quantified the survival of the selected GhABF expressing transgenic Arabidopsis lines, as compared to wild type, following dehydration treatment (Fig. 5A). Substantial differences in survival were apparent between the wild type and transgenic plants after approximately 5.5 h dehydration, and these differences became more pronounced after 6 h (Fig. 5A; Supplementary Table S1). The percent of surviving plants corresponded with ectopic expression level in the majority of the GhABF-expressing lines, with the strongest protective effects seen in the high expressing lines for most gene constructs. Notable exceptions to this trend were seen in the GhABF4 expressing plants, which showed similar survival rates at all expression levels. While survival of the GhABF4A plants was not substantially higher than wild type despite relatively high levels of ectopic expression, GhABF4D lines showed significantly improved survival that correlated more closely with the expression at the protein level.
The most substantial increase in water deficit tolerance was seen in the high GhABF3D line, which showed 71% survival over wild type after 6 h dehydration treatment, and correlated most closely with protein expression levels after ABA treatment.

To corroborate the dehydration survival assay results with osmotic stress, each of the GhABF-expressing lines were subjected to increasingly negative water potentials, and the percent electrolyte leakage was measured (Fig. 5B, Supplementary Table S2). Ectopic expression of the GhABF homologs resulted in reduced electrolyte leakage in nearly all of the lines and, in the majority of the transgenic lines, reduced electrolyte leakage following osmotic stress corresponded with increased plant survival following dehydration. With the exception of the GhABF1A and GhABF2D lines, the highest expressing lines showed the lowest levels of electrolyte leakage. However, this trend was not proportional to the dehydration survival results in all cases. For example, all of the GhABF3A expressing lines showed substantially reduced membrane damage, which contrasts with the plant survival assay, in which the low and medial expressing lines performed similarly to wild type.

Likewise, the GhABF4A and GhABF4D transgenic lines examined exhibited similar survival rates (by homeolog) regardless of expression level, but lines with increasing levels of ectopic expression showed incremental reductions in electrolyte leakage. The GhABF3D lines, on the other hand, showed both substantial increases in survival and substantial reductions in electrolyte leakage corresponding most closely to the level of ectopic expression at the protein level.

Overall, these results indicate that ectopic expression of each of the GhABF homologs in Arabidopsis resulted in protective effects in at least one of the assays used and the magnitude of stress protection was related to transgene expression level in the GhABF1, GhABF2, and GhABF3 expressing lines. However, in the GhABF4 expressing lines, little correlation was evident between transgene expression level and stress protection in the dehydration survival assay, where the highest expressing GhABF4A line, which had transcript levels approximately 25-times higher than highest expressing the GhABF4D line, was much more sensitive to dehydration stress. However, as shown in Fig 3, GhABF4D plants accumulate relatively high levels of GhABF4D protein, in spite of showing relatively low levels of mRNA.

Ectopic GhABF expression can improve cold tolerance, in a gene dependent manner.
Although the AREB/ABFs are generally associated with the osmotic stress response, some studies indicated they can also influence cold responses, directly or indirectly, via crosstalk with cold-responsive signaling pathways (Choi et al., 2000; Oh et al., 2005; Lee et al., 2010; Fujita et al., 2011). Therefore, to determine if ectopic GhABF gene expression in Arabidopsis has an effect on cold tolerance, we analyzed survival following exposure to -7°C over the course of 5 h (Fig. 6A; Supplementary Table S3), and electrolyte leakage (Fig. 6B, Supplementary Table S4) in response to progressively lower freezing temperatures.

Unlike the water deficit tolerance assays where the protective effects were associated with expression level, the effects of ectopic expression of the GhABF homologs in Arabidopsis on freezing temperature survival were gene-specific and largely independent of expression at the mRNA level (Fig. 6A). For example, all of the transgenic Arabidopsis lines expressing either the GhABF1 or GhABF4 homeologs showed significant increases in survival following exposure to -7°C as compared to the wild type plants. However, the ectopic expression of the GhABF2A and GhABF3A appeared to have negative effects on freezing tolerance, and only plants that expressed high levels of GhABF2D or GhABF3D showed increased survival compared to wild type plants.

Similar to the water deficit stress assays, lower levels of electrolyte leakage following exposure to freezing temperatures generally correlated with increased plant survival (Fig. 6B). Relative to wild type plants, the percent of electrolyte leakage measured for all GhABF1 and GhABF4 lines examined was substantially reduced, indicating enhanced cellular tolerance to freezing temperatures. Conversely, expression of GhABF2A, GhABF2A, and GhABF3A appeared to result in significant increases in electrolyte leakage after freezing treatment, relative to the wild type plants. Although plants of the high expressing GhABF2D line showed a small but significant increase in survival, electrolyte leakage assay results show that these plants suffered membrane damage similar to the wild type plants. High expressing GhABF3A line and the low and medial expressing GhABF2A lines showed both reduced survival and increased electrolyte leakage after exposure to freezing temperatures, indicating that freezing tolerance in these plants is likely to be reduced. In summary, ectopic expression of either of the GhABF1A, GhABF1D, and GhABF4D homeologs in Arabidopsis conferred increased tolerance to freezing temperatures while expression of GhABF2A or GhABF3 homeologs appears to compromise freezing tolerance.
Discussion

To determine the functional roles of the GhABF orthologs, we examined their expression patterns in response to various abiotic stressors in cotton and evaluated their effects on development and abiotic stress tolerance by ectopically expressing each in Arabidopsis. Since G. hirsutum is an allotetraploid species, we anticipated that each GhABF ortholog would be present in the cotton genome as a homeologous pair of genes with very similar coding sequences. Eight GhABF coding sequences were isolated, each encoding a putative polypeptide that contains the defining features of the Arabidopsis AREB/ABF proteins, namely, a canonical bZIP domain, and five Ser/Thr kinase phosphorylation sites (Furihata et al., 2006; Fujii et al., 2009). In order to directly compare the expression characteristics of the individual GhABF genes to one another and to the AREB/ABF homologs from Arabidopsis, absolute quantification methods were used to determine the number of transcript copies present in total RNA samples. Furthermore, since the responses of the Arabidopsis AREB/ABFs to cold stress have only been analyzed in a few cases (Choi et al., 2000; Lee et al., 2005), we assayed the expression of these gene in response to low temperatures, in addition to exogenous ABA application and water deficit. We found both the Arabidopsis AREB/ABF and GhABF genes had low levels of basal expression, and each gene was differentially responsive to the various abiotic stress treatments.

In Arabidopsis, expression of AtABF3 is the most responsive to water deficit, chilling temperatures and, along with AtABF4, to ABA treatment, while in G. hirsutum, expression of the GhABF3A is the most highly responsive homeolog to water deficit and ABA treatment, and GhABF1A is most responsive to chilling. These differential expression patterns within the G. hirsutum homeologous pairs could indicate sub-functionalization or silencing of one or the other homeolog due to redundancy. For example, expression of GhABF1 homeologs was only modestly responsive to exogenous ABA or dehydration, and the GhABF4 genes exhibit only a slight induction in response to dehydration, however, GhABF1A and GhABF4D are strongly induced in response to chilling, while expression of GhABF1D responds relatively weakly to chilling and GhABF4A does not respond at all. This increased expression in response to chilling stress could result from cross-talk due to functional interactions between the ABA-dependent and ABA-independent stress response pathways (Yoshida et al., 2014). For example, Arabidopsis AREB1/ABF2 interacts with various AP2 domain proteins, including DREB1A, also known as CBF3, an essential component of the low temperature stress response (Lee et
While ectopic expression of AREB/ABF genes may confer increased stress tolerance, these improvements are often accompanied by delayed growth or reproduction (Kang et al., 2002; Kim et al., 2004; Fujita et al., 2005). Therefore, we analyzed the ability of the GhABFs to confer increased stress tolerance and affect development when ectopically expressed in Arabidopsis. Tradeoffs between stress tolerance and developmental delay were seen with some, but not all, GhABF genes, raising the possibility that negative side-effects on growth and development associated with increased AREB/ABF expression may be gene-specific and it might be possible to mitigate unwanted negative effects by using transgenes that encode specific ABF orthologs and selecting transgenic lines with varying levels of ectopic expression. In this way, it may be possible to find an acceptable balance between positive and negative phenotypes. Therefore, three independent lines with high, low, and medial levels of ectopic expression were selected for each of the eight GhABF gene constructs for physiological examination. Although the gene constructs differed only in their coding sequences, transgene expression levels varied widely among the different GhABF gene constructs. For example, the highest expressing GhABF3A line accumulated more than 6300 transcript copies/ng of total RNA and the medial expressing line had higher transcript levels than the highest expressing line of any of the other constructs. On the other hand, the highest expressing GhABF4D line produced only 63 copies/ng, 1/100th of the level seen in the high expressing GhABF3A line. Yet, these transgenic lines showed similar dehydration stress tolerance phenotypes and the GhABF4D line flowered later and showed stronger cold tolerance than the high expressing GhABF3A line.

The large transgene-specific and event-specific differences in the steady-state levels of the ectopic GhABF transcripts in plants of various transgenic lines does not seem to correspond well with the stress tolerance phenotypes of these lines. A possible explanation for this paradox becomes apparent when protein expression levels are considered. Regardless of the level of mRNA expression, only a very small amount of GhABF protein accumulates in any of the transgenic Arabidopsis plants, as indicated by the requirement for immunoprecipitation to allow detection. This suggests that accumulation of GhABF gene products is under strong post-transcriptional regulation. Chen et al. (2013) reported that AtABF1 and AtABF3 turnover rapidly in the absence of ABA, and degradation is slowed when the plants are pre-treated with ABA and our results indicate that accumulation of GhABF3D is ABA
dependent. Thus, ABA appears to play a role in both the transcriptional and post-transcriptional regulation of some AREB/ABFs in both Arabidopsis and *G. hirsutum*, while protein accumulation in *GhABF2D* and *GhABF3D* lines appears to be relatively insensitive to the levels of mRNA and does not respond to ABA treatment.

The effect of ectopic *GhABF* gene expression on cold tolerance in *Arabidopsis* follows a different pattern to that observed for developmental delay and dehydration tolerance. There are few apparent intragenic or intergenic expression level effects, in fact, the cold tolerance phenotype of the low expressing *GhABF4D* lines is stronger than the much more highly expressed *GhABF4A* lines. However, as with the other characteristics, expression of genes within the homeologous gene pairs generally show similar phenotypes. Interestingly, all *GhABF1A* and *GhABF1D* expressing lines showed substantially increased cold tolerance but no reproductive delay, while the improved cold tolerance of *GhABF4A* and *GhABF4D* expressing lines was associated with severe reproductive delays.

Though possible, it seems unlikely that the large gene-specific differences in transcript abundance result from position effects associated with the stochastic insertion of transgenes into the *Arabidopsis* genome. It seems more probable that the differences in maximal transgene expression are due to the characteristics of the individual *G. hirsutum ABF* coding sequences. These differences could affect transcription, but it is more likely that they affect transcript stability. For example, the attenuating effects of microRNA (miRNA) could differentially affect the accumulation of *GhABF* mRNA from different transgenes. To examine this possibility, the coding sequences of the eight *G. hirsutum ABF* homologs were used to query the *Arabidopsis* miRNA collection in miRBase. Between two and five potential miRNA target sites were found within the coding sequences for all of the *G. hirsutum ABFs*, with the exception of the *GhABF3* homeologs, for which no putative target sites were found. This observation raises the possibility that the high levels of ectopic expression of the *GhABF3* homeologs in transgenic *Arabidopsis* lines could be associated with differential sensitivity to miRNA-dependent transcript destabilization. On the other hand, a unique potential miRNA target site was detected in the *GhABF4D* coding sequence, which might explain its low expression. Interestingly, this miRNA was reported to target transcripts for a MYB transcription factor that interacts with a class of ABRE elements in the promoter of the stress responsive *RD22* gene of *Arabidopsis* (Choi et al., 2000). The possible direct or indirect effects of this or other miRNAs on *GhABF* transcript stability remain to
be investigated.

Overall, our results indicate the isolated GhABF homologs encode functional transcription factors that are likely to play important roles in the regulation of abiotic stress tolerance in cotton. Each homeolog is differentially expressed in response to various abiotic stressors, and the ectopic expression of the majority of these genes confers some degree of increased tolerance to drought or cold stress in Arabidopsis. Keeping in mind that these results represent phenotypic analyses of transgenic Arabidopsis plants that ectopically express cotton ABF genes, it is clear that GhABF3 genes are induced by ABA and dehydration at both the transcriptional and post-transcriptional levels, and together with the GhABF4 genes, may be critical for controlling cellular responses to water deficit in cotton. Likewise, since ectopic expression of the GhABF1 and GhABF4 homeologs provides substantial increases in cold tolerance in Arabidopsis, it seems possible that these factors may also be important for the regulation of cold responsive gene expression in cotton. These data provide a tentative roadmap toward informed decisions regarding the selection of genes for the development of transgenic plants aimed at improving abiotic stress tolerance. However, further functional analyses of the expression of these transgenes in other species, including cotton, will be necessary to confirm these preliminary conclusions.
Table S1. Percent survival of selected GhABF expressing transgenic Arabidopsis lines after 5.5 and 6 h dehydration.

Table S2. Electrolyte leakage (%) of selected GhABF expressing transgenic Arabidopsis lines in response to increasingly negative water potentials.

Table S3. Percent survival of selected GhABF expressing transgenic Arabidopsis lines after 4.5 and 5 hours at -7°C.

Table S4. Electrolyte leakage (%) of selected GhABF expressing transgenic Arabidopsis lines in response to increasingly negative temperatures.

Fig. S1. Multiple sequence alignment of the Arabidopsis AREB/ABFs and GhABFs.

Fig. S2. Maximum likelihood tree of select AREB/ABF subfamily members.

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Yoshida T, Fujita Y, Sayama H, Kidokoro S, Maruyama K, Mizoi J, Shinozaki K, Yamaguchi-Shinozaki, K. 2010. AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. The Plant Journal 61, 672-685.


Table 1. Transcript copy number per ng total RNA and relative expression of selected *GhABF* expressing transgenic *Arabidopsis* lines used for phenotypic and abiotic stress tolerance evaluation. Lines selected represent a relatively low level of ectopic expression, the highest level of ectopic expression of the lines quantified, and an approximate average expression level of the low and high expressing lines. Data are means of three biological replicates and three technical replicates ± SD.

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Figure legends

**Fig. 1.** The AtAREB/ABFs are differentially expressed in response to exogenous ABA, dehydration, and chilling temperatures. Transcript copy number per ng total RNA and relative expression in three week old plants in response to (A-B) 100 μM exogenous ABA application, (C-D) dehydration, and (E-F) chilling temperatures (4 °C). Data are means of three biological replicates and three technical replicates ± SD.

**Fig. 2.** The GhABF homologs are differentially expressed in response to exogenous ABA, dehydration, and chilling temperatures. Transcript copy number per ng total RNA in six to eight week old plants in response to (A-D) 500 μM exogenous ABA application, (E-H) dehydration, and (I-L) chilling temperatures (4 °C). Data are means of three biological replicates and three technical replicates ± SD.

**Fig. 3.** Ectopic GhABF protein expression is largely independent of transcript level. Protein accumulation in eight-day-old seedlings from transgenic lines, compared to WT, expressing GhABF2D, GhABF3D, and GhABF4D treated without and with 50 μM ABA for 6 h. Comassie blue staining was used as the loading control (5% of IP input).

**Fig. 4.** Ectopic expression of the GhABF homologs in Arabidopsis can delay the reproductive transition. (A) Representative images of G. hirsutum ABF expressing transgenic Arabidopsis lines alongside WT Arabidopsis; Δ5 days. (B) Comparison of the reproductive transition of GhABF ectopic expressing Arabidopsis lines relative to WT Arabidopsis. Negative values represent a precocious transition, positive values indicate a delay. Data are means of three independent replicates with an average of five plants each ± SD. Student's t-test; *P <0.05, **P <0.01.

**Fig. 5.** Ectopic GhABF expression in Arabidopsis can improve tolerance to water deficit and osmotic stress. (A) Relative survival (%) of transgenic lines as compared to WT Arabidopsis after 6 h dehydration. Data are means of three independent experiments with an average of ten plants each ± SD. (B) Electrolyte leakage in response to increasingly negative water potentials. Data are means of three independent experiments with three replications each ± SD. Student's t-test; *P <0.05, **P <0.01.
**Fig. 6.** Ectopic *GhABF* expression in *Arabidopsis* can improve cold tolerance, in a gene dependent manner. (A) Relative survival (%) of transgenic lines as compared to WT *Arabidopsis* after 5 h at -7 °C. Data are means of three independent experiments with an average of ten plants each ± SD. (B) Electrolyte leakage in response to increasingly negative temperatures. Data are means of three independent experiments with three replications each ± SD. Student’s *t*-test; * P <0.05, ** P <0.01.
Figures

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**Fig. 3**
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