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

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Running title: Functional characterization of the *ABFs* from upland cotton

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

61 **Introduction**

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

74
 75 Plants have robust abiotic stress responsive networks that include myriad differentially regulated genes
 76 (Wang *et al.*, 2003; Bartels and Sunkar, 2005; Yoshida *et al.*, 2014). Among these, the abscisic acid
 77 (ABA)-responsive element binding proteins/ABRE-binding factors (AREB/ABFs) have been identified
 78 as essential regulators of the osmotic stress response (Yamaguchi-Shinozaki and Shinozaki 2006; Fujita
 79 *et al.*, 2013; Yoshida *et al.*, 2015). The expression of many of the members of this small sub-family of
 80 transcription factors is induced in response to ABA and various other abiotic stressors, and in turn, they
 81 modulate the expression of downstream target genes that ultimately result in the up-regulation of
 82 abiotic stress-protective factors including membrane and protein stabilizing molecules, antioxidants,
 83 and the accumulation of osmocompatible solutes (Wang *et al.*, 2003; Reddy *et al.*, 2004).

84
 85 As ABA-dependent, bZIP transcription factors, the AREB/ABFs interact with the conserved *cis*-acting
 86 ABA-responsive element (ABRE: PyACGTGG/TC) found in the 5' flanking regions of many ABA
 87 responsive genes (Choi *et al.*, 2000; Kang *et al.*, 2002; Fujii *et al.*, 2009; Yoshida *et al.*, 2010; Yoshida
 88 *et al.*, 2014). Nine AREB/ABF family members have been identified in *Arabidopsis*. Of these, three are
 89 induced by osmotic stress: AREB1/ABF2, AREB2/ABF4, and ABF3; a fourth, ABF1, has been shown to
 90 be a functional homolog (Yoshida *et al.*, 2015). These *Arabidopsis* AREB/ABF paralogs contain the
 91 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.*

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

134

135 **qRT-PCR analysis**

136 *Arabidopsis thaliana* Columbia (Col-0) seeds, sown on solid medium containing half strength MS and
 137 1% sucrose, were placed in the dark for 24 h at 4°C, then transferred to a growth chamber at 24°C with
 138 a 15 h light/9 h dark cycle for three weeks. Samples for basal expression level determination were
 139 taken prior to stress treatments. To measure expression levels in response to exogenous ABA, plants
 140 were sprayed to saturation with a 100 µM ABA solution and sampled 0.5 h, 1 h, and 2 h after
 141 application. To measure the dehydration response, plants were removed from the media, keeping the
 142 roots intact, and sampled after 1.5 h, 3 h, and 6 h. To measure the response to chilling temperatures,
 143 plants were transferred to 4°C, and sampled after 1 h, 2 h, and 4 h. *G. hirsutum* (Coker 312) plants were
 144 grown in soil, in 1 L pots, for six to eight weeks under long-day conditions (15 h light/9 h dark) at
 145 30°C. Following pre-treatment sampling for the determination of basal expression, the plants were
 146 subjected to the following treatments. Plants were sprayed to saturation with a 500 µM ABA solution,
 147 and sampled after 0.5 h, 1 h, and 2 h. Water was withheld, and dehydration stress treatment samples
 148 were taken after 48 h (before visible wilting), 72 h (moderate wilting), and 78 h (severe wilting).
 149 Chilling temperature treatment samples were taken after 1 h, 2 h, and 4 h exposure to 4°C. *Arabidopsis*
 150 RNA was extracted using the RNeasy Mini kit (Qiagen). *G. hirsutum* RNA was extracted using the
 151 Spectrum Plant Total RNA kit (Sigma). All RNA concentrations were quantified via Nanodrop,
 152 normalized to 100 ng/µL, and cDNA synthesis was performed using the iScript cDNA synthesis kit
 153 (Bio-Rad). All qRT-PCR reactions were performed using the iTAQ Universal SYBR Green Supermix

(Bio-Rad) in 10 μ L 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 Chemically 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 μ g mL⁻¹ gentamicin, 50 μ g mL⁻¹ kanamycin, and 50 μ g mL⁻¹ rifampicin. Colonies positive for the insert were cultured for 48 h in 25 mL liquid LB supplemented with 10 μ g mL⁻¹ gentamicin, 50 μ g mL⁻¹ kanamycin, and 50 μ g 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 μ g 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

185 *Arabidopsis* seedlings was extracted in immunoprecipitation (IP) buffer (50 mM Tris-HCl (pH 8.1),
186 150 mM NaCl, 1% NP-40 (v/v), 1 mM EDTA, 5% glycerol, 1mM phenylmethylsulfonyl fluoride, and
187 protease inhibitor cocktail (1:100)). The protein extracts (1 mg) were precleared by incubation with
188 Protein A/G beads (Santa Cruz) for 2 h at 4°C, and immunoprecipitated using 20 µl of Anti-FLAG
189 Affinity Gel (Sigma) at 4°C for 1 h. Beads were washed three times with IP buffer for 20 min each at
190 4°C. The precipitated proteins were eluted using 2x SDS sample buffer. Eluted samples were subjected
191 to Western blot analysis using an Anti-FLAG Alkaline Phosphatase antibody (Sigma). Each
192 experiment was replicated three times.

193 194 **Transgenic *Arabidopsis* development and abiotic stress tolerance evaluation**

195 To determine the effects of ectopic expression of the *G. hirsutum* *ABF* homologs in *Arabidopsis* on the
196 reproductive transition, three to four T₃ generation transgenic plants were grown in soil in 15 ml pots
197 alongside wild-type (WT) *Arabidopsis*. The reproductive transition, defined by the initiation of bolting,
198 was monitored for each transgenic line as compared to WT. To measure differential survival following
199 dehydration, homozygous T₃ and WT seeds were surface sterilized in 30% bleach, plated on ½ MS, 1%
200 sucrose solid medium, placed in the dark for 24 hours at 4°C, then transferred to a growth chamber at
201 24°C with a 15 h light/9 h dark cycle for 3 weeks. An average of ten plants from three plates for each
202 transgenic line and WT were removed from the media and transferred to petri dishes lined with glass
203 beads to dehydrate. Plants were re-watered, in 30 min intervals, after a minimum of 4 h dehydration, to
204 a maximum of 6.5 h. Survival was recorded following a 48 h recovery period. Electrolyte leakage, as
205 the result of low water potential-induced damage, was measured as described by Verslues and Bray
206 (2004) and van der Weele *et al.* (2000), with minor modifications. Briefly, three-week-old seedlings
207 were transferred to PEG-infused plates of increasingly negative water potentials (-0.25, -0.50, -0.75,
208 and -1.25 MPa) for 24 h, rinsed in a mannitol solution of the same water potential, and placed in 5 mL
209 deionized water for 1 h. Conductivity was measured, the samples were autoclaved, and conductivity
210 was measured again. Relative electrolyte leakage was calculated by dividing initial conductivity by
211 conductivity following autoclaving. Each genotype and treatment was replicated three times. To
212 measure differential survival following exposure to freezing temperatures, T₃ seeds were sown on soil-
213 filled petri dishes. An average of ten 4 week-old plants per plate were then transferred to a growth
214 chamber at -7°C. After a minimum of 3 h at -7°C, plates were returned to the growth chamber at
215 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* 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

278 increase differed substantially. *AtABF1* expression doubled and *AtAREB1/ABF2* expression tripled
 279 relative to basal levels, while the expression of *AtABF3* increased 6 fold and *AtAREB2/ABF4* increased
 280 7 fold (Fig. 1A,B). Similarly, all *Arabidopsis AREB/ABF* genes were induced in response to water
 281 deficit, though *AtABF1* and *AtAREB1/ABF2* transcript levels increased only slightly, while the
 282 *AtAREB2/ABF4* transcript level increased steadily to 20 times its basal level over the 6 h sampling
 283 period, and the *AtABF3* level increased quickly after 3 h to ultimately reach a level 75 fold greater than
 284 the basal level after 6 h (Fig. 1C,D). Though the *AREB/ABFs* genes are primarily associated with the
 285 response to drought via the ABA-dependent pathway (Lee *et al.*, 2010; Fujita *et al.*, 2013; Yoshida *et*
 286 *al.*, 2014), we also examined their expression in response to low temperature stress. The expression of
 287 *AtABF1* and *AtAREB2/ABF4* did not change in response to chilling, however, the *AtAREB1/ABF2*
 288 transcript level increased gradually to 3 times its basal expression over 4 h at 4 °C, and *AtABF3*
 289 expression rose quickly to 6 six times its basal level after 1 h at 4 °C, then declined after the 2 h time
 290 point (Fig. 1E,F).

291
 292 The expression of each *GhABF* homeolog was induced in response to at least one stress treatment,
 293 though the magnitude of induction varied widely between treatments (Fig. 2), and no consistent bias in
 294 expression of the A or D genome was observed. While expression of each of the eight *GhABF*
 295 homologs increased in response to exogenous ABA application, induction of *GhABF3A* was by far the
 296 strongest, rising to a level 30 times its basal expression over the course of the 2 h assay. Expression of
 297 *GhABF3D* and both *GhABF4* homeologs increased more gradually in response to ABA, reaching levels
 298 8 to 10 fold above basal levels, while expression of the *GhABF2* homeologs increased by about 5 fold,
 299 and the *GhABF1* homeologs increased only by about 2 to 3 fold during the 2 h assay (Fig. 2A-D). In
 300 response to water deficit stress, again, *GhABF3A* expression showed the largest increase in transcript
 301 copy number. In addition, the increase in expression of the *GhABF3* homeologs in response to water
 302 deficit treatment began earlier than the other *GhABF* genes, becoming apparent after 48 h, as compared
 303 to 72 h for the *GhABF1*, *GhABF2*, and *GhABF4* homeologous gene pairs (Fig. 2E-H). Furthermore,
 304 both ABA- and drought-induced expression of *GhABF3A* was considerably stronger than that of
 305 *GhABF3D*, illustrating differential expression among these homeologous pairs.

306
 307 Again, while the *AREB/ABF bZIP* transcription factors are not generally associated with temperature
 308 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 (Table1). 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.

464

465 Discussion

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

481

482 In *Arabidopsis*, expression of *AtABF3* is the most responsive to water deficit, chilling temperatures and,
 483 along with *AtABF4*, to ABA treatment, while in *G. hirsutum*, expression of the *GhABF3A* is the most
 484 highly responsive homeolog to water deficit and ABA treatment, and *GhABF1A* is most responsive to
 485 chilling. These differential expression patterns within the *G. hirsutum* homeologous pairs could
 486 indicate sub-functionalization or silencing of one or the other homeolog due to redundancy. For
 487 example, expression of *GhABF1* homeologs was only modestly responsive to exogenous ABA or
 488 dehydration, and the *GhABF4* genes exhibit only a slight induction in response to dehydration,
 489 however, *GhABF1A* and *GhABF4D* are strongly induced in response to chilling, while expression of
 490 *GhABF1D* responds relatively weakly to chilling and *GhABF4A* does not respond at all. This increased
 491 expression in response to chilling stress could result from cross-talk due to functional interactions
 492 between the ABA-dependent and ABA-independent stress response pathways (Yoshida *et al.*, 2014).
 493 For example, *Arabidopsis* AREB1/ABF2 interacts with various AP2 domain proteins, including
 494 DREB1A, also known as CBF3, an essential component of the low temperature stress response (Lee *et*

495 *al.*, 2010, Zhou *et al.*, 2011).

496

497 While ectopic expression of *AREB/ABF* genes may confer increased stress tolerance, these
 498 improvements are often accompanied by delayed growth or reproduction (Kang *et al.*, 2002; Kim *et al.*,
 499 2004; Fujita *et al.*, 2005). Therefore, we analyzed the ability of the *GhABFs* to confer increased stress
 500 tolerance and affect development when ectopically expressed in *Arabidopsis*. Tradeoffs between stress
 501 tolerance and developmental delay were seen with some, but not all, *GhABF* genes, raising the
 502 possibility that negative side-effects on growth and development associated with increased *AREB/ABF*
 503 expression may be gene-specific and it might be possible to mitigate unwanted negative effects by
 504 using transgenes that encode specific *ABF* orthologs and selecting transgenic lines with varying levels
 505 of ectopic expression. In this way, it may be possible to find an acceptable balance between positive
 506 and negative phenotypes. Therefore, three independent lines with high, low, and medial levels of
 507 ectopic expression were selected for each of the eight *GhABF* gene constructs for physiological
 508 examination. Although the gene constructs differed only in their coding sequences, transgene
 509 expression levels varied widely among the different *GhABF* gene constructs. For example, the highest
 510 expressing *GhABF3A* line accumulated more than 6300 transcript copies/ng of total RNA and the
 511 medial expressing line had higher transcript levels than the highest expressing line of any of the other
 512 constructs. On the other hand, the highest expressing *GhABF4D* line produced only 63 copies/ng,
 513 1/100th of the level seen in the high expressing *GhABF3A* line. Yet, these transgenic lines showed
 514 similar dehydration stress tolerance phenotypes and the *GhABF4D* line flowered later and showed
 515 stronger cold tolerance than the high expressing *GhABF3A* line.

516

517 The large transgene-specific and event-specific differences in the steady-state levels of the ectopic
 518 *GhABF* transcripts in plants of various transgenic lines does not seem to correspond well with the stress
 519 tolerance phenotypes of these lines. A possible explanation for this paradox becomes apparent when
 520 protein expression levels are considered. Regardless of the level of mRNA expression, only a very
 521 small amount of *GhABF* protein accumulates in any of the transgenic *Arabidopsis* plants, as indicated
 522 by the requirement for immunoprecipitation to allow detection. This suggests that accumulation of
 523 *GhABF* gene products is under strong post-transcriptional regulation. Chen *et al.* (2013) reported that
 524 *AtABF1* and *AtABF3* turnover rapidly in the absence of ABA, and degradation is slowed when the
 525 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 the 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

557 be investigated.

558

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

574

575 **Supplementary data**

576 **Table S1.** Percent survival of selected *GhABF* expressing transgenic *Arabidopsis* lines after 5.5 and 6 h
577 dehydration.

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

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

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

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

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

586

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687

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 $\pm$ SD.

<i>Gh</i> homolog	Selected line #	Transcripts / ng total RNA	Relative expression
<i>ABF1A</i>	4	6.5 $\pm$ 1.21	1.0 $\pm$ 0.24
	3	144.7 $\pm$ 25.44	16.4 $\pm$ 0.27
	6	220.1 $\pm$ 52.01	23.9 $\pm$ 0.31
<i>ABF1D</i>	6	19.6 $\pm$ 1.39	1.0 $\pm$ 0.11
	2	121.0 $\pm$ 37.01	5.7 $\pm$ 0.41
	5	309.7 $\pm$ 58.59	13.9 $\pm$ 0.25
<i>ABF2A</i>	3	287.3 $\pm$ 6.29	1.0 $\pm$ 0.03
	5	372.8 $\pm$ 41.11	1.3 $\pm$ 0.15
	9	489.7 $\pm$ 41.33	1.7 $\pm$ 0.11
<i>ABF2D</i>	9	26.6 $\pm$ 5.88	1.0 $\pm$ 0.31
	11	177.5 $\pm$ 37.25	7.1 $\pm$ 0.32
	25	419.5 $\pm$ 114.46	17.3 $\pm$ 0.41
<i>ABF3A</i>	3	175.1 $\pm$ 43.54	1.0 $\pm$ 0.39
	13	1814.5 $\pm$ 358.18	12.3 $\pm$ 0.30
	8	6383.2 $\pm$ 877.40	47.5 $\pm$ 0.21
<i>ABF3D</i>	1	17.2 $\pm$ 3.22	1.0 $\pm$ 0.09
	14	406.8 $\pm$ 60.34	26.2 $\pm$ 0.21
	13	770.7 $\pm$ 49.15	50.7 $\pm$ 0.95
<i>ABF4A</i>	2	224.5 $\pm$ 14.40	1.0 $\pm$ 0.10
	5	887.8 $\pm$ 42.06	4.4 $\pm$ 0.08
	7	1563.3 $\pm$ 190.62	8.1 $\pm$ 0.19
<i>ABF4D</i>	1	21.7 $\pm$ 1.47	1.0 $\pm$ 0.09
	3	42.4 $\pm$ 9.07	1.8 $\pm$ 0.31
	7	62.5 $\pm$ 13.17	2.7 $\pm$ 0.28

695 Figure legends

696 **Fig. 1.** The *AtAREB/ABFs* are differentially expressed in response to exogenous ABA, dehydration, and
697 chilling temperatures. Transcript copy number per ng total RNA and relative expression in three week
698 old plants in response to (A-B) 100 μ M exogenous ABA application, (C-D) dehydration, and (E-F)
699 chilling temperatures (4 °C). Data are means of three biological replicates and three technical replicates
700 $\pm$ SD.

701
702 **Fig. 2.** The *GhABF* homologs are differentially expressed in response to exogenous ABA, dehydration,
703 and chilling temperatures. Transcript copy number per ng total RNA in six to eight week old plants in
704 response to (A-D) 500 μ M exogenous ABA application, (E-H) dehydration, and (I-L) chilling
705 temperatures (4 °C). Data are means of three biological replicates and three technical replicates $\pm$ SD.

706
707 **Fig. 3.** Ectopic *GhABF* protein expression is largely independent of transcript level. Protein
708 accumulation in eight-day-old seedlings from transgenic lines, compared to WT, expressing
709 *GhABF2D*, *GhABF3D*, and *GhABF4D* treated without and with 50 μ M ABA for 6 h. Coomassie blue
710 staining was used as the loading control (5% of IP input).

711
712 **Fig. 4.** Ectopic expression of the *GhABF* homologs in *Arabidopsis* can delay the reproductive
713 transition. (A) Representative images of *G. hirsutum ABF* expressing transgenic *Arabidopsis* lines
714 alongside WT *Arabidopsis*; Δ 5 days. (B) Comparison of the reproductive transition of *GhABF* ectopic
715 expressing *Arabidopsis* lines relative to WT *Arabidopsis*. Negative values represent a precocious
716 transition, positive values indicate a delay. Data are means of three independent replicates with an
717 average of five plants each $\pm$ SD. Student's *t*-test; * *P* <0.05, ** *P* <0.01.

718
719 **Fig. 5.** Ectopic *GhABF* expression in *Arabidopsis* can improve tolerance to water deficit and osmotic
720 stress. (A) Relative survival (%) of transgenic lines as compared to WT *Arabidopsis* after 6 h
721 dehydration. Data are means of three independent experiments with an average of ten plants each $\pm$ SD.
722 (B) Electrolyte leakage in response to increasingly negative water potentials. Data are means of three
723 independent experiments with three replications each $\pm$ SD. Student's *t*-test; * *P* <0.05, ** *P* <0.01.

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726 **Fig. 6.** Ectopic *GhABF* expression in *Arabidopsis* can improve cold tolerance, in a gene dependent
 727 manner. (A) Relative survival (%) of transgenic lines as compared to WT *Arabidopsis* after 5 h at -7
 728 °C. Data are means of three independent experiments with an average of ten plants each $\pm$ SD. (B)
 729 Electrolyte leakage in response to increasingly negative temperatures. Data are means of three
 730 independent experiments with three replications each $\pm$ SD. Student's *t*-test; * $P < 0.05$, ** $P < 0.01$.
 731

Figures

Fig. 1

Fig. 2

Fig. 3

754 Fig. 4

755 **Fig. 5**

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757 **Fig. 6**

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