THE ABFS AND CBFS IN COTTON (*GOSSYPIUM*

HIRSUTUM): A CHARACTERIZATION AND

FUNCTIONAL ANALYSIS IN RESPONSE TO

ABIOTIC STRESS

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Abstract: Abiotic stress is pernicious; every year causing substantial reductions in agricultural yield. Despite this, due to the complex nature of the plant response, our current understanding of the molecular mechanisms and signaling pathways is limited. Among the gene families shown to play a role in dehydration and low temperature tolerance are the *ABFs* and *CBFs*. Both are small families of transcription factors, are expressed in response to abiotic stress, and have been shown to increase abiotic stress tolerance when ectopically expressed. Here, both of these families are isolated and functionally characterized in *G. hirsutum* (cotton)*, the most important global natural fiber source. Gene expression analyses illustrate how these genes respond to abiotic stress, and ectopic expression in *Arabidopsis* illustrates their functionality. Ectopic expression of abiotic stress-related genes has often been shown to increase stress tolerance, however, at a developmental cost. Therefore, a more in-depth understanding of the abiotic stress response is necessary to develop crops able to withstand abiotic stress and at the same time minimize developmental delays.

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CHAPTER I

INTRODUCTION

Though estimates vary as to the extent that abiotic stress negatively impacts

agricultural production and yield (Boyer 1982, Bray *et al.* 2000), it is undeniable that

there is perennial, significant loss associated with abnormally high or low temperatures,

and more significantly, with water deficiency. Exceptional environmental events have

become common in recent years, ranging from extreme drought, often coupled with

above average temperatures, to premature freezing events in the fall, or late frost in the

spring. As extreme climate fluctuations become increasingly common, the sustainability

of the global agricultural system is called into question. Breeding, herbicides, fertilizers,

and efficient practices are all commonly employed methods for increasing yield, and

while the use of these approaches continues to improve productivity, trends indicate that

these practices will not be sufficient to keep up with future demand (Nellemann 2009,

Delmer 2005).

For many crops, the most pernicious abiotic stress is insufficient water. This is

especially true in semi-arid regions which often go weeks without rainfall, combined with

high summer temperatures. Crops like cotton and maize require extensive irrigation, and

particularly in periods of drought, this irrigation is not only economically costly, but also

depletes aquifers or other water sources. Therefore, current practices need continual improvement, and new strategies are needed to increase drought tolerance specifically, and abiotic stress resilience in general. In order to achieve these objectives, a basic understanding of the molecular mechanisms underlying a plant's abiotic stress response is imperative, not only for important agricultural crops, but for plants in general, as an understanding of these intricate pathways will ensure further opportunities to improve the sustainability of future crop production.

One of the many water-intensive crops needing irrigation is upland cotton (*Gossypium hirsutum*). As a tropical species, it is considered relatively drought tolerant, however, when grown in arid and semi-arid regions, extensive irrigation is required to obtain an economic yield. Despite being the most important global natural fiber source (FAO & ICAC 2011), molecular characterization of upland cotton's abiotic stress-responsive pathways has lagged behind that of many other major crops due to the complex nature of its tetraploid genome. Therefore, an examination of some of the critical abiotic stress-responsive pathways of upland cotton (hereinafter, simply cotton) is warranted. Here, two small, but essential families of transcription factors are characterized and functionally analyzed for their roles in abiotic stress tolerance.

Abiotic stress in plants: an overview

Abiotic stress adversely affects plant growth and productivity by altering morphological, physiological, biochemical, and molecular processes (Wang *et al.* 2003). Often interconnected, abiotic stressors induce similar cellular damage; dehydration, high salinity, extreme temperatures, and oxidative stress result in disruption of homeostasis and

ion distribution. Oxidative stress, the result of high temperature, increased salinity, drought, and other abiotic stressors cause denaturation of functional and structural proteins, lipid peroxidation, protein oxidation, DNA damage, etc. (Wang *et al.* 2003). The innate plant response is to activate several signaling pathways and cellular changes, resulting in the production of stress-related proteins, up-regulation of antioxidants, and accumulation of osmocompatible solutes (Reddy 2004).

The genes involved in abiotic stress have generally been divided into three categories: those involved in signaling cascades and transcriptional control including phospholipases and transcription factors, those involved in the protection of membranes and proteins including heat shock proteins and free-radical scavengers, and those involved in water and ion uptake including aquaporins and ion transporters (Wang *et al.* 2003).

Heat, cold, drought, high salinity, and other stressors, and the secondary osmotic and oxidative stresses that result, lead to disruption of ionic homeostasis and damage to structural and functional proteins and membranes. The stress signal is sensed, perceived, and transduced through osmosensors and secondary messengers, leading to the activation of stress-responsive transcription factors, followed by stress-responsive mechanisms which activate downstream genes. The reestablishment of homeostasis and protection of proteins and membranes results in stress tolerance and resistance (Fig. 1; Wang *et al.* 2003).
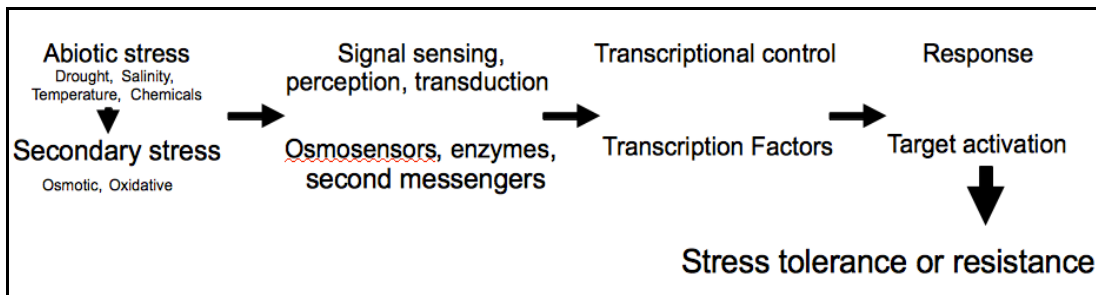
Figure 1. Plant response to abiotic stress (adapted from Wang *et al.* 2003).

As indicated, transcription factors coordinate the abiotic stress response by targeted up- and down-regulation of effector genes, modulating the activity of their associated cellular machinery. The majority of transcription factors can be classified by their common domains into large multi-gene families, *e.g.*, MYB, AP2/EREBP, bZIP, and WRKY. Members of these families may be differentially regulated in response to the same or different stresses (Fujita *et al.* 2005, Wang *et al.* 2003).

The ABF*s and* CBF*s: key transcription factors in the abiotic stress response*

Myriad genes are involved in the abiotic stress response; however, transcription factors are of particular interest as they often target multiple genes (Cutler *et al.* 2010). Two families of transcription factors that have been identified as integral in the response to multiple abiotic stressors are the abscisic acid (ABA)-responsive element (ABRE) binding factors (ABFs; also known as AREBs), and the C-repeat binding factors (CBFs; also known as dehydration-responsive element binding factors (DREB1s)). These gene families have been primarily implicated in dehydration and cold tolerance, respectively; however, they have also been shown to respond to additional abiotic stressors (Fujita *et al.* 2013, Medina *et al.* 2011, Yoshida *et al.* 2015, Zhou *et al.* 2011).

The ABFs are bZIP transcription factors whose expression is modulated in response to ABA levels; ABA levels change in response to various abiotic stressors (Tuteja 2007). This gene family, as characterized in *Arabidopsis*, includes four genes: *ABI5-like 4/ABF1*, *AREB1/ABF2*, *DBPF5/ABF3*, and *AREB2/ABF4* (hereinafter *ABF1-4*; Fujita *et al.* 2005). While predominantly implicated in drought responses, members of this gene family have been shown to respond to various other abiotic stressors including cold, heat, and salinity

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(Choi *et al.* 2000, Fujita *et al.* 2005, Fujita *et al.* 2011, Kim *et al.* 2004, Yoshida *et al.* 2010). Though their target genes overlap, differences in their temporal and spatial expression patterns and modes of action indicate that each is unique (Fujii *et al.* 2009), and up-regulation of many of these genes has been shown to confer abiotic stress tolerance in *Arabidopsis* (Chinnusamy *et al.* 2006, Fujii *et al.* 2009, Fujita *et al.* 2005, Kim *et al.* 2004, Medina *et al.* 2011, Novillo *et al.* 2007, Yoshida *et al.* 2010).

The CBF family is part of the larger AP2/ERF family of transcription factors. *Arabidopsis* has three primary CBFs in the six-member DREB1 gene family shown to respond to low temperatures. A fourth CBF responds to ABA and drought, though not to low temperatures (Zhou *et al.* 2011). Though primarily implicated in response to low temperatures, CBFs are also subject to regulation by the circadian clock (Dong *et al.* 2011, Medina *et al.* 2011), and are responsive to various other abiotic stressors (Zhou *et al.* 2011).

Osmotic stress leads to the accumulation of ABA, however, osmotic stress induces gene expression in both ABA-dependent and ABA-independent pathways. The ABFs, via the ABRE *cis*-element, act in an ABA-dependent manner, while the CBFs, via the DRE/CRT *cis*-element, act in an ABA-independent manner (Fig. 2; Yoshida *et al.* 2014). Each of these modes of action is essential in the abiotic stress response. It has also been shown that some CBF and ABF proteins physically interact (Lee *et al.* 2010), indicating that these seemingly distinct transcription factor families may act to coordinate abiotic stress responses.

The ABFs: ABA-dependent and dehydration-responsive

The ABFs have been extensively examined in *Arabidopsis* due to their crucial role in drought stress tolerance. Many studies have examined the response of these genes to various
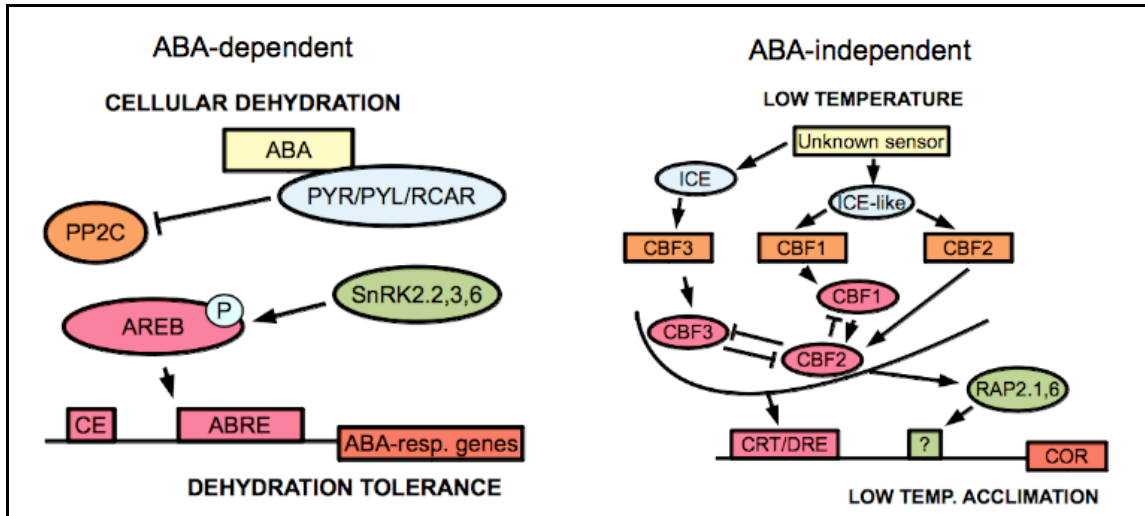
Figure 2. Simplified ABF and CBF signaling pathways (adapted from Fujii *et al*. 2009 & Zhou *et al*. 2011).

stressors, and though not all agree at the individual gene level, it is clear that, in *Arabidopsis*, these genes are differentially expressed in response to abiotic stressors. Choi *et al*. (2000), one of the early reports to examine these genes using RNA gel blot hybridization assays, showed an increase in expression of all *ABF* genes in response to ABA, though to varying extents. However, their research indicated that only *ABF4* was up-regulated in response to drought. Differential expression changes were also seen under cold and high salinity treatments. Fujita *et al*. (2005), also using RNA gel blots, found strong up-regulation of *ABF2-4* in dehydration and ABA treated plants, but no change in *ABF1* expression.

More recent research, using quantitative real-time polymerase chain reaction (qRT-PCR) assays, agrees with the consensus of many earlier examinations. Yoshida *et al.* (2015) measured the relative expression change of all four *Arabidopsis ABF* genes under dehydration, high salinity, and exogenous ABA treatments; while all showed changes in relative expression, these changes were not consistent over all treatments. *ABF1* showed only

a slight change, and while *ABF3* was strongly increased in plants undergoing dehydration treatment, the relative increase in transcript levels in salinity and ABA treatments was much less pronounced. Various members of the *ABF* gene family have also been examined in other species. In tomato, two *ABF* orthologs were shown to be induced by dehydration and high salinity. Similarly, in millet, *ABF* orthologs responded to dehydration, salt, and ABA treatments (Orellana *et al.* 2010, Li *et al.* 2014). Overall, the change in expression of the *ABF* genes in multiple species indicates their importance in the abiotic stress response.

In addition to the examination of gene expression, many of these same studies have shown the physiological effects of ectopic expression on abiotic stress resilience. Kang *et al.* (2002) ectopically expressed *Arabidopsis ABF3* and *ABF4* in *Arabidopsis*, resulting in ABA-hypersensitivity and increased dehydration tolerance, however, growth rate and the reproductive transition were delayed, most severely in the *ABF4* over-expressing plants. Comparatively, Kim *et al.* (2004) found plants with defective *ABF3* and *ABF4* had decreased sensitivity to ABA and salt, and were more susceptible to dehydration stress. It was also shown that *ABF2* over-expression increased dehydration tolerance, though there was a two- to three-week delay in reproductive development. Fujita *et al.* (2005) found similar results in *ABF2* over-expressing plants. Finally, recently, Fujita *et al.* (2013), showed that an *ABF* quadruple mutant is insensitive to ABA, more susceptible to water deficit, and that there is little effect on growth or reproduction. A triple mutant, where *ABF1* is still active, showed little difference from the quadruple mutant. While over-expression of *Arabidopsis ABF*s in *Arabidopsis* often leads to growth inhibition, Oh *et al.* (2005) found the over-expression of *Arabidopsis ABF3* in rice had no effect on growth, but did confer increased tolerance to dehydration.

The ABA-dependent pathway leading to the activation of *ABF* gene expression has been reconstituted (Fig. 2; Fujii *et al.* 2009). START domain proteins, known as PYR/PYLs, are ABA receptors. In the presence of ABA, these receptors interact with and inhibit clade-A PP2Cs. In the absence of ABA, these PP2Cs negatively regulate ABA responses. The inhibition of the PP2Cs allows the SnRK2 kinases to be activated, which are then free to phosphorylate, thus activating the *ABF*s. The activated ABFs then bind ABRE promoter elements in their target genes, inducing the expression of ABA-responsive genes (Fujii *et al.* 2009).

The CBFs: ABA-independent and cold temperature-responsive

The CBFs have also been extensively examined in *Arabidopsis* for their role in cold and freezing tolerance. Medina *et al.* (1999) identified *CBF2* and *CBF3* as homologs of *CBF1*. Expression measured by RNA blot assays indicated that all three *CBF* genes responded within an hour of exposure to 4 degrees Celsius, though by six hours the transcript levels had returned to pre-treatment levels. In addition, there was no change observed in response to ABA application or dehydration. Fowler *et al.* (2005) showed that expression of *CBF* genes is also regulated by the circadian clock; when plants were transferred to 4 degrees Celsius four hours after dawn, *CBF1-3* were all strongly up-regulated thereafter, as shown on RNA gel blots. By eight hours at low temperatures, expression for all had dropped significantly. However, when transferred to 4 degrees Celsius sixteen hours after dawn, *CBF1* and *CBF3* expression was little changed over a 24 hour period. *CBF2* expression did increase after four hours, but not nearly to the extent of the four hours after dawn assay. More recent reviews have complied and confirmed by consensus that *CBF1-3* are induced

under low temperature conditions, however, indicating that *CBF1* and *CBF3* expression peaks around one hour at 4 degrees Celsius, while *CBF2* peaks at about two hours and is relatively much more highly expressed. The *CBF* genes have been examined in many other species; interestingly, a study in grape found differential induction at low temperatures, but also varying up-regulation of the grape *CBF* genes under ABA and dehydration treatments (Xiao *et al.* 2006). Similarly in soybean, the *CBF* orthologs have been shown to respond to multiple abiotic stressors (Kidokoro *et al.* 2015).

Many studies have also shown the effects of ectopic expression of the *CBF*s on abiotic stress tolerance. Gilmour *et al.* (2000) showed that ectopic expression of *CBF3* slows growth, while improving low temperature tolerance. Morphological abnormalities and delayed flowering were also observed in *CBF1* and *CBF2* over-expressing lines in combination with increased levels of certain sugars and an increase in freezing tolerance (Gilmour *et al.* 2004). Furthermore, Gilmour *et al.* (2004) also concluded that all three CBFs were functionally redundant. Similar to studies of the ABFs, there are conflicting reports as to which abiotic stressors influence *CBF* expression levels, and which stressors are affected by ectopic expression. Novillo *et al.* (2007) determined that *CBF* genes are not functionally redundant based on RNAi and antisense suppressed transgenic lines. A review by Zhou *et al.* (2011) lists a large set of studies examining *CBF* transgenes and which abiotic stressors are affected.

The signaling pathway leading to the low temperature activation of the *CBF*s is initiated by an unknown sensor. While there are many elicitors and inhibitors, this sensor activates ICE and ICE-like proteins, which induce the expression of the *CBF* genes. Accumulation of CBF proteins then leads to an increase in transcription of target genes

containing the CRT/DRE *cis*-element in their promoter (Zhou *et al.* 2011). However, the *CBF* genes also regulate themselves. While reports of these interactions differ, each is induced upon exposure to low temperatures, however, upon reaching a certain level, *CBF2* acts as an inhibitor on *CBF1* and *CBF3* to attenuate their response. Additionally, *CBF2* expression declines soon after *CBF1* and *CBF3*, indicating regulatory feedback or inhibition through other means (Medina *et al.* 2011, Novillo *et al.* 2007, Zhou *et al.* 2011).

Cotton: the subject of investigation

As described, the ABF and CBF transcription factors have an essential role in the response to abiotic stress, and have been extensively examined in model species and some agricultural crops. However, to date, these gene families have not been characterized in cotton. This is likely, at least in part, due to the tetraploid nature of the *Gossypium hirsutum* genome. As the world's primary source of natural fiber, 90% of all cotton comes from *G. hirsutum* (Meyer *et al.* 2007, Osakwe 2009). While other *Gossypium* species are cultivated, primarily *G. barbadense* and *G. arboreum*, these account for only 10% of commercial cotton fiber. As previously indicated, most cotton cultivation is concentrated in regions with less than optimal rainfall, necessitating irrigation to provide economically sufficient yield. Increasingly unpredictable weather patterns, and no foreseeable decrease in demand, necessitate that various strategies are employed to mitigate abiotic stress and improve yield in cotton, and in all essential agricultural crops (Meyer *et al.* 2007).

While not unique to *G. hirsutum,* genomic examination and sequencing has lagged behind that of many diploid crops due to its tetraploid nature. While it has been established that *G. hirsutum* is the result of a polyploidy event between an Old World species and a New

World species, it has not been definitively established that both progenitors are extant

species, though *G. arboreum* and *G. raimondii* are considered to be the closest extant species,

designated as the AA and DD genomes, respectively (Guan *et al*. 2014).

Transgenic manipulation: advantages and pitfalls

Critical to the characterization of a gene or gene family, as illustrated, is the change in

expression in response to various treatments, in this case abiotic stress. While sequence-

based predictions and expression patterns can indicate possible functions, ectopic expression

or mutant knockouts are necessary to determine the physiological roles of specific genes. A

gene may be expressed endogenously, within a species, *e.g.* an *Arabidopsis* gene ectopically

in *Arabidopsis*, or exogenously between species. In either case, ectopic expression has the

potential to alter homeostasis, exhibited through a difference in any trait.

Ectopic expression of an endogenous gene, while still potentially altering

homeostasis, may have the benefit that the endogenous transcript is recognized, and correctly

post-transcriptionally processed. However, ectopic expression, by definition, increases

transcript levels and presumably protein levels. Therefore, any functional effect observed

should be caused by the overwhelming of the post-transcriptional/translational processing

machinery, with the excess then over-activating downstream regulatory pathways, altering

molecular and physiological outcomes.

Alternatively, the functional effect of ectopically expressing an exogenous gene may

be the result of many different scenarios. An exogenous gene can either be recognized and

post-transcriptionally processed correctly, thus being regulated similarly to an endogenous

gene, or only partially recognized. In this case, varying degrees of mis-regulation lead to

unpredictable outcomes. If translated, and not properly post- translationally modified or regulated, the result may be the over-activation of orthologous targets, inefficient activation, or no activation at all. Similarly, if not properly post-transcriptionally modified, it may be improperly translated, again leading to unpredictable functional effects. Transgenic expression, therefore, is inconsistent, often unpredictable, and dependent on the gene and extent of expression.

Ectopic expression in **Arabidopsis** *and cotton: conflicting results*

These aforementioned reports of ectopic expression of *Arabidopsis ABF* genes in *Arabidopsis* to determine the function of these abiotic stress-responsive genes show that indeed, dehydration tolerance is improved. Allen (2012), exploring strategies to increase drought tolerance in cotton, ectopically expressed *Arabidopsis ABF3* under control of the strong constitutive cauliflower mosaic virus 35S (CaMV35S) promoter in *G. hirsutum.* While dehydration tolerance was significantly improved, there was a significant decrease in growth rate and reproductive development under greenhouse conditions. If similar effects are seen when plants are grown in the field, it would take nearly twice as long to reach maturity. This example illustrates the unpredictable nature of ectopic expression. Kang *et al.* (2002) reported that over-expression of *Arabidopsis ABF3* in *Arabidopsis* resulted in mild growth retardation, though still similar to wild type, and slightly shorter and thicker siliques.

Gilmour *et al.* (2000) over-expressed *Arabidopsis CBF3* in *Arabidopsis* and found an increase in low temperature tolerance, but with a two-to three-week delay in reproductive development. Allen (2012) attempted to over-express *Arabidopsis CBF3* in cotton, and while plantlets were regenerated, all failed to grow or produce roots. However, transgenic cotton

plants expressing an *Arabidopsis CBF3* gene under control of a weaker, stress inducible promoter showed only a slight delay in growth, but improved dehydration tolerance.

As outlined, *Arabidopsis* engineered to ectopically express endogenous *ABF* and *CBF* genes often involves a trade-off: while the target trait is improved, it is at the expense of another trait. Over-expression of a stress-responsive gene causes the plant, via the activation of the over-expressed gene's targets, to be in a persistent state of stress response, causing a delay in development due to an altered allocation of resources, or an overall lack of resources. Exogenous expression in cotton produced similar results, however, the negative effects were mitigated through the use of a stress-inducible promoter (Allen 2012).

Endogenous expression within a species, or exogenous expression between species, clearly does not produce predictable results, nor does native expression as measured under abiotic stress predict the functional effect of ectopic expression. However, in each of these cases, abiotic stress tolerance, specifically dehydration tolerance, has been improved, though the extent varies. Similarly, variation has been observed for the negative, unintended consequences. Therefore, is there a scenario in which a specific trait can be improved without unintended negative effects? Or, more practically, is there a balance by which the negative consequences of ectopic expression can be minimized, while still resulting in a significant improvement in the desired trait?

Towards a greater understanding of abiotic stress: the cotton ABFs and CBFs

The results of endogenous expression in *Arabidopsis* and exogenous expression in cotton illustrate the unpredictability of the effects of transgenic manipulation. Many studies have examined the effects of ectopic expression in a unidirectional fashion; however, seldom

have the effects of ectopic expression in two species been compared bi-directionally. The effects of ectopic expression of *Arabidopsis ABF* and *CBF* genes in *Arabidopsis* have been well documented, and Allen (2012) has shown the effect of exogenous expression of *Arabidopsis ABF3* and *CBF3* in cotton. Regardless, all of the *ABF* and *CBF* transgenes examined in relation to cotton have focused on expression of *Arabidopsis* genes.

The cotton *ABF* and *CBF* genes have not previously been characterized or functionally examined. Here, these genes are isolated, their expression patterns in response to various stressors are detailed, and they are ectopically expressed in *Arabidopsis* to help determine their role in the regulation of abiotic stress responses. Comparison of these results with the aforementioned ectopic expression of the endogenously-expressed *Arabidopsis* genes in *Arabidopsis*, and the exogenously-expressed *Arabidopsis* genes in cotton, will show how orthologous genes from two different plant families functionally respond to abiotic stress. In addition, over-expression of a cotton *ABF2* ortholog in cotton was examined to determine if this endogenous ectopic expression combination can increase drought tolerance, while minimizing negative developmental effects.

CHAPTER II

MATERIALS AND METHODS

Isolation of* Gossypium ABF *and* CBF *coding sequences

Published *Arabidopsis ABF* and *CBF* sequences were used to query BLAST in the NCBI database for *G. hirsutum ABF* and *CBF* gene sequences. No annotated *Gossypium hirsutum ABFs* or *CBFs* existed, though the EST database revealed small portions of putative homologs. Again, BLAST was queried using these initial results, in various databases, limited to the *Gossypium* genus, resulting in significantly longer putative sequences, however, none represented full coding regions. RACE-PCR was employed 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* and *CBF* coding regions were derived in a similar fashion for those sequences not found in the NCBI database.

Gossypium ABF *and* CBF *protein alignments*

Isolated coding regions from the *G. arboreum, G. hirsutum*, and *G. raimondii ABF* and *CBF* homologs, and the published *Arabidopsis ABF* and *CBF* nucleotide sequences, were imported into MEGA6.06-mac (Tamura *et al.* 2013), translated, and

aligned by ClustalW. The aligned amino acid sequences were imported into Jalview 2.9.Ob2 (Waterhouse *et al.* 2009) for visualization and annotation.

Phylogenetic analyses

Isolated *Gossypium* and published *Arabidopsis ABF* and *CBF* coding sequences were imported into MEGA6.06-mac (Tamura *et al.* 2013), and aligned by ClustalW. The resulting multiple sequence alignment was used to derive an initial maximum likelihood tree, using default settings, with the following changes: gaps/missing data were given a partial deletion site coverage cutoff of 90%, and the tree was bootstrapped 250 times. Additional *ABF* and *CBF* sequences were obtained from NCBI to derive the second tree; parameters were the same as for the primary tree.

Gene expression analyses in **Arabidopsis** *and* **Gossypium**

Wild type *Arabidopsis thaliana* Columbia (Col-0) were plated to ½ MS, 1% sucrose solid medium, placed in the dark for 24 hours at 4 degrees Celsius, then transferred to a growth chamber at 24 degrees Celsius with a 15 hour light / 9 hour dark cycle for three to four weeks. Prior to exogenous ABA application, plants were sampled, then sprayed to saturation with a 100µM ABA solution. Further samples were taken 30 minutes, one hour, and two hours after application. To test the dehydration response, plants were sampled, then removed from media keeping the roots intact. Further samples were taken after 1.5, three, and six hours. To test the low temperature expression response, plants were sampled, transferred to 4 degrees Celsius, and sampled after one, two, and four hours at low temperature.

Gossypium hirsutum Coker 312 was grown in soil in 1/4 gallon pots, for six to eight weeks under long-day conditions (15 hours light / 9 hours dark) at an average 30 degrees Celsius. Prior to ABA application, pre-treatment samples were taken, then plants were sprayed to saturation with a 1mM ABA solution and sampled after 30 minutes, one hour, and two hours. Dehydration treatment samples were taken prior to treatment, then water was withheld. Further samples were taken after 48 hours (before visible wilt), after 72 hours (moderate wilt), and after 78 hours (severe wilt). Low temperature test samples were taken prior to the treatment, plants were transferred to 4 degrees Celsius, and sampled after one, two, and four hours.

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 was quantified via Nanodrop. cDNA synthesis was performed using the iScript cDNA synthesis kit (Bio-Rad), and the provided random primer mix for both *Arabidopsis* and *G. hirsutum*. RNA samples were normalized to 100ng/µL. All reactions were conducted as per the manufacturers' instructions.

All qRT-PCR reactions used the iTAQ Universal SYBR Green Supermix (Bio-Rad) 10uL pre reaction. Standard curves were derived using the pGWB12 plasmid constructs (detailed in the following section). Dilutions ranged from 100ng per reaction to .001ng, at varying increments. All abiotic stress samples were then analyzed according to the manufacturers' instructions, at a volume of 10µL per reaction.

Ectopic expression of the G. hirsutum ABFs *and* CBFs *in* Arabidopsis

Coding regions of the *G. hirsutum ABFs* and *CBFs* 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* as per manufacturer's instructions. Colonies positive for the insert were cultured overnight at 37 degrees Celsius in LB supplemented with 50µg/mL kanamycin. The plasmid was purified using the QIAprep Spin Miniprep kit (Qiagen). LR recombination (Invitrogen) was used to transfer the *ABF* and *CBF* homologs to the pGWB12 expression vector (provided by T. Nakagawa, Research Institute of Molecular Genetics, Shimane University, Japan), then transformed into Library Efficiency DH5-alpha *E. coli* (Invitrogen). Positive colonies were cultured overnight at 37 degrees Celsius in LB supplemented with 50µg/mL kanamycin, and the pGWB12 plasmid purified as above. 5µL purified plasmid was added to 25µL *Agrobacterium tumefaciens* C58 on ice, transferred to a water bath at 47 degrees Celsius for seven minutes, then returned to ice. 1mL LB was added and the culture was incubated at 30 degrees Celsius with shaking for three hours, then plated to solid LB supplemented with 10µg/mL gentamicin, 50µg/mL kanamycin, and 50µg/mL rifampicin.

Colonies positive for the insert were cultured for 48 hours in 25 mL LB supplemented with 10µg/mL gentamicin, 50µg/mL kanamycin, and 50µg/mL rifampicin at 30 degrees Celsius with shaking, then transferred to 250mL LB for 24 hours. Cells were pelleted, then resuspended in a 400mL 5% sucrose, .01% Silwet L-77 solution.

Flowering *Arabidopsis thaliana* Col-0 were dipped for 20 seconds with agitation, then placed under cover in the dark for 24 hours before being transferred to growth conditions at 24 degrees Celsius with a 15 hour light / 9 hour dark cycle.

Harvested seeds were plated onto solid ½ MS, 2% sucrose, 50µg/mL kanamycin

media. Independent transformed lines were transferred to soil, verified via PCR, and relative expression was determined via qRT-PCR for a minimum of ten lines. Three lines, representing low, median, and high expression of the ectopically-expressed target were selected for further examination. These T2 seeds were plated onto solid ½ MS, 1% sucrose, 50µg/mL kanamycin media, transferred to soil, and T3 seeds were harvested for physiological testing.

Developmental and abiotic stress tests

T3 or T4 generation seeds of transgenic *Arabidopsis* used in early development and dehydration tests were surface sterilized in 50% bleach, plated to ½ MS, 1% sucrose solid medium, placed in the dark for 24 hours at 4 degrees Celsius, then transferred to a growth chamber at 24 degrees Celsius with a 15 hour light / 9 hour dark cycle. Plants used to examine development were monitored and photographed weekly. To test dehydration tolerance, an average of ten plants from three plates for each transgenic line and wild type, after three weeks' growth, were removed from the media and transferred to petri dishes lined with glass beads to dehydrate. Plants were re-watered, in half hour increments, after a minimum of four hours, to a maximum of six and a half hours. After 48 hours recovery, all plants, for all time intervals, were recorded for survival per plate as compared to the total number of plants on each plate.

T3 or T4 generation seeds of transgenic *Arabidopsis* used for later development and cold tolerance tests were sown in soil in small pots or petri dishes, respectively, at the same time as wild type, placed in the dark for 24 hours at 4 degrees Celsius, then transferred to growth conditions at approximately 24 degrees Celsius with a 15 hour

light/ 9 hour dark cycle. Plants used to examine development were photographed and monitored for reproductive transition. To test low-temperature tolerance, each soil-filled petri plate, containing an average of ten plants for each line and wild type were transferred to -7 degrees Celsius. After three hours, one plate for each transgenic line and wild type were removed every half-hour up to five hours. After 48 hours' recovery, all plants, for all time intervals, were recorded for survival per plate as compared to the total number of plants on each plate.

***Ectopic expression of the* G. hirsutum ABF2-like D *homolog in* G. hirsutum**

The following transformation protocol was adapted from Gould & Magallanes-Cedeno (1998), Kumria *et al*. (2003), and Leelavathi *et al.* (2004). *G. hirsutum* Coker 312 seeds were surface sterilized for ten minutes, with agitation in 50% bleach, then placed on ½ MS (1% agar) in mason jars. One- to two-week-old plants were removed from jars, hypocotyls were cut into one centimeter sections, and plated to embryogenic induction media (4.4g/L MS with vitamins, 3% maltose, .1mg/L 2,4-D, .5mg/L kinetin, 1% agar) for eight to 12 weeks. Embryogenic calluses were transferred to callus induction medium (4.4g/L MS with vitamins, 3% maltose, 1% agar) for four to eight weeks, sub-culturing every four weeks to maintain the callus pool. For transformation, 25mL *Agrobacterium tumefaciens* C58 was cultured as previously described, pelleted, and resuspended in *Agrobacterium* inoculation medium (.4g/L yeast extract, 10g/L mannitol, .1g/L NaCl, .2g/L MgSO4, .5g/L K2HPO4, 50mg/L kanamycin). Mature callus was broken up, suspended in this medium, and shaken for 30 minutes. Excess liquid was drawn off, and the callus was plated on filter paper to full MS with vitamins (1% agar) and placed in the

dark at room temperature for 48 to72 hours. The callus-covered filter paper was then transferred to kanamycin selection medium 1 (4.4g/L MS, 3% maltose, 50mg/L kanamycin, 500mg/L cefotaxime, 1% agar) for three to six weeks. Globular embryos were then transferred to kanamycin selection medium 2 (1.1g/L MS with vitamins, 1% maltose, 50mg/L kanamycin, 250mg/L cefotaxime, 1% agar). Developing embryos were transferred to kanamycin selection medium 3 (4.4g/L MS with vitamins, 1% maltose, 25mg/L kanamycin, 250mg/L cefotaxime, 1% agar) two to six weeks later. Cotyledonary embryos were transferred to MSGA medium (4.4g/L MS with vitamins, .05mg/L GA, 1% agar) for seven to 14 days (until root emergence), then transferred to ½ MS (1% agar). Independent transgenic plantlets were then transferred to soil under cover and grown under long day conditions (15 hours light / 9 hours dark) at an average 30 degrees Celsius.

Three ectopically-expressing *G. hirsutum ABF2-like D* lines were used to test development and dehydration tolerance (2-1, 1-1, and 1-3). T3 generation seeds were planted alongside *G. hirsutum* Coker 312. Development was monitored and photographed weekly. To test dehydration tolerance, all lines and wild type were fully watered, then water was withheld, and plants were monitored for wilting. All plants either reached the permanent wilting point, or were re-watered soon before permanent wilt was reached and monitored for recovery.

CHAPTER III

ABF RESULTS

***Isolation of cDNAs that encode* ABF *homologs from* Gossypium hirsutum*, G. arboreum*, *and* G. raimondii**

cDNAs that encode putative *G. hirsutum AREBs/ABFs* (hereinafter simply *ABFs*) were initially queried using published *Arabidopsis AREB/ABF* nucleotide sequences (*sensu* NCBI). No corresponding annotated genes were found, therefore, EST contigs were compiled using the top 100 ESTs corresponding to each *Arabidopsis ABF*. Four distinct contigs resulted, largely concentrated around conserved regions (*e.g.* the bZIP domain), representing only a portion of the target cotton genes. Secondary contigs were then derived using the initial contigs limited to *G. hirsutum*. This extended the length of the putative cDNA sequences, and revealed the presence of potential homeologous pairs within the cotton ESTs. However, none of these contigs represented the majority of the coding regions of any cotton *ABF* as predicted by translated protein alignment of the *Arabidopsis* homologs. RACE-PCR was employed to determine the sequence of both the 5' and 3' ends of the coding regions. Eight distinct *G. hirsutum ABF* coding sequences, four homeologous pairs, with a pairwise similarity in excess of 90%, were revealed. These results further strengthen the prediction that *G. hirsutum*, as a tetraploid cotton

species, has two distinct orthologous genes for every single *Arabidopsis* homolog. In addition to the isolation of cDNAs for the *G. hirsutum ABFs*, cDNAs for the *ABF* orthologs from *G. arboreum* and *G. raimondii* were also isolated using similar methods. These gene sequences, from old world and new world diploid cotton, help to define which *G. hirsutum* homeolog can be attributed to the AA or DD progenitor genome, respectively.

Predicted protein structure of the **Gossypium hirsutum** *ABFs*

A multiple protein sequence alignment of the eight *G. hirsutum* ABFs was performed in MEGA 6.06-mac together with the *Arabidopsis*, *G. arboreum,* and *G. raimondii* ABFs and then visualized in Jalview 2.9.Ob2 (Fig. 3). The combined aligned length of all sequences was 527 amino acids, including gaps; the longest *G. hirsutum* ABF was found to be 425 amino acids in length, while the shortest was 388. Amino acids at79 positions were completely conserved throughout the 20 sequences compared. When comparing only the *Arabidopsis* and *G. hirsutum* ABF sequences, 92 positions were found to be completely conserved. In both of these iterations, a significant proportion of the completely conserved positions centered around the bZIP domain, found near the C-terminus of the protein. Each *Gossypium* ABF sequence isolated contains the requisite basic region and leucine repeats characteristic of the bZIP domain.
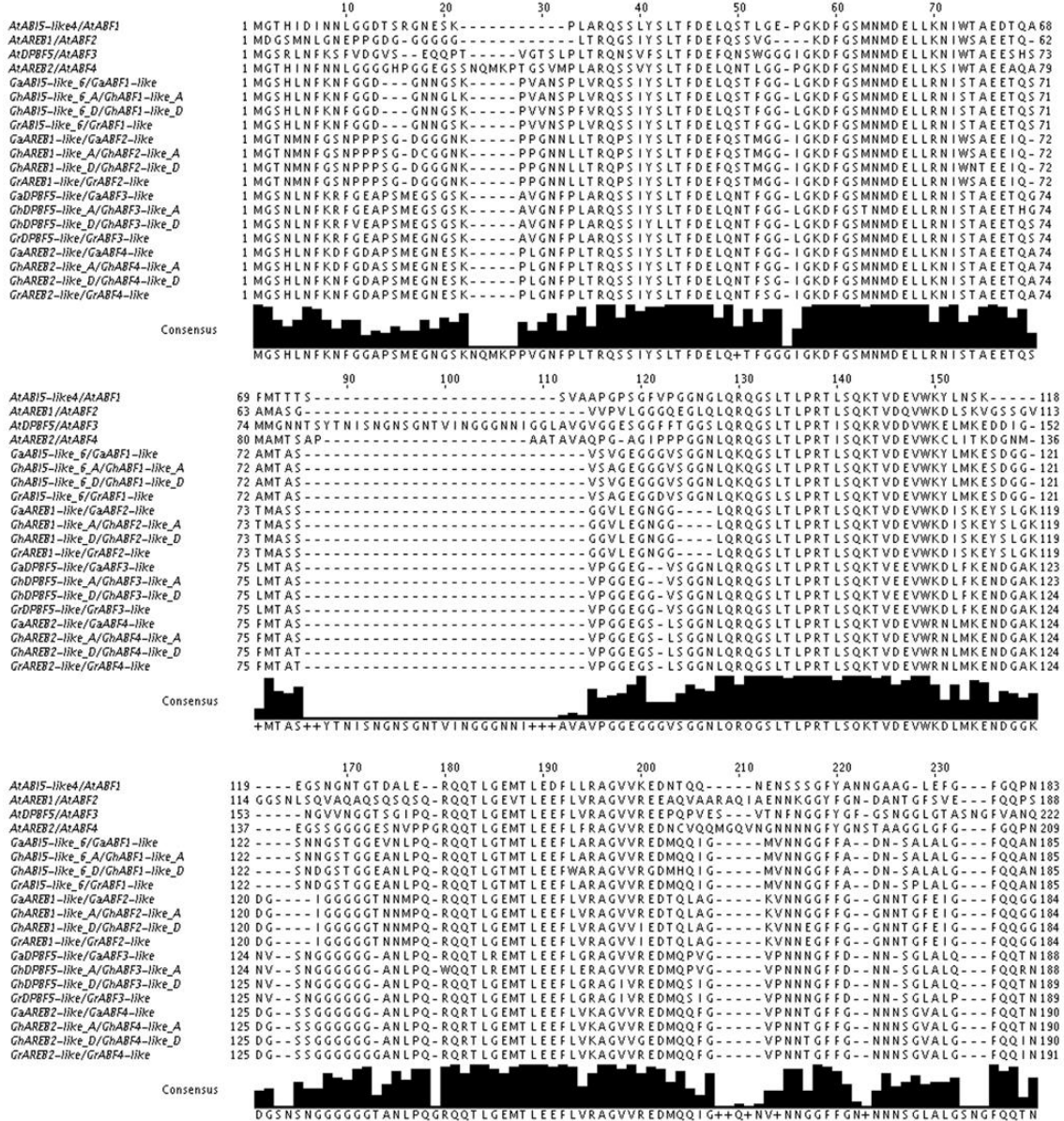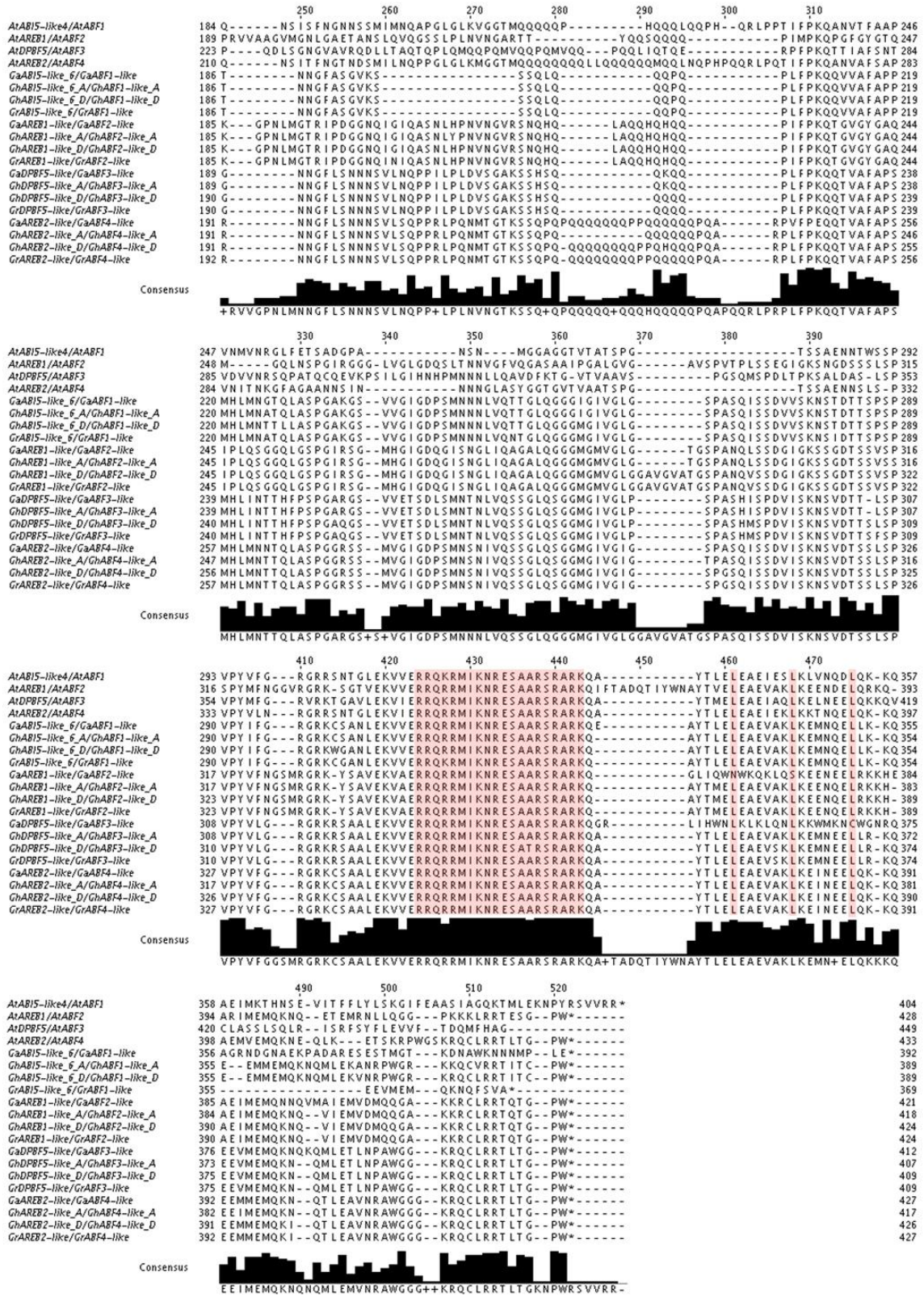
```
                        10        20        30        40        50        60        70
AtABI5-like4/AtABF1    1 MGTHIDINNLGGDTSRGNESK-----------PLARQSSLYSLTFDELQSTLGE-PGKDFGSMNMDELLKNIWTAEDTQA 68
AtAREB1/AtABF2         1 MDGSMNLGNEPPGDG-GGGGG-------------LTRQGSIYSLTFDEFQSSVG----KDFGSMNMDELLKNIWSAEETQ- 62
AtDPBF5/AtABF3         1 MGSRLNFKSFVDGVS--EQQPT-----VGTSLPLTRQNSVFSLTFDEFQNSWGGGIGKDFGSMNMDELLKNIWTAEESHS 73
AtAREB2/AtABF4         1 MGTHINFNNLGGGGHPGGEGSSNQMKPTGSVMPLARQSSVYSLTFDELQNTLGG-PGKDFGSMNMDELLKSIWTAEEAQA 79
GaABI5-like_6/GaABF1-like       1 MGSHLNFKNFGGD---GNNGSK-----PVANSPLVRQSSIYSLTFDELQSTFGG-LGKDFGSMNMDELLRNISTAEETQS 71
GhABI5-like_6_A/GhABF1-like_A   1 MGSHLNFKNFGGD---GNNGLK-----PVANSPLVRQSSIYSLTFDELQSTFGG-LGKDFGSMNMDELLRNISTAEETQS 71
GhABI5-like_6_D/GhABF1-like_D   1 MGSHLNFKNFGGD---GNNGSK-----PVVNSPFVRQSSIYSLTFDELQSTFGG-LGKDFGSMNMDELLRNISTAEETQS 71
GrABI5-like_6/GrABF1-like       1 MGSHLNFKNFGGD---GNNGSK-----PVVNSPLVRQSSIYSLTFDELQSTFGG-LGKDFGSMNMDELLRNISTAEETQS 71
GaAREB1/GaABF2-like             1 MGTNMNFGSNPPPSG-DGGGNK-----PPGNNLLTRQPSIYSLTFDEFQSTMGG-IGKDFGSMNMDELLRNIWSAEEIQ- 72
GhAREB1-like_A/GhABF2-like_A    1 MGTNMNFGSNPPPSG-DCGGNK-----PPGNNLLTRQPSIYSLTFDEFQSTMGG-IGKDFGSMNMDELLRNIWSAEEIQ- 72
GhAREB1-like_D/GhABF2-like_D    1 MGTNMNFGSNPPPSG-DGGGNK-----PPGNNLLTRQPSIYSLTFDEFQSTMGG-IGKDFGSMNMDELLRNIWNTEEIQ- 72
GrAREB1-like/GrABF2-like        1 MGTNMNFGSNPPPSG-DGGGNK-----PPGNNLLTRQPSIYSLTFDEFQSTMGG-IGKDFGSMNMDELLRNIWSAEEIQ- 72
GaDPBF5/GaABF3-like             1 MGSNLNFKRFGEAPSMEGSGSK-----AVGNFPLARQSSIYSLTFDELQNTFGG-LGKDFGSMNMDELLRNISTAEETQS 74
GhDPBF5-like_A/GhABF3-like_A    1 MGSNLNFKRFGEAPSMEGSGSK-----AVGNFPLARQSSIYSLTFDELQNTFGG-LGKDFGSTNMDELLRNISTAEETHG 74
GhDPBF5-like_D/GhABF3-like_D    1 MGSNLNFKRFVEAPSMEGSGSK-----AVGNFPLARQSSIYLLTFDELQNTFGG-LGKDFGSMNMDELLRNISTAEETQS 74
GrDPBF5/GrABF3-like             1 MGSNLNFKRFGEAPSMEGSGSK-----AVGNFPLARQSSIYSLTFDELQNTFGG-LGKDFGSMNMDELLRNISTAEETQS 74
GaAREB2/GaABF4-like             1 MGSHLNFKDFGDAPSMEGNESK-----PLGNFPLTRQSSIYSLTFDELQNTFGG-IGKDFGSMNMDELLKNISTAEETQA 74
GhAREB2-like_A/GhABF4-like_A    1 MGSHLNFKDFGDASSMEGNESK-----PLGNFPLTRQSSIYSLTFDELQNTFGG-IGKDFGSMNMDELLKNISTAEETQA 74
GhAREB2-like_D/GhABF4-like_D    1 MGSHLNFKDFGDAPSMEGNESK-----PLGNFPLTRQSSIYSLTFDELQNTFSG-IGKDFGSMNMDELLKNISTAEETQA 74
GrAREB2-like/GrABF4-like        1 MGSHLNFKGDAPSMEGNESK-----PLGNFPLTRQSSIYSLTFDELQNTFSG-IGKDFGSMNMDELLKNISTAEETQA 74

Consensus
MGSHLNFKNFGGAPSMEGNGSKNQMKPPVGNFPLTRQSSIYSLTFDELQ+TFGGGIGKDFGSMNMDELLRNISTAEETQS


                         90       100       110       120       130       140       150
AtABI5-like4/AtABF1   69 FMTTTS-------------------------SVAAPGPSGFVPGGNGLQRQGSLTLPRTLSQKTVDEVWKYLNSK-----118
AtAREB1/AtABF2        63 AMASG----------------------------VVPVLGGGQEGLQLQRQGSLTLPRTLSQKTVDQVWKDLSKVGSSGV113
AtDPBF5/AtABF3        74 MMGNNTSYTNISNGNSGNTVINGGGNN-IGGLAVGVGGESGGFFTGGSLQRQGSLTLPRTISQKRVDDVWKELMKEDDIG-152
AtAREB2/AtABF4        80 MAMTSAP--------------------AATAVAQPG-AGIPPPGGNLQRQGSLTLPRTISQKTVDEVWKCLITKDGNM-136
GaABI5-like_6/GaABF1-like       72 AMTAS---------------------------VSVGEGGGVSGGNLQKQGSLTLPRTLSQKTVDEVWKYLMKESDGG-121
GhABI5-like_6_A/GhABF1-like_A   72 AMTAS---------------------------VSAGEGGGVSGGNLQKQGSLTLPRTLSQKTVDEVWKYLMKESDGG-121
GhABI5-like_6_D/GhABF1-like_D   72 AMTAS---------------------------VSVGEGGGVSGGNLQKQGSLTLPRTLSQKTVDEVWKYLMKESDGG-121
GrABI5-like_6/GrABF1-like       72 AMTAS---------------------------VSAGEGGDVSGGNLQKQGSLSLPRTLSQKTVDEVWKYLMKESDGG-121
GaAREB1/GaABF2-like             73 TMASS---------------------------GGVLEGNGG----LQRQGSLTLPRTLSQKTVDEVWKDISKEYSLGK119
GhAREB1-like_A/GhABF2-like_A    73 TMASS---------------------------GGVLEGNGG----LQRQGSLTLPRTLSQKTVDEVWKDISKEYSLGK119
GhAREB1-like_D/GhABF2-like_D    73 TMASS---------------------------GGVLEGNGG----LQRQGSLTLPRTLSQKTVDEVWKDISKEYSLGK119
GrAREB1-like/GrABF2-like        73 TMASS---------------------------GGVLEGNGG----LQRQGSLTLPRTLSQKTVDEVWKDISKEYSLGK119
GaDPBF5/GaABF3-like             75 LMTAS---------------------------VPGGEG--VSGGNLQRQGSLTLPRTLSQKTVEEVWKDLFKENDGAK123
GhDPBF5-like_A/GhABF3-like_A    75 LMTAS---------------------------VPGGEG--VSGGNLQRQGSLTLPRTLSQKTVEEVWKDLFKENDGAK123
GhDPBF5-like_D/GhABF3-like_D    75 LMTAS---------------------------VPGGEGG-VSGGNLQRQGSLTLPRTLSQKTVEEVWKDLFKENDGAK124
GrDPBF5/GrABF3-like             75 LMTAS---------------------------VPGGEGG-VSGGNLQRQGSLTLPRTLSQKTVEEVWKDLFKENDGAK124
GaAREB2/GaABF4-like             75 FMTAS---------------------------VPGGEGS-LSGGNLQRQGSLTLPRTLSQKTVDEVWRNLMKENDGAK124
GhAREB2-like_A/GhABF4-like_A    75 FMTAS---------------------------VPGGEGS-LSGGNLQRQGSLTLPRTLSQKTVDEVWRNLMKENDGAK124
GhAREB2-like_D/GhABF4-like_D    75 FMTAT---------------------------VPGGEGS-LSGGNLQRQGSLTLPRTLSQKTVDEVWRNLMKENDGAK124
GrAREB2-like/GrABF4-like        75 FMTAT---------------------------VPGGEGS-LSGGNLQRQGSLTLPRTLSQKTVDEVWRNLMKENDGAK124

Consensus
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                        170       180       190       200       210       220       230
AtABI5-like4/AtABF1  119 ----EGSNGNTGTDALE--RQQTLGEMTLEDFLLRAGVVKEDNTQQ-----NENSSSGFYANNGAAG-LEFG---FGQPN183
AtAREB1/AtABF2       114 GGSNLSQVAQAQSQSQSQ-RQQTLGEVTLEEFLVRAGVVREEAQVAARAQIAENNKGGYFGN-DANTGFSVE--FQQPS188
AtDPBF5/AtABF3       153 -----NGVVWNGGTSGIPQ-RQQTLGEMTLEEFLVRAGVVREEPQPVES---VTNFNGGFYGF-GSNGGLGTASNGFVANQ222
AtAREB2/AtABF4       137 ----EGSSGGGGESNVPPGRQQTLGEMTLEEFLFRAGVVREDNCVQQMGQVNGNNNNGFYGNSTAAGGLGFG---FGQPN209
GaABI5-like_6/GaABF1-like       122 ----SNNGSTGGEANLPQ-RQQTLGTMTLEEFLARAGVVREDMQQIG-----MVNNGGFFA--DN-SALALG---FQQAN185
GhABI5-like_6_A/GhABF1-like_A   122 ----SNNGSTGGEANLPQ-RQQTLGTMTLEEFLARAGVVREDMQQIG-----MVNNGGFFA--DN-SALALG---FQQAN185
GhABI5-like_6_D/GhABF1-like_D   122 ----SNDGSTGGEANLPQ-RQQTLGTMTLEEFWARAGVVRGDMQQIG-----MVNNGGFFA--DN-SALALG---FQQAN185
GrABI5-like_6/GrABF1-like       122 ----SNDGSTGGEANLPQ-RQQTLGTMTLEEFLARAGVVREDMQQIG-----MVNNGGFFA--DN-SPLALG---FQQAN185
GaAREB1/GaABF2-like             120 DG----IGGGGTNNMPQ-RQQTLGEMTLEEFLVRAGVVREDTQLAG-----KVNNGGFFG--GNNTGFEIG---FQQGG184
GhAREB1-like_A/GhABF2-like_A    120 DG----IGGGGTNNMPQ-RQQTLGEMTLEEFLVRAGVVREDTQLAG-----KVNNGGFFG--GNNTGFEIG---FQQGG184
GhAREB1-like_D/GhABF2-like_D    120 DG----IGGGGTNNMPQ-RQQTLGEMTLEEFLVRAGVVIEDTQLAG-----KVNNEGFFG--GNNTGFEIG---FQQGG184
GrAREB1-like/GrABF2-like        120 DG----IGGGGTNNMPQ-RQQTLGEMTLEEFLVRAGVVIEDTQLAG-----KVNNEGFFG--GNNTGFEIG---FQQGG184
GaDPBF5/GaABF3-like             124 NV--SNGGGGGG-ANLPQ-RQQTLGEMTLEEFLERAGVVREDMQPVG-----VPNNNGFFD--NN-SGLALQ---FQQTN188
GhDPBF5-like_A/GhABF3-like_A    124 NV--SNGGGGGG-ANLPQ-WQQTLREMTLEEFLERAGVVREDMQPVG-----VPNNNGFFD--NN-SGLALQ---FQQRN188
GhDPBF5-like_D/GhABF3-like_D    125 NV--SNGGGGGG-ANLPQ-RQQTLGEMTLEEFLGRAGIVREDMQSIG-----VPNNNGFFD--NN-SGLALQ---FQQTN189
GrDPBF5/GrABF3-like             125 NV--SNGGGGGG-ANLPQ-RQQTLGEMTLEEFLGRAGIVREDMQSIG-----VPNNNGFFD--NN-SGLALP---FQQTN189
GaAREB2/GaABF4-like             125 DG--SSGGGGGG-ANLPQ-RQRTLGEMTLEEFLVKAGVVREDMQQFG-----VPNNTGFFG--NNNSGVALG---FQQTN190
GhAREB2-like_A/GhABF4-like_A    125 DG--SSGGGGGG-ANLPQ-RQRTLGEMTLEEFLVKAGVVREDMQQFG-----VPNNTGFFG--NNNSGVALG---FQQTN190
GhAREB2-like_D/GhABF4-like_D    125 DG--SSGGGGGG-ANLPQ-RQRTLGEMTLEEFLVKAGVVGEDMQQFG-----VPNNTGFFG--NNNSGVALG---FQQIN190
GrAREB2-like/GrABF4-like        125 DG--SSGGGGGGANLPQ-RQRTLGEMTLEEFLVKAGVVREDMQQFG-----VPNNTGFFG--NNNSGVALG---FQQIN191

Consensus
DGSNSNGGGGGGTANLPQGRQQTLGEMTLEEFLVRAGVVREDMQQIG++Q+NV+NNGGFFGN+NNNSGLALGSNGFQQTN
```

Figure 3. Multiple sequence alignments of the *Arabidopsis, G. arboreum, G. hirsutum,* and *G. raimondii ABFs.* The consensus histogram defines the degree of conservation at each site. The highlighted sites (see next page) correspond to the basic region and the leucine repeats of the bZIP domain.

Figure 3, continued.

Phylogenetic analyses of the **Gossypium hirsutum ABF***s*

To elucidate the phylogenetic relationships between *Arabidopsis* and the three isolated *Gossypium* species' *ABFs*, a maximum likelihood phylogenetic tree was constructed (Fig. 4). Rooted by *Arabidopsis ABI5*, which is closely related to the *Arabidopsis ABFs*, clear *Gossypium* AA and DD genome relationships were defined in a one-to-one fashion. However, no definitive correlations were resolved between the *Arabidopsis* and *Gossypium ABF* sequences. A second phylogenetic tree was derived including additional plant species in an attempt to resolve these relationships (Fig. 5).
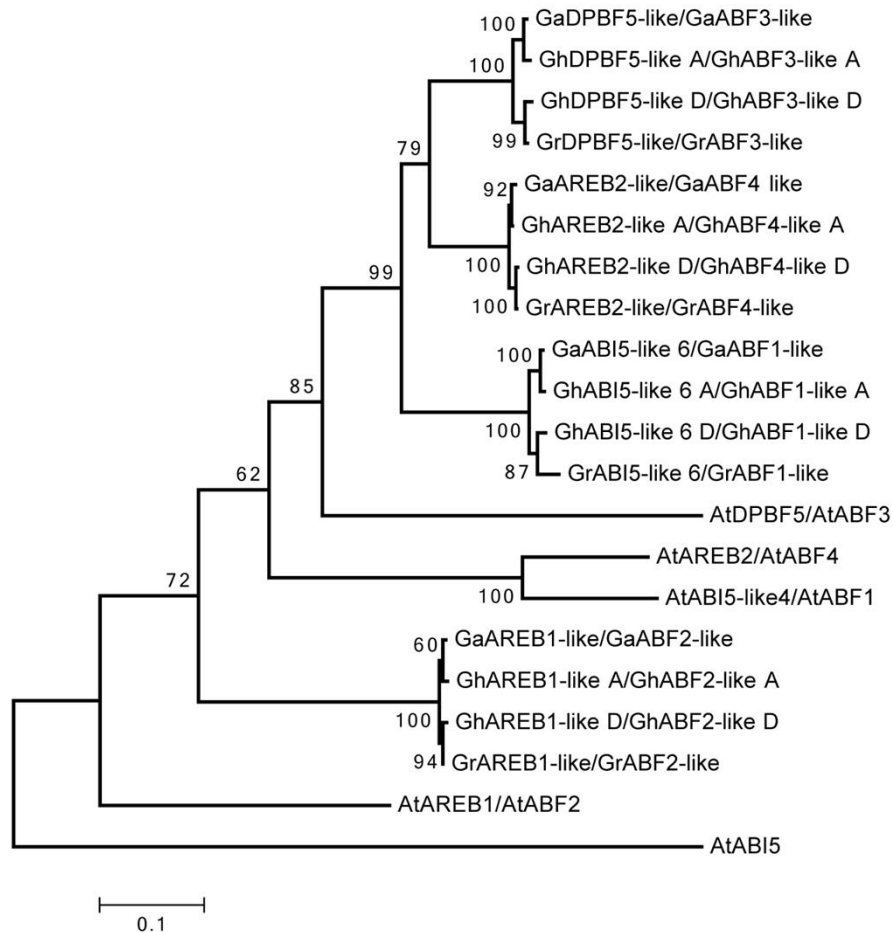
Figure 4. Maximum likelihood tree of the *Arabidopsis, G. arboreum, G. hirsutum, and G. raimondii ABFs*; 250 bootstrap replications.

26
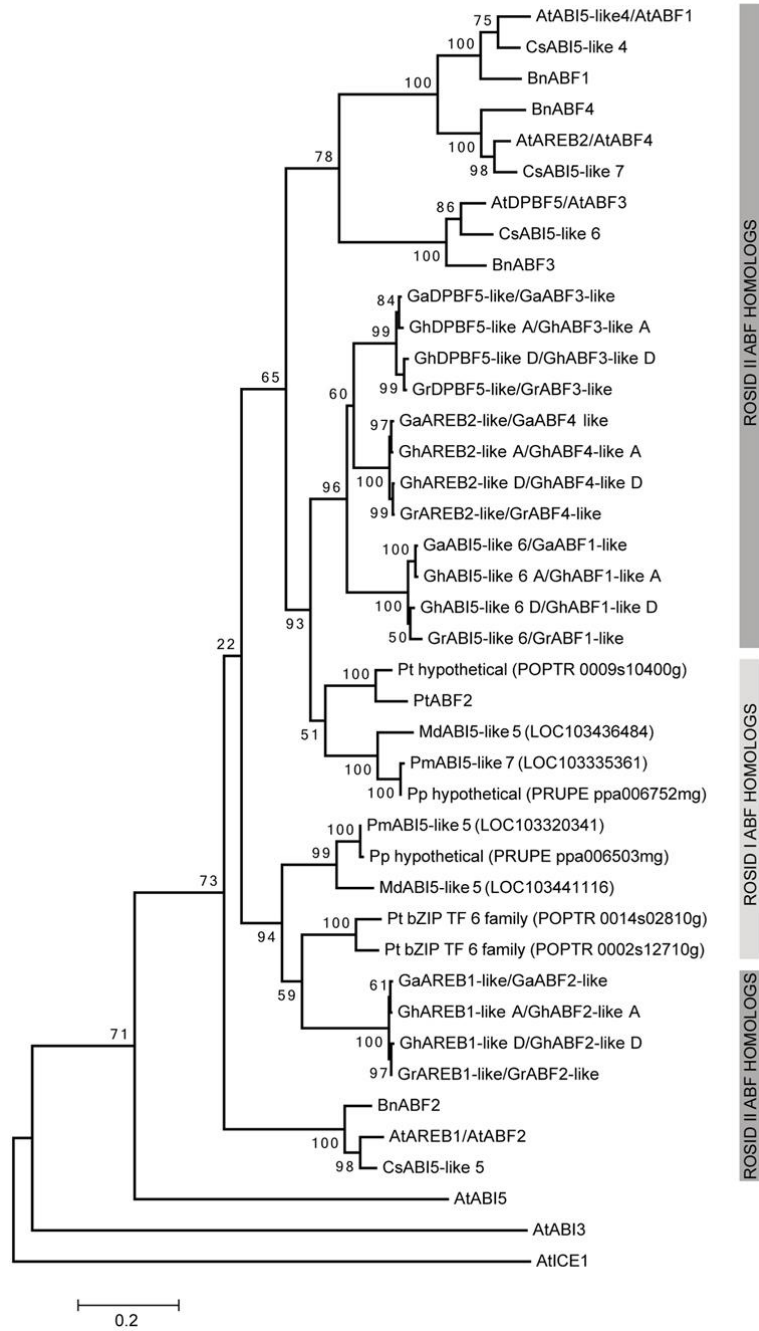
Figure 5. Maximum likelihood tree of *ABF* homologs from multiple species; 250 bootstrap replications. Rosid I and rosid II clade members are delineated on the right by grey bars.

Again, this analysis did not resolve the *Arabidopsis/Gossypium ABF*

relationships; however, it did clarify broader *ABF* relationships. Three species of the Brassicales (*Arabidopsis thaliana, Brassica napus, Camelina sativa*) were all clustered corresponding to their *ABF* homologs. As described, so did the three *Gossypium* species analyzed. However, as noted, the examined Brassicales and Malvales *ABF* homologs did not resolve between orders. The addition of further, more distantly-related *ABF* sequences were then integrated from the Malpighiales and Rosales. These *ABFs* did not change the structure of the Brassicales/Malvales relationship, however, all of these rosid I/Fabidae representatives clustered separately from the rosid II/Malvidae clade members.

Expression patterns of the **Arabidopsis ABF***s in response to abiotic stress*

Previous examination of ectopic expression of the *Arabidopsis ABFs* has shown each is differentially regulated in response to various abiotic stressors (Choi e*t al.* 2000, Fujita *et al.* 2005, Kim *et al.* 2004, Oh *et al.* 2005, Yoshida *et al.* 2015). However, these studies describe only relative changes in expression levels. In order to develop a more complete understanding of the response of these genes to abiotic stress, and for a more direct comparison to *G. hirsutum*, absolute qRT-PCR expression levels were measured in response to the following stressors in *Arabidopsis*: exogenous ABA application, dehydration, and low temperature (Fig. 6).

To determine the *Arabidopsis ABF* transcriptional response to ABA, three week old wild-type *Arabidopsis* plants grown on ½ MS media were sampled prior to application, then sprayed to the point of saturation with a 100μM ABA solution. Further samples were taken after thirty minutes, an hour, and after two hours. The response of the four *Arabidopsis ABFs* to exogenous ABA application was comparable to that previously

28
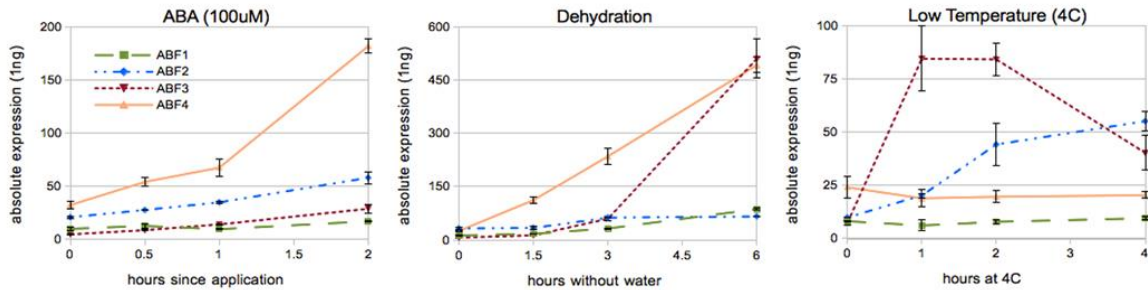
Figure 6. Expression patterns of the *Arabidopsis ABFs* in response to exogenous ABA application (100μM), dehydration, and low temperature (4°C)

reported in semi-quantitative and relative expression studies; however, the absolute quantification of these transcripts illustrates the magnitude of change in expression as well as the basal transcript level. Relative expression studies, while informative, can be deceptive. For example, a ten-fold change in expression may be an increase from one to ten copies, or an increase from ten to 100 copies in response to the exogenous treatment. Following the application of 100 μM ABA, *Arabidopsis ABF1* expression was largely unchanged. *ABF2* and *ABF3* expression roughly doubled from an average 20 copies to an average of 40 copies per one nanogram of total RNA (hereafter, copies/ng), while *ABF4* expression increased seven-fold from approximately 35 to 180 copies/ng.

To test the absolute change in expression of the *Arabidopsis ABFs* in response to dehydration, four-week-old wild type *Arabidopsis* plants were sampled, then removed from soil with roots intact and allowed to dry for two, four, and eight hours. In response to dehydration, *ABF3* and *ABF4* exhibited the most dramatic changes in expression, again, commensurate with aforementioned studies. Expression of *ABF1* and *ABF2* also increased; though to a much lesser degree. After four hours, *ABF3* expression rose from 6 copies to 59 copies/ng; by eight hours, 510 copies/ng were measured. *ABF4* exhibited a

similar rise, from a basal level of 25 copies/ng, to 492 copies/ng at eight hours.

As noted, the *ABFs* are ABA dependent, and generally associated with dehydration stress, however, they have been shown to respond to other abiotic stressors (Choi *et al.* 2000; Fujita *et al.* 2005, Yoshida *et al.* 2015). Thus, each of the *Arabidopsis ABFs* were also tested for their response to low temperatures. Plants were first sampled, then exposed to 4 degrees Celsius for one, two, and four hours, with sampling at each time point. As in the previous physiological tests, changes in expression were measured by qRT-PCR. While the magnitude of change was far less than that seen with the ABA and dehydration treatments, *ABF2* and *ABF3* expression increased significantly after one hour at 4 degrees Celsius, and continued to rise or remained elevated after two hours. After four hours at 4 degrees Celsius, *ABF2* had risen slightly, while *ABF3* had declined significantly.

Expression patterns of the **Gossypium hirsutum ABF** *genes in response to abiotic stress*

The phylogenetic analyses defined the AA and DD genome origins of the *G. hirsutum* homeologous *ABF* pairs, but it did not resolve the relationship of the orthologous *Arabidopsis* and *G. hirsutum* genes. Therefore, assignation of the *G. hirsutum ABFs* in a one-to-one fashion to the *Arabidopsis ABFs* necessitated an alternate measurement. Thus, the qRT-PCR expression patterns of the *G. hirsutum ABFs* were used. Each of the *G. hirsutum* homeologs were exposed to various abiotic stressors similar to the *Arabidopsis* treatments (Fig. 7). Their absolute expression was then quantified from standard curves derived from their corresponding construct plasmids,
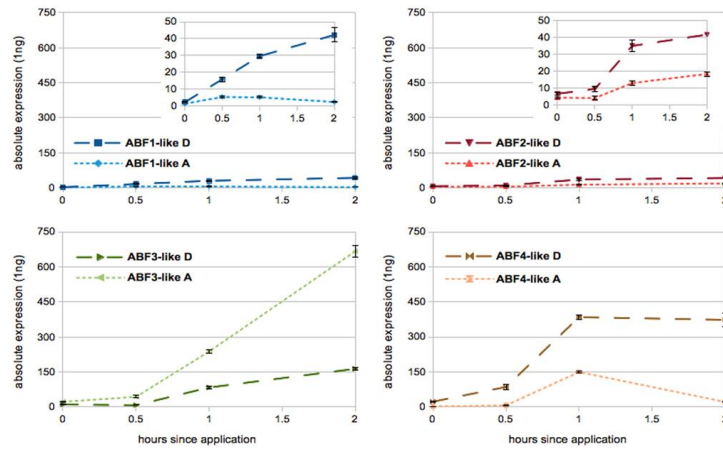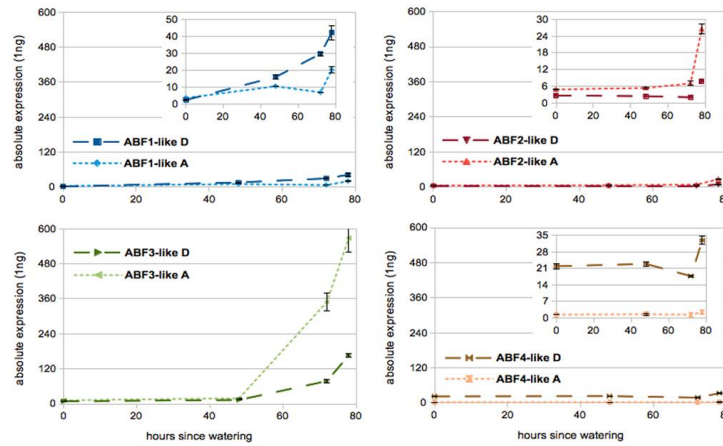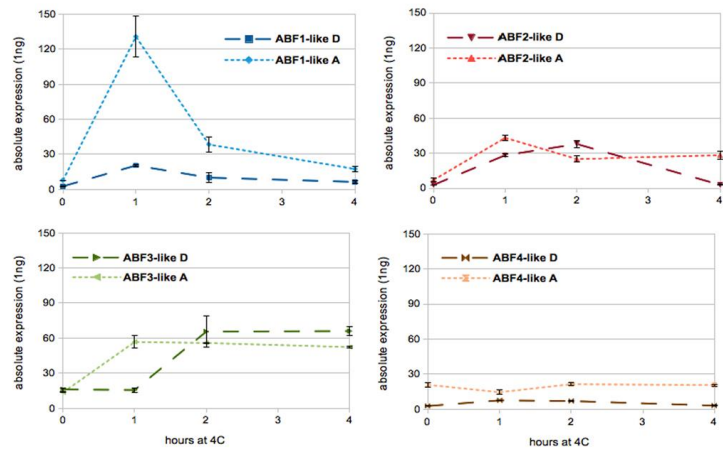
Figure 7. Absolute expression of the *G. hirsutum ABF* homologs in response to abiotic stress. (a) Exogenous ABA application (1mM) over two hours. (b) Dehydration over multiple days to the permanent wilting point. (c) Low temperature (4°C over 4 hours).

similar to the *Arabidopsis ABFs*. Time-zero expression levels were not uniform across the examined stress treatments due to biological variation, however, in all cases they were similar. Additionally, basal expression level of all of the *G. hirsutum ABFs* was relatively low, as is expected with induced transcription factor genes.

To determine the response of the *G. hirsutum ABF* genes to ABA, four-week-old plants were sprayed with a 1mM ABA solution and sampled over a two-hour period. Within each homeologous gene pair, a differential expression response was exhibited. In addition, between paralogs there were substantial differences in the magnitude of expression change (Fig. 7a). To illustrate, expression of *ABF1-like A* nearly quadrupled in the first half-hour after application, from an average 1.4 to 5.4 copies/ng, but returned to pre-treatment levels by two hours. Alternatively, expression of its homeolog *ABF1-like D* increased eight-fold in the first half hour, and 18-fold by two hours; from 2 to 42 copies/ng. While this is a significant differential expression change within the homeologous pair, comparatively, the *ABF3-like A* paralog increased 30-fold over the two hour treatment period, from an average of 22 copies/ng prior to application, to 660 copies/ng after two hours. *ABF3-like D* homeolog expression also increased, but to a much lesser extent:16-fold, from 10 to 160 copies/ng. The *ABF2* and *ABF4* paralogs also responded to exogenous ABA application.

Differential, and drastic, expression changes were also observed in response to dehydration (Fig. 7b). Similarly to the ABA treatment, there was substantial differential expression both within the homeologous pairs, and across all of the *G. hirsutum ABF* genes. Expression of the *ABF3-like A* paralog increased 47-fold, from an average basal level of 12 copies/ng, to 570 copies/ng by the last sample taken when the plants exhibited

severe wilting. The expression level of its homeolog, *ABF3-like D*, also increased, however, the final sample indicated only 165 copies/ng. In contrast, although the expression of the majority of the six remaining *ABFs* did increase in response to dehydration, none showed more than 40 transcript copies/ng at the time of extreme wilt.

The ABFs, being ABA-dependent and primarily implicated in dehydration tolerance, are not typically examined for their response to low or freezing temperatures, however, expression changes were observed in the *G. hirsutum ABFs* when exposed to low temperature (4 degrees Celsius; Fig. 7c). While the magnitude was far less than that of the ABA and dehydration treatments, significant changes were still observed in a majority of the genes. Expression of *ABF1-like D* increased 16-fold, from a basal level of 8 copies to 130 copies/ng, within the first hour at 4 degrees Celsius, before dropping back to near basal levels by four hours. Expression of *ABF1-like A* rose from an average basal level of 2.5 copies to 20 copies/ng in the first hour, then declined, mirroring its homeolog. Expression of the *ABF2-like* homeologs both rose at least 6-fold within an hour. By four hours *ABF2-like D* had returned to basal levels, whereas *ABF2-like A* remained at roughly the same elevated level. The *ABF3-like* homeologs were both elevated by two hours, and remained so after four hours. Neither *ABF4-like* homeologs showed any notable change in expression.

Functional analysis of the **Gossypium hirsutum ABF***s expressed in* **Arabidopsis**

In order to investigate the function of the cotton *ABF* genes, all eight of the *ABF* orthologs isolated from *G. hirsutum* were ectopically expressed in stable transgenic *Arabidopsis* plants under control of the strong constitutive cauliflower mosaic virus 35S

(CaMV35S) promoter. Plants were transformed using the floral dip transformation method (Clough & Bent 1998). A minimum of ten independent transgene-expressing lines were obtained for each *G. hirsutum ABF* gene construct. The level of ectopic expression in each plant was determined by relative qRT-PCR. Three lines were chosen for functional testing for each construct representing high, low, and median expression as per the relative qRT-PCR results. The vast majority of lines were fertile, and T3 or T4 generation plants were subsequently used to test the functional effects of ectopic expression on development, dehydration tolerance, and freezing tolerance.

The primary negative consequence of ectopic expression of the *Arabidopsis ABFs* in *Arabidopsis* has been shown to be delayed growth and reproduction (Kang *et al.* 2002, Kim *et al.* 2004, Fujita *et al.* 2005). Allen (2012) also showed that ectopic expression of *Arabidopsis ABF3* in cotton significantly slows growth and delays flowering. Therefore, the primary physiological test of the functional effect of ectopic expression of the *G. hirsutum ABF* orthologs in *Arabidopsis* was to determine the extent to which this ectopic *G. hirsutum ABF* expression affected seedling growth. The three lines examined for each of the ectopically-expressed *G. hirsutum ABFs* were sown on plates containing artificial media alongside wild type *Arabidopsis*, and monitored for any difference in early growth rate for three weeks (Fig. 8).

Figure 8 illustrates the developmental differences representative of *Arabidopsis* ectopically expressing the *G. hirsutum ABF*s after an average of 13 days. While many transgenic lines show no observable difference, a few stand out for their slower growth habit; namely *ABF2-like D* and *ABF3-like D*. Other lines seem to display more rapid growth (*e.g.*, *ABF1-like D*, *ABF4-like A*). These differences are, however, minor, and all

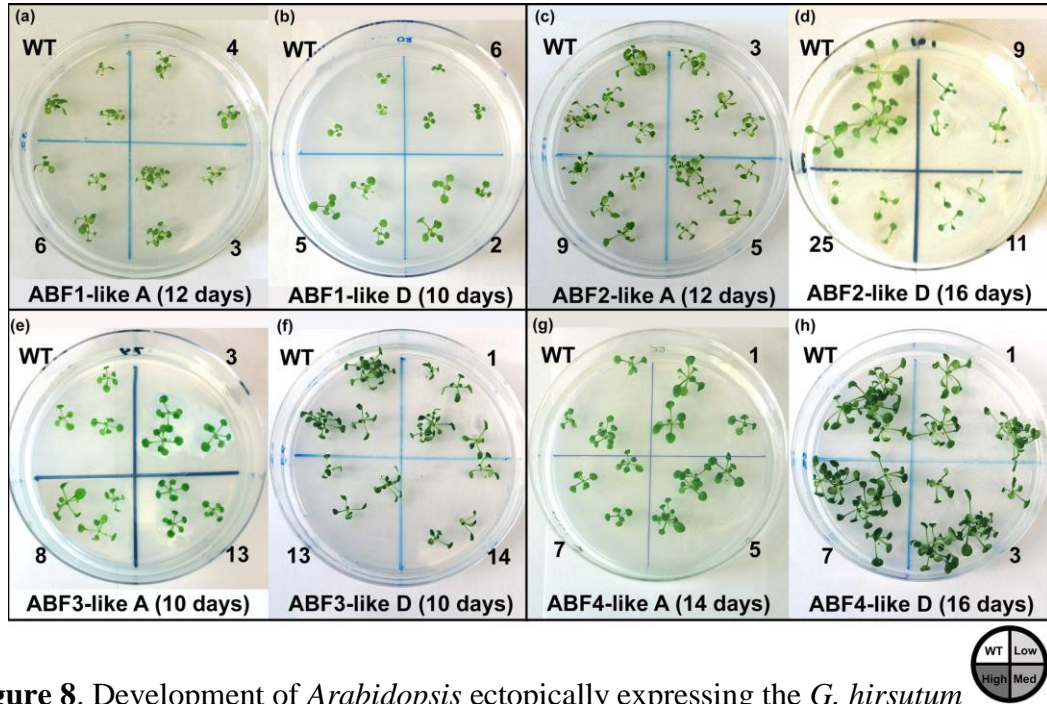plants attained similar size after three or four weeks.

Figure 8. Development of *Arabidopsis* ectopically expressing the *G. hirsutum ABF* homologs on media. Each homeologous pair (ABF1-like (a,b), ABF2-like (c,d), ABF3-like (e,f,), and ABF4-like (g,h)) was plated and monitored for any difference in growth from wild type over three weeks. All plates were divided into quadrants, with wild type in the upper left. Clockwise from wild type are the low, median, and high ectopically-expressing lines. Days since sowing are noted for each.

The above examination of the development of transgenic lines on plates was limited to early differences prior to the reproductive transition; therefore, to determine if there was any delay in this transition, plants were also grown on soil (Fig. 9).

The effect of ectopic expression of the *G. hirsutum ABFs* in *Arabidopsis* on reproduction was not consistent across all genes or lines, however, differences in the time of bolting, as compared to wild type, were observed. Three pots for each line were

monitored, each containing an average of four plants; representative differences are illustrated in Figure 9. The *ABF1-like A* lines examined showed no substantial difference from wild type in time to bolting, however, the median-expressing line generally began to bolt prior to the rest (Table 1). The *ABF1-like D* low-expressing line transitioned to reproduction one day before the other lines and wild type. The median- and high-
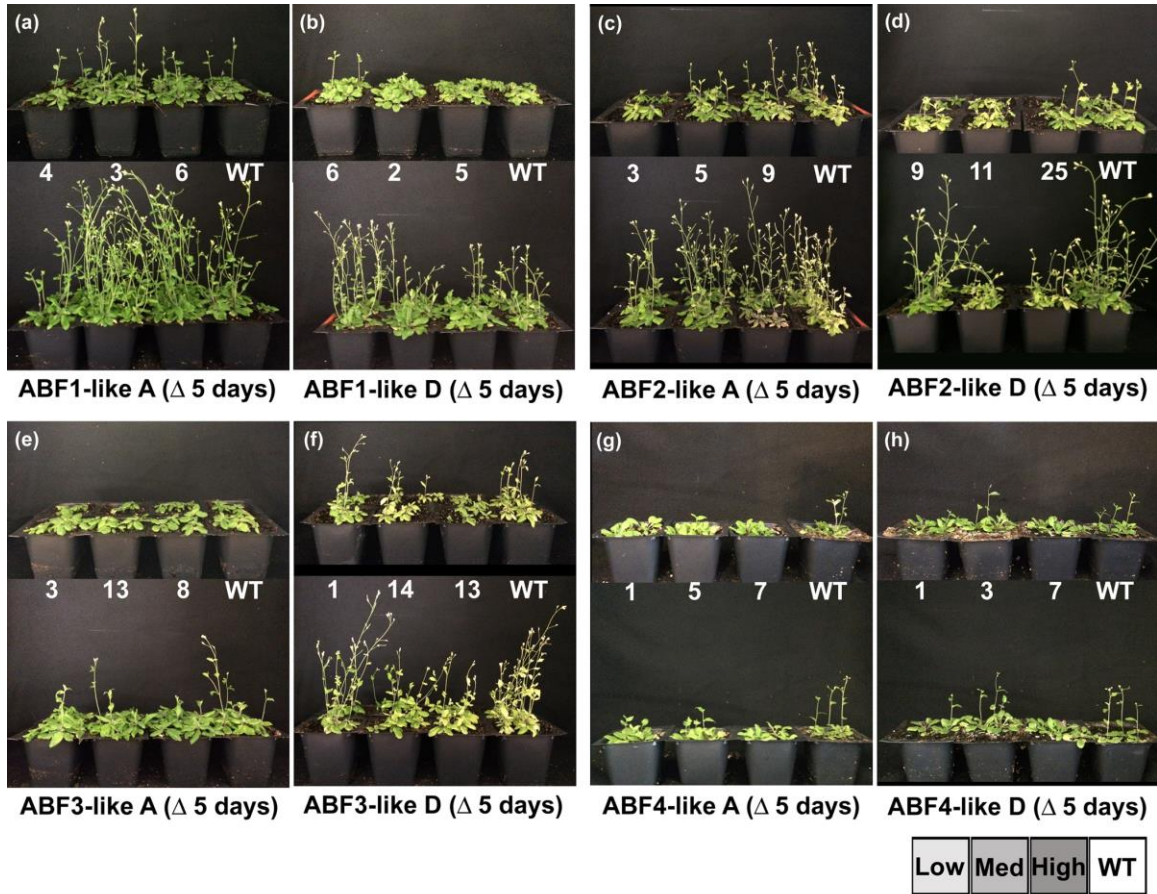

Figure 9. Development of *Arabidopsis* ectopically expressing the *G. hirsutum ABF* homologs in soil. Each homeologous pair (ABF1-like (a,b), ABF2-like (c,d), ABF3-like (e,f,), and ABF4-like (g,h)) was grown in soil for three to six weeks and monitored for the transition to reproduction (bolting). All images are ordered with the lowest ectopically-expressing line to the left, then the median and high expressing lines, with wild type on the right. Days between photographs are indicated.

expressing lines were no different than wild type. The *ABF2-like A* lines examined violated the assumption that the high-expressing line should have the largest delay in development; it began to bolt the same day as wild type, followed a day later by the median-expressing line. The low-expressing line was two days' delayed as compared to the high-expressing line and wild type. All the *ABF2-like D* lines bolted 1.5 to two days later than wild type. All of the *ABF3- like A* lines bolted on average two days after wild type, while the *ABF3-like D*-expressing lines transitioned one, two, and three days later on average, low to high, respectively. The *ABF4-like A* low- and median-expressing lines were delayed three days, while the high-expressing line was delayed by five. The *ABF4-like D* lines were delayed four, two, and five days, low to high, respectively.

Table 1. Effect of ectopic expression of the *G. hirsutum ABF* homologs in *Arabidopsis* on the reproductive transition. Average values represent the difference from wild type in day of bolting in three pots, containing an average of four plants each.

Gh homeolog	Ectopically expressing lines					
	Low		Median		High	
	Ave	SD	Ave	SD	Ave	SD
ABF1-like A	-0.08	0.67	-0.17	0.83	0.00	0.95
ABF1-like D	-0.75	1.14	-0.08	0.79	0.08	0.90
ABF2-like A	1.67	0.89	0.75	0.62	0.08	0.67
ABF2-like D	1.58	0.90	1.67	0.98	1.83	0.83
ABF3-like A	1.75	1.14	1.75	0.87	2.50	1.17
ABF3-like D	0.50	0.52	1.75	0.97	2.58	1.16
ABF4-like A	2.83	1.11	2.67	1.30	4.50	1.57
ABF4-like D	3.83	1.53	1.50	0.80	4.75	1.06

The *ABFs* are linked to dehydration tolerance, therefore, *Arabidopsis* plants ectopically expressing each *G. hirsutum ABF* were examined for increased drought tolerance. An average of ten plants per plate from each of the transgenic lines and wild

type were grown on artificial media for three weeks before being removed, and allowed

to dehydrate for a minimum of four hours, to a maximum of six and a half hours, in half-

hour intervals, then re-watered. After 48 hours recovery, all plants, at all time intervals,

were examined for survival. Results were recorded as living plants over total plants per
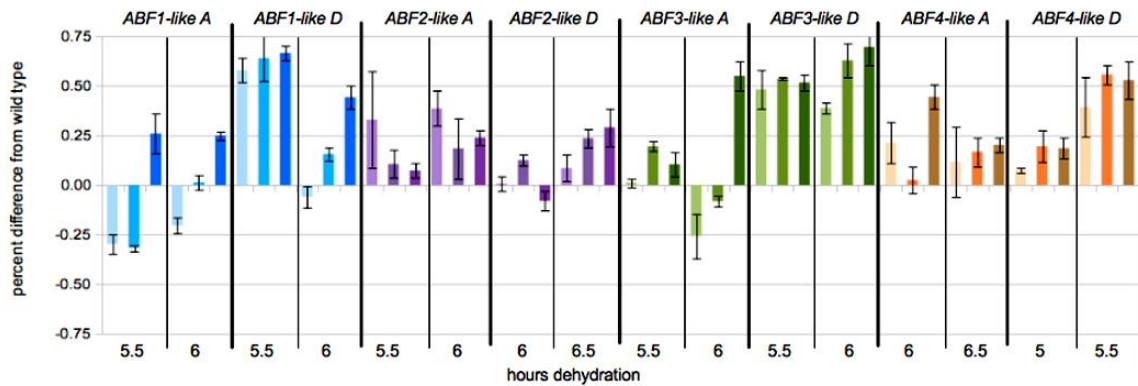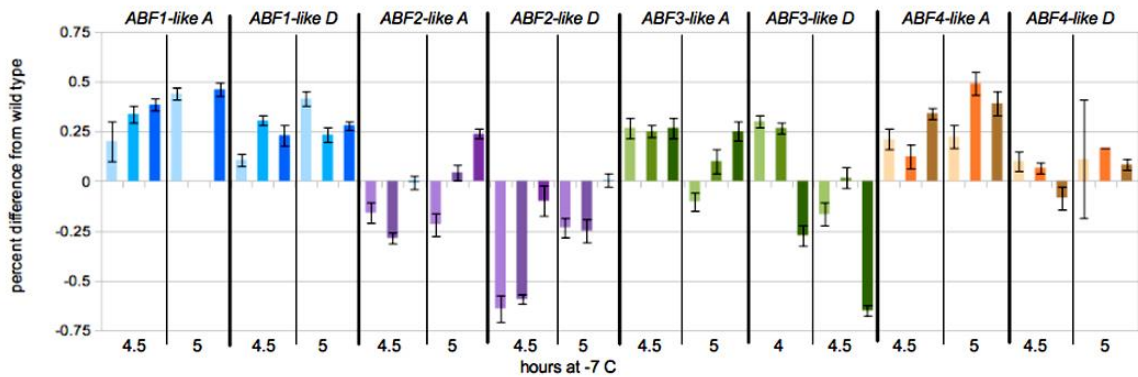
plate at each time interval (Fig. 10).

Figure 10. Percent survival of *Arabidopsis* ectopically expressing *G. hirsutum ABFs*

following dehydration stress relative to wild type; lines are ordered from low- to high-

expressing for each gene. Plants were removed from media and dehydrated from four to

6.5 hours, in half-hour increments, re-watered, and assayed for survival after 48 hours

As compared to wild type, the majority of transgenic lines tested showed

increased rates of survival, indicating ectopic expression of the *G. hirsutum ABFs* in

Arabidopsis can improve dehydration tolerance. At the longest dehydration period

examined, six or 6.5 hours, depending on the gene, only four of the 24 lines examined

exhibited lower survival than wild type. The low-expressing lines of the *ABF1-like*

homeologs exhibited decreased survival, though the median- and high-expressing lines

were improved. The *ABF2-like* and *ABF4-like* all performed better than wild type. While

the *ABF3-like D* lines also showed increased dehydration tolerance, the *ABF3-like A* low-and median-expressing lines exhibited decreased survival to wild type. Overall, increased ectopic expression leads to increased survival.

The *G. hirsutum ABF*-expressing lines in *Arabidopsis* were also examined for increased cold temperature tolerance. An average of ten plants for each line and wild type were grown in soil in petri dishes for four weeks, then transferred to -7 degrees Celsius to determine if freezing tolerance was improved in *G. hirsutum ABF*-expressing *Arabidopsis* plants. Plates for each transgenic line and wild type were removed from freezing temperatures at half-hour intervals after a minimum of three hours, to a maximum of five hours. After 48 hours recovery, all plants, at all time intervals, were examined for survival. Results were recorded as living plants over total plants per plate for each time interval (Fig. 11).

Figure 11. Percent survival of *Arabidopsis* ectopically expressing *G. hirsutum ABFs* following low temperature stress relative to wild type; lines are ordered from low- to high-expressing for each gene. Four-week-old plants were transferred to -7°C, and removed at set intervals from three to five hours, in half-hour increments, and assayed for survival after 48 hours.

While the majority of ectopically expressed *ABF* homologs performed significantly better than wild type in dehydration tests, only the *ABF1-like* homeologs displayed consistently improved freezing tolerance. Specific individual lines from other paralogs did perform better than wild type, though the improvement was not consistent across homeologous pairs, or between the transgenic lines examined. On average, ectopic expression of the *G. hirsutum ABFs* did not lead to substantially improved low temperature tolerance.

Ectopic expression of a **Gossypium hirsutum ABF** *gene in* **Gossypium hirsutum**

As the literature previously outlined has shown, ectopic expression of the *Arabidopsis ABFs* in *Arabidopsis* can increase dehydration tolerance, though this improvement is often associated with developmental and reproductive delays. Ectopic expression of *Arabidopsis ABF3* in cotton yielded similar results. As shown above, ectopic expression of some of the *G. hirsutum ABFs* in *Arabidopsis* leads to increased dehydration tolerance though with some negative impacts on development. Therefore, the final remaining combination was to examine ectopic expression of the *G. hirsutum ABFs* in *G. hirsutum*. The *ABF2-like D* homeolog responded to all of the abiotic stress treatments quantified as by qRT-PCR, but the magnitude of change in expression was moderate. Additionally, though transgenic *Arabidopsis* plants ectopically expressing this cotton *ABF* did not exhibit the largest improvement in dehydration tolerance, and had slower growth initially, only minor delays were observed in the reproductive transition, so it was hypothesized that ectopic expression of *G. hirsutum ABF2-like D* could increase dehydration tolerance and minimize developmental delay.

Three independent transgenic cotton lines ectopically expressing the *G. hirsutum ABF2-like D* gene under the control of the CaMV35S promoter were used to examine the functional effects of this gene in its native background. Basal ectopic expression levels were measured for each of the lines, ordering each from relatively low to relatively high expression. In the absence of abiotic stress, absolute qRT-PCR showed that line 2-1 had an average of 1130 copies/ng of *ABF2-like D* transcript, line 1-1 had an average of 3527 copies/ng, and line 1-3 had an average of 11,195 copies/ng. Each line was exposed to at least one of the aforementioned stress treatments, and no significant change in absolute expression level was observed, as would be expected with this constitutive promoter.

As all of the discussed combinations of *ABF* ectopic expression within and between *Arabidopsis* and *G. hirsutum* have resulted in some degree of developmental delay, T3 generation *G. hirsutum ABF2-like D* over-expressing lines were examined for any delay in growth or reproduction (Fig. 12). The higher *ABF2-like D* expressing lines, 1-3 and 1-1, exhibited slightly delayed germination, and slightly delayed growth in the first 30 days. However, after the first month, growth of all lines was similar to the wild type plants. Reproductive development was monitored through flowering. No significant delay was recorded in flowering for any of the lines as compared to wild type.

These developmental data indicate no significant delay in reproduction when the *G. hirsutum ABF2-like D* gene is ectopically expressed in cotton despite the substantially increased transcript level. Each line was subsequently tested to determine if there was any increase in dehydration tolerance (Fig. 13). Line 2-1, the lowest ectopically-expressing line, did not show any significant increase in dehydration tolerance despite the nearly 300-fold increase in transcript level (Fig. 13a). However, line 1-1, which ectopically

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Figure 12. Development of *G.hirsutum* ectopically expressing the *G. hirsutum ABF2-like D* gene. Two plants representative of each line are shown, from low to high expressers, followed by wild type. Days since planting indicated.
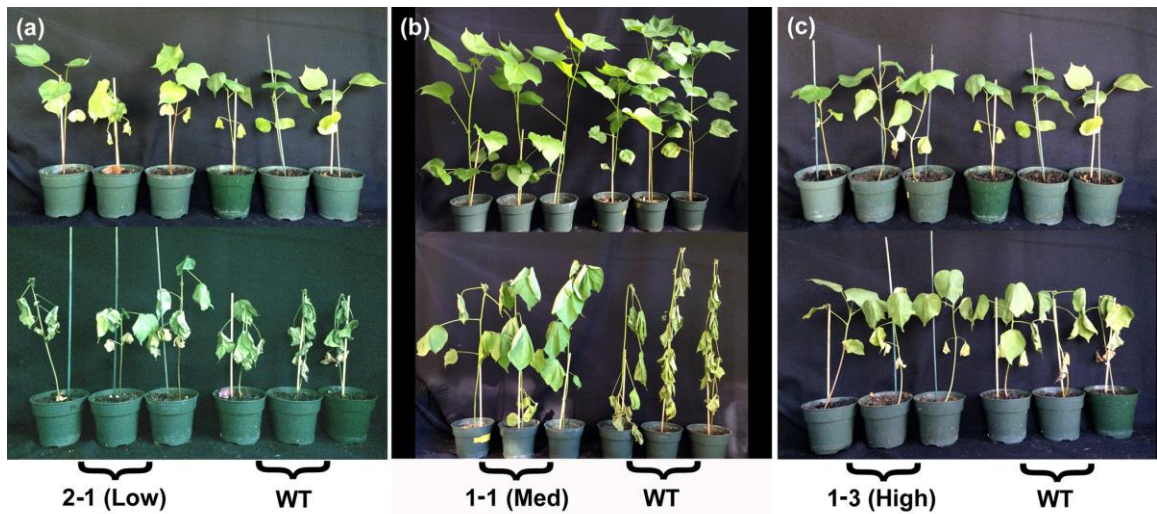

Figure 13. Dehydration tolerance of *G. hirsutum* ectopically expressing the *G. hirsutum ABF2-like D* gene in three transgenic lines, as compared to wild type. Top panels depict fully-watered plants. Bottom panels depict water-stressed plants where the wild type are at or near their permanent wilting points.

expresses *ABF2-like D* at a significantly higher level than 2-1, in the absence of any

stress, at an average of 3527 copies/ng, exhibited significantly delayed wilting. After a

slow drying of the soil, under the same conditions as line 2-1, and after re-watering, the

ABF2-like D-expressing line 1-1 plants recovered up to three days after the wild type

controls had reached their permanent wilting point (Fig. 13b). Line 1-3, the highest-

expressing line, also showed a significant improvement in dehydration tolerance (Fig.

13c). Similar to line 1-1, line 1-3 plants recovered up to three days after the wild type

plants had reached their permanent wilting point. Even though the 1-3 line had more than

double the basal ectopic expression level of line 1-1, the increase in dehydration tolerance

was not observed to be significantly higher than that of line 1-3.

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CHAPTER IV

CBF RESULTS

***Isolation of cDNAs that encode* CBF *homologs from* Gossypium hirsutum, G. arboreum, *and* G. raimondii**

cDNAs that encode putative *G. hirsutum DREB1/CBFs* (hereinafter simply *CBF*s) were initially queried using published *Arabidopsis CBF* nucleotide sequences (*sensu* NCBI). As no corresponding annotated genes were found at that time, EST contigs were compiled using the top 100 ESTs corresponding to *Arabidopsis* C*BF1-3*. Three distinct contigs resulted, largely centered around the characteristic, conserved AP2 domain, though representing only a portion of the target cotton coding sequences. Secondary contigs were then derived using the initial contigs but limited to *G. hirsutum*. This extended the length of the putative cDNA sequences, and revealed the presence of potential homeologous pairs within the isolated cotton *CBF* ESTs. However, none of these contigs represented the majority of the coding regions of any cotton C*BF* as predicted by translated protein alignment of the *Arabidopsis* homologs. RACE-PCR was then employed to determine the sequence of both the 5' and 3' ends of the coding regions. Six distinct *G. hirsutum CBF* coding sequences, three homeologous pairs, with a pair-

wise similarity in excess of 90%, were revealed. As *G. hirsutum* is a tetraploid cotton species, the presence of these homeologous *CBF* pairs further strengthens the prediction that *G. hirsutum* has two distinct orthologous *CBFs* for every single *Arabidopsis CBF*. In addition to the isolation of cDNAs for the *G. hirsutum ABFs*, cDNAs for the *ABF* orthologs from *G. arboreum* and *G. raimondii* were also isolated using similar methods. These gene sequences, from Old World and New World diploid cotton, help to define which *G. hirsutum* homeolog can be attributed to the AA or DD progenitor genome, respectively.

Predicted protein structure of the Gossypium hirsutum *CBF*s

A multiple protein sequence alignment of the six *G. hirsutum* CBFs was performed in MEGA 6.06-mac together with the *Arabidopsis*, *G. arboreum,* and *G. raimondii* CBFs and then visualized in Jalview 2.9.0b2 (Fig. 14). The combined aligned length of all sequences was 325 amino acids, including gaps; the longest *G. hirsutum* CBF was found to be 227 amino acids in length, while the shortest was 217. Amino acids at 88 positions were completely conserved throughout the 15 sequences compared. A significant proportion of the completely-conserved positions centered around the AP2/ERF domain, found near the center of the protein. Each *Gossypium* CBF sequence contains the requisite alpha-helix and beta-sheets characteristic of the domain.

Phylogenetic analyses of the Gossypium hirsutum CBFs

To elucidate the phylogenetic relationships between the *Arabidopsis* and the three isolated *Gossypium* species' *CBFs*, a maximum likelihood phylogenetic tree was

constructed (Fig. 15). Anchored by *Arabidopsis ICE1*, found upstream in the *CBF*

signaling pathway, clear *Gossypium* AA and DD genome relationships were defined in a

one-to-one fashion. However, no correlations were resolved between the *Arabidopsis* and

Gossypium CBF sequences. A second phylogenetic tree was derived from a wider array

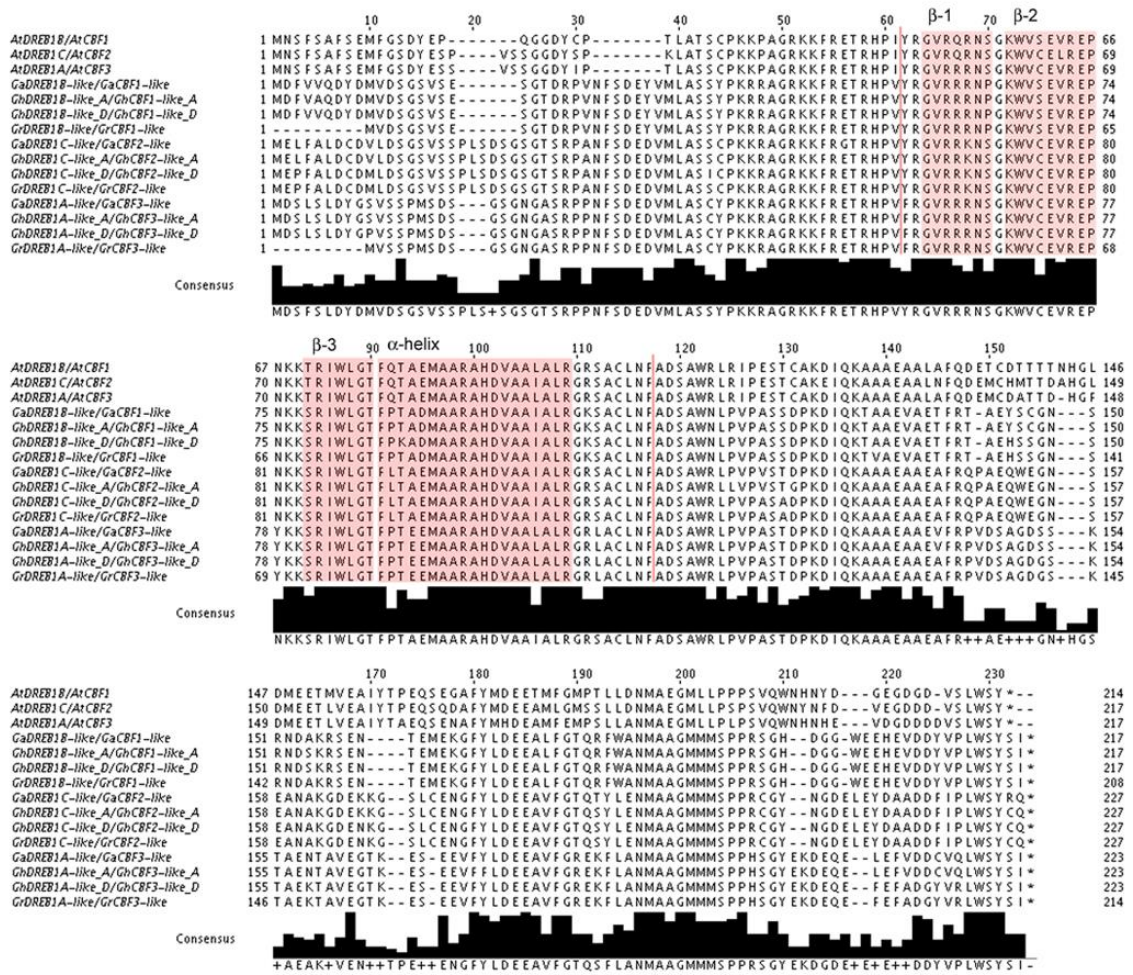of plant species in an attempt to resolve these relationships (Fig. 16).

Figure 14. Multiple sequence alignments of the *Arabidopsis, G. arboreum, G. hirsutum,*

and *G. raimondii CBFs.* The consensus histogram defines the degree of conservation at

each site. The highlighted sites correspond to the alpha-helix and beta-sheets of the

AP2/ERF domain.
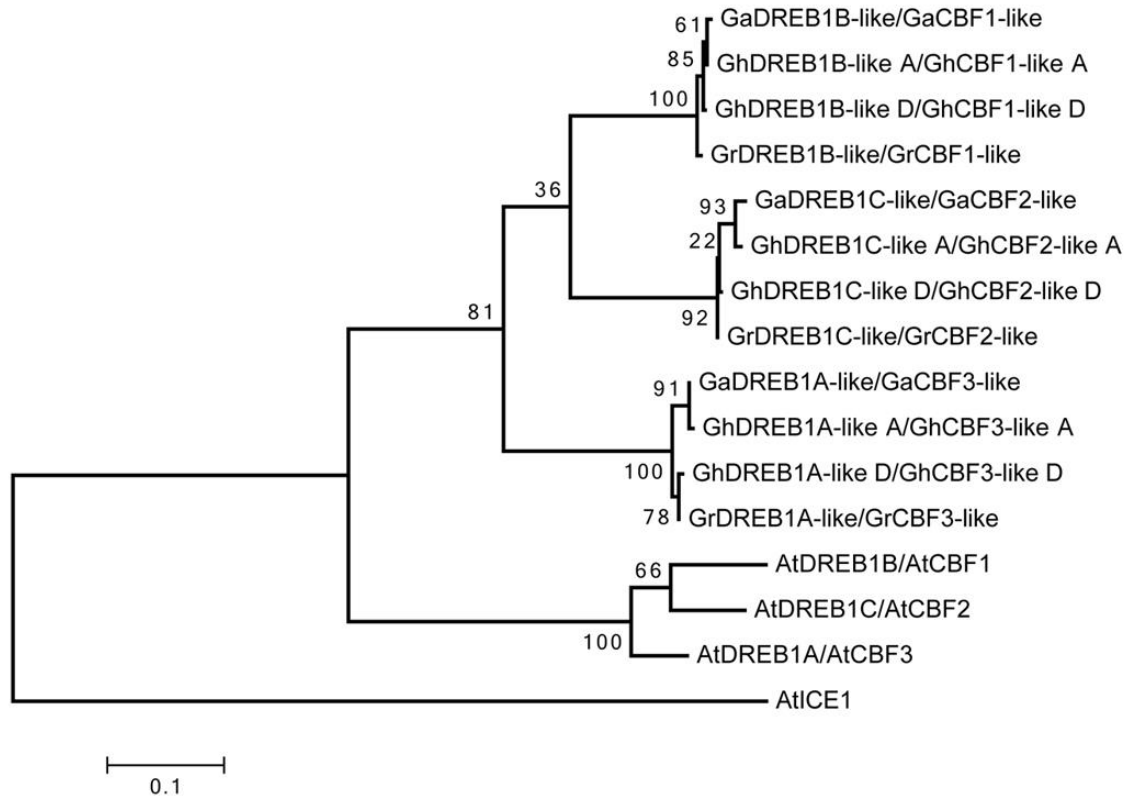
Figure 15. Maximum likelihood tree of the *Arabidopsis, G. arboreum, G. hirsutum,* and *G. raimondii CBFs*; 250 bootstrap replications.

Again, this analysis did not resolve the *Arabidopsis/Gossypium CBF* relationships. The addition of two additional Brassicaceae species' *CBFs* (*Brassica napus, Camelina sativa*) further complicated these relationships, as even within this family one-to-one relationships were not resolved. *CBF* homologs from further distantly-related species were also included in this expanded tree (*Malus, Populus*; rosid I/Fabidae). While these orthologs did group separately from the rosid II/ Malvidae *CBFs*, they too did not resolve in a one-to-one fashion, and did not further clarify the *CBF* relationships between orders.
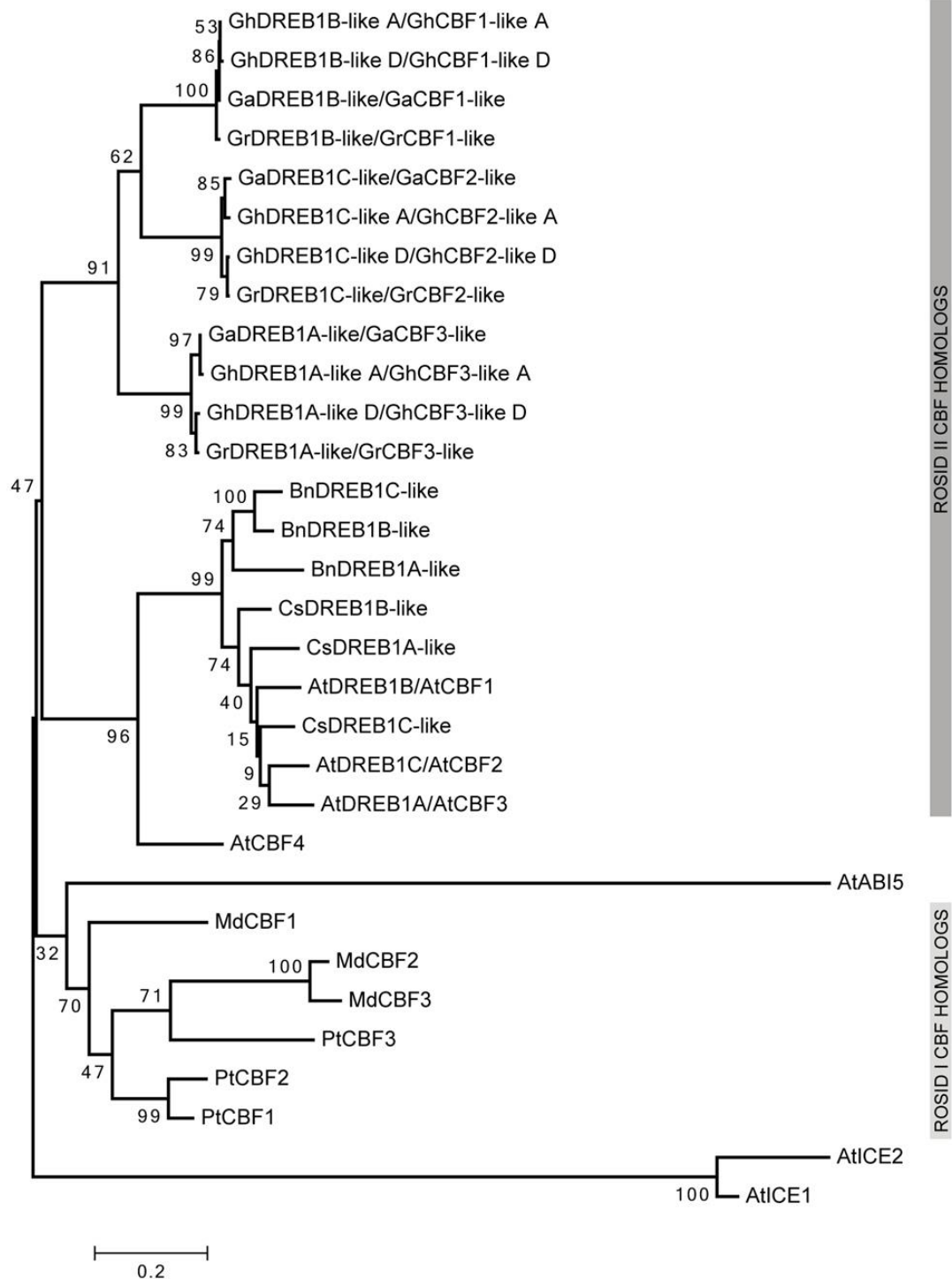
Figure 16. Maximum likelihood tree of *CBF* homologs from multiple species; 250 bootstrap replications. Rosid I and rosid II clade members are delineated on the right by grey bars.

Expression patterns of the **Arabidopsis CBFs** *in response to abiotic stressors*

Previous examination of ectopic expression of the *Arabidopsis CBFs* has shown each is differentially regulated in response to various abiotic stressors (Medina *et al.* 2011, Xiao *et al.* 2006). However, these studies describe only relative changes in expression levels. Relative expression studies, while informative, can be deceptive. For example, a ten-fold change in expression may be an increase from one to ten copies, or an increase from ten to 100 copies in response to the exogenous treatment. In order to develop a more complete understanding of the response of these genes to abiotic stress, and for a more direct comparison to *G. hirsutum*, absolute qRT-PCR expression levels were measured in response to the following stressors in *Arabidopsis*: exogenous ABA application, dehydration, and low temperature (Fig. 17).
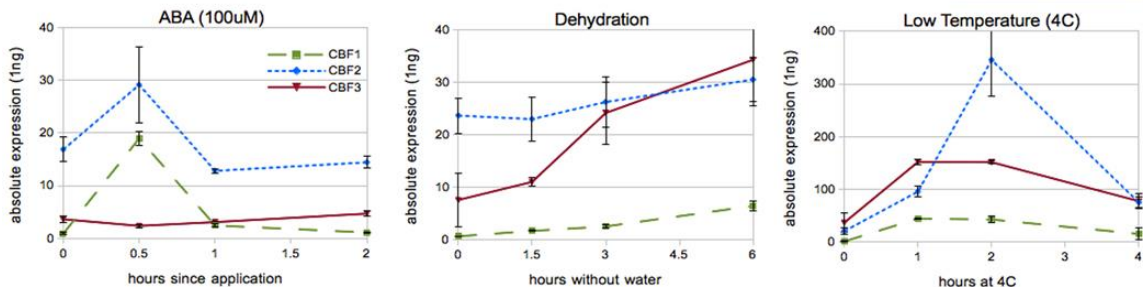

Figure 17. Expression patterns of the *Arabidopsis CBFs* in response to exogenous ABA application (100μM), dehydration, and low temperature (4°C).

While the *CBFs* are primarily implicated in the low temperature response, they have also been shown to respond to other abiotic stressors (Medina *et al.* 2011, Xiao *et al.* 2006). To determine the *Arabidopsis CBF* transcriptional response to ABA, three

week old wild type *Arabidopsis* plants grown on ½ MS media were sampled prior to application, then sprayed to the point of saturation with a 100μM ABA solution. Further samples were taken after thirty minutes, an hour, and after two hours. Following the application of 100μM ABA, *Arabidopsis CBF1* and *CBF2* expression was largely unchanged, though slight increases in both were observed after 30 minutes. *CBF3*, however, increased dramatically from about 30 copies per one nanogram of total RNA (hereafter, copies/ng) before treatment, to around 135 copies/ng at 30 minutes, before dropping off after one and two hours.

To test the absolute change in expression of the *Arabidopsis CBFs* in response to dehydration, four week old wild type *Arabidopsis* plants were sampled, then removed from soil with roots intact, and allowed to dry for one and a half, three, and six hours. In response to dehydration, expression of *CBF3* rose slightly after eight hours, roughly doubling, while expression of *CBF1* and *CBF2* showed no significant change.

Each of the *Arabidopsis CBF* were also tested for their response to low temperatures. Plants were first sampled, then exposed to 4 degrees Celsius for one, two, and four hours, with sampling at each time point. Each showed significant changes within the first hour, however, the magnitude and timing differed over the four-hour treatment period. *CBF1* expression quickly rose 40-fold in one hour from a pre-stress level of one copy/ng, and after two hours began declining. *CBF3* expression followed a similar pattern, though the initial transcript level was 20 copies/ng, and the increase was only four-fold. *CBF2* had the most dramatic rise in expression; while *CBF1* and *CBF3* expression plateaued at one hour, *CBF2* expression continued to rise sharply for two hours before declining from an initial 20 copies/ng to a peak of 345 copies/ng.

*Expression patterns of the* **Gossypium hirsutum CBF** *genes in response to abiotic stress*

Phylogenetic analyses defined the AA and DD genome origins of the *G. hirsutum* homeologous *CBF* pairs, but it did not resolve the relationship of the orthologous *Arabidopsis* and *G. hirsutum* genes. Therefore, assignation of the *G. hirsutum CBFs* in a one-to-one fashion to the *Arabidopsis CBFs* necessitated an alternate measurement. Thus, each of the *G. hirsutum* homeologs were exposed to various abiotic stressors similar to the *Arabidopsis* treatments. Their absolute expression was then quantified from standard curves derived from their corresponding construct plasmids similar to the *Arabidopsis CBFs*. Time-zero expression levels were not uniform across the examined stress treatments due to biological variation, however, they were similar (Fig. 18).

To determine the response of the *G. hirsutum CBF* genes to ABA, four-week-old plants were sprayed with a 1mM ABA solution and sampled over a two-hour period. Within each homeologous gene pair, a differential expression response was exhibited. In addition, between paralogs there were substantial differences in the magnitude of expression change (Fig. 18a). In response, the *CBF1-like A* homeolog expression quickly rose, peaking at one hour after a 19-fold rise from an initial copy level of 8/ng. However, expression of its homeolog *CBF1-like D* showed little change. This pattern was mirrored by the *CBF3-like* homeologs, however, the magnitude of expression change was roughly three times greater. The *CBF2-like D* and *CBF2-like A* homeologs each showed a significantly higher basal level of expression, 100 and 432 copies/ng, respectively, as compared to the other *G.hirsutum CBFs*. Each also rose substantially over the two hour treatment period.
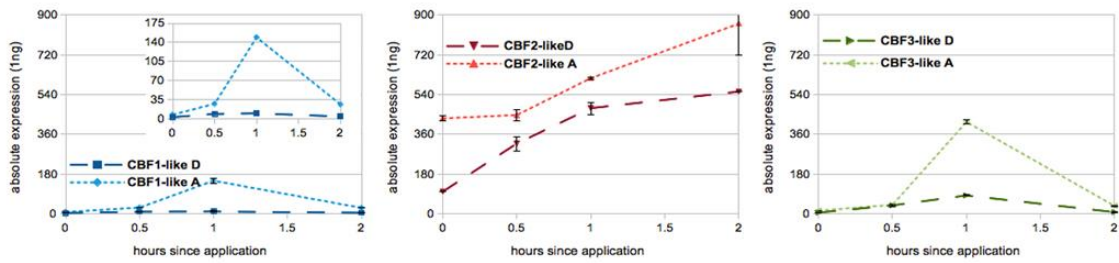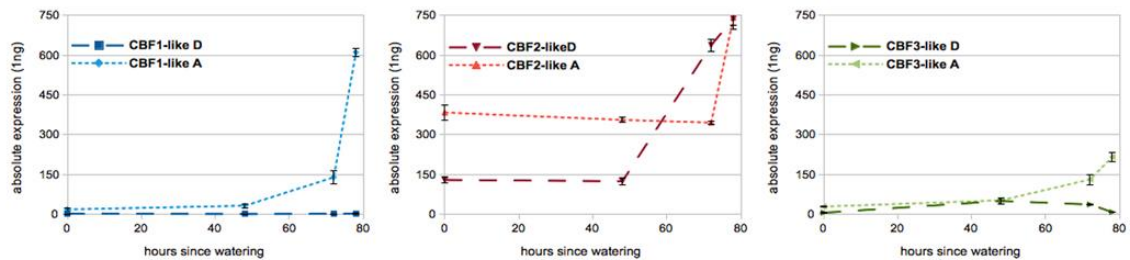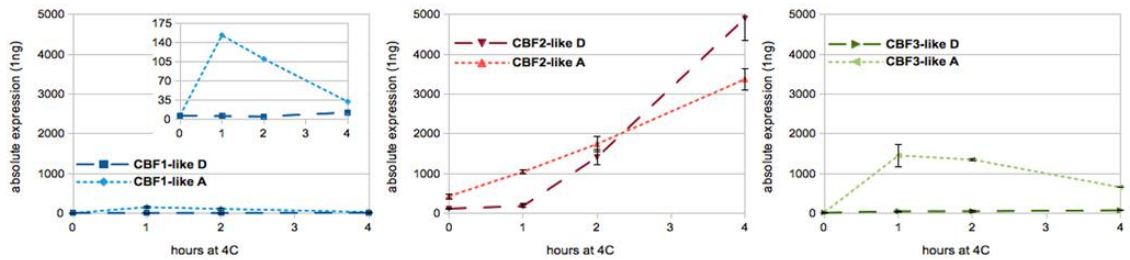
Figure 18. Absolute expression of the *G. hirsutum CBF* homologs in response to abiotic stress. (a) Exogenous ABA application (1mM) over two hours. (b) Dehydration over multiple days to the permanent wilting point. (c) Low temperature (4°C) over 4 hours.

Significant changes in expression of most *G. hirsutum CBF homologs* were also observed in response to dehydration (Fig. 18b). Expression of the *CBF1-like D* homeolog rose substantially when the plants were experiencing extreme water deficit, while the *CBF1-like A* homeolog's expression remained unchanged. The expression of the *CBF3-like D* homeolog also increased under extreme water deficit, however, not nearly to the

extent of *CBF1-like D*. The expression of *CBF3-like A* did increase slightly with mild

dehydration, however, expression declined when this stress became extreme.

While differential responses were observed in the *G. hirsutum CBFs* in response

to exogenous ABA application and dehydration, the magnitude pales in comparison to

the expression change for some of the *CBFs* in response to low temperature (Fig. 18c).

The *CBF1-like* homeologs, in response to low temperature, are dissimilar in their

expression response to exogenous ABA, with *CBF1-like D* rising substantially, while

CBF1-like A shows little change. Expression of the *CBF3-like D* homeolog increased

four-fold over the treatment period, while the *CBF3-like A* homeolog's expression

increased 55-fold, from an initial copy number of 26/ng, to 1453 copies/ng after one

hour, before plateauing and then declining after two hours. Expression of the *CBF2-like*

homeologs, taking into considering their high basal copy number, rose consistently for

each time point tested. After four hours at 4 degrees Celsius, *CBF2-like A* had an average

expression of 3372 copies/ng, and *CBF2-like D* had an average of 4903 copies/ng.

Functional analysis of the **Gossypium hirsutum CBFs** *expressed in* **Arabidopsis**

In order to investigate the function of the cotton *CBF* genes, all six of the *CBF*

orthologs isolated from *G. hirsutum* were ectopically expressed in stable transgenic

Arabidopsis plants under the control of the strong constitutive cauliflower mosaic virus

35S (CaMV35S) promoter. Plants were transformed using the floral dip transformation

method (Clough & Bent 1998). A minimum of ten independent transgene-expressing

lines were obtained for each *G. hirsutum CBF* gene construct. The level of ectopic

expression in each plant was determined by relative qRT-PCR. Three lines were chosen

for functional testing for each construct representing high, low, and median expression as per the relative qRT-PCR results. The vast majority of lines were fertile, and T3 or T4 generation plants were subsequently used to test the functional effects of ectopic expression on development, dehydration tolerance, and freezing tolerance.

Ectopic expression of the *Arabidopsis CBFs* in *Arabidopsis* has been shown to delay growth and reproduction (Gilmour *et al.* 2000, Gilmour *et al.* 2004, Zhou *et al.* 2011). Allen (2012) ectopically expressed the *Arabidopsis CBF3* homolog in cotton under the control of the *Arabidopsis* APX promoter, reporting no significant delay in reproduction, and slightly improved dehydration tolerance. Therefore, all *G. hirsutum CBF* homologs were examined not only for an increase in freezing tolerance, but also for developmental abnormalities and increased dehydration tolerance.

The primary physiological test of the functional effects of ectopic expression of the *G. hirsutum CBF* orthologs in *Arabidopsis* was to determine the extent to which this ectopic *G. hirsutum CBF* expression affected seedling growth. The three lines selected for each of the ectopically expressed *G. hirsutum CBFs* were sown on plates containing artificial media alongside wild type *Arabidopsis*, and monitored for any difference in early growth rate for three weeks (Fig. 19). No substantial differences in development were observed between the *Arabidopsis* ectopically expressing the *G. hirsutum CBF* homologs and wild type.

The examination of the development of *G. hirsutum CBF* transgenic lines on plates was confined to early differences prior to the reproductive transition; therefore, to determine if there was any delay in this transition, each of the three lines being examined for every *G. hirsutum CBF* were also grown in soil alongside wild type plants (Fig. 20).
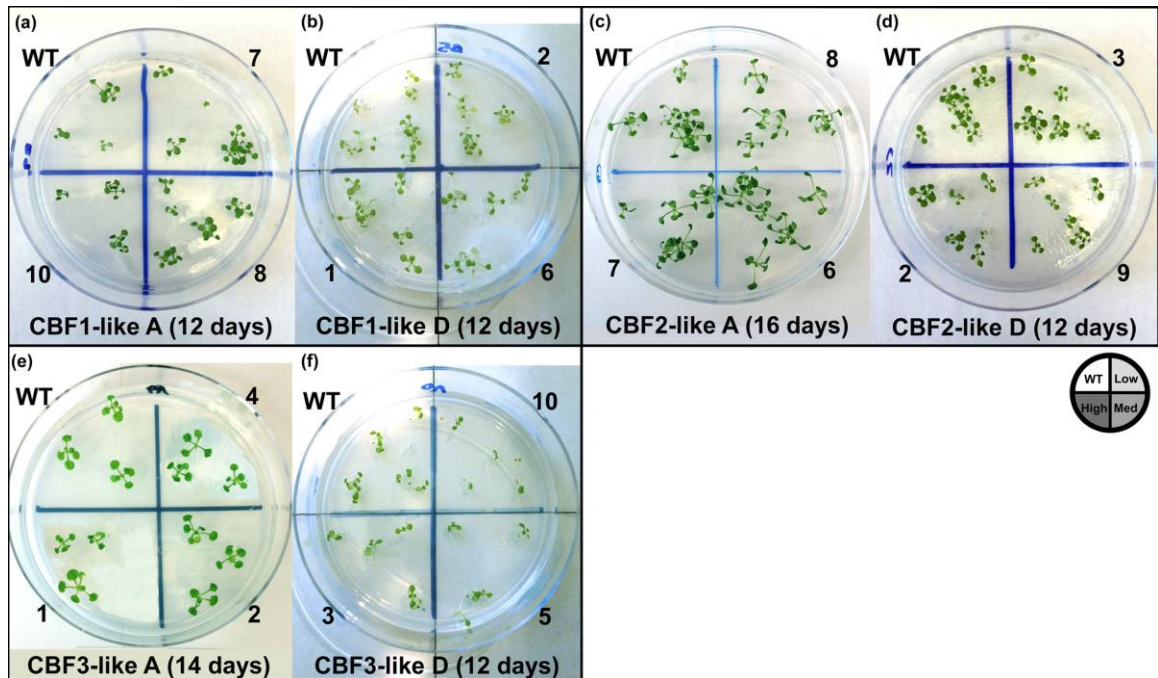
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Figure 19. Development of *Arabidopsis* ectopically expressing the *G. hirsutum CBF* homologs on media. Each homeologous pair (CBF1-like (a,b), CBF2-like (c,d), and CBF3-like (e,f,)) was plated and monitored for any difference in growth from wild type over three weeks. All plates were divided into quadrants, with wild type in the upper left. Clockwise from wild type are the low, median, and high ectopically-expressing lines. Days since sowing are noted for each.

The effects of ectopic expression of the *G. hirsutum CBFs* in *Arabidopsis* on the reproductive transition were not consistent across all genes or lines, however, the high expressing lines, for each homeolog, were delayed. Three pots for each line were monitored, each containing an average of four plants; representative plants are illustrated in Figure 20. The developmental impact of the low- and median-expressing lines for all homologs was mixed: the *CBF1-like* homeologs were no more than a day delayed from wild type, as with the *CBF2-like A* homolog (Table 2). The *CBF2-like D* low- and

median-expressing lines alternatively, bolted before the wild type plants. The *CBF3-like*

low and median lines either bolted at the same time as wild type, or one to three days

later. The highest expressing lines for each *CBF* were all delayed three to seven days.
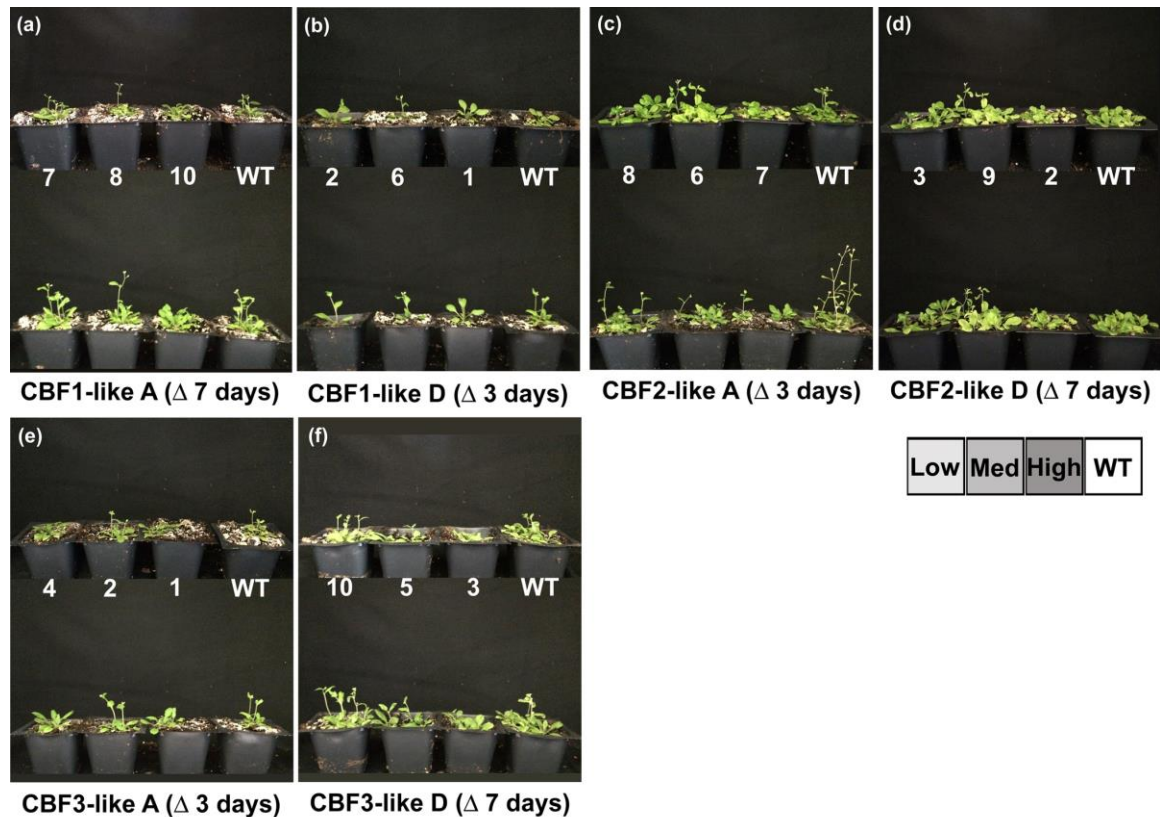

Figure 20. Development of *Arabidopsis* ectopically expressing the *G. hirsutum CBF*

homologs in soil. Each homeologous pair (CBF1-like (a,b), CBF2-like (c,d), and CBF3-

like (e,f,)) was grown in soil for three to six weeks and monitored for the transition to

reproduction (bolting). All images are ordered with the lowest ectopically-expressing line

to the left, then the median- and high- expressing lines, with wild type on the right. Days

between photographs are indicated.

Though the *CBFs* are primarily implicated in freezing tolerance, *Arabidopsis*

plants that ectopically expressed each *G. hirsutum CBF* were examined for increased

dehydration tolerance as previous studies have indicated that the ectopic expression of the

CBF genes can improve dehydration tolerance (Allen 2012, Zhou *et al.* 2011). An

average of ten plants per plate from each of the transgenic lines and wild type were

grown on artificial media for three weeks before being removed from the media and

allowed to dry for a minimum of four hours, to a maximum of six and a half hours, in

half-hour intervals, then re-watered. After 48 hours' recovery, all plants, at all time

intervals, were examined for survival. Results were recorded as surviving plants over

total plants per plate, for each time interval (Fig. 21).

Table 2. Effect of ectopic expression of the *G. hirsutum CBF* homologs in *Arabidopsis*
on the reproductive transition. Average values represent the difference in day of bolting
from wild type over three pots, containing an average of four plants each.

Gh homolog	Ectopic expressing lines					
	Low		Median		High	
	Ave	SD	Ave	SD	Ave	SD
CBF1-like A	0.08	0.79	0.75	0.75	4.75	1.36
CBF1-like D	0.75	0.62	0.83	0.58	3.50	0.90
CBF2-like A	0.67	0.65	0.33	0.78	4.83	1.19
CBF2-like D	-0.50	0.90	-0.67	0.98	7.25	1.29
CBF3-like A	2.58	1.00	0.58	0.51	4.18	0.98
CBF3-like D	0.25	0.62	0.83	0.58	6.75	1.14

Dehydration treatment results were mixed; the ectopic expression of the *G.*

hirsutum CBFs in *Arabidopsis* did not increase dehydration tolerance in the majority of

lines examined. The *CBF3-like* homeologs showed the greatest improvement, though half

the lines examined were still below wild type levels. All lines for both *CBF1-like*

homeologs exhibited survival rates similar to wild type, except for the *CBF1-like D*
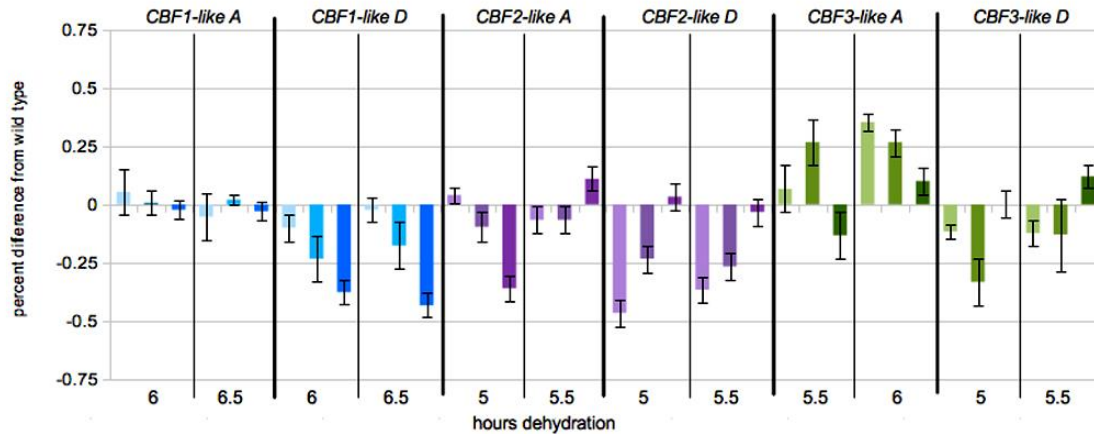
Figure 21. Percent survival of *Arabidopsis* ectopically expressing *G. hirsutum CBFs* following dehydration stress relative to wild type; lines are ordered from low- to high-expressing for each gene. Plants were removed from media and dehydrated from four to 6.5 hours, in half-hour increments, re-watered, and assayed for survival after 48 hours.

homeolog. The majority of the *CBF2-like* homeologs exhibited lower survival than their wild type comparisons. Overall, ectopic expression of the *G. hirsutum CBF* homologs was not found to significantly increase dehydration tolerance.

The *G. hirsutum CBF*-expressing lines were also examined for increased cold temperature tolerance. An average of ten plants grown in soil in petri dishes for four weeks were then transferred to -7 degrees Celsius to determine if freezing tolerance was improved in *G. hirsutum CBF*-expressing *Arabidopsis* plants. These plates were then removed from freezing temperatures at half-hour intervals after a minimum of three hours, to a maximum of six hours (Fig. 22).

The majority of lines examined performed better than wild type. All *CBF1-like* and *CBF3-like A* lines were improved, as were the majority of the *CBF2-like* lines. As observed in other treatments, a linear improvement in tolerance from low- to high-

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expressing lines was not always the case, however, overall improvements in freezing

tolerance were observed in the majority of lines.
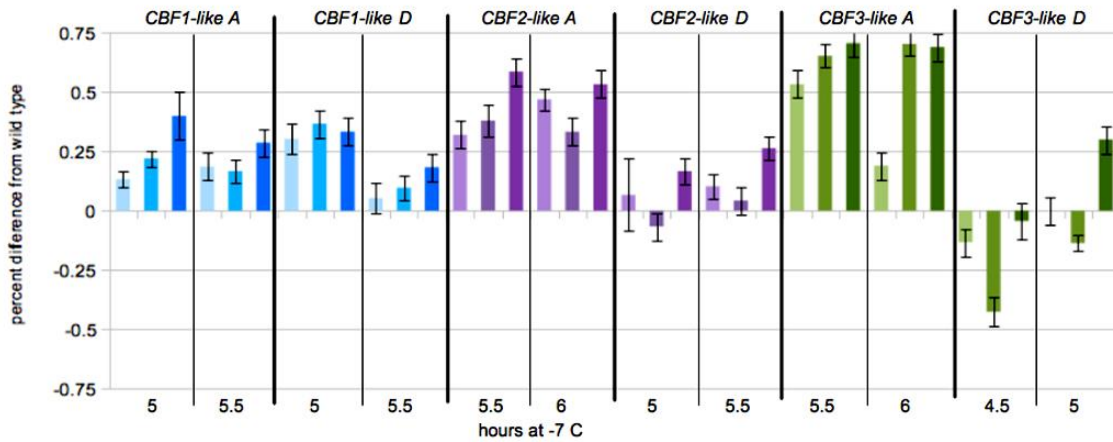

Figure 22. Percent survival of *Arabidopsis* ectopically expressing *G. hirsutum CBFs* following low temperature stress relative to wild type; lines are ordered from low- to high-expressing for each gene. Four-week-old plants were transferred to -7°C, and removed at set intervals from three to six hours, in half-hour increments, and assayed for survival after 48 hours.

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CHAPTER V

DISCUSSION AND CONCLUSIONS

Drought, extreme temperatures, salinated soil—abiotic stressors are a perennial obstacle to agriculture (Boyer 1982, Bray *et al.* 2000). Some abiotic stressors can be mitigated, *e.g,.* dehydration stress via irrigation. Others cannot, such as unpredictable temperature extremes. When addressing drought, even irrigation has its limits, be it aquifer depletion or excessive cost. Therefore, strategies to minimize the perennial impact of abiotic stress are necessary (Nellemann 2009, Delmer 2005), and those solutions will increasingly rely on the informed manipulation and control of plant physiological responses to abiotic stress.

The profound complexity of the abiotic stress response—multiple signaling pathways, myriad genes, feedback and crosstalk—makes this a daunting challenge, and the results of the manipulation of any one component unpredictable. However, to elucidate these processes, and determine which variables play critical roles, identified elements need to be examined, at a basic level, in model species and in target agricultural species, and analyzed functionally between species to determine their role and effects, and how these may differ from species to species.

Two of the many families of abiotic stress-responsive genes, chosen for their influential roles in abiotic stress (as primarily documented in *Arabidopsis*), were examined here in *Gossypium hirsutum* in order to not only determine their influence on abiotic stress in cotton, but also to investigate how these two families compare to their homologs in *Arabidopsis*. Previous and current expression and transgenic analyses were used to compare the action of these genes endogenously; further exogenous ectopic expression was used to elucidate functionality.

Ectopic expression, compared between *Arabidopsis* and *G. hirsutum*, using previously published studies of ectopically-expressed *Arabidopsis* genes in *Arabidopsis* and ectopically-expressed *Arabidopsis* genes in *G. hirsutum*, alongside the *G. hirsutum* genes ectopically expressed in *Arabidopsis* and the *G. hirsutum* genes ectopically expressed in *G. hirsutum* described in this research, corroborates the observation that the functional effects of ectopic expression are, to a certain extent, unpredictable. The *ABFs*, described as master transcription factors (Tuteja 2007), are up-regulated in response to dehydration and other various abiotic stressors in *Arabidopsis* (Choi *et al.* 2000, Fujita *et al*. 2005, Fujita *et al*. 2011, Kim *et al*. 2004, Yoshida *et al*. 2010) and cotton. Similarly, the *CBFs* have been described as key responders to low temperature stress (Zhou *et al.,* 2011, Medina *et al*. 2011), but are also responsive to other abiotic stressors.

Isolation and phylogenetic analyses of the G. hirsutum ABFs *and* CBFs

A preliminary NCBI search for annotated *G. hirsutum ABF* or *CBF* coding sequences was unsuccessful; annotated *Arabidopsis* sequences, and some other species, however, were returned. Further searches, querying annotated *Arabidopsis* sequences,

returned small, incomplete, putative homologous sequences. Despite the importance of cotton as the most economically-important natural fiber in the world (FAO & ICAC 2011), genome sequencing and annotation has lagged behind that of other important crops, likely because of the tetraploid genome of *G. hirsutum.* Together, RACE-PCR and contig assembly revealed multiple highly similar, though consistently distinct sequences—homeologous pairs resulting from a polyploidization event. Translation and alignment to their *Arabidopsis* counterparts revealed multiple conserved regions in the *G. hirsutum ABF* and *CBF* homologs, primarily centered around functional domains. The *G. hirsutum ABFs* exhibited high conservation around putative phosphorylation sites and the bZIP DNA binding domain. The *CBFs* were highly conserved in the AP2/ERF domain region. The *G. arboreum* and *G. raimondii* isolated coding sequences further strengthened these correlations, and together confirm the predicted isolated *G. hirsutum* nucleotide sequences as *ABF* and *CBF* homologs.

Phylogenetic analyses of the isolated *Gossypium* sequences yielded unexpected results; none of the *G. hirsutum* homologs resolved, one-to-one, with the *Arabidopsis ABFs* or *CBFs.* The incorporation of additional species added further complications to the *CBF* phylogeny, while the *G. arboreum* and *G. raimondii* nucleotide sequences resolved the AA and DD origin of the *G. hirsutum* homeologs for both the A*BFs* and C*BFs.* Additional Brassicales added to the *ABF* phylogeny resolved, as expected, to the *Arabidopsis ABFs.* However, these did not change the cotton/*Arabidopsis* relationships. Addition of *Malus, Populus,* and *Prunus* homologous sequences, even more distantly-related, also did not clarify the relationships.

The *G. arboreum* and *G. raimondii* sequences resolved the *CBF* AA and DD *G.*

hirsutum homeologs as with the *ABFs.* Though, again, the *Gossypium* and *Arabidopsis* sequences did not resolve. The addition of more Brassicales not only did not improve the aforementioned relationships, but did not even resolve in a one-to-one fashion. Addition of rosid I/Fabidae *CBF* homologs, as with the *ABFs,* did not resolve the target species.

Arabidopsis ABF *and* **CBF** *expression in response to abiotic stress*

Previous studies examining the relative expression of the *Arabidopsis ABFs* in response to abiotic stress do not agree, and the absolute expression results presented here add additional complexity. In response to exogenous ABA, this study did not show any significant change in *ABF1* expression, agreeing with Fujita *et al.* (2005), though Choi *et al.* (2000) and Yoshida *et al.* (2015) both indicate a relative rise in expression. Choi *et al.* (2000) did not report any change in expression for any *ABF* in response to dehydration except for *ABF4.* These results, and those of Fujita *et al.* (2005) and Yoshida *et al.* (2015), show a significant increase in expression for *ABF2-4.* Medina *et al.* (1999) and a review by Medina *et al.* (2011) describe a relative increase in *CBF1-3* expression in *Arabidopsis* in response to low temperature, however, Medina *et al.* (1999) reported no response to ABA or dehydration. While these results agree with these previous low temperature examinations, they show *CBF1* and *CBF2* did respond to ABA, though only briefly before returning to basal levels, and *CBF3* exhibited a definite rise in response to dehydration. These examples, though not comprehensive, illustrate not only differing results from previous studies, but how absolute expression can agree with relative expression examinations at face value, while at the same time the results may be contradictory. For example, a ten-fold relative expression change is the same for a

transcript that increases from one to ten copies, or another that increases from ten to 100 copies. A relative two-fold increase may indicate an increase from 100 to 200 copies, while at the same time, a five-fold increase indicates a change in copy from ten to 50.

Cotton* ABF *and* CBF *expression in response to abiotic stress

As with the *Arabidopsis ABFs* and *CBFs,* all of the cotton *ABF* and *CBF* homologs were examined for expression change in response to exogenous ABA, dehydration, and low temperature. While there are correlations between dehydration and the *ABFs*, and low temperature and the *CBFs*, many members of both gene families respond to all of these stressors, though to vastly differing magnitudes.

The *G. hirsutum ABF* and *CBF* homeologous pairs display significant differential expression in most cases; these results are not surprising due to the tetraploid nature of *G. hirsutum.* Basal copy number varied slightly across treatments due to biological variation, and the AA genome homeologs, on average, are three-fold higher than the corresponding DD genome homeologs, however, this was not necessarily a predictor of expression change in response to abiotic stress. As the *ABF* and *CBFs* did not resolve one-to-one phylogenetically, assigning names to the *G. hirsutum* homologs was based on expression patterns as compared to *Arabidopsis.* The response to dehydration was used primarily for the *ABFs,* and the response to low temperature was used primarily for the *CBFs.* This method was not perfect due to differential expression and the response to multiple stressors, however.

The ABA receptors and the pathway to *ABF* activation have been described (Fujii *et al.,* 2009), defining the association of ABA to the *ABFs.* As in *Arabidopsis*, many of

the *G. hirsutum ABF* homologs responded to exogenous ABA application, and the

magnitude of change was substantial across the eight *G. hirsutum* homeologs, a common

theme among the majority of the *G. hirsutum ABFs* in response to stress treatments. The

ABF3-like A homeolog exhibited the largest absolute change in response to 1mM ABA

application, from 22 to 666 copies per one nanogram of RNA (copies/ng). This compared

to *ABF1-like* and *ABF2-like* homeologs, though having a lower basal copy number (on

average seven copies/ng), where the largest increase in expression was only to 42

copies/ng (*ABF1-like D*). These results illustrate that each of the four homeologous pairs

respond differently to stress, and at least at the expression level, are not redundant. More

interesting are the differences in expression within the homeologous pairs. As noted, the

ABF3-like A homeolog had the largest absolute change, however, the *ABF3-like D*

homeolog, while still expressing a larger absolute increase than the majority of the others,

only rose from 10 to 163 copies/ng. This pattern of differential expression within pairs is

seen in all of the homeologous pairs in response to abiotic stress. Additionally, many *G.*

hirsutum ABF homologs either plateaued or began to decrease their rate of increase an

hour after ABA application.

In response to dehydration, there were similar patterns of differential expression

change between the four homeologous pairs, and also within these pairs. *ABF3-like A* and

ABF3-like D both began to show a significant rise in expression between 48 and 72 hours

of drying, rising to 566 and 165 copies/ng, respectively. While the majority of the

remaining homologs also increased in expression as the dehydration stress became more

severe, none had an absolute rise comparable to the *ABF3-like* homeologs. The low

temperature test had the smallest absolute change of the three treatments: *ABF1-like A*

peaked after one hour at 4 degrees Celsius from 7 to 130 copies/ng, but fell dramatically by two hours. The *ABF2-like* and *ABF3-like* homologs also rose in response to low temperature, but to a lesser degree, and either plateaued or began to decline by two hours.

Importantly to note, all of the *G. hirsutum ABFs* responded to various abiotic stressors, and the expression patterns for each was unique. The absolute magnitude of change, though comparable for the ABA and dehydration treatments, was at least four-fold lower for the low temperature treatment, indicating a lesser role for the *ABFs*. In comparison to the *Arabidopsis ABFs*, and across all treatments, there were a few trends, though overall, due to their divergence and unresolved phylogenetic relationships, and despite attempts to match orthologs based on expression, all of these genes respond in a unique fashion. The cotton *ABF1-like* homeologous pair is relatively quiet in response to ABA and dehydration, though *ABF1-like A* spikes early in in response to low temperature. Though on different scales, the *Arabidopsis ABF1* homolog also exhibits minimal change for all treatments. The *ABF2-like* homeologs do respond to the treatments, however, relative to other cotton *ABFs*, their absolute change in expression is also minimal. If scaled, the *Arabidopsis ABF2* homolog also shows little change. The *ABF3-like* and *ABF4-like* homeologs rise significantly in response to ABA, though the *ABF4-like A* homeolog drops off before the others. As described, the *ABF3-like* homeologs also rise significantly in response to dehydration, but the *ABF4-like* homeologs do not, in contrast to their response to ABA. *Arabidopsis ABF3* and *ABF4* rise significantly in response to dehydration.

Relatively small expression changes may seem insignificant when compared to homologs with large expression changes, though this is not necessarily true. Ectopic

expression of the *Arabidopsis ABF2* homolog in *Arabidopsis* confers increased

dehydration tolerance (Choi *et al.* 2000, Fujita *et al*. 2005, Yoshida *et al*. 2015), though

these results indicate a relatively minor increase in transcript levels; of course, the

ectopically-expressed transcript may be many times higher.

These overall observations also apply to the *CBF* expression analyses in

Arabidopsis and *G. hirsutum.* The *G. hirsutum CBF* homeologs, as with the *ABFs*, all had

differential responses to the various abiotic stress treatments; however, unlike the *ABFs,*

whose basal expression levels ranged from one to 22 copies/ng, the *CBF2* homeologs had

ten to 40 times the basal level of the *CBF1* and *CBF2* homeologs, which had an average

pre-treatment level of 7 to 16 copies/ng. The *CBFs* are primarily associated with low-

temperature tolerance, and are ABA independent (Yoshida et al. 2014), however, these

results show that *Arabidopsis CBF1* and *CBF2* both have a significant spike in

expression in the first half hour after exogenous ABA application, though they return to

basal levels soon after. At least one homeolog from each *G. hirsutum CBF* also increases

in response to exogenous ABA; *CBF1-like A* and *CBF3-like A* both peak one hour after

application, then drop to pre-treatment levels by two hours, while both *CBF2-like*

homeologs continually increased over the treatment period.

In response to dehydration, only the *Arabidopsis CBF3* homolog rose

substantially. *G. hirsutum CBF1-like A* and *CBF3-like A* both increased as well, though

with a large difference in absolute magnitude. However, both *CBF2-like* homeologs had

the highest expression level at the last time point sampled, when the plants were

experiencing extreme wilt, though they did not have the largest absolute change. Low

temperature stress also induced the expression of *CBF1-like A* and *CBF3-like A* within an

hour, with no change in their respective homeologous partner, however, their expression began declining after this first hour. *CBF2-like A* and *CBF2-like D* also began to rise after an hour, and their absolute expression levels, by four hours, dwarfed the absolute levels of all of the genes examined in all abiotic stress tests analyzed here at 3372 and 4903 copies/ng, respectively.

As stated, study of the *CBFs* has primarily focused on cold tolerance, however, these results show that there are significant changes in many of the homologs, in both *Arabidopsis* and *G. hirsutum,* in response to ABA and dehydration as well, though the absolute change in expression is greatest in response to low temperatures. Interestingly, the *G. hirsutum CBF1-like* and *CBF3-like* homeologous pairs have nearly identical patterns of expression, though to different scales, to both ABA and low temperature, and the *CBF2-like* homeologs respond substantially to all stressors examined.

Overall, all of the *ABF* and *CBF* homologs examined respond to varying extents to the abiotic stressors examined. While the *ABFs* respond to ABA and dehydration, so too do they respond to low temperature. While the *CBFs* respond to low temperature, so too do they respond to ABA and dehydration. This said, the absolute copy number, and the absolute change in copy number, must be considered in terms of scale such that the relatively smaller changes are not discounted. Finally, no iteration of the *Arabidopsis* and *G. hirsutum ABF* and *CBF* homolog expression profiles can be directly matched, indicating that, since the divergence of their respective orders, and the polyploidization of *G. hirsutum*, all of these genes have acquired unique roles in the abiotic stress response, though often these roles may overlap.

***Ectopic expression of the* G. hirsutum ABF*s and* CBF*s in* Arabidopsis**

Ectopic expression and mutant examination, complimented by gene expression analyses, and coupled to tests of the traits being studied, is a primary method for determining the functionality of a gene. Ectopic expression of the *Arabidopsis ABFs* and *CBFs,* have revealed, to varying degrees, developmental defects, yet at the same time increases in dehydration and low temperature tolerance, respectively (Fujita *et al.* 2013, Medina *et al.* 2011, Yoshida *et al.* 2015, Zhou *et al.* 2011). Ectopic expression of *Arabidopsis* homologs in *G. hirsutum*, have also shown developmental delays, while increasing dehydration tolerance (Allen, 2012). The results presented here of the ectopic expression of the *G. hirsutum ABFs* and *CBFs* in *Arabidopsis,* and a *G. hirsutum ABF* ectopically-expressed in *G. hirsutum* indicate developmental differences along with improvements in abiotic stress tolerance. Together, all of these results illustrate the functions of these important abiotic stress-responsive gene families both within and between these species. While each gene tested has its own unique expression pattern and physiological effects, overall, improvements in dehydration and low temperature tolerance can be achieved; however, the extent of these improvements may be countered by delays in development.

Each cotton *ABF* and *CBF* homolog was ectopically expressed in *Arabidopsis* to determine their effect on development, dehydration tolerance, and cold tolerance. While weeks of delay in reproduction were reported for some ectopically expressed endogenous *Arabidopsis ABFs,* the largest average delay in the reproductive transition of any of the ectopically-expressed *G. hirsutum ABFs* was five days. The largest average delay in the reproductive transition of any of the ectopically-expressed *G. hirsutum CBFs* was eight

days.

The observations of growth rate for the vast majority of the three ectopically-expressing lines examined for each gene, on artificial media, showed no substantial difference. The few differences were minor, and disappeared over time. These results indicate that over-expression of these cotton genes in *Arabidopsis* may have a small effect on early development, but over time, these differences are insignificant. However, when the time of bolting as compared to wild type was examined, substantial differences were observed for many of the lines examined. All *ABF3-like* and *ABF4-like* lines were delayed at least one day. *ABF2-like D* lines also showed a delay. The most significant delay was five days. In comparison to the *ABF-like* expressing lines, which can be, for the most part, separated by gene for reproductive delay, the *CBF-like* expressing lines largely diverge along the low/median/high-expressing lines. All of the *CBF* high-expressing lines were four to eight days delayed. Ectopic expression, as previously reported, of endogenous *Arabidopsis* genes in *Arabidopsis* shows reproductive delays (Fujita *et al.* 2013, Medina *et al.* 2011, Yoshida *et al.* 2015, Zhou *et al.* 2011). These delays, while predicted, strengthen the observation that, whether endogenous or exogenous, ectopic expression to improve an abiotic stress-related trait, has a tradeoff.

Ectopic expression of the *G. hirsutum ABFs* and *CBFs* in *Arabidopsis* improved dehydration tolerance and low temperature tolerance, respectively. While keeping in mind the aforementioned described tradeoffs, the majority of the *ABF-like* over-expressing lines exhibited substantially increased dehydration tolerance. The most improved lines, the *ABF3-like* lines and *ABF4-like* lines, also had the biggest developmental delays. The *ABF1-like* and *ABF2-like* lines on average displayed a more

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equal transition to reproduction, though overall were not as dehydration tolerant. The majority of the *CBF-like* over-expressing lines exhibited substantially increased freezing tolerance. The majority of these lines were the high-expressing lines, which also displayed the largest delays in the reproductive transition. While respective improvements in abiotic tolerance were observed, the *ABF* homologs seem to correlate by gene, while the *CBF* homologs seem to relate to the level of expression. Overall, the increase of abiotic stress tolerance appears to imply a larger delay in reproductive development.

Ectopic expression of a **G. hirsutum ABF** *gene in* **G. hirsutum**

As described by Allen (2012), the ectopic expression of the *Arabidopsis ABF3* homolog in *G. hirsutum* caused substantial reproductive delay, at the same time resulting in a substantial increase in dehydration tolerance. However in the current study, ectopic expression of the *G. hirsutum ABF2-like D* homolog in *G. hirsutum* yielded an increase in dehydration tolerance, with no substantial delay in reproductive development.

The three lines ectopically-expressing the *G. hirsutum ABF2like D* homolog under the control of the CaMV35S promoter examined for development and dehydration tolerance initially exhibited a slight retardation in growth. However, after one to two months' growth (in controlled conditions), no substantial ultimate differences were observed. Continuous monitoring of development showed a minimal difference in the timing of the transition to reproductive development, and no more than a week's difference was recorded in the reproductive transition to flowering. Dehydration tolerance, however, was substantially improved in in all but the lowest-expressing line,

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which was similar to wild type. The median- and high-expressing lines, on average, recovered three days after their wild type counterparts had reached their permanent wilting points. Though the high-expressing line had an unstressed expression level twice that of the median-expressing line, dehydration tolerance was not substantially different, possibly indicating saturation of the pathway and downstream genes. These results, examined under controlled conditions, need to be further examined in field conditions, though they do indicate the possibility that dehydration tolerance can be improved with minimal negative impacts on development.

Conclusions

Here, both the *G. hirsutum ABFs* and *CBFs* were isolated, matched to their AA or DD progenitor genomes, phylogenetically compared to their *Arabidopsis* homologs, expressionally compared to their *Arabidopsis* homologs through various abiotic stress treatments, and heterologously ectopically expressed in *Arabidopsis* to elucidate their function. Gene expression was increased in response to dehydration and low temperatures, in a mixed fashion, for both the *G. hirsutum ABFs* and *CBFs.* Functional analyses revealed a delay in the reproductive transition, correlated either with the gene ectopically expressed, or the level of expression. This correlation was also observed with increased abiotic stress tolerance. Ectopic expression of the *G. hirsutum ABF2-like D* homolog in *G. hirsutum* also produced minor developmental delays but increased dehydration tolerance. Ectopic expression of endogenous *Arabidopsis ABFs* and *CBFs* improved their target traits. Exogenous ectopic expression of *Arabidopsis* in *G. hirsutum* improved the target trait. Exogenous expression of *G. hirsutum* in *Arabidopsis* improved

the target traits. Ectopic expression of an endogenous *G. hirsutum ABF* improved the

target trait. All of these improvements, however, were accompanied by varying negative

consequences in development. Therefore, compromises may be necessary to balance

minor delays in reproductive development with improvements in abiotic stress tolerance.

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