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ORIGINAL PAPER

Yan Sun · Randy D. Allen

Functional analysis of the *BIN2* genes of cotton

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Abstract Brassinosteroids (BR) promote the elongation of cotton fibers and may be a factor in determining their final length. To begin to understand the role of BR-mediated responses in the development of cotton fibers we have characterized the *BIN2* genes of cotton. BIN2 is a member of the shaggy-like protein kinase family that has been identified as a negative regulator of BR signaling in Arabidopsis. Sequence analyses indicate that the tetraploid cotton genome includes four genes with strong sequence similarity to *BIN2*. These genes fall into two distinct subclasses based on sequence and expression patterns. Sequence comparisons with corresponding genes from cotton species that have the diploid A and D genomes, respectively, shows that each pair of genes comprises homeologs derived from the A and D subgenomes. Transgenic Arabidopsis plants that express these cotton *BIN2* cDNAs show reduced growth and similar phenotypes to the semi-dominant *bin2* mutant plants. These results indicate that the cotton *BIN2* genes encode functional BIN2 isoforms that can inhibit BR signaling. Further analyses of the function of BIN2 genes and their possible roles in determining fiber yield and quality are underway.

Keywords BIN2 · Brassinosteroid · *Gossypium hirsutum* · Protein kinase · Signal transduction

G. Jürgens

Y. Sun
Department of Biological Sciences,
Texas Tech University, Lubbock
TX, 79409-3131, USA

R. D. Allen (✉)
Department of Plant and Soil Sciences,
Texas Tech University, Lubbock
TX, 79409, USA
E-mail: randy.allen@ttu.edu
Tel.: +1-806-7422711
Fax: +1-806-7422963

Introduction

More than 14 billion kilogram of cotton fiber are produced annually world-wide. Cotton fibers are seed trichomes that undergo rapid and extensive elongation and can typically reach lengths of 30 mm. Phytohormones, including brassinosteroids (BR), are critical regulators of cotton fiber development. Analysis of fiber development on cotton ovules grown in vitro in liquid medium indicates that exogenous auxin and gibberellic acid (GA) are required for maximal fiber elongation (Beasley and Ting 1974). Likewise, BR treatments increase fiber growth both in cultured ovules and in planta (Ashcraft 1996; Kasukabe et al. 2000). Furthermore, the brassinosteroid biosynthesis inhibitor brassinazole (Asami et al. 2000; Sekimata et al. 2001) strongly inhibits fiber development in ovule culture (Sun et al. 2004). These results indicate that BR plays an important role in promoting cotton fiber initiation and elongation. Therefore, to better understand the role of BR in cotton development, we have undertaken an analysis of the BR signaling pathway in this species.

Brassinosteroids are the only steroid-like growth regulators found throughout the plant kingdom that are required for normal plant development. These molecules have wide ranging effects on plants, which are similar to those of auxin in many respects. Like auxin, BR promotes growth of shoots and inhibits root elongation (Mandava 1988). BR also promotes seed germination and pollen development. Recent transcriptional profiling studies have shown that BR and auxin affect the expression of overlapping sets of genes, but genes that respond specifically to BR or auxin were also identified (Goda et al. 2004). In addition, mutant plants with lesions in genes that disrupt the BR signaling pathway generally remain responsive to auxin and vice versa (Goda et al. 2002).

Several components of the BR signal transduction pathway have recently been identified. Li and Chory (1997) described a gene from Arabidopsis, *BRASSINOSTEROID INSENSITIVE 1* (*BRI1*), which encodes a

putative membrane-associated BR receptor. *BRI1* codes for a leucine-rich-repeat receptor-like kinase (LRR-RLK) that is localized in the plasma membrane (Friedrichsen et al. 2000; Oh et al. 2000). *BRI1 ASSOCIATED RECEPTOR KINASE1* (*BAK1*) encodes an LRR-RLK distinct from BRI1 that is a component of the BR signaling pathway and may interact with BRI1 (Li et al. 2002). *BRASSINOSTEROID INSENSITIVE 2* (*BIN2*) was identified upon re-examination of BR insensitive dwarf mutants of Arabidopsis. *bin2-1* represents a semi-dominant gain-of-function mutation that causes a *bri1*-like dwarf phenotype (Li et al. 2001). Therefore, *BIN2* was proposed to encode a negative regulator of BR signaling. The protein encoded by the gene *BRASSINAZOLE-RESISTANT 1* (*BZR1*) is a positive regulator of BR signaling that acts downstream of BRI1 and BIN2. BZR1 was found to be stabilized and accumulates in the nucleus in response to treatment with BR, and in plants that carry the dominant allele *bzr1-1D* (Wang et al. 2002). *BRI1-EMS-SUPPRESSOR 1* (*BES1*) encodes a BZR1-like protein that also accumulates in the nucleus in response to BR, and its accumulation is negatively regulated by BIN2. BES1 has been shown to interact directly with BIN2 in both yeast two-hybrid and GST pull down assays, and BIN2 efficiently phosphorylates BES1 in vitro (Yin et al. 2002). *BRASSINOSTEROID SUPPRESSOR1* (*BSU1*) was recently found to encode a nuclear protein phosphatase that counteracts the action of BIN2 by dephosphorylating BES1 and stabilizing it (Mora-Garcia et al. 2004).

The BR signaling cascade is similar in many respects to the Wingless/Wnt signaling pathway of vertebrates and invertebrates (Kim and Kimmel 2000) with BIN2 acting as a central switch. Under steady-state conditions, BIN2 is active and phosphorylates BZR1 and BES1, which then become targets for degradation. Interaction with BR triggers hetero-dimerization and kinase activation of the BRI1/BAK1 receptor complex (Nam and Li 2002). Although BIN2 does not appear to be a direct target of BRI1/BAK1 (Nam and Li 2004), exposure to BR presumably leads to inactivation of this negative regulator. Reduced BIN2 activity allows hypo-phosphorylated forms of BZR1 and BES1 to accumulate and translocate to the nucleus, where they affect BR-responsive gene expression (He et al. 2002; Zhao et al. 2002).

The Arabidopsis *BIN2* (*AtBIN2*) gene encodes a member of the glycogen synthase kinase-3/SHAGGY-like kinase (GSK3) family, a highly conserved family of serine/threonine kinases found in many eukaryotes (Li and Nam 2002). Embi et al. (1980) first identified GSK3 as an enzyme that phosphorylates glycogen synthase. Members of this family often function as negative regulators of signal transduction pathways (Kim and Kimmel 2000). GSK3 was recently found to be an important and versatile switch in target discrimination and pathway insulation (Kim and Kimmel 2000). The mutant form of AtBIN2 encoded by *bin2-1* was shown to have increased kinase activity when expressed in *Escherichia coli*. This protein kinase apparently blocks BR signal transduction by hyperphosphorylation of BRZ1/BES1, leading to a pronounced BR-insensitive dwarf phenotype. On the other hand, reduced expression of BIN2 partially rescues the phenotype of the weak *bri1-5* mutant (Li and Nam 2002). Therefore, BIN2 appears to be a negative regulator of BR signaling in plants, which is constitutively active in the absence of BR and phosphorylates other BR signaling regulators, targeting them for degradation (Clouse 2002).

BRI1 homologs have been identified in rice (Yamamuro et al. 2000), tomato (Montoya et al. 2002), pea (Nomura et al. 2003) and barley (Chono et al. 2003), and we recently characterized two *BRI1* genes from cotton (*Gossypium hirsutum*; Sun et al. 2004). Here we report the functional analysis of four putative *BIN2* genes from cotton. Overexpression of each of the cotton *BIN2* genes in wild-type Arabidopsis plants resulted in severe stunting and phenotypes similar to those of strong BR-insensitive mutants, indicating they can indeed function as negative regulators of BR signal transduction. We anticipate that further dissection of the BR signaling pathway in cotton will reveal the role of BR in regulating cotton fiber development.

Materials and methods

Plant growth condition

Cotton plants [*Gossypium hirsutum* cv. Coker 312, *G. arboreum* (accession A2-347) and *G. thurberi* (accession D1-4)] were grown in potting soil in a greenhouse. Arabidopsis plants (*Arabidopsis thaliana* ecotype Ws-2) were grown under continuous fluorescent light in potting soil at room temperature.

Isolation of genomic DNA and total RNA

Cotton and Arabidopsis genomic DNA samples were isolated from leaf tissues using the DNeasy Plant Mini Kit (Qiagen, Santa Clara, CA, USA).

RNA was isolated from leaves, buds and sepals of mature cotton plants and from hypocotyls and roots of seedlings (7–10 days post-imbibition). Flowers were tagged on the day of anthesis, and bolls were harvested at 0 DPA (days post-anthesis), 5 DPA, 10 DPA and 20 DPA. Immature ovules (5–8 DPA), 20-DPA fibers, and 30-DPA fibers were also collected. Samples were ground to a powder in liquid nitrogen and stored at −80°C. Total RNA was extracted using the method of Wan and Wilkins (1994). Arabidopsis RNA was isolated from whole plant tissues using the RNeasy Plant Mini Kit (Qiagen).

Cloning of *GhBIN2* cDNAs

Two cotton ESTs (Genbank Accession Nos. AI726755 and AI731919) with high sequence homology to

Arabidopsis *BIN2* (Li and Nam 2002) were identified in the NCBI GenBank dbEST database using the BLAST program. These ESTs correspond to the 5′-end of the *AtBIN2* sequence (96–666 and 1–600) and were used as starting points to amplify *GhBIN2* cDNAs from a cotton boll cDNA library. Vector-specific primers that annealed to the lambda ZAP cDNA vector were SK-P (5′-CGCTCTAGAACTAGTGGATC-3′) and T7 (5′-GTAATACGACTCACTATAGGGC-3′). Primers B1 (5′-CTTGATAGCAACCTCGTGCC-3′) and B2 (5′-GAGTTGATGGAAGAAGACCCC-3′), derived from the putative *GhBIN2* ESTs were used as *BIN2*-specific primers. PCR was performed, and the amplified fragments were subcloned into the vector pGEM-T Easy (Cat. No. A1360; Promega, Madison, WI, USA) and sequenced for comparison with *AtBIN2*. Fragments that shared high sequence similarity were selected for the design of further primers, and PCR amplification was carried out to isolate full length *GhBIN2* cDNAs from the cDNA library. Four different *GhBIN2* cDNA clones (*GhBIN2-B*, *GhBIN2C*, *GhBIN2-D*, and *GhBIN2-E*) were sequenced to obtain the four full-length sequences.

Quantitative real-time PCR

Reverse transcription PCR (RT-PCR) assays were carried out on total RNA samples using TaqMan Reverse Transcription Reagents (Cat. No. N808-0234; Applied Biosystems, Foster City, CA, USA). Cotton RNA samples were quantified using a Nanodrop spectrophotometer. All cotton RNA samples were diluted to 100 ng/μl. A total of 0.5 μg of RNA was used for each 50-μl RT-PCR. Random hexamers were used for first-strand cDNA synthesis. Levels of the *GhBIN2* mRNAs in total RNA samples from different cotton tissues and transgenic Arabidopsis plants were quantified with an ABI (Applied Biosystems) PRISM 7000 Sequence Detection System. The *GhBIN2*-specific FAM-labeled TaqMan probes and forward and reverse primers were derived from the 3′ ends of the coding sequences, which are sufficiently divergent to distinguish between the four *GhBIN2* sequences. They were designed using ABI Primer Express 2.0 and synthesized by Applied Biosystems (their sequences are listed in Table 1). An 18S rRNA-specific VIC-labeled TaqMan probe and 18S rRNA forward and reverse primers were used for RNA normalization. The TaqMan PCR Core Reagent Kit (N808-0228; Applied Biosystems) was used for quantitative real-time PCR. Control reactions without reverse transcriptase were included to check each RNA sample for possible amplification of contaminating genomic DNA. Each of the four *GhBIN2* FAM-labeled probes (first probe), forward and reverse primers, the 18S rRNA VIC-labeled probe (second probe), 18S rRNA forward and reverse primers, AmpErase UNG (uracil-it N-glycosylase), AmpliTaq Gold DNA polymerase and other components were used in the same tube for multiplex PCR. Each RNA sample was assayed three times.

The relative expression levels of all the samples were calculated according to the method recommended in User Bulletin No. 2 for the ABI PRISM 7700 Sequence Detection System. In brief, the threshold cycle (Ct) values for *GhBIN2* mRNA and 18S RNA in different samples were obtained by quantitative real-time PCR. The Ct value for the normalizer 18S RNA was subtracted from the *GhBIN2* Ct value to obtain the dCt value of the sample. The dCt value of the calibrator sample (the sample with the highest dCt value) was subtracted from every other sample to produce the ddCt value. The relative expression levels are equivalent to $2^{-\mathrm{ddCt}}$ for each sample.

Functional analysis of *GhBIN2* cDNAs in transgenic Arabidopsis

The four *GhBIN2* cDNAs were separately inserted into pBI121 in place of the *uidA* gene. The plant gene expression cassettes were constructed as follows. The pGEM-T plasmids containing *GhBIN2-B* and *GhBIN2-D* were cleaved with *Sac*II and the ends were blunted by a 3′ fill-in reaction using T4 DNA polymerase, then digested with *Sac*I and the resulting fragments were ligated into pBI121 DNA that had been digested with *Sma*I and *Sac*I. The pGEM-T plasmids containing *GhBIN2-C* and

Table 1 Primers and probes used for real-time PCR

Gene	Primer/probe	Sequences (5′ → 3′)
GhBIN2-B	Forward primer	AAATGTCGGCCCCTGTTGT
	Probe	^{6FAM} ACGTCAATGATCCGGTTACCGGTCAC^{TAMRA}
	Reverse primer	CCTCCAATCGTTGTGGAAATG
GhBIN2-C	Forward primer	GGGCCCCGGAACTCATC
	Probe	^{6FAM} TGGTGCCACTGAATACACAACCTCCATTG^{TAMRA}
	Reverse primer	AGAACACAACCAGCAGACCAGATA
GhBIN2-D	Forward primer	GAAATGTCGGCCCTTGTTGT
	Probe	^{6FAM} ATGTCAATGATCCGGTTACCGGTCACA^{TAMRA}
	Reverse primer	CCTCCAATCGTTGTGGAAATG
GhBIN2-E	Forward primer	TCGGGCCCCAGAACTCAT
	Probe	^{6FAM} TTTGGTGCAACTGAATACACAACCTCCATTG^{TAMRA}
	Reverse primer	AGAACACAACCAGCTGACCAGATA

GhBIN2-E were digested with *Sac*II and *Stu*I, and the ends blunted by 3′ fill-in reaction using T4 DNA polymerase. The resulting fragments were then ligated into pBI121 DNA that had been digested with *Sma*I and *Sac*I, and blunted by 3′ fill-in using T4 DNA polymerase. The ligated plasmids were transformed into *E. coli*. pBI121-GhBIN2 plasmids were recovered from bacterial transformants, and transformed into *Agrobacterium tumefaciens* GV3101 (C58). *A. tumefaciens* cells containing pBI121-GhBIN2 constructs were selected on LB plates containing kanamycin (50 μg/ml), gentamycin (25 μg/ml) and rifampicin (100 μg/ml). The plasmids were isolated and the inserts were sequenced to confirm the presence and accuracy of the *GhBIN2* ORFs.

The *GhBIN2* transgenes were introduced into Ws-2 Arabidopsis plants, using the *Agrobacterium*-mediated-flower infiltration transformation method (Clough and Bent 1998). The plants in pots were placed in trays and covered with plastic wrap for 16–24 h to maintain high humidity. Plants were watered from the bottom of the tray and watering was reduced as seeds matured. Mature seeds were harvested from individual plant lines.

Arabidopsis seeds were sterilized with 75% ethanol for 1 min and 50% bleach solution for 10 min. The seeds were then rinsed with sterile water (three to five times) and plated. Transformed seedlings were selected on antibiotic-containing MS plates (250 μg/ml carbenicillin, 250 μg/ml cefotaxime, 50 μg/ml kanamycin). The PCR analysis using specific primers designed from the CaMV 35S promoter and the *GhBIN2* coding sequences were performed to confirm the insertion of the *GhBIN2* transgenes.

Results

Characterization of *GhBIN2* cDNAs

The NCBI dbEST database was screened for cotton ESTs that show sequence similarity to *BIN2* from *A. thaliana* (Li and Nam 2002), using the BLAST program. Two cotton ESTs (Genbank Accession Nos. AI726755 and AI731919) from *G. hirsutum* with high sequence identity to *AtBIN2* were identified. These ESTs, which correspond to the 5′-end of the *AtBIN2* coding sequence, were used as starting points to amplify *GhBIN2* sequences from cotton.

Primers derived from the putative *GhBIN2* ESTs were used, together with 5′ and 3′ primers based on the lambda ZAP vector, to amplify corresponding cDNAs from a cotton cDNA library. The DNA fragments obtained by PCR were subcloned, sequenced and compared to *AtBIN2*. DNA fragments that shared high sequence identity with *AtBIN2* were then used for the next amplification step. New primers were designed on the basis of the elongated DNA sequence and, after several such steps, primers were designed to amplify the complete *GhBIN2* coding sequences from the cotton cDNA library. Finally, full-length cDNAs were amplified. The amplified cDNA fragments were cloned and twelve representative cDNAs were sequenced. Each of these cDNAs includes a 1143-bp ORF that encodes a derived polypeptide of 381 amino acids—one amino acid longer than AtBIN2.

Comparisons of *BIN2* cDNA sequences from cotton and Arabidopsis using the Vector NTI Align X program showed that the ORFs of the *GhBIN2* cDNAs share between 77.7 and 79.4% nucleotide sequence identity with *AtBIN2*. Comparisons of the derived amino acid sequences of GhBIN2 proteins with AtBIN2 showed 87.4–90.6% sequence identity (Fig.1). From these alignments, it became clear that the *GhBIN2* cDNAs fell into four distinct sequence classes. Representative cDNAs for each class were named *GhBIN2-B*, *GhBIN2-C*, *GhBIN2-D*, and *GhBIN2-E*. *GhBIN2-B* and *GhBIN2-D* share 96.6% nucleotide sequence identity (including the 3′ untranslated region) and their products are 97.4% identical. Although *GhBIN2-C* and *GhBIN2-E* share only 93.4% nucleotide sequence identity, their derived amino acid sequences are 99.7% identical, differing only by a single conservative substitution in the 381-amino acid polypeptide. One of the ESTs (AI726755) used as starting points for the amplification of these cDNAs corresponds to *GhBIN2-D*, while the other (AI731919) corresponds to *GhBIN2-E*.

BLAST analyses of the four *GhBIN2* sequences indicated that they are all very similar to GSK3/SHAGGY-like kinase sequences. The alignment scores (bits) are as high as 600–700. Sequence comparison with mammalian GSK3β (Strausberg 2002) and the *Drosophila* SHAGGY kinase (Bourouis et al. 1990) showed that derived GhBIN2 sequences share many conservative regions with these two proteins (data not shown). Based on the extensive identity between the *GhBIN2* sequences and *AtBIN2*, we predicted that these cDNAs encode functional homologs of *AtBIN2*, and are likely to play important roles in the BR signal transduction pathway in cotton.

Expression of *GhBIN2* genes

Quantitative real-time PCR assays were performed to compare the relative expression levels of *GhBIN2* genes in cotton plants. Total RNAs isolated from a variety of tissues from cotton plants including leaves, buds, hypocotyls, roots, sepals, 5-day to 8-day ovules, and samples from fibers at different developmental stages were used for this analysis. As shown in Fig.1, GhBIN2-B and GhBIN2-D fall into one sequence type, while GhBIN2-C and GhBIN2-E can also be grouped together. Interestingly, gene expression analyses also show that the *GhBIN2* genes can be classified into two groups. *GhBIN2-C* and *GhBIN2-E* mRNAs are expressed in similar patterns throughout the cotton plant. Highest expression levels were seen in hypocotyls and in buds, with lower levels in sepals. In cotton bolls, these mRNAs were expressed at higher levels in 5-DPA

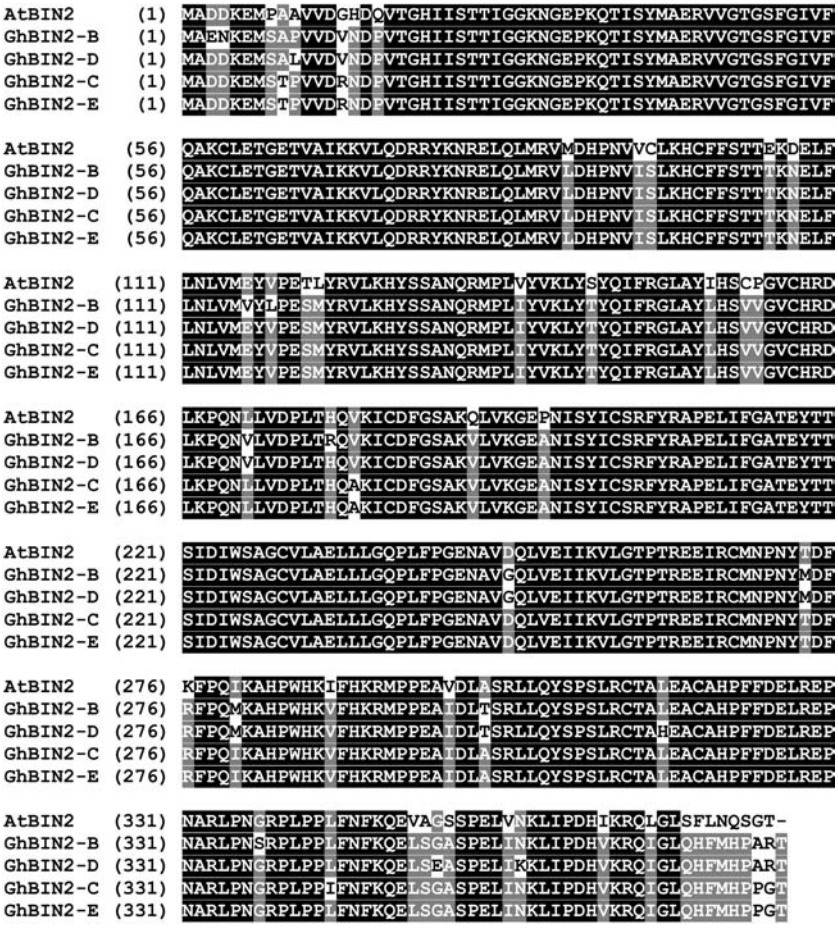

Fig. 1 Comparison of derived amino acid sequences of BIN2 orthologs from Arabidopsis (AtBIN2) and cotton (GhBIN2s), using Vector NTI. Amino acids that are identical in all four sequences are shaded in *black*, blocks of similar amino acids are shaded in *gray*

and 10-DPA bolls than in 0-DPA bolls. They were expressed at high levels in 5–8 DPA ovules, and at lower levels in 30-DPA fibers. The expression of *GhBIN2-E* in 30-DPA fiber was used as the standard for comparative expression analyses. In general, *GhBIN2-C* and *GhBIN2-E* are expressed in patterns that are similar to that of *GhBRI1* (Sun et al. 2004). Expression of these mRNAs is higher in tissues that are undergoing rapid cell expansion and/or vascular development (Fig.2, lower panels). Conversely, mRNAs corresponding to *GhBIN2-B* and *GhBIN2-D* are expressed constitutively at relatively high levels in all of the cotton tissues tested (Fig.2, upper panels). With the exception of the 30-DPA fiber sample, the levels of both *GhBIN2-B* and *GhBIN2-D* mRNAs were not significantly different from tissue to tissue and the expression levels of these mRNAs were between 3-fold and 20-fold higher than that of *GhBIN2-C* or *GhBIN2-E* in corresponding RNA samples.

Identification of the two homeologous *GhBIN2* loci in the tetraploid cotton genome

Cultivated cotton varieties (*G. hirsutum* and *G. barbadense*) are allotetraploids (2 n = 4 x = 52, AADD) that originated from diploid cotton ancestors (Wendel et al. 1992). The two extant diploid cotton species with the A-genome (2 n = 2 x = 26, AA), *G. arboreum* and *G. herbaceum*, are thought to be closely related to the A sub-genome of the tetraploid cottons. *G. thurberi* and *G. raimondii* are among several extant D-genome diploid cotton species (2 n = 2 x = 26, DD) and *G. raimondii* is considered to be most closely related to the D sub-genome of the tetraploid cotton species (Iqbal et al. 2001). As shown in Fig.1, *GhBIN2-B* and *GhBIN2-D* fall into one sequence type, while *GhBIN2-C* and *GhBIN2-E* represent a distinct type, and gene expression data further support the grouping of these genes (see Fig.2). Based on these results, it is apparent that these cDNAs represent four *GhBIN2* genes that fall into two distinct structural and functional classes. Therefore, we speculated that one member of each pair of *GhBIN2* cDNAs is derived from the A sub-genome while the second originates from the D sub-genome. If this were the case, we would expect diploid cottons to contain two *BIN2* loci; one locus corresponding to the *GhBIN2-B*/*D* class of genes and a second corresponding to the *GhBIN2-C*/*E* genes. To test this hypothesis, *BIN2* cDNA fragments were amplified from both *G. arboreum* (*GaBIN2*) and *G. thurberi* (*GtBIN2*), using specific primers corresponding to each of the *GhBIN2* cDNA classes. The resulting cDNA amplicons were sequenced and compared to the *GhBIN2* cDNAs. As predicted, two distinct *BIN2* sequences were identified from *G. arboreum* (*GaBIN2-1*

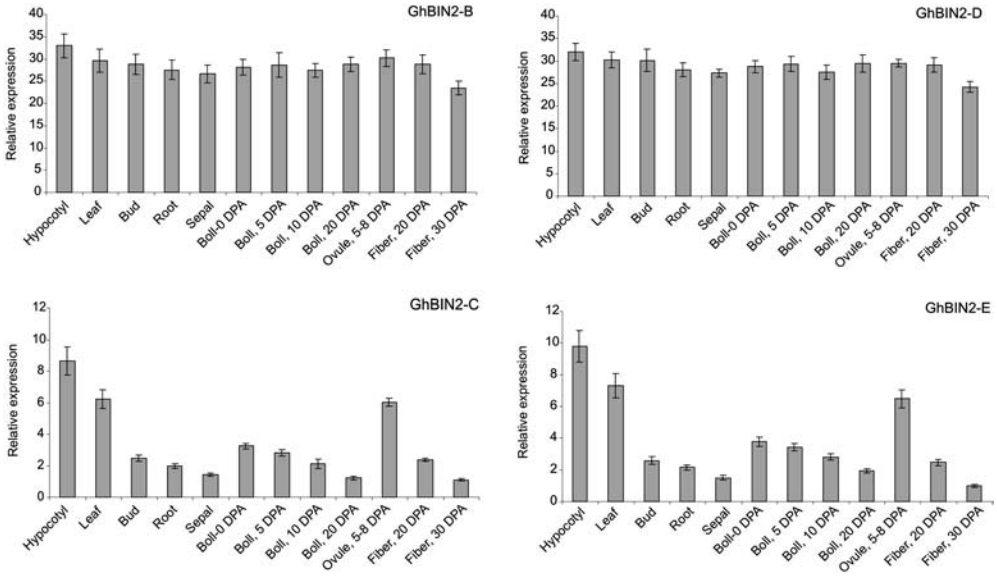

Fig. 2 *GhBIN2s* expression levels in cotton plants. The levels of *GhBIN2* mRNAs in total RNA samples were assayed by a duplex TaqMan quantitative real-time PCR assay using 18S rRNA as a reference. The sample with the lowest ratio of *GhBIN2-E* mRNA to 18S RNA (30 DPA fiber) was set to 1 and the values for the other samples are expressed relative to that value. Values are means (±SD) of three independent assays

and GaBIN2-2) and from *G. thurberi* (*GtBIN2-1* and *GtBIN2-2*). Nucleotide sequence comparisons showed that *GaBIN2-1* is most closely related to *GhBIN2-B* (99.3% identity) and *GtBIN2-1* is nearly identical to *GhBIN2-D* (99.4% identity). Likewise, *GtBIN2-2* shares 99.8% identity with *GhBIN2-C* and *GaBIN2-2* is 99.8% identical to *GhBIN2-E*. A phylogenetic tree based on AlignX analyses of these eight sequences, together with the coding sequences of 10 Arabidopsis shaggy-like protein kinase genes, is shown in Fig.3. *AtSK21* corresponds to *AtBIN2* while *AtSK22* and *AtSK23* are the most closely related shaggy-like protein kinase genes. This analysis places *GhBIN2-B* and *GhBIN2-D* within a clade together with *GaBIN2-1* and *GtBIN2-1*. *GhBIN2-C* and *GhBIN2-E* form a second clade with *GaBIN2-2* and *GtBIN2-2* (Fig.3). We interpret these data to indicate that *GhBIN2-B* and *GhBIN2-E* represent orthologous gene loci derived from the A sub-genome ancestor of cotton, while *GhBIN2-D* and *GhBIN2-C* represent the D sub-genome homeologues.

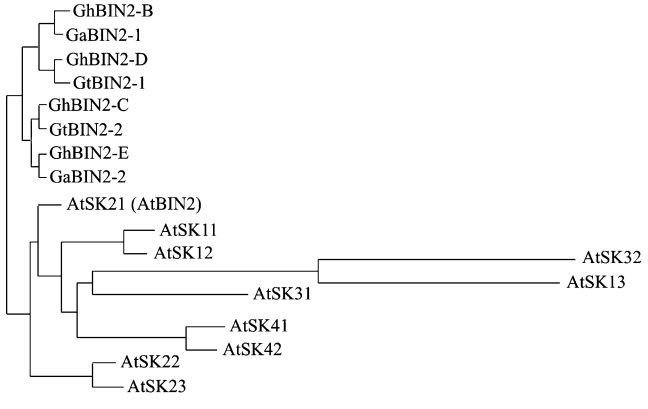

Fig. 3 Phylogenetic tree based on the alignment of amino acid sequences derived from BIN2-encoding cDNAs from *G. hirsutum* (*GhBIN2-B, GhBIN2-D, GhBIN2-C*, and *GhBIN2-E*), and from the A-genome diploid species *G. arboreum* (*GaBIN2-1* and *GaBIN2-2*) and the D-genome species *G. thurberi* (*GtBIN2-1* and *GtBIN2-2*). Sequences derived from 10 Arabidopsis genes for shaggy-like kinases were also included in the analysis, which was performed using AlignX (Vector NTI)

Functional analysis of *GhBIN2* gene products in Arabidopsis

To investigate the functional characteristics of these *GhBIN2* genes, transgenic Arabidopsis plants were developed that express each of the four *GhBIN2* cDNAs under the control of the strong, constitutive CaMV 35S promoter. Since overexpression of *AtBIN2* in otherwise wild-type Arabidopsis was sufficient to produce a strong BR-insensitive phenotype (Li and Nam 2002), we predicted that, if functional, expression of the *GhBIN2* cDNAs should also produce a dwarf and *bin2* mutant phenotype.

The *GhBIN2* transgenes were transformed into wild-type Ws-2 plants via floral inoculation, and T_1 seedlings were selected on kanamycin-containing medium. Although many of the kanamycin-resistant plants had near-normal phenotypes, some of the *GhBIN2*-overexpressing plants were stunted to various degrees (Fig.4). The most severely affected had phenotypes similar to those of *bin2* mutant plants. These plants grew as small, tightly compact rosettes with curled leaves that tended to be darker in color than wild-type plants. The shoot heights of these transgenic Arabidopsis plants were measured after 5 weeks of growth and compared to that of the wild-type Ws-2 plants. PCR analyses using specific primers designed from CaMV 35S promoter and *GhBIN2* coding sequences were used to confirm the presence of *GhBIN2* transgenes, and quantitative real-time PCR was used to assay the expression levels of the four *GhBIN2s* in individual transgenic plants.

Fig. 4 Comparison of a wild-type Arabidopsis plant (Ws-2) with selected transgenic plants that express *GhBIN2* gene constructs. Plants representing a series of *bin2*-like phenotypes are shown. The phenotypes range from mild growth inhibition to severe stunting. Photographs were taken 25 days after sowing. *Scale bar* 4 mm

Expression of each of the four *GhBIN2* transgenes in Arabidopsis plants resulted in phenotypes ranging from mild to severe dwarfism and *bin2* phenotypes. Quantitative real-time PCR analysis showed the level of *GhBIN2* expression was negatively correlated with the height of the transgenic plants. As shown in Fig.5 (upper panels), *GhBIN2-B* and *GhBin2-D* expression levels were 4-fold to 6-fold higher in severely dwarfed transgenic plants than in moderately dwarfed plants. In addition, as also shown in Fig.5 (lower panels), *GhBIN2-C* and *GhBin2-E* expression levels were 3-fold to 4-fold higher in severely dwarfed plants than in plants with moderate growth inhibition. Correlation analysis showed a strong inverse correlation between *GhBIN2* expression levels and the heights of the transgenic plants (mean correlation, $r = -0.90$). These results show that expression of each of the *GhBIN2* genes in Arabidopsis results in a phenotype that is indistinguishable from that of the *bin2-1* mutant or *AtBIN2*-overexpressing transgenic plants, indicating that all four *GhBIN2* genes encode functional proteins that can act as negative regulators of the BR signal transduction pathway.

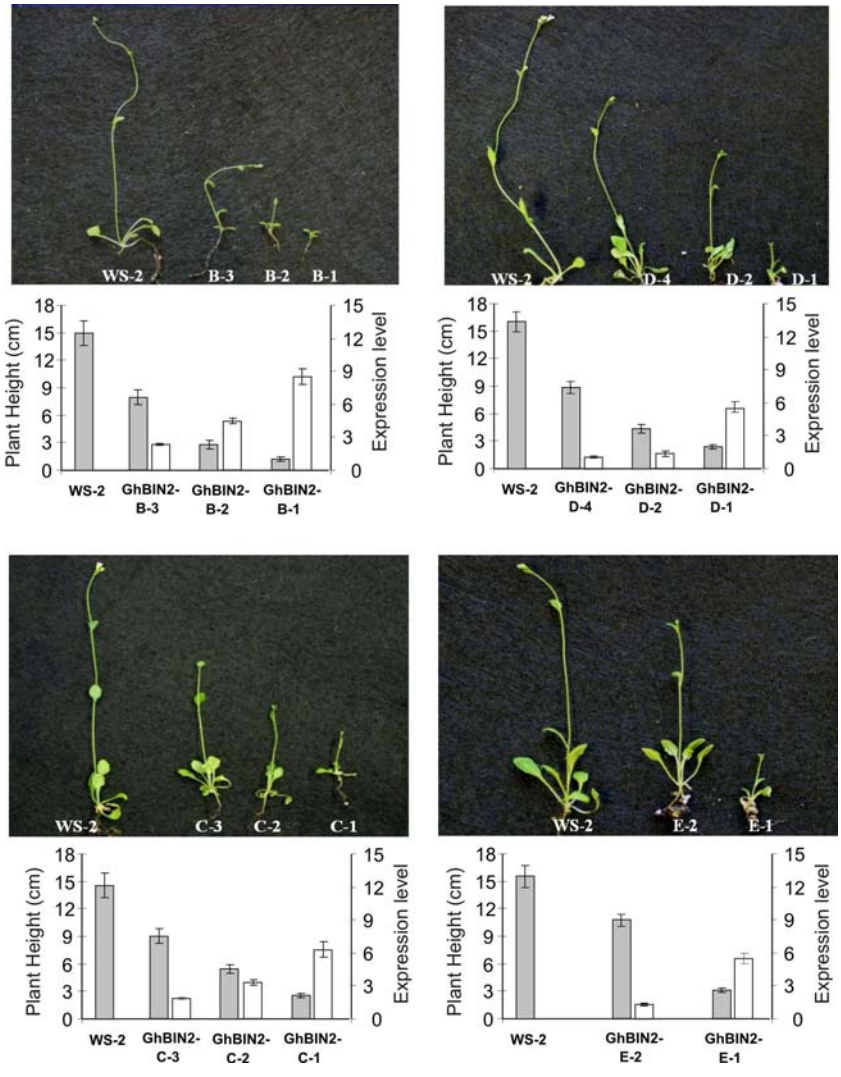

Fig. 5 *GhBIN2* expression levels are inversely correlated with the height of transgenic Arabidopsis plants. The mean height of wild-type plants (Ws-2) is shown. Several independent T0 *GhBIN2* transgenic plants of similar heights were measured, then assayed for transgene expression. Plant heights are shown as *shaded bars* and *GhBIN2* expression levels are shown as *open bars*. The *error bars* represent standard deviation (SD)

Discussion

Four cotton cDNAs were identified that display strong sequence identity to *BIN2*, a negative regulator of the BR signaling pathway in Arabidopsis. These four cDNAs encode putative proteins that fall into two classes; two (*GhBIN2-B* and *GhBIN2-D*) are more than 97% identical to each other, and the members of the second pair (*GhBIN2-C* and *GhBIN2-E*) differ from each other by only a single amino acid. The identification of these subgroups is further supported by differences in their expression profiles.

There are at least 10 genes for GSK3/SHAGGY-like protein kinases (*AtSK*) in Arabidopsis (Dornelas et al. 1998) and Charrier et al. (2002) divided the entire *AtSK* gene family into four subgroups based on sequence similarity. In this scheme, *AtSK22* and *AtSK23* are most closely related to *AtBIN2* (*AtSK21*). Our phylogenetic tree analysis showed that the *GhBIN2* genes are more closely related to *AtBIN2* than to *AtSK22, AtSK23* or other *AtSK*s (Fig.3), which supports the hypothesis that the *GhBIN2s* are bona fide *BIN2* homologs.

GSK3/SHAGGY-like protein kinases such as BIN2 are a group of conserved serine/threonine kinases that are involved in a number of signaling pathways (Li and Nam 2002). These enzymes are reported to control metabolism, cell fate determination, and tissue patterning in many organisms. Catalytic domains of the GhBIN2 family share 70% sequence identity to mammalian GSK3β (Strausberg 2002) and *Drosophila* SHAGGY kinase (Bourouis et al. 1990), and they also share many conservative regions including the eight subdomains found in other serine/threonine kinases (Hanks et al. 1988).

Expression analysis using real-time PCR revealed that these genes, including *BIN2*, are expressed in all tested Arabidopsis organs, with higher levels being found in flower buds and open flowers than in the other organs (Charrier et al. 2002). We found that each of the *GhBIN2* genes is also expressed in all cotton tissues tested. However, *GhBIN2-B* and *GhBIN2-D* transcripts are expressed at consistent levels throughout the plants, while the *GhBIN2-C* and *GhBIN2-E* mRNAs are differentially expressed—with highest levels occurring in hypocotyls, leaf buds, and in immature bolls and ovules. Generally, *GhBIN2-C* and *GhBIN2-E* are expressed in rapidly growing tissues in a pattern similar to that of *GhBRI1*, the putative BR receptor gene in cotton (Sun et al. 2004). Since our experiments show that all four *GhBIN2* genes encode proteins that can negatively regulate BR signaling in transgenic Arabidopsis plants, the significance of the differential expression patterns among these genes is not clear. However, it is important to remember that mRNA levels do not necessarily correlate with levels of functional BIN2 within the cells and, that the expression could be limited to specific tissue or cell types within the organs tested. Therefore, more detailed expression analyses to determine the spatial patterns of *GhBIN2* expression should provide a more complete understanding of BIN2 function.

Upland cotton (*G. hirsutum*) is a tetraploid plant that originated from A-genome and D-genome cotton species (Iqbal et al. 2001). Sequence comparison of the four *GhBIN2* genes with those of diploid A-type (*G. arboreum*) and D-type (*G. thurberi*) cotton suggests a specific ancestral relationship. Based on this analysis, we predict that *GhBIN2B*, which is most similar to *GaBIN2-1*, represents the A sub-genome homeolog of the constitutively expressed *GhBIN2* pair. Likewise, *GhBIN2E*, which most closely matches *GaBIN2-2*, represents the A sub-genome homeolog of the developmentally regulated *GhBIN2* pair. On the other hand, constitutively expressed *GhBIN2-D*, and developmentally regulated *GhBIN2-C*, which most closely resemble sequences from G . *thurberi* (*GtBIN2-1*, and *GtBIN2-2* respectively) represent the corresponding D sub-genome homeologs. If this is the case, the cotton *BIN2* gene family was probably derived via a gene duplication event that predates the divergence of the A-genome and D-genome diploid *Gossypium* species. Based on their distinct expression patterns, these genes may have diverged functionally, as well as structurally. The subsequent polyploidization event that resulted in tetraploid *G. hirsutum* produced the current gene family consisting of two homeologous pairs of genes. This contrasts with the *BRI1* genes: here, single A sub-genome and D sub-genome homeologs were identified (Sun et al. 2004).

Current models of the BR signal transduction pathway indicate that BIN2 negatively regulates BR-mediated responses (Clouse 2002). According to this model BIN2 is constitutively active in the absence of BR and it phosphorylates the positive downstream regulators BZR1 and BES1 (BZR2), targeting them for degradation (Clouse 2002). Li and Nam (2002) reported that about 10% of the kanamycin-resistant plants transformed with a wild-type *BIN2* gene construct displayed BR-insensitive phenotypes. These *BIN2* transgenic plants formed an allelic series of *bin2* phenotypes, ranging from minor reductions in growth to the severe dwarfing characteristic of the semi-dominant *bin2* mutant plants. Northern hybridization analysis revealed a correlation between the level of *BIN2* expression and severity of the phenotype in these transgenic Arabidopsis plants (Li and Nam 2002). Overexpression of each of the *GhBIN2* cDNAs in Arabidopsis gave similar results. About 10% of the transgenic plants showed *bin2*-like phenotypes, of varying severity (see Fig.4) and quantitative real-time PCR analysis showed that the heights of the transgenic plants were inversely correlated with the *GhBIN2* expression levels. We interpret these results to indicate that the *GhBIN2* genes encode proteins that are functionally homologous to *AtBIN2*, and we speculate that these genes may negatively regulate BR signal transduction in cotton.

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