

An ATP-Binding Cassette Transporter GhWBC1 from Elongating Cotton Fibers¹

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We have isolated a cDNA (*GhWBC1*) from cotton (*Gossypium hirsutum*) that encodes an ATP-binding cassette transporter of the WBC (white/brown complex) subfamily. Members of this subfamily are half-sized transporters and are reported to mediate lipid and drug excretion in human (*Homo sapiens*). *GhWBC1* is highly expressed in developing fiber cells, but transcripts were also detectable in other tissues except roots. The transcript level peaked in rapidly expanding fibers from 5 to 9 DPA and then decreased. The *GhWBC1* expression was weak in fiber cells of an *li* (*ligon-lintless*) mutant, which is defective in fiber cell elongation. These data indicate that *GhWBC1* gene expression correlates with cotton fiber elongation. Transient expression of enhanced green fluorescence protein-GhWBC1 fusion protein in onion (*Allium cepa*) epidermal cells revealed plasma membrane localization. The *GhWBC1* cDNA driven by a constitutive 35S promoter was introduced into Arabidopsis. About 13% of the transformants produced short siliques (SSs), whereas others had normal siliques (long siliques [LSs]). In siliques of SS lines, most embryos were severely shriveled, and only several seeds per silique could be found at maturity. The transgene expression level was higher in SS lines than in LS lines. Expression of *AtWBC11*, the closest homolog of *GhWBC1* in Arabidopsis, was not altered in either SS or LS transgenic plants examined. These data suggest that GhWBC1 interferes with substance translocation that is required for Arabidopsis seed and silique development. Characterization of Arabidopsis WBC members, particularly *AtWBC11*, will help to dissect the role of GhWBC1 in cotton fiber development and elongation.

The ATP-binding cassette (ABC) transporters, known for their function of translocating a broad range of substances across biological membranes powered by ATP hydrolysis, are ubiquitously present in bacteria, fungi, plants, and animals, and they constitute one of the largest protein families (Higgins, 1992; Henikoff et al., 1997; Theodoulou, 2000).

Functional ABC transporters typically consist of two transmembrane domains (TMDs) and two nucleotide-binding folds (NBFs). In eukaryotic organisms, these four domains are often arranged into a single polypeptide, forming a full-sized transporter. However, a number of eukaryotic genes also encode half-sized transporters, with each peptide containing one NBF and one TMD (Higgins, 1992). These half-sized transporters fulfill their functions through formation of homodimers or heterodimers with other partners.

In bacteria, many ABC transporters are involved in nutrient acquisition, such as His and maltose (Higgins, 1992). For eukaryotic ABC transporters, much attention has been paid to their roles in multidrug

resistance. For example, overexpression of *STS1* in yeast (*Saccharomyces cerevisiae*) and *BCRP/MXR/ABC2* in cancer cells increased cellular resistance to cytotoxic agents (Bissinger and Kuchler, 1994; Doyle et al., 1998; Litman et al., 2000). In addition, a number of eukaryotic ABC transporters have been shown to participate in other distinct biological processes, such as translocation of lipids, excretion of yeast mating factors, and regulation of ion channel activities (Higgins, 1995; Ketchum et al., 2001; Oram, 2002).

Plants, with a sessile life-style, have a particularly large number of ABC transporters. The genome of Arabidopsis contains 129 open reading frames (ORFs) capable of encoding ABC proteins (Sánchez-Fernández et al., 2001). Based on their size, orientation, domain organization, and resemblance to ABC proteins from other organisms, these proteins can be classified into 12 subfamilies: multidrug resistance proteins, multidrug resistance-associated proteins (MRPs), pleiotropic drug resistance proteins, and ABC1 homolog are full-sized transporters; the peroxisomal membrane proteins (PMPs), white/brown complex proteins (WBCs), ABC2 homologs, ABC transporters of the mitochondrion (ATMs), and transporters associated with antigen processing are half-sized transporters. In addition, three groups of soluble proteins are also categorized within this superfamily (Sánchez-Fernández et al., 2001).

Plant ABC transporters have been found to be involved in detoxification by means of vacuolar se-

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questration of various metabolites or excretion of toxic compounds out of the cell (Rea et al., 1998; Theodoulou, 2000; Jasinski et al., 2001; Martinoia et al., 2002). Members of the MRP subfamily (AtMRP1, AtMRP2, AtMRP3, and AtMRP5) exhibit glutathione S-conjugates transport activity (Lu et al., 1997, 1998; Tommasini et al., 1998; Gaedeke et al., 2001). Two pleiotropic drug resistance proteins, NpABC1 from *Nicotiana plumbaginifolia* and SpTUR2 from *Spirodela polyrrhiza*, are able to excrete diterpenoid antifungal agent, the sclareolide derivatives (Jasinski et al., 2001; van den Brûle et al., 2002). However, function of plant ABC proteins is not restricted to detoxification because genetic analysis of several loss-of-function mutants has revealed their diverse roles in plant growth and development, including metabolite translocation, far-red light response, stomata movement, auxin transport, maturation of cytosolic Fe/S proteins, and seed germination (Sidler et al., 1998; Debeaujon et al., 2001; Gaedeke et al., 2001; Kushnir et al., 2001; Møller et al., 2001; Noh et al., 2001; Footitt et al., 2002). Despite progresses made in the last several years, functions of most plant ABC transporters remain unknown.

Cotton (*Gossypium hirsutum*) fibers are single-celled seed hairs developed from the outmost layer of the integument. After anthesis, differentiated fiber cells begin to expand and undergo rapid elongation, followed by overlapping stages of secondary cell wall synthesis and dehydration (Applequist et al., 2001; Kim and Triplett, 2001). Fiber quality is of paramount importance for industry, and quality characters are mostly controlled by genetic factors (Paterson et al., 2003). Several spontaneous mutants defective in fiber development were found during cotton breeding (Kim and Triplett, 2001); however, none of the mutated genes has been cloned, partly because of the large genome size of cotton. Quite a number of cDNAs, on the other hand, have been isolated from developing cotton fibers with expression patterns characterized (John and Crow, 1992; Orford and Timmis, 1998; Li et al., 2002; Cedroni et al., 2003; Ji et al., 2003). Although precise mechanisms controlling fiber initiation and growth are still elusive, the popular assumption for fiber cell elongation is, to date, turgor-based cell expansion (Smart et al., 1998). Recent investigation showed the importance of plasmodesmatal gating, substance transport (Suc and K⁺), and carbon partitioning in cotton fiber development and expanding (Haigler et al., 2001; Ruan et al., 2001, 2003). Therefore, the cell-cell communication and substance exchange between fibers and adjacent cells of ovules are critical to fiber elongation.

Here, we report cDNA cloning and expression analysis of a half-sized ABC transporter, *GhWBC1*, from cotton. *GhWBC1* is predominantly expressed in developing fibers and exhibited a temporal expression pattern correlating with fiber elongation. Enhanced green fluorescence protein (EGFP)-*GhWBC1*

fusion protein was localized to plasma membrane. Interestingly, overexpression of *GhWBC1* in Arabidopsis plants resulted in impaired seed development and short siliques (SSs), suggesting that this transporter plays an important role in plant development.

RESULTS

Isolation of *GhWBC1*

Upland cotton is a tetraploid species widely cultivated in the world. An *fl* (*fuzzless-lintless*) cotton mutant, which was isolated from cotton cv Xu-142, has seeds that are nearly glabrous, and the mutant plants do not show other phenotypic changes (Zhang and Pan, 1991; Yu et al., 2000). To isolate mRNAs that are enriched in fiber cells, we performed comparative reverse transcription (RT)-PCR analysis of ovules of the wild-type cotton cultivar and the *fl* mutant. By analyzing transcripts corresponding to randomly selected clones from a cDNA library of cotton ovules (−3–15 DPA), we found one of the cDNAs, *ZHU93*, that showed a much higher level of transcripts in wild type than in *fl* mutant ovules at 5 DPA (Fig. 1).

The *ZHU93* cDNA is 2,372 bp in length (Gene Bank accession no. AY255521). An in-frame stop codon was present upstream to the translation start codon ATG, suggesting a complete ORF. This ORF encodes a protein of 705 amino acid residues with a predicted molecular mass of 77 kD. BLAST analysis indicated that the deduced protein was highly similar to ABC transporter proteins, and the highest sequence identities were found with a group of WBC proteins of the ABC transporter superfamily, including AtWBC11 (At1g17840) of Arabidopsis, with an identity of 82%, and a putative transporter of rice (*Oryza sativa*; AAM92819), with an identity of 79%. In this cotton protein, and in AtWBC11 and the putative WBC transporter of rice, an NBF is located near the N-terminal, where there are three conserved motifs: the Walker motif A and B and motif C (Fig. 2). Motif C is situated between two Walker motifs, and the sequence here (ISGGEKRRVSIA) is consistent with the consensus of ABC signature (Bairoch, 1992). Hydrophobicity analysis showed that the C-terminal one-half of this cotton protein is highly hydrophobic,

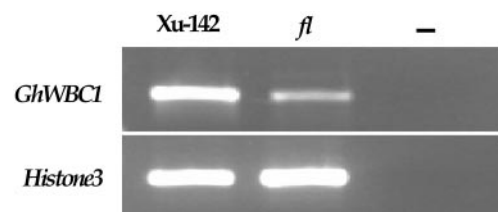


Figure 1. RT-PCR analysis of *GhWBC1* (*Zhu93*) gene expression in developing ovules of cotton cv Xu-142 and *fl* mutant, collected at 5 DPA. The cotton *Histone-3* (*GhHIS3*) was amplified as a control; in negative control (−), no template DNA was added. The PCR was performed with primers GH1 and GH2.

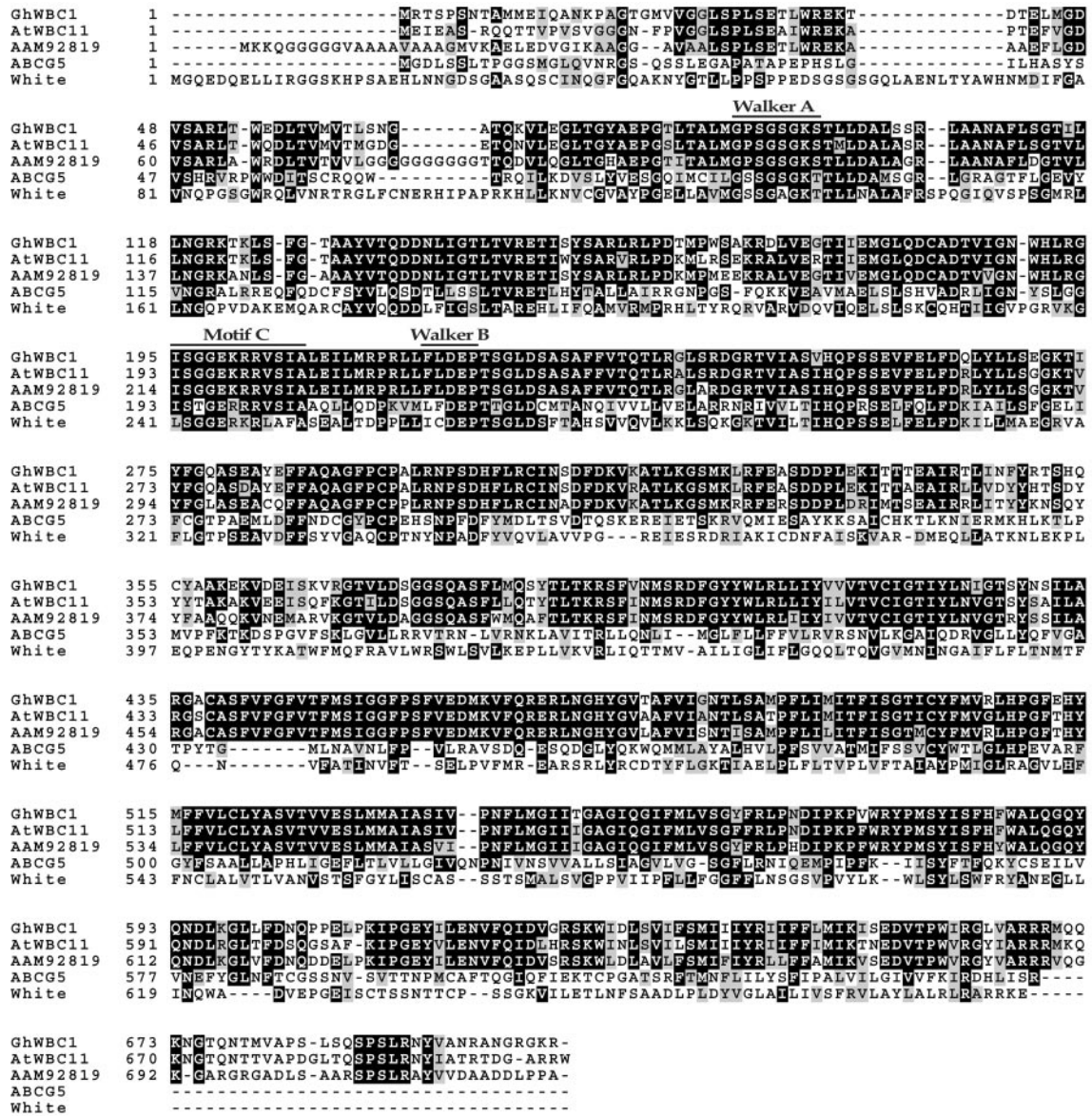


Figure 2. Alignment of amino acid sequences of GhWBC1, Arabidopsis AtWBC11 (At1g17840), a rice putative ABC transporter (AAM92819), human ABCG5 (AAG40003), and fruitfly White protein (P10090). All the proteins aligned here are WBC members of the ABC transporter superfamily. The sequences were aligned with ClustalW (Thompson et al., 1994) using default parameters through EMBnet (<http://www.ch.embnet.org/software/ClustalW.html>), and the box shade was created by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

and six putative transmembrane segments are predicted to be present (data not shown). As revealed by protein sequence alignment (Fig. 2), this reverse orientation (NBF-TMD) is shared by all the WBC proteins (Sánchez-Fernández et al., 2001), including fruitfly (*Drosophila melanogaster*) WBC proteins (Dreesen et al., 1988), human (*Homo sapiens*) BCRP (Doyle et al., 1998), ABCG5, and ABCG8 (Berge et al., 2000; Klucken et al., 2000). Taken together, sequence information indicates that this cotton cDNA encodes a half-sized ABC transporter of the WBC subfamily, and it is then named *GhWBC1*.

Southern blot showed that the diploid genome of *Gossypium arboreum* contains a small family of two to three genes encoding GhWBC1 homologous proteins (data not shown).

Spatial and Temporal Expression Pattern

As shown in Figure 1, RT-PCR analysis of *ZHU93* revealed a significantly higher level of *GhWBC1* gene expression in wild-type ovules containing fibers than in fiberless *fl* ovules, both at 5 DPA. We then examined *GhWBC1* expression in other tissues. RNA blot

showed that, in contrast to a high level of *GhWBC1* transcripts in cotton fibers of 9 DPA, only very low levels were present in leaves, cotyledons, and hypocotyls of the 10-d-old seedlings. The transcripts were undetectable in either roots or *fl* ovules (Fig. 3A). These results indicate that *GhWBC1* is regulated in a fiber-preferential manner.

Accumulation of *GhWBC1* mRNA in fibers at different developmental stages was also analyzed. In

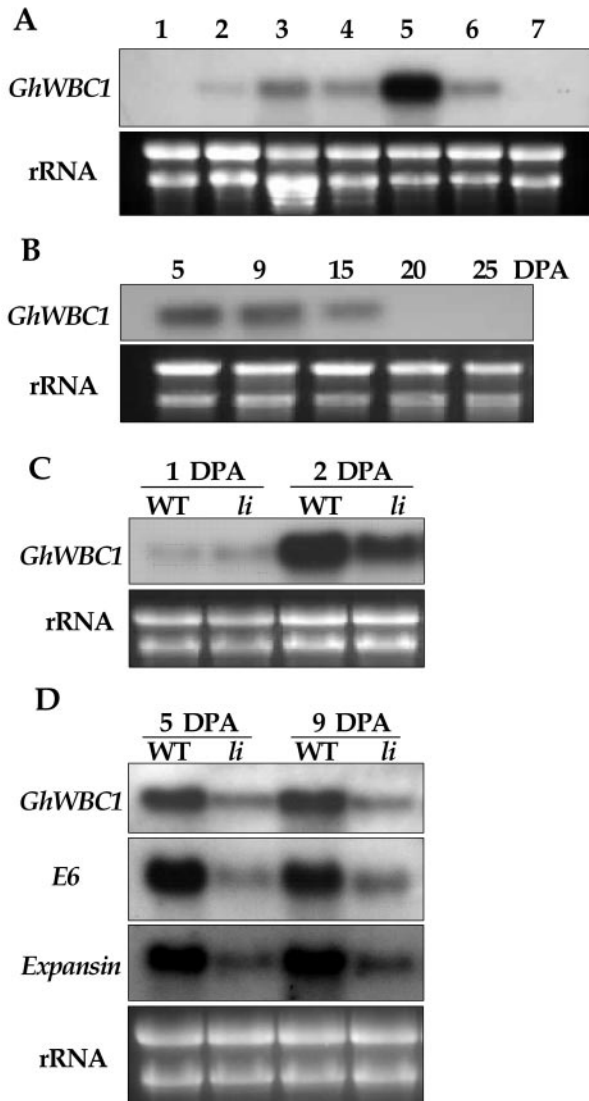


Figure 3. Northern-blot analysis of spatial and temporal pattern of *GhWBC1* expression. **A**, Expression in seedlings (10 d old) and developing ovules (9 DPA); total RNA was isolated from roots (1), hypocotyls (2), cotyledons (3), young true leaves (4), fiber cells (5), wild-type intact ovules (6), and *fl* ovules (7); 20 μ g of total RNA was loaded each lane. **B**, Expression of *GhWBC1* in developing fibers of 5, 9, 15, 20, and 25 DPA, respectively; 1.25 μ g of total RNA was loaded each lane. **C** and **D**, expression of *GhWBC1*, *E6*, and *Expansin* genes in *li* mutant and wild-type cotton cv Xu-142 ovules. **C**, *GhWBC1* expression in 1- and 2-DPA ovules; **D**, *GhWBC1*, *E6*, and *Expansin* expression in 5- and 9-DPA ovules. Ten micrograms of total RNA was loaded each lane.

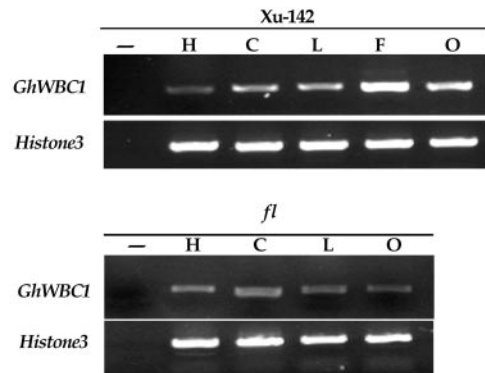


Figure 4. RT-PCR analysis of *GhWBC1* expression in different aerial organs of cotton cv Xu-142 and *fl* mutant. Total RNAs were isolated from hypocotyls (H), cotyledons (C), and young true leaves (L) of the 10-d-old seedlings and from 9-DPA ovules (O) or fibers (F). The PCR was conducted by 27 cycles of PCR amplification with primers GH3 and GH4.

rapidly elongating fibers (5 and 9 DPA), the *GhWBC1* transcript level was high. The transcript abundance decreased significantly at 15 DPA and diminished at 20 and 25 DPA (Fig. 3B), a stage when fibers entered the secondary cell wall formation. The results indicate that *GhWBC1* gene expression is up-regulated in rapidly elongating fibers, with the mRNA level peaking around 5 to 9 DPA, the stage in which the fastest elongation of fibers occurs (Basra and Malik, 1984; Smart et al., 1998).

Then, we investigated *GhWBC1* expression in fiber cells of an *li* (*ligon-lintless*) mutant of cotton, which has fibers that are not longer than 6 mm (Karaca et al., 2002), significantly shorter than the fibers of wild-type cotton cultivars (approximately 30 mm long). For both the wild type and *li* mutant, expression level of *GhWBC1* was low in 1-DPA ovules (Fig. 3C). Immediately afterward (at 2 DPA), the transcript abundance increased dramatically in wild-type cotton ovules and also to a lesser extent in *li* ovules. However, a more profound difference of transcript abundance appeared in 5- and 9-DPA ovules, with a much lower level in the *li* mutant (Fig. 3D). At the same time, two fiber-enriched genes, *E6* and *Expansin*, were also down-regulated in *li* mutant fibers at 5 and 9 DPA (Fig. 3D). *E6* is the first identified fiber-specific gene (John and Crow, 1992), and *Expansin* protein has been proposed to function in cell wall loosening and cell expansion (Orford and Timmis, 1998; Cosgrove, 2000). These results further support the notion that *GhWBC1* expression correlates with fiber elongation.

In northern analysis described above, the probe used covered part of the coding region, and it may have hybridized with transcripts of other members of the family. We then conducted the more specific RT-PCR with primers GH3 and GH4. The latter was designed according to the sequences of the 3'-untranslated region of the *GhWBC1* cDNA. As shown in Figure 4, RT-PCR again showed the highest level

of transcripts in developing fibers and a lower level in cotyledons, hypocotyls, and leaves. In addition, RT-PCR also revealed a weak expression of *GhWBC1* in aerial organs of the *fl* mutant (Fig. 4). This indicates that, except for the fiberless ovule in which the *GhWBC1* transcript abundance is significantly reduced, the *GhWBC1* gene expression in the *fl* mutant is not altered.

Subcellular Localization

Cellular and subcellular localization of a transporter is often related to its function. To examine in which cellular compartment GhWBC1 is present, a 35S:*EGFP-GhWBC1* expression cassette was transferred into onion (*Allium cepa*) epidermal cells by particle bombardment for transient expression of the fusion protein. After an overnight incubation, the green fluorescence signal of EGFP-GhWBC1 was observed around the cell surface, whereas the signal of EGFP alone was ubiquitously spread in the cell (data not shown). To determine whether GhWBC1 is localized to the cell wall or to the plasma membrane, a plasmolysis experiment was performed. As shown in Figure 5, GhWBC1-directed green fluorescence signal was internalized and associated with plasma membrane after plasmolysis, whereas no strong signal remained present around the cell wall. This observation suggests that GhWBC1 is likely a plasma membrane protein.

Phenotypic Changes of Transgenic Arabidopsis

To get further clues to its function, *GhAWC1* cDNA driven by a 35S promoter was introduced into Arabidopsis. About 400 primary transformants were obtained on the basis of kanamycin resistance and PCR identification. In the early stage of vegetative growth, no phenotypic changes were observed. In the reproductive stage, however, 53 of 400 (13.3%) T₁ transgenic plants produced SSs, whereas other lines

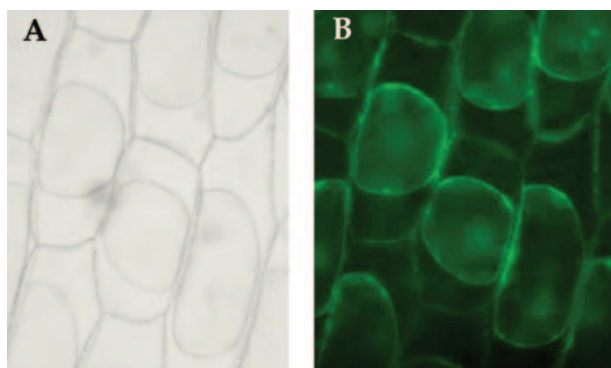


Figure 5. Subcellular localization of EGFP-GhWBC1 fusion protein in onion epidermal cells. A, Cell structure under light microscopy. B, Green fluorescence signal of EGFP-GhWBC1 after plasmolysis with 20% (w/v) Suc for 5 min. Plasma membrane and GFP signal shrank together from cell wall.

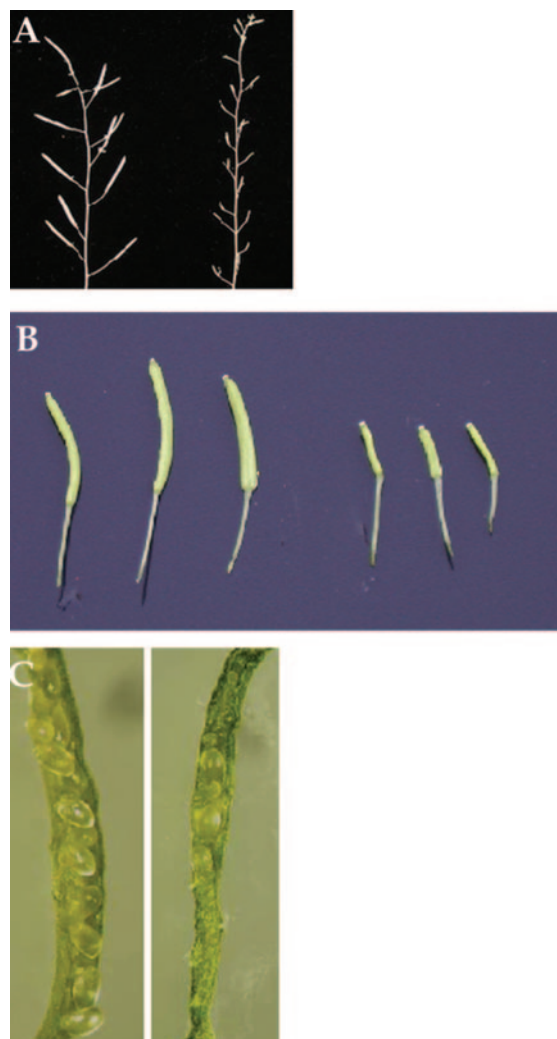


Figure 6. Phenotype of transgenic Arabidopsis plants overexpressing *GhWBC1* under a 35S promoter. A, Inflorescence of mature plants of WT (left) and SS-29 transgenic line (right); B, siliques of WT (left) and SS-29 line (right); C, seeds in the 3-d-old siliques of WT (left) and of SS-29 line (right).

had siliques with similar length to the wild type (long siliques [LSs]; Fig. 6, A and B). The SS phenotype was also observed in T₂ and T₃ generations, although with a percentage lower than expected (data not shown).

Several transgenic plant lines were used for further measurements. In our greenhouse conditions, the average silique length of SS lines was around 0.5 cm, significantly shorter than that of LS lines (e.g. LS-7, 1.14 ± 0.16 cm) and wild-type plants (1.20 ± 0.06 cm; Fig. 7A). The SS plants produced fewer seeds per silique (Fig. 7B). In SS-29 plants, for example, there were three to four seeds per silique, whereas the numbers for WT and LS-7 plants were 44.9 ± 3.38 , and 35.87 ± 4.9 , respectively. In developing siliques of the wild type (about 3 DPA), the embryos were regularly lined. In siliques of SS lines, most embryos were severely shriveled (Fig. 6C).

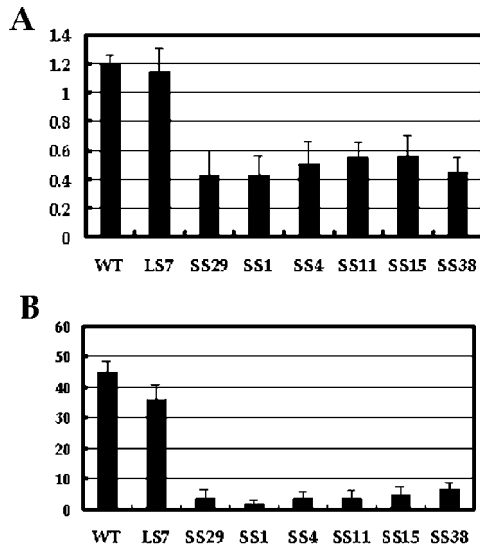


Figure 7. Silique length and seed number per silique of WT and *GhWBC1*-transgenic lines of *Arabidopsis*. A, Length of siliques; B, seed number per silique. LS and SS represent the long (normal) silique lines and the SS lines of transgenic *Arabidopsis*, respectively. For each line, around 10 siliques were measured.

The SS-29 and SS-11 lines (T_3) then were selected for further molecular examination. RT-PCR analysis of transgenic plants was performed to see if the phenotypic changes were related to transgene expression. Higher levels of *GhWBC1* transcripts were found in both SS-29 and SS-11 lines, whereas in LS lines (LS-7 and LS-2) that showed a wild-type phenotype, the *GhWBC1* expression level was lower (Fig. 8A). We further investigated whether the SS phenotype was caused by cosuppression of an *Arabidopsis* homolog. The closest homolog of *GhWBC1* in the *Arabidopsis* genome is *AtWBC11*, which is predominantly expressed in inflorescences and siliques according to our preliminary analysis (Fig. 8). RT-PCR showed that *AtWBC11* transcript levels in siliques of LS and SS transgenic lines and of the wild-type *Arabidopsis* were comparable (Fig. 8A). These data suggest that a high-level (and probably ectopic) expression of *GhWBC1* in transgenic *Arabidopsis* plants may have impeded seed and silique development.

DISCUSSION

GhWBC1 encodes a half-sized ABC transporter of the WBC subfamily. In *Arabidopsis* genome, the full- and half-sized ABC transporters are equally represented. The largest group of *Arabidopsis* ABC proteins is the WBC subfamily, with 29 genes annotated (Sánchez-Fernández et al., 2001), but to date, none of them has a known function reported. In fruitfly, three WBC transporter genes are expressed in the eye pigimentary cells, and they form two types of heterodimers that mediate transport of guanine (WBC) and Trp (White/Scarlet complex), respectively

(Dreesen et al., 1988). In the human genome, there are four WBC proteins that have been identified. ABCG1 (ABC8) regulates the transport of macrophage cholesterol and phospholipids (Klucken et al., 2000), ABCG5 and ABCG8 form a functional complex that mediates efflux of cholesterol and phospholipids (Berge et al., 2000), and BCRP forms homodimer to extrude drugs (Kage et al., 2002).

GhWBC1 transcripts are enriched in developing fibers during the elongation stage. This expression pattern suggests that *GhWBC1* protein may be involved in the transport of a substance during fiber growth. If *GhWBC1* protein is present in plasma membrane, as revealed by the EGFP-*GhWBC1* fusion protein, it is unlikely that this transporter is directly involved in vacuolar sequestration that contributes to maintaining turgor pressure of expanding fiber cells. The *GhWBC1* protein may not transport cell wall building blocks, either, because during the stage of secondary cell wall deposition, *GhWBC1* transcripts in fibers were hardly detectable. Several genes whose products are involved in lipid biosynthesis and translocation are actively transcribed in rapidly expanding fibers, including an acyl carrier protein gene, *ACP* (Song and Allen, 1997); a lipid transfer protein gene, *GH3* (Ma et al. 1995); and a putative β -ketoacyl-CoA synthase (Fiddlehead-like protein) gene, *GhFDH* (Li et al., 2002). All three genes show similar expression pattern to *GhWBC1*, with the highest level of expression taking place in fast-expanding fiber cells. Three of the four human WBCs are found to mediate lipid efflux (Berge et al., 2000; Klucken et al., 2000). Whether *GhWBC1* also participates in lipid translocation is worthy of further investigation. Clearly, our

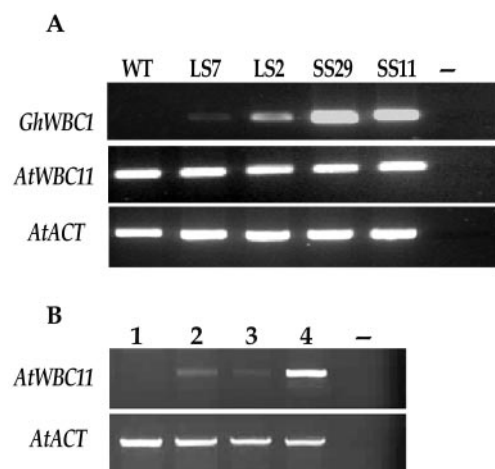


Figure 8. A, RT-PCR analysis of transcripts of *GhWBC1* and *AtWBC11* in siliques of *GhWBC1* transgenic lines of *Arabidopsis*; higher level of *GhWBC1* gene expression was detected in siliques of SS lines. B, RT-PCR analysis of *AtWBC11* expression in WT *Arabidopsis* plants. 1, Roots; 2, leaves; 3, stem; 4, inflorescence; -, negative control without template DNA. The PCR was performed by 30 cycles of amplification. *Arabidopsis ACTIN2* (*AtACT*) was amplified as a control.

results presented here do not naturally lead to an assumption that it is the mutation of *GhWBC1* that has caused the mutant phenotype of either *fl* or *li* plants. However, based on its expression pattern, *GhWBC1* may play an important role in cotton fiber development.

It is interesting that a high-level expression of *GhWBC1* in *Arabidopsis* driven by the 35S promoter resulted in phenotypic changes: Seed development was largely impaired, and the siliques were significantly shorter. Severity of the phenotype was related to the transgene expression level, and low levels of expression did not cause visible phenotypic changes. In addition, expression of the closest homolog *AtWBC11* was not altered in SS lines; thus, cosuppression is unlikely the cause of developmental abnormalities, although possibilities of suppressing other genes cannot be fully excluded. It is possible that the impeded seed and silique development of transgenic SS lines was caused by excessive and/or ectopic activities of the introduced transporter *GhWBC1*, which resulted in disrupted homeostasis of related component(s), such as membrane or extracellular lipids. Alternatively, *GhWBC1* might form heterodimers with *Arabidopsis* half-sized ABC protein(s), such as *AtWBC11*, thus reducing activities of endogenous transporter(s) or acquiring novel activities. As mentioned above, different heterodimer complexes of WBCs in fruitfly mediate intercellular transport of different substances (Dreesen et al., 1988). Knockout lines of several *Arabidopsis* WBC members are now available. Detailed investigation of these knockout lines and further analysis of transgenic *Arabidopsis* plants will be greatly helpful in elucidation of *GhWBC1* function in cotton fiber development.

MATERIALS AND METHODS

Plant Materials

Cotton (*Gossypium hirsutum* L. cv Xu-142) plants, the *fl* mutant (Zhang and Pan, 1991), and an *li* mutant of cotton (Karaca et al., 2002) were grown in the field. Cotton bolls were tagged on the day of anthesis. Ovules were harvested at various developing stages and frozen in liquid nitrogen. Plants of *Arabidopsis* ecotype Columbia were grown in soil under continuous light at 22°C.

DNA Analysis

Genomic DNA of the diploid cotton *G. arboreum* was extracted from etiolated seedlings (10 d old) as described by Tan et al., (2000). After an overnight digestion with *Hind* III, *Eco*R V, *Eco*R I, and *Xba*I, respectively, 40 µg of DNA in each lane was separated by electrophoresis and blotted onto nylon Hybond-XL (Amersham-Pharmacia Biotech, Uppsala). PCR performed on *GhWBC1* cDNA with primers GH1056 (5'-TCCCCGGGCC-AAAGTTAGAGGAACCGTGC-3') and GHR1850 (5'-CGGGATCCTCCATTTCGACCGTCCG-3'), and the amplified product was ³²P labeled by using the Prime-a-Gene labeling system (Promega, Madison, WI) and used as a probe. Hybridization and washing were performed according to a standard protocol (Sambrook et al., 2001). The membrane was exposed to x-ray film for 2 d.

cDNA Library Construction and RNA Analysis

Arabidopsis total RNA was isolated by using the TRIzol method (Invitrogen, Carlsbad, CA). Cotton Total RNA was isolated from different organs using a cold phenol method (Groppe and Morse, 1993). For cDNA library construction, total RNA was isolated from ovules of cotton cv Xuzhou-142, which were collected at -3, -1, 0, 1, 3, 5, 7, 9, 11, 13, and 15 DPA, respectively. The RNA samples were then pooled with equal quantity for mRNA extraction. The library was constructed by using a ZAP Express cDNA Synthesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's manual.

For RNA blots, total RNA was separated by denaturing agarose gel and transferred onto Hybond-XL membranes (Amersham-Pharmacia Biotech). The blots were hybridized with ³²P-labeled DNA probes of *GhWBC1*, *E6*, and *Expansin*, respectively. Hybridization and washing were performed according to a standard protocol (Sambrook et al., 2001). The blots were finally exposed to x-ray film for 2 d.

The DNA probe of *GhWBC1* was obtained as described for Southern blot. For *E6* and *Expansin*, PCR was performed on corresponding cDNA clones. The *E6* fragment was amplified with primers E60 (5'-CCATGGCTCCTCACAA-3') and E63 (5'-GGCATTCTGTGTAGTAG-3'), and the *Expansin* fragment was amplified with primers EXP1 (5'-CGAACCAACCGTACAGC-3') and EXP2 (5'-AATTTCTGGAC-ATAGGTAG-3').

For RT-PCR analysis, the first strand cDNA was synthesized with 1 µg of total RNA in a volume of 20 µL using the RNA PCR kit (TaKaRa, Dalian, China). Reaction mixture of 1 µL was used as template in PCR amplification with gene-specific primers. The primers used were: GH1 (5'-CTTATTAACCTTACCGTACATCAC-3'), GH2 (5'-AACACGAAGAA CATATAATGTTCA-3'), GH3 (5'-TTCCTGGCGAATACATCCTT-3'), and GH4 (5'-AACTTCAAACGCACAATACAGAT-3') for *GhWBC1* (Zhu93); HIS1 (5'-GAAGCCTCATCGATACCGTC-3') and HIS2 (5'-CTACCACTACCATCATGGC-3') for *GhHIS3* (AF024716); AT1 (5'-TTGGTTGACTATTACCACACTTCTG-3') and AT2 (5'-AGAACAAGAAGAGGTAGTGAGTG-3') for *AtWBC11*; and ACT3 (5'-GACCTGCATCACTACTCG-3') and ACT5 (5'-TTCCTCAATCTCATCTTCTCC-3') for *AtACT* (*ACTIN2*, At3g18820).

Subcellular Localization

The coding sequence of EGFP was amplified from the plasmid pEGFP-1 (CLONTECH, Palo Alto, CA) and in-frame fused to the 5'-terminal of *GhWBC1*. An intervening unstructured region (GPGGGG; Peck et al., 2001) was introduced between *EGFP* and *GhWBC1*. The resultant chimerical fusion gene *GFP-GhWBC1* and the *EGFP* coding region alone were inserted into pBI121 vectors (CLONTECH) behind the 35S promoter, respectively. For transient expression, inner onion (*Allium cepa*) peels (2 × 2 cm²) were placed on a filter soaked with liquid Murashige and Skoog medium and bombarded with 2 µg of each plasmid under a vacuum of 27 inches Hg and a helium pressure of 1,100 psi. After incubation on Murashige and Skoog medium for about 16 h, green fluorescence was examined under a microscope (BX51, Olympus, Tokyo).

Arabidopsis Transformation

PCR was performed with Pyrobest DNA polymerase (TaKaRa) on *GhWBC1* cDNA to introduce a *Sac*I site after the stop codon; then, the ORF of *GhWBC1* was inserted into a pBI121 vector (CLONTECH) between the *Sma*I and *Sac*I restriction sites, replacing the *GUS* reporter gene. The 35S: *GhWBC1*-NOS expression cassette was also cloned into pCAMBIA2301. The resultant binary vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 via electroporation. Transformation of *Arabidopsis* was performed by a floral dip method (Clough and Bent, 1998). T₁ transgenic plants were selected on one-half-strength Murashige and Skoog agar plates containing kanamycin (50 µg mL⁻¹). One-week-old resistant seedlings were transferred to soil.

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