Cotton GhHB1 gene and its expression profiling in root development and in response to stresses and phytohormones

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Homeodomain-leucine zipper (HD-Zip) proteins are transcriptional factors involved in plant development. In this study, one cDNA clone (Gossypium hirsutum homeobox1, designated GhHB1) encoding HD-Zip protein was isolated from a cotton root cDNA library. The GhHB1 cDNA is 1132 bp in length, including an 828 bp open reading frame that encodes a peptide with 275 amino acids, and 5′-/3′- untranslated regions. The predicted GhHB1 protein containing a homeodomain and a leucine-rich zipper motif shares relatively high identity with other plant HD-Zip proteins. Analysis using quantitative real-time RT-PCR indicated that the GhHB1 gene is predominantly expressed in roots and hypocotyls. Furthermore, GhHB1 transcripts were largely accumulated in early root development, and significantly reduced to very low levels as roots further developed, suggesting that the gene might function in the early development of roots. Under treatment with 1% NaCl, the expression level of the GhHB1 gene was dramatically increased in roots. Likewise, GhHB1 activity in roots was up-regulated by abscisic acid. These results imply that GhHB1 might play an important role in response to salt stress and to abscisic acid signaling.

Keywords: cotton; homeodomain protein; gene expression; development; stress

Homeodomain-leucine zipper (HD-Zip) proteins are transcriptional factors involved in plant development, and can be classified into four groups, HD-Zip I, HD-Zip II, HD-Zip III, and HD-Zip IV [1]. HD-Zip I and II are very similar in their domain structures, and might be related to the signal transduction networks of light, dehydration-induced abscisic acid (ABA), and auxin [2–5], whereas HD-Zip III and IV display slightly different sequences in their domains [6,7].

The homeobox (HB) genes encoding HD-Zip proteins are identified only in plants [8–10]. The expression of the HB genes is regulated by different external factors [11–13]. For example, the expression of AtHB-2/HAT4 is regulated by far-red light [14]. The antisense-transgenic plants of AtHB-2/HAT4 were shorter and developed more slowly than wild-type plants, whereas AtHB-2/HAT4 sense-transgenic plants showed a shade-avoidance phenotype with elongated hypocotyls and petioles, as well as earlier flowering, compared with the wild-type [14–17]. Sunflower Hahb-10 gene is predominantly expressed in mature leaves and up-regulated by etiolation and gibberellin in seedlings [18]. Overexpression of the Hahb-10 gene in Arabidopsis showed altered responses to illumination quality and intensity, indicating that Hahb-10 plays a role in light-dependent responses of plants [18]. The study revealed that ATHB7 expression in Arabidopsis is induced by drought as well as by ABA [19]. Although some HB genes have been well characterized in a few plant species (such as Arabidopsis), little is known about cotton HB genes.

In this study, we isolated a novel homeobox gene (Gossypium hirsutum homeobox1, designated GhHB1) in cotton and we report its expression profiling in cotton root development and in response to stresses and phytohormones.

Materials and Methods

Collection of plant materials

Cotton (Gossypium hirsutum) seeds were surface-sterilized with 70% (V/V) ethanol for 60 s and 10% (V/V) H₂O₂ for
Isolation of GhHB1 cDNA

To identify the genes that might be involved in regulation of root development, over 1,000 cDNA clones were randomly selected from a root cDNA library of cotton for sequencing. Some cDNA clones, including GhHB1, encoding the HD-Zip proteins were identified for further characterization.

DNA sequencing and protein analysis

Nucleotide and amino acid sequences were analyzed using DNAStar (DNAStar, Madison, USA). The GhHB1 protein structure was analyzed by MotifScan (http://myhits.isb-sib.ch/cgi-bin/motif_scan). The HD-Zip peptide sequences were aligned with the ClustalW program (http://www.ebi.ac.uk), and phylogenetic analysis was used to investigate the evolutionary relationships between GhHB1 and other plant HD-Zip proteins. A neighbor-joining tree was generated in MEGA3.1 [20]. A bootstrap analysis with 1000 replicates was carried out to assess the statistical reliability of the tree topology.

Treatments with NaCl, polyethylene glycol (PEG), and phytohormones, and at 4 °C

After seeds germinated and grew on basic MS semisolid medium with 0.4% agar without phytohormone at 28 °C in light for 5 d, the cotton seedlings were transferred to a cold environment, at 4 °C, for 12 h, or into MS liquid medium containing 1% NaCl, 16% PEG, 10 μM zeatin, 10 μM gibberellin3, 10 μM indole acetic acid, or 10 μM ABA for 12 h. For further experiments, the 5-d-old cotton seedlings were treated with various concentrations of ABA for 12 h, or with 10 μM ABA and 1% NaCl for 3, 6, 12, 24, or 48 h in MS liquid medium. Roots were collected from the seedlings for total RNA isolation.

Real-time RT-PCR

Total RNA was isolated from fibers, ovules, anthers, petals, leaves, stems, cotyledons, hypocotyls, and roots of cotton using modified CTAB acerbic phenol and hot phenol methods. Concentration of the isolated total RNA was determined using a NanoDrop spectrophotometer (NanoDrop, Wilmington, USA) and agarose gel electrophoresis. The real-time RT-PCR reaction was carried out according to our previous method, using a cotton polyubiquitin gene (GhUBI) as a standard control [21]. First, total RNA samples (2 μg per reaction) from fibers, ovules, anthers, petals, leaves, stems, cotyledons, hypocotyls, and roots were reversely transcribed into cDNAs by AMV reverse transcriptase (Roche, Nutley, USA) according to the manufacturer’s instructions. The cDNAs were used as templates in real-time PCR reactions with gene-specific primers. The specific primers of GhHB1 were GhHB1-Up (5′-GCATGACTCAACTCC-TTCAG-3′) and GhHB1-Down (5′-GCCAACCAACTC-TCCATATTG-3′). The real-time PCR reaction was carried out using Real-time PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The amplification of the target genes was monitored every cycle by SYBR-Green fluorescence. The Ct, defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, was used as a measure for the starting copy numbers of the target gene. Relative quantification of the target GhHB1 expression level was carried out using the comparative Ct method. The relative value for the expression level of the GhHB1 gene was calculated using the equation:

\[ Y = 10^{\frac{\Delta Ct}{3.7}} \times 100\% \]

where, \( \Delta Ct \) is the difference in Ct between the control GhUBI products and the target GhHB1 products; i.e., \( \Delta Ct = Ct_{GhUBI} - Ct_{GhHB1} \). PCR products were confirmed on an agarose gel. The primer efficiency was detected using GhHB1 cDNAs as the standard template, and the RT-PCR data were normalized with the relative efficiency of the primer pair.

Results

Isolation and characterization of GhHB1 gene

To isolate the genes that might be involved in regulation of root development, we randomly sequenced over 1000 cDNA clones from a root cDNA library of cotton. Clones likely to be involved in regulation of root development were chosen for further study. Of these, one cDNA clone (designated GhHB1; GenBank accession No. EF151309) was identified to encode an HD-Zip protein. It was 1132 bp in length, including an 828 bp open reading frame encoding an HD-Zip protein with 275 amino acids (molecular weight 31.28 kDa; pI 6.665). Protein structure analysis revealed that the deduced GhHB1 protein contains the typical homeodomain and leucine zipper motif located at Ser73–Lys133 and Leu135–Leu177, respectively (Fig. 1). Similarity comparison between GhHB1 and three known cotton polyubiquitin gene (GhUBI) as a standard control [21]. First, total RNA samples (2 μg per reaction) from fibers, ovules, anthers, petals, leaves, stems, cotyledons, hypocotyls, and roots were reversely transcribed into cDNAs by AMV reverse transcriptase (Roche, Nutley, USA) according to the manufacturer’s instructions. The cDNAs were used as templates in real-time PCR reactions with gene-specific primers. The specific primers of GhHB1 were GhHB1-Up (5′-GCATGACTCAACTCC-TTCAG-3′) and GhHB1-Down (5′-GCCAACCAACTC-TCCATATTG-3′). The real-time PCR reaction was carried out using Real-time PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The amplification of the target genes was monitored every cycle by SYBR-Green fluorescence. The Ct, defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is first detected, was used as a measure for the starting copy numbers of the target gene. Relative quantification of the target GhHB1 expression level was carried out using the comparative Ct method. The relative value for the expression level of the GhHB1 gene was calculated using the equation:

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HD-Zip proteins is shown in Fig. 2. GhHB1 shares 51% identity with HAHB1, 56% identity with HAT7, and 49% identity with ATHB13. All of the proteins contain the conserved homeodomain and leucine zipper motif. Although the homeodomain is quite conserved, there are 13 positions in which amino acids substitutions occurred among the homeodomains of the four HD-Zip proteins. In the homeodomain of GhHB1, the amino acid substitutions are involved in seven positions (74, 75, 84, 89, 93, 106, and 110), and five out of the seven substitution locations belong to conservative interchanges (Leu$^{75}$/Ala/Met, Leu$^{84}$/Met, Ala$^{89}$/Thr, Val$^{106}$/Met/Ile, and Lys$^{110}$/Arg). However, there are two positions at which dissimilar amino acid substitutions (His$^{74}$/Gln and Ser$^{93}$/Asn) occurred on the homeodomains between GhHB1 and the other HD-Zip proteins. Such interchanges could affect the HD-Zip protein structure and its function.

Fig. 2 Comparison of protein sequences among cotton GhHB1 and other known HD-zip proteins in plants

The homologous parts of the homeodomain structure are highlighted in black. The leucine zipper structure is denoted by gray box. ATHB13 (Arabidopsis thaliana): NP_177136; GhHB1 (Gossypium hirsutum) in this work; HAHB1 (Helianthus annuus): AAA63765; HAT7 (Arabidopsis thaliana): AAF26152.

Phylogenetic analysis of GhHB1

To analyze the phylogenetic relationships of GhHB1 with the known HD-Zip I proteins in plants, 11 HD-Zip proteins from different plant species were used for constructing a phylogenetic tree with MEGA 3.1. As shown in Fig. 3, these HD-Zip proteins could be divided into two clades. The first clade contains six members (ATHB7, ATHB12, ATHB13, HAT7, HAHB1, and GhHB1) and the second...
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The clade possesses five members (Oshox4, Oshox5, ATHB5, ATHB6, and ATHB1). All the 11 HD-Zip I proteins might have common provenance, and each member located in the same subgroup might have diverged relatively late during evolution. The cotton GhHB1 belongs to the first clade, and has the closest evolutionary relationship with HAHB1 (Fig. 3).

GhHB1 gene preferentially expressed in young roots and hypocotyls

To investigate the expression pattern of GhHB1 in cotton, we carried out real-time quantitative RT-PCR. The experimental results indicated that GhHB1 was strongly expressed in roots and hypocotyls, but its transcripts were detected at very low levels in other tissues (such as leaves, stems, cotyledons, petals, anthers, fibers, and ovules), suggesting that GhHB1 might function mainly in root and hypocotyl development. Furthermore, the highest accumulation of GhHB1 transcripts was detected in 3-day old roots, then the gene expression declined significantly to a relatively low level in 6-day old roots and gradually to much lower levels in 9- and 11-day old roots, suggesting that GhHB1 expression is regulated during the development of cotton roots.

GhHB1 expression is up-regulated by NaCl stress in roots

To investigate GhHB1 expression under various stresses, we treated the cotton seedlings with 1% NaCl, cold (4 °C), and 16% PEG. The experimental results revealed that GhHB1 expression was significantly increased in roots under NaCl stress, but was only slightly changed under cold and PEG treatments. Furthermore, the level of GhHB1 expression in the roots of the NaCl-treated seedlings was 2.5 to 3 folds higher than in the control seedlings, and its highest activity was detected in the roots under 1% NaCl treatment for 6–12 h, suggesting that the GhHB1 gene might be involved in response to salt signaling in early development of roots.

GhHB1 expression is induced by ABA in roots

To investigate whether phytohormones influence GhHB1

Fig. 3 Phylogenetic relationships between cotton GhHB1 protein and other HD-Zip proteins in plants

The neighbor-joining tree was constructed in MEGA3.1 from 1,000 bootstrap replicates. ATHB1, 5, 6, 7, 12, and 13 (Arabidopsis thaliana): NP_186796, NP_201334, NP_565536, NP_182191, NP_191748, and NP_177136, respectively; GhHB1 (Gossypium hirsutum) in this work; HAHB1 (Helianthus annuus): AAA63765; HAT7 (Arabidopsis thaliana): AAF26152; Oshox4 and 5 (Oryza sativa, japonica cultivar group): AAD37697 and AAD37698, respectively.

Fig. 4 Real-time RT-PCR analysis of GhHB1 expression in cotton tissues

(A) Expression of GhHB1 in different cotton tissues. 1, 6-day old root; 2, hypocotyls; 3, stem; 4, cotyledon; 5, leaf; 6, petal; 7, anther; 8, 10-day post-anthesis fiber; 9, 10-day post-anthesis ovule. (B) Expression of GhHB1 in 3-, 5-, 9-, and 11-day old roots. Relative value of GhHB1 expression in cotton tissues is shown as the percentage of GhUBI expression activity (n=3). Error bars represent standard deviation.
expression, several exogenous phytohormones were added in MS medium for cultivating cotton seedlings, and then \textit{GhHB1} expression activity in roots of the seedlings was detected by real-time RT-PCR. The experimental results revealed that under ABA treatment, the accumulation of \textit{GhHB1} mRNAs was significantly increased in roots. Unlike ABA, however, indole acetic acid and zeatin treatments did not affect \textit{GhHB1} activity in roots (data not shown). Compared with the control seedlings, the expression of \textit{GhHB1} was significantly enhanced by over 2-fold in roots when the seedlings were treated by ABA for 3 h, and kept its high expression levels in the roots till 48 h [Fig. 6(A)]. Furthermore, all the treatments with 2–25 μM ABA were effective on promoting \textit{GhHB1} expression activity in roots of cotton seedlings [Fig. 6(B)]. The experimental results suggest that \textit{GhHB1} might be involved in the ABA signaling pathway.

**Discussion**

HD-Zip proteins are characterized by the presence of a DNA-binding homeodomain and an adjacent leucine zipper motif mediating protein-dimer formation [10]. HD-Zip proteins, which are unique to plants, might play important roles in the development of high plants. In this study, an HB gene \textit{GhHB1} encoding an HD-Zip protein was identified in cotton. Structural analysis of the deduced protein showed that \textit{GhHB1} contains an HD and a Zip motif (Fig. 1). Comparison of protein sequences showed that \textit{GhHB1} shares relatively high identity with the other plant HD-Zip I proteins (Fig. 2). The phylogenetic analysis also showed that \textit{GhHB1} has a close evolutionary
relationship with the HD-Zip I proteins (Fig. 3). Thus, cotton GhHB1 belongs to the HD-Zip I subfamily.

The expressions of many plant HB genes are regulated by internal and external factors, and are involved in ABA signaling pathway. A previous study revealed that the expression of ATHB5, as a positive regulator of ABA responsiveness, is developmentally controlled and dependent on ABA signal transduction in Arabidopsis seedlings [22]. Similarly, the expression of ATHB7 and ATHB12 genes are induced by exogenous ABA or stimuli (such as water deficit or osmotic stress) that increase the endogenous levels of ABA, indicating that these genes might act in signaling pathways mediating responses to drought in Arabidopsis [23–25]. Sunflower Habb-4 is up-regulated by water stress, suggesting that it might function in signaling cascades of ABA-dependent responses to water stress [13]. Arabidopsis ABI1 and ABI2, the type 2C protein serine/threonine phosphatases, act as key regulators in response to ABA, whereas the transcriptional regulator ATHB6, as a target of ABI1, linked the protein phosphatase 2C for the regulation of ABA signaling [4].

The study showed that CpHB6 and CpHB7 are up-regulated by dehydration, whereas CpHB3, CpHB4, and CpHB5 are down-regulated by dehydration in both leaves and roots of Craterostigma plantagineum [26]. In oilseed rape (Brassica napus), BnHB6 expression in shoots is significantly up-regulated by ABA, mannitol, NaCl, cold, H2O2, and salicylic acid treatments, implying that it plays a positive role as a regulator of biotic and abiotic stresses on seedling growth [27]. In this study, likewise, the presented data indicated that GhHB1 expression was up-regulated by ABA and NaCl treatments (Figs. 5 and 6), implying that GhHB1 might play a role in response to salt stress and to ABA signaling. Furthermore, the GhHB1 transcripts were largely accumulated in 3-day old roots, but its expression activity gradually declined to very low levels as roots further developed (Fig. 4), suggesting that it might function in early root development of cotton.

In summary, the isolated GhHB1 gene is a new member of the plant HB gene family, and its expression is regulated in root development of cotton and in response to stresses and phytohormone signaling. Thus, the results of this study contribute to the understanding of the regulation of GhHB1 expression in cotton. However, the role of this gene in cotton development still remains to be explored in the future.

References

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