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Expression analysis of two novel cotton *14-3-3* genes in root development and in response to salt stress

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Abstract

14-3-3 proteins are phosphoserine-binding proteins that regulate the activities of a wide array of targets via direct protein–protein interactions, and may play an important role in response to biotic and abiotic stresses. In this study, two cDNAs (designated as *Gh14-3-3b* and *Gh14-3-3c*) encoding putative 14-3-3 proteins were isolated from cotton cDNA libraries. *Gh14-3-3b* gene encodes a protein of 268 amino acids, while *Gh14-3-3c* gene encodes a protein of 261 amino acids. Real-time RT-PCR analysis revealed that both the *Gh14-3-3b* and *Gh14-3-3c* genes were preferentially expressed in roots. The transcript levels of both the genes were the highest in 3-day-old roots, and then dramatically decreased to relatively low levels in parallel with root development. In addition, the expression of these *Gh14-3-3* genes in roots was significantly up regulated by salt treatments, suggesting that they may be involved in the signaling pathways in response to salt stress in cotton.

Keywords: Cotton (*Gossypium hirsutum*); *14-3-3* genes; Gene expression; Root development; Salt stress

1. Introduction

The 14-3-3 protein family was originally identified in 1967 by Moore and Perez [1]. The name 14-3-3 was given to this abundant mammalian brain protein family due to its particular elution and migration pattern on two-dimensional DEAE-cellulose chromatography and starch gel electrophoresis [2]. Members of the 14-3-3 protein family form a group of highly conserved 30 kDa acidic proteins expressed in a wide range of organisms and tissues. These molecules form homo- and hetero-dimers that can interact with a wide variety of cellular proteins [3,4]. 14-3-3 proteins associate with so many different molecules in large part because of their specific phosphoserine/phosphothreonine-binding activity [5]. For example, the tobacco transcription factor RSG binds to 14-3-3 proteins by its specific phosphoserine/phosphothreonine-binding activity, and is involved in gibberellins biosynthesis [6,7]. Analysis of known 14-3-3-binding sites, together with the use of peptide libraries, has defined two high affinity phosphorylation-dependent binding motifs that are recognized by all 14-3-3 isotypes: RSXpSXP (mode 1) and RXXXpSXP (mode 2), where pS represents phosphoserine [8,9]. As 14-3-3 binding is primarily phosphorylation dependent, 14-3-3 interactions are largely regulated by the kinases and phosphatases that modulate the phosphorylation state of the target protein.

In plants, various 14-3-3 isoforms have differential affinities to certain target proteins [10]. *Arabidopsis* has the most completely elucidated and the largest *14-3-3* family, which consists of 15 members and at least 12 expressed members classified into two evolutionary branches [11]. These 14-3-3

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isoforms exhibit a highly cellular and tissular specificity, and are localized within the cytoplasm and in organelles such as nuclei, plastids and mitochondria. The expression specificity and subcellular compartmentalization of 14-3-3 isoforms contribute to their diverse interactions with their partners and differential functions in cellular activities. However, our understanding of the significance of 14-3-3 proteins in the regulation of cotton development and environmental adaptation is still in its infancy, and little attention has been focused on cotton 14-3-3 proteins to date.

Cotton (*Gossypium hirsutum*) has been cultivated worldwide for centuries as an important crop (cultivated primarily for its fibers and seeds). Environmental conditions affect the growth state of cotton. For instance, mineral nutrient deficiencies and salinity constitute major limitations for crop growth in agricultural soils around the world [12,13]. Plant roots are the primary organ involved in mineral acquisition and salt tolerance. Plant roots perceive and respond to their environment using signaling systems, particularly adapted to mineral nutrient deficiencies and salinity in soils. The recent studies suggest that the plant phosphoserine-binding 14-3-3 proteins may play an important role in response to salt stress. Although the expression of *14-3-3* genes under biotic and abiotic stress has been investigated in a few plant species [14], the expression profile of the *14-3-3* genes in response to salt stress in cotton has not been studied. In this study, two cDNAs (designated as *Gh14-3-3b* and *Gh14-3-3c*) encoding putative 14-3-3 proteins were isolated from cotton cDNA libraries. The molecular characterization of the two *Gh14-3-3* genes and their expression in cotton tissues and in response to salt stress were investigated to further analyze the roles of these cotton 14-3-3 proteins in cotton development and in response to abiotic stress.

2. Materials and methods

2.1. Collection of plant materials

Cotton (*Gossypium hirsutum*) seeds were surface sterilized with 70% ethanol for 60 s and with 10% H_2O_2 for 1–2 h, followed by washing with sterile water. The sterilized seeds were germinated on 1/2MS medium under a 16 h light/8 h dark cycle at 28 °C for 5–6 days. Roots, cotyledons and hypocotyls were cut from sterile seedlings. The seedlings were transplanted into soil for further growth to maturation. Other tissues for RNA extraction were derived from cotton plants grown in fields.

In the stress experiments, the sterilized seeds were germinated on 1/2MS medium under a 16 h light/8 h dark cycle at 28 °C for 3 days. Control plants were maintained under the same conditions of light and temperature. For salt stress treatments, the plants were grown for 6 days in 1/2MS medium, and then transferred into the fresh medium supplemented with 0.25%, 0.5%, 0.75%, and 1% NaCl separately for 12 h. The parallel control plants were grown in the same medium without NaCl. Cotton roots (stressed and control) were harvested 12 h after the plants were exposed to these treatments. Total RNAs from roots were reverse transcribed to cDNAs, which were subjected to comparative real-time PCR quantification.

2.2. Isolation of 14-3-3 cDNAs

More than 4000 cDNA clones were randomly selected from the cotton cDNA libraries that we constructed (unpublished) for sequencing. Two cotton *14-3-3* cDNAs (designated *Gh14-3-3b* and *Gh14-3-3c*) were identified from these clones, and then completely sequenced.

2.3. RNA isolation and real-time RT-PCR analysis

Total RNA was extracted from roots, hypocotyls, cotyledons, leaves, petals, anthers, 10 DPA (day post anthesis) ovules and 10 DPA fibers of cotton by CTAB/acerbic phenol and hot-phenol methods. The concentration and purity of total RNA were identified by spectrophotometry and agarose gel electrophoresis.

Expression of the *Gh14-3-3* genes in cotton tissues was analyzed by real-time quantitative RT-PCR using the fluorescent intercalating dye SYBR-Green in the detection system (MJ Research, Option 2). A cotton polyubiquitin gene (*GhUBI1*, GenBank Accession No. EU604080) was used as a standard control in the RT-PCR. A two-step RT-PCR procedure was performed in all experiments using a method described earlier [15]. In brief, total RNA was reverse transcribed into cDNA and used as templates in real-time PCR with gene-specific primers as follows: *GH14-3-3b*, 5′-AGCCAGAGCAGTAGTGAGGGTC-3′ and 5′-GTAGGGAGCCACTCAGGGGCAC-3′; *GH14-3-3c*, 5′-ATCGCCAATCTTCACGGTAC-3′ and 5′-CATCTCCATCATCACTCCCT-3′; *GhUBI1*, 5′-CTGAATCTTCGCTTTCACGTTATC-3′ and 5′-GGGATGCAAATCTTCGTGAAAAC-3′. The real-time PCR was performed using SYBR Green Real time PCR Master Mix (Toyobo, Japan) according to the manufacturers' instructions. The cycle threshold (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 number of the target gene. Relative quantity of the target *Gh14-3-3* expression level was determined using the comparative *Ct* method. The relative value for the expression level of each *Gh14-3-3* gene was calculated by the equation $Y = 10\Delta^{Ct/3.75} \times 100\%$ (ΔCt is the differences of *Ct* between the control *GhUBI* products and the target *Gh14-3-3* products, i.e. $Ct = CtGhUBI - CtGh14\text{-}3\text{-}3$). To achieve optimal amplification, PCR conditions for every primer combination were optimized for annealing temperature and Mg^{2+} concentration. PCR products were confirmed on an agarose gel. The efficiency of each primer pair was detected by using *Gh14-3-3* cDNAs as the standard templates, and the RT-PCR data were normalized with the relative efficiency of each primer pair.

2.4. DNA sequencing and protein analysis

The sequences of the isolated cotton *14-3-3* genes (cDNAs) and their deduced proteins were analyzed by DNASTAR software (DNA Star Co.), and protein sequence homology analysis was performed with Clustal W (http://www.ebi.ac.uk/clustalw/). Phylogenetic analysis was carried out to investigate the evolutionary relationships among the Gh14-3-3 proteins and other plant 14-3-3 proteins. A neighbor-joining tree was generated by MEGA3.1 software. A bootstrap analysis with 1000 replicates was performed to assess the statistical reliability of the tree topology.

3. Results

3.1. Isolation and characterization of Gh14-3-3 cDNA

We randomly sequenced over 4000 cDNA clones from cotton cDNA libraries, and two cDNA clones were identified which encode putative 14-3-3 proteins. Both the cDNAs, designated as *Gh14-3-3b* and *Gh14-3-3c* (GenBank accession numbers: EU189221 and EU189222), contain a coding region, and a 5′-UTR and 3′-UTR. *Gh14-3-3b* cDNA contains an open reading frame (ORF) of 807 bp encoding a protein of 268 amino acids (30.24 kDa, pI 4.625), including 33 basic amino acids, 50 acidic amino acids, 70 polar amino acids, and 89 hydrophobic amino acids. *Gh14-3-3c* cDNA contains an ORF of 786 bp encoding a protein of 261 amino acids (29.51 kDa, pI 4.68), including 34 basic amino acids, 50 acidic amino acids, 63 polar amino acids and 89 hydrophobic amino acids. *Gh14-3-3b* and *Gh14-3-3c* proteins share a high homology with the *Gh14-3-3L* at the amino acid level [16].

3.2. Homology analysis of 14-3-3 proteins

By comparing the amino acid sequences (Fig. 1) of Gh14-3-3b and Gh14-3-3c with Gh14-3-3L proteins, we found that the amino acid sequences of these proteins are highly conserved except in the C-terminal and N-terminal regions. All the proteins contain nine antiparallel alpha-helices, and a dimeric structure which forms a groove for potential interaction with a substrate, and two highly conserved phosphorylation-dependent binding motifs, as found in known plant 14-3-3 proteins [17], which were found to lie within helices 3 and 5. The dimeric structure of 14-3-3 proteins contains many potential protein-binding sites, which are specific to the phosphorylation status of its targets. Furthermore, this conserved structure may be related to the regulation pathway of Gh14-3-3 proteins.

3.3. Phylogenetic relationship analysis of 14-3-3 proteins

To investigate divergence of the Gh14-3-3 proteins with other known 14-3-3 proteins during evolution, the phylogenetic relationships of sixteen 14-3-3 proteins were generated by MEGA3.0. As shown in Fig. 2, the tree is clearly divided into two distinct branches: the eight proteins (including Gh14-3-3b and Gh14-3-3c) form one branch, while the remaining eight proteins (including Gh14-3-3L) form the other branch. The two groups can be further divided into seven subgroups. Among them, clade 2, clade 3 and clade 6 have only one member (GRF5, GRF4 and GRF3), respectively, indicating that the divergence of the three proteins occurred early during evolution. In contrast, clade 1 contains four members, clade 4 has two members, and clade 5 has three members, while clade 7 consists of four members. Gh14-3-3b and GRF1 together form clade 4, indicating that divergence between these two proteins occurred relatively late. On the other hand, Gh14-3-3c, GRF6, GRF8 and GRF12 together form a single branch, indicating that they may have a close phylogenetic relationship.

3.4. Expression analysis of Gh14-3-3b/c genes in different tissues

To investigate expression patterns of the isolated *Gh14-3-3* genes, total RNA was extracted from roots, hypocotyls, cotyledons, leaves, petals, anthers, 10 DPA ovules and 10 DPA fibers of cotton. The expression patterns of two *Gh14-3-3* cDNA clones were analyzed by a real-time quantitative SYBR-Green RT-PCR using gene-specific primers as described in Methods. The relative transcript levels of the two *Gh14-3-3* genes are shown in Fig. 3(a) and (b), using the cotton polyubiquitin gene (*GhUBI1*) as a standard control to normalize differences in RNA template concentrations [15]. The results indicated that both *Gh14-3-3b* and *Gh14-3-3c* genes are preferentially expressed in roots. The *Gh14-3-3b* mRNA was detected mainly in roots and hypocotyl, and the gene showed relatively low expression levels in other tissues. *Gh14-3-3c* was predominantly expressed in roots, but also expressed at a relatively high level in ovules. These tissue-wide patterns of *Gh14-3-3* expression are probably associated with their differential functions, implicating their multifunctional roles in plant development processes.

3.5. Developmental-regulated expression of Gh14-3-3b/c genes in cotton roots

From Fig. 3(a) and (b), we found that *Gh14-3-3b* and *Gh14-3-3c* are preferentially expressed in roots. To investigate whether expressions of the Gh14-3-3 proteins are developmentally regulated in root, expression profiles of the two *Gh14-3-3* genes during root development were analyzed by real-time quantitative RT-PCR. The experimental results (Fig. 3(c) and (d)) revealed that *Gh14-3-3b* and *Gh14-3-3c* possess similar expression profiles in root development. The transcripts of the two *Gh14-3-3* genes were accumulated at the highest quantity at an early stage (3-day-old), and then decreased to relatively low levels in parallel with root development, suggesting that the expression

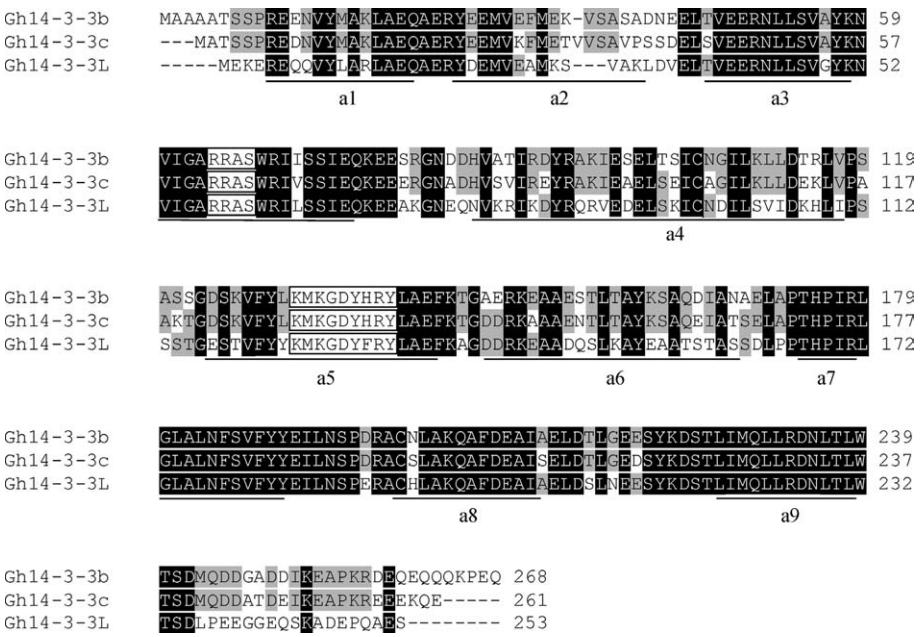

Fig. 1. Sequence alignment among the three cotton 14-3-3 proteins. Multiple sequence alignment was performed with the Clustal W program. The amino acid residues identical among the sequences are indicated in black, while similar residues are shown in grey. Dashes indicate gaps in the sequences to allow for maximal alignment, and the regions of the conversed antiparallel α-helices are underlined. Two white frames represent two phosphorylation motifs conserved among the sequences.

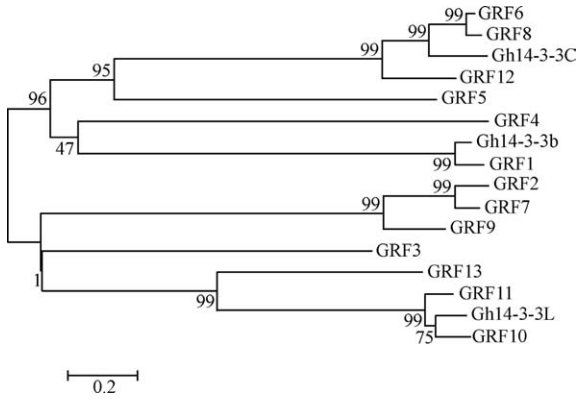

Fig. 2. Phylogenetic relationships of cotton 14-3-3 proteins and *Arabidopsis* 14-3-3 proteins. The accession numbers of various *Arabidopsis* 14-3-3 proteins are GRF1: NP_567344; GRF2: NP_565176.1; GRF3: NP_001031982.1; GRF4: NP_564453.1; GRF5: NP_568325.1; GRF6: NP_001031868.1; GRF7: NP_566174.1; GRF8: NP_569012.2; GRF9: NP_001031532.1; GRF10: NP_564167.1; GRF11: NP_001077649.1; GRF12: NP_564249.1; GRF13: NP_001106044.1.

of the *Gh14-3-3s* is highly developmentally regulated in roots.

3.6. Expression analysis of Gh14-3-3b/c genes under NaCl stress

We analyzed the expression of these *Gh14-3-3* genes in response to abiotic stresses, including salinity, drought and cold treatments. The results indicated that both *Gh14-3-3b* and *Gh14-3-3c* genes were differentially regulated by the stress stimuli. The transcript levels of *Gh14-3-3s* rapidly increased in roots when treated with 1% NaCl for 12 h, but their expression was hardly altered under PEG 6000 and 4 °C cold treatments (data not shown), suggesting that the *Gh14-3-3b/c* might play a role in response to NaCl stress.

We further analyzed the expression of the two *Gh14-3-3* genes in response to salt stress (Fig. 3(e) and (f)). Expression of the two *Gh14-3-3* genes was rapidly up regulated by the salt treatments. They exhibited slightly similar expression patterns in response to salt stress in young roots. Expression levels of *Gh14-3-3b* and *Gh14-3-3c* were up regulated to the highest level under 0.25% salt treatment. This result suggests that these *Gh14-3-3* genes may be involved in the salt stress signaling pathways in cotton roots.

4. Discussion

The 14-3-3 proteins form a family of ubiquitous regulatory molecules, which have been found in virtually every eukaryotic organism and tissue [2]. The 14-3-3 proteins have been known to play important roles in many plant biological processes. 14-3-3 proteins were the first studied in mammalian nervous tissues, but in the past decade their indispensable role in higher plants adapted to abiotic stress had been increasingly established [14].

In *Arabidopsis*, tobacco and tomato, many 14-3-3 isoforms have been found. So far, 14-3-3 proteins are thought to be involved in a large range of abiotic signaling processes and to interact with many target molecules, including plasma membrane H^+-ATPase, ion channels,

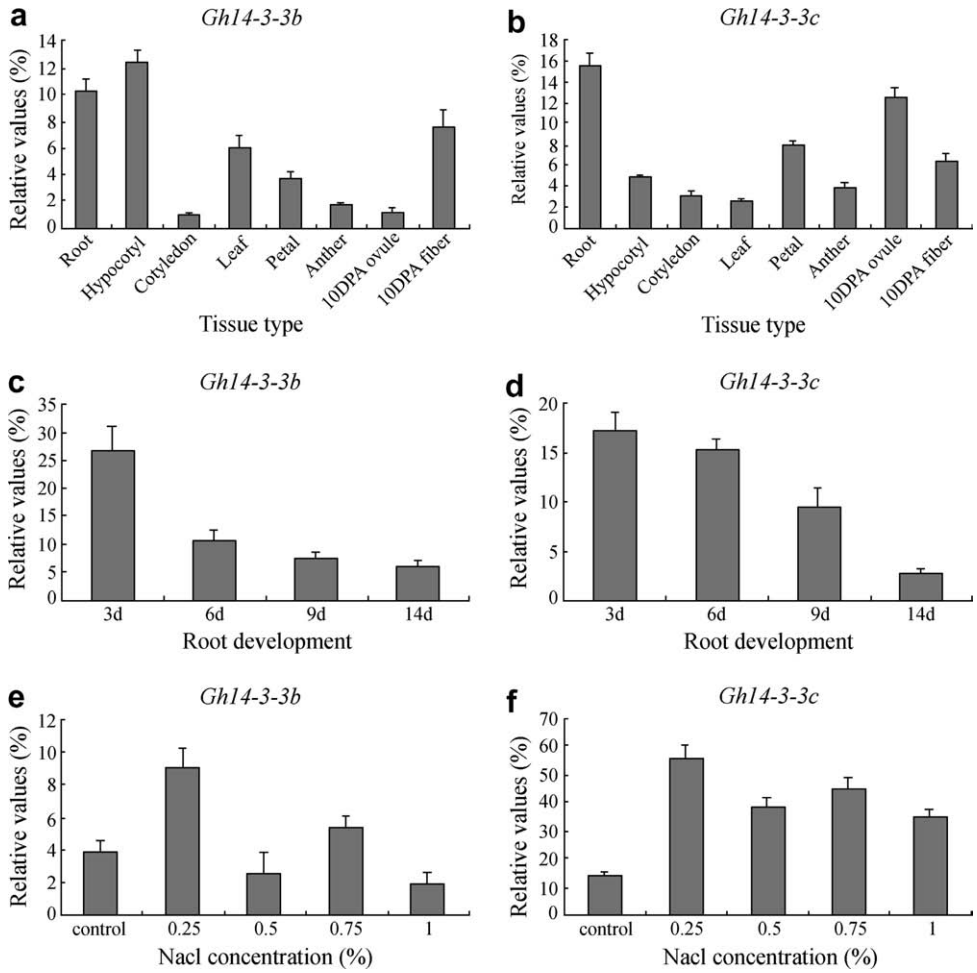

Fig. 3. Expression analysis of *Gh14-3-3b* and *Gh14-3-3c* genes. (a and b): expression of Gh14-3-3b/c in cotton tissues; (c and d): expression of Gh14-3-3b/c in root development of cotton (d: day old); (e and f): expression of *Gh14-3-3b/c* genes in cotton roots under NaCl stress.

ascorbate peroxidase (APX) and abscisic acid (ABA). All these targets are very important for plants adapted to salinity [18–21]. Thus, 14-3-3 proteins may be involved in regulation of the higher plant response to the soil abiotic stresses. In this study, we performed a real-time RT-PCR to investigate the expression profile of the isolated cotton *14-3-3* genes in response to salt stress. Our experimental results indicated that the expression levels of the two *Gh14-3-3* genes were significantly enhanced under NaCl treatment compared to the control conditions, implicating that these 14-3-3 proteins may be involved in response to salt stress in cotton roots.

Under high salt conditions, plant growth is severely inhibited due to ionic, osmotic and oxidative stresses [22]. It is well known that in higher plants, the plasma membrane H^+-ATPase plays an important role under salt stress and is activated by binding of a 14-3-3 protein to the phosphorylated C-terminus [23,24]. Under osmotic stress, an increase in the enzyme activity of the plasma membrane H^+-ATPase was accompanied by accumulation of 14-3-3 proteins in plasma membranes in maize roots [25]. A previous study revealed that increased 14-3-3 protein levels were detected in the plasma membrane of tomato cells upon osmotic shock [26]. APX, which interacts with 14-3-3 proteins, also plays a very important role in protecting plants from osmotic and oxidative stresses [18]. Furthermore, overexpression of the 14-3-3 protein in potato improves the total antioxidant potential [27]. Therefore, 14-3-3 proteins may take part in the signaling pathways regulating plants in response to biotic and abiotic stresses, including salt stress. Similarly, we found that the expression of the isolated *Gh14-3-3* genes was significantly up regulated in root development and under salt stress (Fig. 3). These results suggest that the cotton 14-3-3 isoforms may be involved in the signaling pathways in root development and in response to salt stress; playing similar roles to the known 14-3-3 proteins in other plants.

In conclusion, two novel *14-3-3* genes were identified in cotton, and their expression patterns were revealed in root development and in response to salt stress. Understanding the actual roles and the complexity of the interactions of 14-3-3 proteins are the challenging goals for future

research. Complete understanding of Gh14-3-3 roles will require the determination of cellular and subcellular localizations and targets of specific isoforms. Therefore, searching for and identifying functions of the target proteins of a given 14-3-3 protein through the forward or reverse genetic approach would provide a clue for the 14-3-3-mediated cellular activities.

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