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Identification and characterization of a novel gene encoding myb-box binding zinc finger protein in *Gossypium arboreum*

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Abstract

Zinc finger proteins are encoded by the genes chiefly involved in stress resistance hormone signal transduction of plants. In this study, a cDNA encoding a zinc finger transcription factor was isolated by the yeast one-hybrid system from *Gossypium arboreum* using the MYB-box element of the universal stress gene (GUSP1) promoter as bait. The corresponding protein (named GaZnF) can bind specifically to a 13 bp MYB-box region. The *GaZnF* cDNA is 1093 bp in length, including a 510 bp open reading frame. The predicted GaZnF protein contains ANI-A20 motifs and shares a high sequence similarity with zinc finger proteins from other plants. Spatial expression pattern of *GaZnF* was studied under drought, heavy metals and salt stresses through real-time PCR. The gene showed enhanced expression under each stress treatment with maximum transcript abundance in root tissues. The results support the hypothesis that *G. arboreum* zinc finger proteins are involved in plant response to drought, salt and heavy metal stresses.

Additional key words: drought, gene expression, GaZnF protein, heavy metals, salinity.

Introduction

One important way to study multiple stress tolerance is to overexpress transcription factor gene(s) regulating multiple genes from various pathways (Oh *et al.* 2005, Ito *et al.* 2006) or by overexpressing genes involved in abiotic signal perception and transduction (Teige *et al.* 2004). Zinc finger transcription factors in plants are chiefly involved in stress resistance correlated with hormone signal transduction. Several zinc finger proteins have been reported in alfalfa (Bastola *et al.* 1998), *Arabidopsis* (Sakamoto *et al.* 2000, Ciftci-Yilmaz *et al.* 2007) and rice (Mukhopadhyay *et al.* 2004, Liu *et al.* 2007).

Universal stress protein (USP) is a small bacterial cytoplasmic protein that is upregulated when the bacterium is exposed to stress agents (Nystrom *et al.* 1994). A cotton homologue universal stress gene *GUSP1* enhanced the stress response of cotton in roots, shoots and leaves during the drought treatment (Maqbool *et al.* 2008). Strong activation of the *GUSP1* full promoter region occurred in tobacco leaves following drought, abscisic acid, salt and heavy metals treatments. There are several stress responsive *cis*-acting elements in *GUSP1* promoter including MYB, ABRE and metal response elements (Zahur *et al.* 2009). Binding motifs for plant MYB proteins are involved in the drought stress-induced gene expression (Urao *et al.* 1993).

In this study, we performed yeast one-hybrid screening with MYB motif as bait to isolate the nuclear protein interacting with this motif present in *GUSP1* promoter. We obtained a full length cDNA clone (acc. No. GQ169757) which encodes a protein named as GaZnF. This protein has two zinc finger domains, ANI and A-20 that bind to the MYB-box sequence (TAACTG). Real-time expression studies suggest that cotton zinc finger *GaZnF* may function as a stress responsive transcription factor and may be involved in the control of stress tolerance gene expression.

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Abbreviations: ABRE - abscisic acid response elements; GAPDH - glyceraldehyde-3-phosphate dehydrogenase; GaZnF - *Gossypium arboreum* zinc finger protein; MRE - metal response elements; NCBI - National Center for Biotechnology Information; q-PCR - quantitative real time polymerase chain reaction; RT-PCR - reverse transcriptase PCR; SAPs - stress associated proteins; USP - universal stress protein; UTRs - un-translated regions.
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Materials and methods

Plants and yeast strains: Seeds of cotton (*Gossypium arboreum* cv. FDH-171) were grown in peat, sand and soil mixture (1:1:1) in pots placed in greenhouse at temperature 25 ± 2 °C, relative humidity near 50 % and irradiance at midday 1 500 μmol m^{-2} s^{-1}. Plants (8-week-old) were subjected to drought stress for 10 d till the leaves showed wilting symptoms. For heavy metal and salt stresses, plants were placed in the solution with 500 μM $PbCl_2$, $CoCl_2$, and $NiCl_2$ or 500 μM NaCl solutions, respectively, for 3 d. Leaf samples were collected from control and stressed plants, immersed in liquid nitrogen and stored at -70 °C. Yeast Y187 strain was used for one hybrid library construction and transformation (Lopato *et al.* 2006).

Construction of target reporter strains: The reporter constructs were prepared by inserting a trimer of MYB motif (TCCAA**TAACTG**CC) into pHIS2.1 vector (*Clontech*, Palo Alto, CA, USA). A pair of primers was synthesized, one representing the sense strand and the other its antisense complement. To ligate the double stranded oligos into pHIS2.1 vector, 5'-overhangs compatible with *Eco*RI and *Sac*I restriction sites were inserted. The oligonucleotides were annealed in 1:1 ratio by heating at 95 °C for 30 s, 72 °C for 2 min, 37 °C for 2 min, 25 °C for 2 min and then slowly cooled to room temperature. The annealed fragments were fused to pHIS2.1 vector that had been digested with *Eco*RI and *Sac*I. The structure of the fusion construct (bait vector) was confirmed by sequencing.

Yeast one-hybrid screening: For *in vivo* recombination double-stranded cDNA prepared from dehydrated cotton leaves and pGADT7-Rec2 AD cloning vector (*Clontech*) were co-transformed into competent yeast cells along with bait vector through matchmaker one hybrid library construction and screening kit (*Clontech*) according to the protocol. Obtained positive colonies were re-streaked on SD/-Leu/-Tryp/-His/5mM 3-amino-1,2,4-triazole (3-AT) plates. Yeast colonies were selected and analyzed for presence of insert by specific PCR amplification using *Matchmaker AD LD-insert Amplimer* set (*Clontech*). Plasmid DNA from yeast cells was prepared according to Hoffman and Winsten (1987) and Kaiser and Auer (1993), as described in the one-hybrid system protocol (*Clontech*) and yeast protocols handbook (*Clontech*).

Sequence and phylogenetic analysis: The clones obtained after screening were sequenced (*ABI prism* dye terminator kit and *ABI 3100* automated DNA sequencer). Database search for homology was carried out using the *BLAST* tools provided by *NCBI* (Altschul *et al.* 1990). To find out the untranslated regions (UTRs), and *Poly-A tail* softberry server was used (http://www.softberry.com/berry.phtml). The conceptual translation of nucleotide sequence was made using the open reading frame finder program (ORF; www.ncbi.nlm.nih.gov/gorf/gorf.html). Multiple sequence alignment was performed using the *CLUSTALW* (Thompson *et al.* 1994) with default parameters through *EMBnet* (http://www.ch.embnet.org/software/ClustalW.html).

A phylogenetic analysis (Fig. 2) was performed with full length zinc finger sequences publicly available for *Citrus, Zea mays, Oryza sativa, Medicago truncatula, Camellia sinensis*, *Ricinus communis*, *Brassica rapa* and *Chlamydomonas reinhardtii*. A rooted Neighbor-joining phylogenetic tree was generated using the *MEGA* software package *v. 4.0* (http://www.megasoftware.net) (Kumar *et al.* 2004) from the previously aligned amino acid sequences. For rooting the tree, the *Chlamydomonas reinhardtii* sequence was designated as outgroup. To determine relative level of support for the tree topology bootstrap values were generated from 1 050 replicates.

Expression studies: A Jaakola *et al.* (2001) method was used for RNA extraction from leaf and root samples from control and stressed plants. Total RNA (1 μg) from each tissue was reverse transcribed with oligo-dT primer using *RevertAid*^{TM} *H minus* first strand cDNA synthesis kit (*Fermentas*, Germany) according to the manufacturer's protocol after DNase1 treatment.

For expression studies quantitative real-time PCR (q-PCR) reactions were carried out in an *iQ5* cycler with a 96-well plate using the *IQTM SYBR-Green* super mix (*Bio-Rad*, Hercules, USA). The reactions were performed using a pair of gene specific primers: RTSR-F: 5′-TCA GGATCATTGCCTTGTGA-3′ and RTSR-R: 5′-AGG TGGTTTTGTTGCAGGAG-3′ designed on the basis of the *G. arboreum* cDNA sequence encoding a product of 162 bp. The cotton glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene using the specific primers: Gap-F: 5′-TGGGGCTACTCTCAA AGGGTTG-3′ and Gap-R: 5′-TGAGAAATTGCTGAAG CCGAAA-3 (Maqbool *et al.* 2008). Standard curve was prepared with purified PCR product of *GaZNF* gene and its dilutions to validate the *iQ5* cycler reaction and to determine the range of quantification. In each reaction 200 ng of cDNA was used. Thermal cycling conditions were: 95 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 30 s. A melting curve analysis was carried out by continuously monitoring fluorescence between 60 and 95 °C with 0.5 °C increments every 30 s.

Statistical analysis of q-PCR results was performed using *iQ5* software *v. 1.0* (*Bio-Rad*) on the basis of ct values of the gene in different samples that were converted to their linear form using the mathematical term $2^{-\Delta\Delta CT}$ (Livak and Schmittgen 2001) normalized with *GAPDH* gene. The relative gene expression analysis was carried out by *SDS 3.1* software. Each individual experiment was performed in triplicate.

Results

The yeast reporter constructs, purified cDNA and pGAD-T7-Rec2 vector were co-transformed into yeast Y187 cells. A library of 0.213×10^6 independent clones was obtained. Colony PCR was performed from different plates. Four colonies were isolated from the plate carrying the MYB reporter strains as bait all of which were resistant to 5 mM 3-AT. The cDNA inserts of the isolated plasmids and purified colony PCR were studied after DNA sequencing.

One of the clones designated as A-19 contained a fragment of 1 093 bp encoding a putative nucleic acid binding zinc finger protein named as GaZnF (acc. No. GQ169757; Fig. 1).

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   1                                    ctctggtgtactgttgatt
  20 gatgaatcatgatggagatagttagtcctaggtatgagttcttat
  65 ttagtaaaatgatcttttacatgcttaatatgtgctcatgtctgt
 110 ttattgatatccatcttttggagttgaatcactttgttgttcttc
 155 ctctcttccttttgctcttattttcttaagttcctccttttatttt
 200 ggcttatcagtagtaaggtggttttgttgcaggagattagagaag
 245 atggaccctcatgatgagacgggccgccaagcttcagaaggcccc
     M  D  P  H  D  E  T  G  R  Q  A  S  E  G  P
 290 atcctgtgtgttaataactgtggtttctttggaagtgcggcgacc
     I  L  C  V  N  N  C  G  F  F  G  S  A  A  T
 335 atgaatatgtgctccaaatgtcacaaggcaatgatcctgaagcag
     M  N  M  C  S  K  C  H  K  A  M  I  L  K  Q
 380 gaacaggcacaactcgtagcatcatccatcgatagcattgttaat
     E  Q  A  Q  L  V  A  S  S  I  D  S  I  V  N
 425 ggcagcactagcgggaatggtaaggaaccatctgttgctgctgct
     G  S  T  S  G  N  G  K  E  P  S  V  A  A  A
 470 ttggctgtgcaatgtggaaattttgggtcaaaaattgaatcatcc
     L  A  V  Q  C  G  N  F  G  S  K  I  E  S  S
 515 atcgatccatcccatatgaccttcggtgggatgaaaacaaaagag
     I  D  P  S  H  M  T  F  G  G  M  K  T  K  E
 560 ggtcccaacaggtgtaatgcatgccacaagcgtgttggtttgaca
     G  P  N  R  C  N  A  C  H  K  R  V  G  L  T
 605 ggatttagctgcaggtgtggaaacatcttctgtgcagcacatagg
     G  F  S  C  R  C  G  N  I  F  C  A  A  H  R
 650 tactctgacaaacacaactgtcctttcgattataggacagctgcc
     Y  S  D  K  H  N  C  P  F  D  Y  R  T  A  A
 695 cgcgatgctattgctaaagccaaccccgtagtgagggctgagaaa
     R  D  A  I  A  K  A  N  P  V  V  R  A  E  K
 740 cttgataaaatctaaaaagagtcgaggatgaaggtgaaggtgaag
     L  D  K  I  *
 785 aattgtgtaatgaagtatgggggtttatgggtcctacccattgtg
 830 tcgtcagctcatcatagtgaaaggtttatttaaagttgggtgctt
 875 tgtctcattaggtgtccccttgatttgatataaaaacagtttgg
 920 taaagtggatatagggaaggacacaattgcagtgtaggaagtcta
 965 tatcatgtcattgtggcgatcatcagtcgccctgttggtagtcat
1010 tggattgggtgcctggtttgatggaacaatcgatatgatcccctt
1055 gtaatgcttcttttcaaaaaaaaaaaaaaaaaaaaaaaa 1093
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Fig. 1. Nucleotide and derived amino acid sequence of *GaZnF*. *Grey shading* indicates the zinc finger ZF-A20 domain (N-terminal) and ZF-AN1 domain (C-terminal).

The full length cDNA of *GaZnF* contained 244 bp of a 5' UTR a 339 bp 3' UTR and a 24 bp polyA tail. The coding sequence of *GaZnF* spans 510 bp (Fig. 1) encoding a predicted 18.1 kDa protein consisting of 170 amino acids. The N-terminal zinc finger domain is 34 amino acids long ranging from 12 - 46. It belongs to ZF-A20 super family. The C-terminal zinc finger of ZF-AN1 type super family was 43 amino acids long ranging from 107 - 150 and contains six conserved cysteine and two histidine residues. The protein has no detected signal sequence and the pSORT analysis showed that the protein was hydrophilic. On the basis of *BLAST* homology search, the prey protein was assigned to the ZnF-ANI-AN20 TF superfamily.

Homology search with the full-length amino acid sequences showed significant similarity to stress-associated proteins (SAPs) from rice and *Arabidopsis* (Vij and Tyagi 2006). The conserved domain search of GaZnF amino acid sequence in *NCBI* and the *Inter-proscan* in *EBI* server predicted the presence of two zinc finger domains characteristic of SAP gene family in plants with the consensus sequence of Cx2Cx9-12Cx1-2Cx4Cx2Hx5HxC, where x represents any amino acid.

The deduced 170 amino acid protein coded by the 1 093 bp coding sequence of the *GaZnF* was compared to the known sequences of zinc finger proteins: *Citrus* (73 %), *Zea mays* and *Oryza sativa* (65 %), *Ricinus communis* and *Medicago truncatula* (60 %), *Camellia sinensis* (57 %), *Brassica rapa* (52 %), and *Chlamydomonas reinhardti* (36 %). A-20 domain of GaZnF showed 91, 97, 76, 79, 85, 79, 82 and 88 % identity and AN1 domain showed 77, 81, 84, 77, 84, 68, 66 and 79 % identity to its homologues from *Zea, Citrus, Camellia, Medicago, Ricinus, Brassica, Arabidopsis* and *Oryza*, respectively. AN1 domain of GaZnF also showed 65 % similarity to *Phaseolus vulgaris* pathogen related protein (PVPR3; Sharma *et al.* 1992).

A phylogenetic tree was constructed based on the multiple sequence alignment of zinc finger protein sequences of plants. From the phylogenetic tree GaZnF was found to be closely related to zinc finger proteins of *Camellia sinensis* rather than that of *Oryza sativa* and *Zea mays* (Fig. 2).

Spatial expression patterns of cotton zinc finger protein were determined by q-PCR analysis. The used cDNAs were derived from cotton stems, roots and leaves under normal condition and after drought, salt and metals treatments.

The *GaZnF* gene was strongly expressed under drought treatment in roots (6-fold), shoots (4-fold) and leaves (3-fold) as compared to control plants. The expression pattern observed under salt treatment was 7-fold, 2-fold and 5-fold higher in roots, shoots and leaves, respectively. In contrast, *GaZnF* induction was six times higher in leaves after metals treatments, four times higher in roots and three times higher in shoots as compared to control. Overall the mRNA of the gene has a very low level of abundance in all examined unstressed tissues of cotton (Fig. 3).

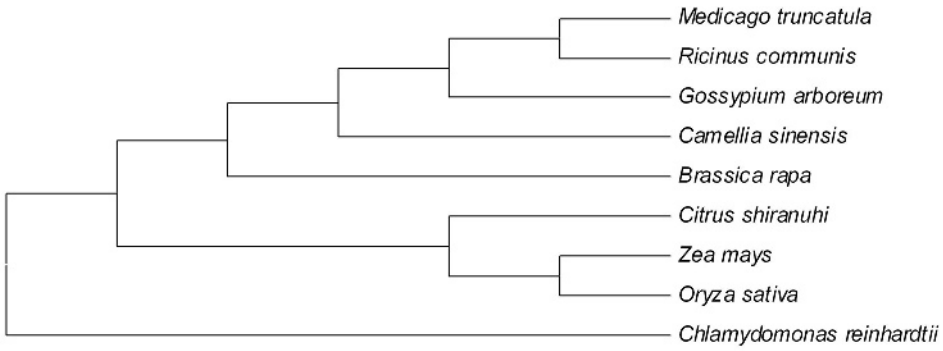

Fig. 2. Phylogenetic relationships between cotton GaZnF protein and other ZF proteins in plants. The distance-based Neighbor-joining tree was constructed in *MEGA4* from 1 050 bootstrap replicates. Sequences were aligned with the *ClustalW*. ABN08135: *Medicago truncatula*; EEF48869: *Ricinus communis*; *GQ169757: Gossypium arboreum*; ABI31653: *Camellia sinensis*; ABV89666: *Brassica rapa*; ABL67658: *Citrus shiranuhi*; ABS83245: *Zea mays*; AAQ84334: *Oryza sativa*; EDP01908: *Chlamydomonas reinhardtii* (used as an outgroup).

Discussion

Using the yeast one-hybrid screening method, a cDNA encoding GaZnF transcription factor was identified from *G. arboreum*. Zinc finger proteins can function as stress responsive transcription factors in plants, suggesting that they can regulate the activation of downstream target genes in stressed tissues.

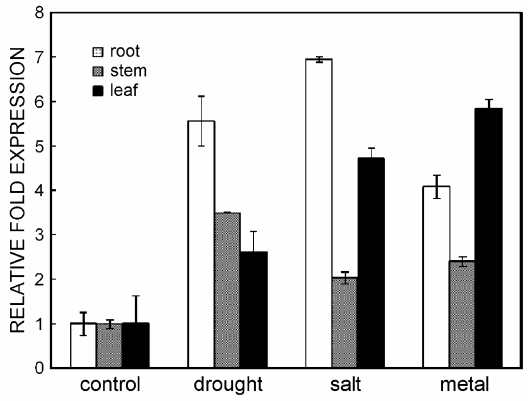

Fig. 3. Expression of *GaZnF* in root, stem and leaves (values relative to GADPH) of control and stressed cotton plants (10-d drought stress, 500 μM NaCl or 500 μM $PbCl_2$, $CoCl_2$ and $NiCl_2$ for 3d) determined by Q-PCR. Three independent experiments were performed having triplicate samples. *Error bars* indicate SD.

There are a number of DNA binding proteins containing different types of zinc finger domains. Binding specificity of each zinc finger protein differs among different plants. The three representative drought-responsive Q-type 2-fingered ZFP proteins from *Triticum aestivum* (TaZFP46h) were reported to bind preferably to a dimer of a G-rich motif (5'-GGGAGTGA) (Kam 2008). Sakamoto *et al.* (1996) reported the binding of WZF-1 zinc-finger protein to a CACTC sequence in the wheat histone gene promoter. Zinc finger protein MNB la with single putative zinc finger is known to interact with AAGG motifs (Yanagisawa 1995). A plant GATA-type zinc finger protein *AGP1* mediated transcriptional activation by binding to the AG-motif (AGATCCAA) of the wound-inducible *Myb* gene *NtMyb2* (Sugimoto *et al.* 2003). Here a novel zinc finger gene was first reported to binds to specific MYB elements (TCCAATAACTGCC) present in *GUSP1* promoter.

Table 1. Relative expression of zinc finger protein in cotton roots stems and leaves after 10-d drought stress, 500 μM NaCl or 500 μM $PbCl_2$, $CoCl_2$ and $NiCl_2$ for 3d as compared to control.

Treatments	Roots	Stems	Leaves
Control	1.00	1.00	1.00
Drought	5.56	3.50	2.62
NaCl	6.96	2.04	4.72
Metals	4.08	2.41	5.84

The amino acid sequence showed the presence of potential protein kinase phosphorylation site at 67 - 69 aa region and an N-myristoylation site (Towler *et al.* 1998, Grand 1989) at 127 - 133 position (Kanneganti and Gupta 2008). The human immune system protein *ZNF216* has the similar domain architecture (Huang *et al.* 2004). There are 18 rice and 14 *Arabidopsis* genes coding for SAP related proteins. SAP gene family members are characterized by the presence of A20/AN1 domains in their putative encoded proteins. The proteins with A20/AN1 zinc finger domains are presented in all eukaryotes, well characterized in animals. In accordance to the finding from animal systems that A20 and AN1 zinc finger domains are usually found linked with each

other (Evans *et al.* 2004), the majority of plant SAP gene family members have both the A20 (present at the N-terminus) and AN1 (present at the C-terminus) zinc finger domains. Genes showing homology in GaZnF conserved regions have been annotated in other plant species such as *Zea* (ABS83245.1), *Citrus* (ABL67658.1), *Camellia* (ABI31653.1), *Medicago* (ABN08135.1), *Oryza* (AAQ84334.1), *Brassica* (ABV89666) and *Arabidopsis* (AAK68811).

The cysteine and histidine residues of A20 and AN1 that are presumed to be involved in zinc finger formation were shown to be conserved between the *GaZnF* and mammalian zinc finger proteins as predicted by Kanneganti and Gupta (2008).

GaZnF is a multiple stress inducible gene. All the *SAP* gene family members were reported to be stress inducible (Vij and Tyagi 2006). Several zinc finger proteins from plant species were responsive to drought like petunia *ZPT2-3* gene encoding a Cys_2/His_2-type zinc finger protein (Shoji *et al.* 2003). Focusing on the drought related zinc finger proteins, Kam *et al* (2007) analyzed the expression profile of seven RING zinc finger genes from *Triticum aestivum* (designated *TaRZF*) and found that four genes were responsive to drought stress in various organs (leaf, root, stem, spike, endosperm and embryo). The q-PCR analysis of the mRNA distribution of 44 Q-type TaZFP genes in various organs revealed that 30 genes were predominantly expressed in the roots. These data are consistent with our findings that the transcript abundance increased in root tissues during drought stress.

The expression pattern of *GaZnF* was observed under salt treatment where it showed 2- to 7-folds over-expression in roots, shoots and leaves as compared to control (Table I). Similar induction was seen in *PSTZ*, a zinc finger protein from *Populus euphratica* (Wang *et al.* 2008) and AtSZF1 and AtSZF2 zinc finger proteins from *Arabidopsis thaliana* (Sun *et al.* 2007). The genes showed enhanced expression under salt stress in transgenic tobacco and *Arabidopsis*. Expression of *AZF2* and *STZ* of cys-2/his-2-type zinc-finger family from *A. thaliana* was strongly induced by drought, salt and cold stress. Like the *GaZnF* expression in leaves under drought and salt stress, histochemical analysis of β-glucuronidase activities driven by the *AZF2* or *STZ* promoters revealed that both genes were induced by different stresses in leaves of *Arabidopsis* (Sakamoto *et al.* 2004). The zinc-finger protein *Zat12* was involved in responses of *Arabidopsis* to oxidative, osmotic, high irradiance and heat stress (Davletova *et al.* 2005). Previous research indicated that most of the drought-inducible genes were also induced by high salinity (Shinozaki *et al.* 2003). *Artemisia desertorum AdZFP1* gene expressed in transgenic tobacco plants was induced by salinity, cold and heat stress (Yang *et al.* 2008). Another finding suggested that *ZFP252*, a TFIIIA-type zinc finger protein gene from rice played an important role in rice response to salt and drought stresses and was useful in engineering crop plants with enhanced tolerance (Xu *et al.* 2008). Possible role of zinc finger proteins under stress was also seen in *Saccharomyces cerevisiae* (Görner *et al.* 1998). The differential drought responsiveness in different organs has also been reported for other genes (Alexandersson *et al.* 2005, Chen *et al.* 2002). However, the molecular mechanisms responsible for this differential regulation require further investigation. *TaRZF70* from *T. aestivum* was predominantly expressed in the root under non-stress conditions. Similar to *GaZnF*, its mRNA level in the root was 30 times higher than that in the leaf (Kam *et al.* 2007).

It was found that *GaZnF* was also responsive to different heavy metal treatments. The expression was highest in leaves and lowest in shoots (Table 1). A similar response was observed for petunia *ZPT2-3* gene (Shoji *et al.* 2003). *OsiSAP1* gene from rice was also found to be responsive to different heavy metals, where, treatment of rice plants with zinc, copper, cadmium and calcium salts led to significant increase in transcription level (Kanneganti and Gupta 2008). The observations that expression of *GaZnF* is induced by Pb, Co and Ni makes it a transcription factor involved in upregulation of metal stress responsive genes.

The results of this study led to the novel observation that a *Gossypium arboreum* zinc finger protein shows specific expression patterns during abiotic stresses and possibly presents a valuable source for transformation of cotton cultivars leading to multiple stress tolerance.

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