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Full Length Research Paper

Molecular cloning, structural analysis and expression of a zinc binding protein in cotton

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The full-length zinc-binding protein (ZnBP) gene was cloned from a normalized cDNA library constructed from a cotton mutant (Xiangmian-18) during the gland-forming stage. The clone was sequenced and analysed. BLASTP analysis showed that the deduced amino acid sequence of ZnBP in Xiangmian-18 is similar to that in *Arabidopsis thaliana* (GenBank accession no. EFH46337.1) with an overall similarity of 77%. The cDNA insert comprises 654 base pairs (bp) and 217 amino acid residues. Its molecular weight is 24.6 kDa, and the theoretical pI is 9.33. The cotton ZnBP gene was cloned from the gDNA from Xiangmian-18 leaves. After sequencing the two fragments, a 1731 bp cotton ZnBP gene with three introns was identified. Using pET-28a(+) as a prokaryotic expression vector, the gene was expressed in *Escherichia coli* BL21(DE3). The conditions for achieving optimal ZnBP expression were 37°C, IPTG 1 mmol/L, 8 h and a shaker speed of 150 rpm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis confirmed the correct expression of the protein. pCAMBIA2300-35S-OCS was used as a eukaryotic expression vector. The recombinant plasmid pCAMBIA2300-ZnBP was used to transform competent *Agrobacterium* GV3101 by the freeze-thaw method. Then, *A. thaliana* plants were transformed by the floral dipping method. Transformed plants were grown to maturity in a growth chamber. After screening on kanamycin-resistant half-strength Murashige and Skoog plates and polymerase chain reaction (PCR) analysis, two transgenic plant strains were obtained. Northern blot analysis showed that ZnBP expression was higher in homozygous plants than in wild-type plants. The differences between the phenotypes of homozygous and wild-type plants indicate that the ZnBP gene affects the growth and development of *A. thaliana*. The results of prokaryotic expression of ZnBP and overexpression of the ZnBP gene in *A. thaliana* improve our understanding of the function of this gene. Future studies should investigate the molecular mechanisms involved in gland morphogenesis in cotton.

Key words: *Gossypium hirsutum*, pigment gland, zinc binding protein, prokaryotic expression, overexpression.

INTRODUCTION

Metal ions are essential in various biochemical functions. They are incorporated into or associate with proteins in living cells, and can have purely stabilizing roles or be central to protein function (Wintz et al., 2003; Cox, 2000; Michel and Berg, 2002; Jensen et al., 2005). About 30% contain at least one metalloenzyme that requires Mg, K, Ca, Fe and Zn to sustain life. Other elements in cluding Cu, Mo, Ni, Se and Co are required by more primitive organisms (Arya et al., 1998; Dupont et al., 2010). The bound amino acids are almost always Cys, His, Glu and Asp residues (Golovin, 2005; Auld, 2001), which ligate metal ions through polar side chain atoms (Alberts, 1998). Metal-binding proteins play fundamental roles in plant processes, acting as cofactors in enzymes, osmotic regulators and current carriers in structural functions of proteins, as well as in protein – protein interactions (Kraemer and Clemens, 2005; Passerini et al., 2006). In

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plant mitochondria, key functions of metal cofactors include metabolism, electron transport, ATP synthesis and the detoxification of reactive oxygen species (Bridgewater, 2006; Harding, 2004; Tan, 2010).

Zinc is one of the most abundant and important metal ions (Dupont et al., 2010; Rachline et al., 2005; Auld, 2001). It is essential for the growth and development of not only plants (Kraemer and Clemens, 2005; Passerini et al., 2006; Bridgewater, 2006; Harding, 2004; Tan, 2010; Bixby, 1999), but also animals including humans (Frederickson, 1989; Earl et al., 1988) and microorganisms (Stempniak et al., 1997; Muhlberger et al., 1999). More than three hundred enzymes/proteins that require zinc to function have been identified (Vallee, 1999; Coleman, 1992). Zinc is required for the protein import apparatus, for both carrier protein transport to the inner membrane and pre-sequence degradation (Kuznetsova et al., 2005). Zinc readily forms complexes with amino acids, peptides, nucleotides and proteins in biological media (Vallee and Auld., 1990). Physical and chemical properties of zinc, such as its stable association with proteins and its co-ordination flexibility, make it highly adaptable to the requirements of proteins and enzymes that carry out diverse biological functions (Vallee, 1993, 1999; Coleman, 1992). The amino acids involved in zinc–protein interactions are histidine, glutamate, aspartate and cysteine (Golovin, 2005; Auld, 2001; Alberts, 1998; Golovin, 2005). For a structural zinc-binding site, Cys and His are the preferred coordinating residues, and there are usually no water molecules in the primary coordination sphere (Auld, 2001; Patel et al., 2007). In numerous zinc-binding proteins (ZnBPs), zinc ions are organized into tetrahedral or distorted tetrahedral structures according to negatively charged groups (carboxylates and thiolates) by charge–charge interactions, and/or neutral dipolar groups (example, carbonyls and imidazoles) through orientation-dependent charge–dipole interactions (Vallee and Falchuk, 1993; Wooltorton et al., 1997; Rachline et al., 2005).

Plant AT-rich sequence and zinc-binding protein 1 (PLATZ1) was isolated from peas (Inaba, 1999). Expression of PLATZ1 represses the expression of reporter constructs containing the coding sequence of a luciferase gene driven by the cauliflower mosaic virus (CaMV) 35S90 promoter fused to tandem repeat A/T-rich sequences (Sandhu et al., 1998; Schwabe and Klug, 1994; Nagano, 2001). Zinc also help to modulate the properties of channels such as voltage-gated channels (Bixby, 1999; Anumonwo, 1999; Wang, 2007), NMDA receptor channels (Rachline et al., 2005), GABA channels (Wooltorton et al., 1997) and chloride channels (Chen, 1998). MTI-II was isolated as ZnBP and found to be identical to parathymosin. It functions not only in the nucleus but also in the cytoplasm, playing essential and important roles in cell differentiation, although its specific functions and precise molecular mechanisms remain unknown (Okamoto and Isohashi, 2000). To date, there have been no reports of ZnBP in cotton.

We previously isolated the complete sequence of ZnBP from a normalised cDNA library constructed from a cotton mutant (Xiangmian-18) during the gland-forming stage (Xie et al., 2007). The structure of the gene was analysed, a ZnBP prokaryotic expression vector was constructed and expressed in *Escherichia coli* BL21(DE3), and a ZnBP overexpression vector was constructed and transfected into *Arabidopsis thaliana*. In this study, the differences in the phenotypes of homozygous and wild type (WT) plants were compared to gain an initial understanding of the functions of the gene, and to provide a scientific basis for studying related biological functions such as the molecular mechanism of gland formation and the metabolism of gossypol.

MATERIALS AND METHODS

Seeds from *Xiangmian-18* (with low gossypol seeds and glanded plants) were obtained from the National Research Center for Cross-Cotton of China (Hunan Changde, China), while seeds from WT *A. thaliana* (ecotype "Columbia") were provided by the Crop Institute of the Chinese Academy of Agricultural Sciences.

Strains and plasmid

E. coli DH5a, *E. coli* BL21(DE3), *Agrobacterium* GV3101 and pET-28a(+) were available in our laboratory. pMD19-T vector was obtained from TaKaRa (Japan) and pCAMBIA2300-35S-OCS was provided by the Chinese Academy of Agricultural Sciences.

Enzymes and reagents

Restriction endonucleases (*Xba*I, *Sal*I, *Xho*I, *Bam*HI, and *Pst*I), T4 DNA ligase, DNase I (RNase-free), RNase H and TaKaRa Ex Taq™ were obtained from TaKaRa (Japan). The plant gDNA isolation mini kit and plant RNA isolation mini kit were purchased from Watson Biotech (Shanghai, China). TIAN prep mini plasmid kit, TIAN gel mini purification kit, TIAN script RT kit, anti-His antibody, Pro-light HRP chemiluminescence detection reagent, and DNA marker III were obtained from TIANGEN Biotech Co., Ltd (Beijing, China).

Molecular cloning and sequence analysis of the cotton ZnBP gene

Total RNA extraction and synthesis of the first-strand cDNA

Cotton seeds were disinfected in 70% ethanol and 5% NaClO solution, dipped in sterilized water, and allowed to bud in plates containing sterilized filter paper and water. The pigment gland begins to develop 36 h after budding. Total RNA was extracted using the plant RNA isolation mini kit (Watson Biotech) according to the manufacturer's instructions. RNA yield was measured using an ultraviolet spectrometer and through electrophoresis on a denaturing formaldehyde agarose gel. First-strand cDNA was synthesized using the TIAN script RT kit (TIANGEN Biotech Co., Ltd).

Cloning of the ZnBP gene and sequence analysis

The primary cDNA synthesised was used as a template for

Table 1. The amplification PCR primers in structural analysis.

Primer	Sequence of primer (5'—3')
ZnBP-1F	TCCGTGAAATCAAGCCCAAA
ZnBP-1R	GAGAACAGAAGCGAAATGAG
ZnBP-2F	GACCGTAGCCTTGTCG
ZnBP-2R	GTAGGGTAAATATATATC

polymerase chain reaction (PCR) amplification using the ZnBP gene-specific primers ZnBPF (5'-ATGGGAGCTGGTGGGCCTGATG-3') and ZnBPR (5′-TTAATATTCTATGATTAGACC-3′), which were designed based on the ZnBP gene of normal upland cotton (*Gossypium hirsutum*, GenBank accession no. EU372997.1). Reactions (total volume, 20 µL) contained the following: template, 25 ng; 10× Taq buffer, 2 µL; dNTP mix, 1.6 µL; $MgCl_2$, 1.2 µL; F and R primers (20 mmol), 1 µL each; Taq DNA polymerase, 0.2 µL; and H_2O, 12 µL. PCR was performed with the following thermal conditions: 94°C for 4 min followed by 30 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 60 s, and a final extension at 72°C for 10 min. The amplified cDNA fragment was isolated by agarose gel electrophoresis and then purified. The purified fragment was cloned into the pMD19-T vector (TaKaRa) and then transfected into *E. coli* DH5α. The inserted fragment was sequenced by Beijing Sunbiotech Co., Ltd.

Primer design and the identification of open reading frames in the novel cotton ZnBP cDNA sequence were performed using DNAman software. BLASTN and BLASTP were used to identify sequences in GenBank that are homologous to the cotton ZnBP cDNA and protein sequences (http://www.ncbi.nlm.nih.gov/blast).

Structural analysis of the cotton ZnBP gene

Genomic DNA extraction, PCR amplification, and sequencing

Xiangmian-18 leaves were collected and total DNA was extracted using a plant gDNA isolation mini kit (Watson Biotech). The primers, ZnBP-1F/ZnBP-1R and ZnBP-2F/ZnBP-2R, were designed based on the sequence of the cotton ZnBP cDNA (Table 1). PCR amplification was performed using 2× Taq Master Mix (TIANGEN Biotech Co., Ltd). Reactions (total volume, 20 µL) contained the following: template 100 ng; 2× Taq Master Mix, 10 µL; F and R primers (ZnBP-1F/ZnBP-1R or ZnBP-2F/ZnBP-2R), 1 µL each; and H_2O, 8 µL. PCR was performed with the following thermal conditions: 94°C for 5 min followed by 30 cycles of 94°C for 60 s, 56°C for 60 s and 72°C for 2 min, and a final extension at 72°C for 10 min. The amplified fragment was isolated by agarose gel electrophoresis and then purified. The purified fragment was cloned into the pMD19-T vector (TaKaRa) and then transfected into *E. coli* DH5α. The inserted fragment was sequenced by Beijing Sunbiotech Co. Ltd.

Structural analysis of ZnBP from cotton

DNAman software was used to compare the gDNA sequence to the cDNA sequence to determine the number, length and position of introns in the cotton ZnBP gene.

Prokaryotic expression analysis of cotton ZnBP

pET-28-ZnBP vector construction and E. coli BL21(DE3) transformation

The primary cDNA synthesised was used as a template for PCR amplification using the ZnBP gene-specific primers YHZnBPF (5′-CGGATCCATGGGAGCTGGTGGGCCTGATG-3′) and YHZnBPR (5′-CCCTCGAGTTAATATTCTATGATTAGACC-3′), which were designed based on the ZnBP gene of normal upland cotton. (The underlined sequences represent *Bam*HI and *Xho*I restriction sites.) The inserted fragment was isolated and ligated into *Bam*HI- and *Xho*I-digested pET-28a (+) expression vector and then transfected into *E. coli* BL21 (DE3) cells (Novagen). The recombinant plasmid was named pET-28-ZnBP and the inserted fragment was sequenced by Beijing Sunbiotech Co. Ltd.

Expression and analysis of pET-28-ZnBP

The confirmed clone was grown in kanamycin-supplemented Luria-Bertani (LB) medium to an OD_{600} of 0.8 to 1.0 at 37°C and 150 rpm, and then induced with isopropyl-β-D-thiogalactopyranoside (IPTG). Culture samples (20 ml) for different induction times (0, 2, 4, 8 and 12 h) were processed by the ultrasonic method, and the cell pellets were collected by centrifugation at 12,000 rpm. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 4% (v/v) stacking gel and a 15% (v/v) separating gel. Separated proteins were visualised by Coomassie Brilliant Blue staining and gel images were captured using a Gel Doc 2000 Gel Documentation System (Bio-Rad). For Western blot analysis, recombinant protein samples were separated by SDS-PAGE and then electrically transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with membrane-confining liquid (25°C, 300 mA, 2 h), and then incubated overnight at 4°C with anti-His antibody (dilution 1:2,000). After washing for 10 min with PBST, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG in membrane-confining liquid (dilution 1:200). The blot was washed three times with phosphate-buffered saline (PBST) (10 min per wash). Finally, the blot was incubated in the dark with Pro-Light HRP detection reagent, and images were captured using a Gel Doc 2000 gel documentation system.

Overexpression of the cotton ZnBP gene

pCAMBIA2300-ZnBP vector construction and A. thaliana transformation

The primary cDNA synthesised was used as a template for PCR amplification using the ZnBP gene-specific primers GBZnBPF (5′-GCTCTAGAATGGGAGCTGGTGGGCCTGATG-3′) and GBZnBPR (5′-AACTGCAGTTAATATTCTATGATTAGACC-3′), which were designed based on the ZnBP gene of normal upland cotton (the underlined sequences represent *Xba*I and *Pst*I restriction sites). The plant expression vector pCAMBIA2300-35S-OCS containing the CaMV 35S promoter and the *npt*II kanamycin-resistance gene was used to clone the cotton ZnBP gene. The amplified ZnBP fragment and pCAMBIA2300-35S-OCS vector were digested using *Xba*I and *Pst*I, isolated by agarose gel electrophoresis and then purified. The purified fragment was cloned through incubation

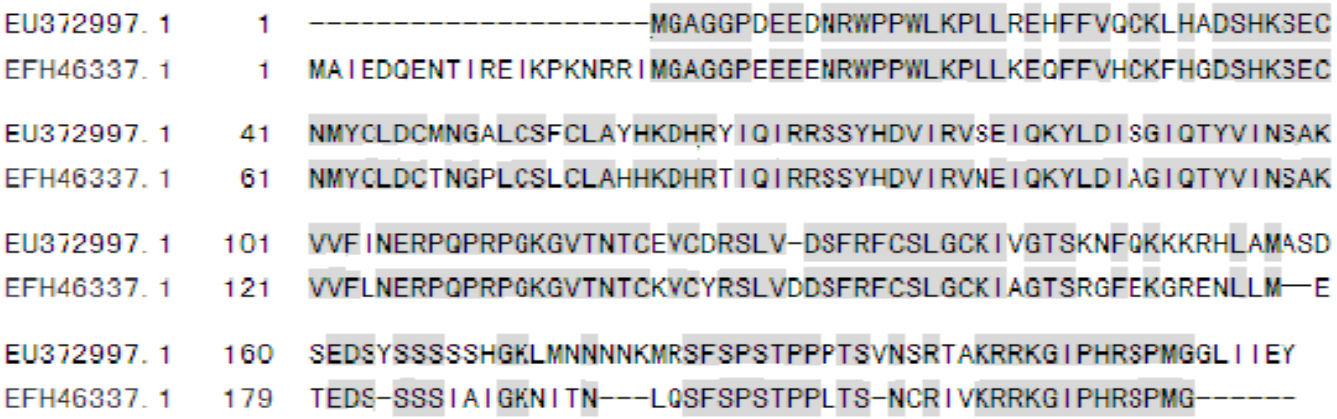

Figure 1. Alignment of the deduced amino acid sequences of ZnBP in *cotton* and *A. thaliana*. Amino acids are numbered on the left. Identical residues are shown with gray shading.

overnight at 16℃ with T4 DNA ligase, and then transfected into *E. coli* DH5α. The recombinant plasmid was sequenced by Beijing Sunbiotech Co. Ltd. Subsequently, the recombinant plasmid pCAMBIA2300-ZnBP was used to transform competent *Agrobacterium* GV3101 by the freeze-thaw method. A recombinant *Agrobacterium* strain was selected and used to transform *A. thaliana* plants by the floral dipping method. Transformed plants were grown to maturity in a growth chamber.

Selection and analysis of transgenic A. thaliana plants

Seeds (T_1) were collected from the transformed plants and checked by growing them on kanamycin-resistant half-strength Murashige and Skoog (1/2MS) plates containing 50 μg/ml. Resistant plants were transferred to pots containing vermiculite, perlite, soil (3:1:1 v/v/v), and ½ MS liquid medium and grown to maturity in a growth chamber. Kanamycin-resistant plants were screened for the presence of the transgene by PCR using the pCAMBIA2300-ZnBP-specific primers 2300F (5′-GCTATGACCATGATTACGAAT-3′) and 2300R (5′-GCAAGGCGATTAAGTTGGG- TAAC-3′), which were designed based on the sequence of the pCAMBIA2300-35S-OCS vector. DNA was isolated from the leaves of transgenic and WT plants using a plant gDNA isolation mini kit (Watson Biotech). The amplified fragments were analysed on a 1% agarose gel. Furthermore, Seeds (T_2) were harvested and sown on ½ MS agar to obtain homozygous plants. Homozygous plants were transferred to soil and were grown for the purpose of seed production. Seeds (T_3) from these homozygous plants were used for Northern blot analysis.

Northern blot analysis of ZnBP expression

Total RNA was isolated from the seedlings of homozygous and WT plants using a plant RNA isolation mini kit (Watson Biotech) and analysed on a 1% agarose gel. Next, total RNA was fractionated on a denaturing agarose gel and transferred to a positively charged nylon membrane (Maarten and Feng, 1995) according to the method described in the DIG-High Prime DNA Labelling and Detection Starter Kit (F. Hoffmann-La Roche, Ltd).

Phenotype analyses of transgenic A. thaliana plants

Seeds (T_3) were sown on ½ MS agar after disinfection. Seven days later, the seedlings from transgenic and WT *A. thaliana* plants were transferred to pots containing vermiculite, perlite, soil (3:1:1 v/v/v), and ½ MS liquid medium. Differences between the transgenic and WT *A. thaliana* plants were compared 21 and 30 days later.

RESULTS

Molecular cloning and sequence analysis of the cotton ZnBP gene

cDNA encoding the ZnBP gene from an upland cotton gland mutant (*Xiangmian-18*) during the pigment gland-forming stage was amplified by RT-PCR. A 654 base pair (bp) open reading frame encoding a 217 aa protein with a calculated molecular mass of 26.4 kDa and an isoelectric point of 9.33 was identified. The DNA sequence of the ZnBP gene was deposited in GenBank (accession no. EU372997.1). BLASTP analysis showed that the deduced amino acid sequence of ZnBP in *Xiangmian-18* is similar to that in *A. thaliana* (accession no. EFH46337.1), with an overall similarity of 77% (Figure 1).

Structural analysis of the cotton ZnBP gene

Genomic DNA extracted from *Xiangmian-18* leaves was used as the template for PCR using the primers ZnBP-1F/ZnBP-1R and ZnBP-2F/ZnBP-2R. After sequencing the two fragments cloned from the cotton ZnBP gene, the cotton ZnBP gene, 1731 bp in length and containing three introns, was identified (Figure 2).

Prokaryotic expression analysis of cotton ZnBP

The recombinant plasmid pET-28- ZnBP was transfected into *E. coli* BL21(DE3) and the expression of recombinant ZnBP protein was induced through treatment with IPTG.

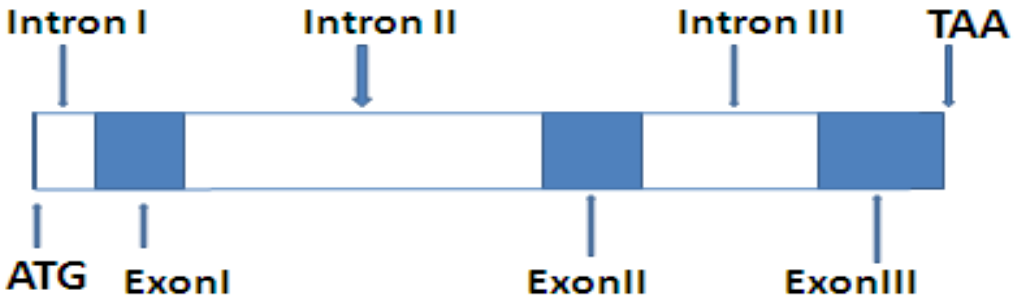

Figure 2. The sketch map of ZnBP gene. Intron I =99 bp; Intron II = 714 bp; Intron III = 264 bp; Exon I = 198 bp; Exon II = 216 bp; Exon III = 237 bp.

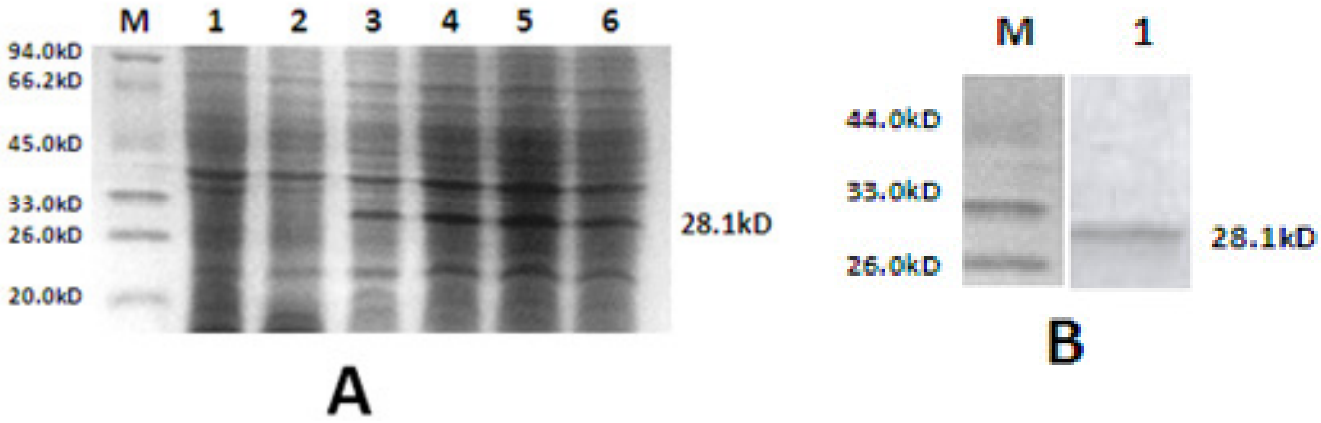

Figure 3. (**A)** SDS-PAGE analysis of the recombinant ZnBP. SDS-PAGE (15%) was loaded with *E. coli* extract expressing the ZNBP before (lane 2) and after 2, 4, 8 and 12 h IPTG induction (lanes 3, 4, 5, 6), and lane 1 was the protein product by vector of pET-28a(+). **(B)** Western blotting analyses with lane 5. Molecular weight markers in KDa are indicated on the right. The larger size of the fusion protein is due to the N-terminal leader peptide of 3.5 kDa encoded by the expression vector.

The estimated molecular mass of recombinant ZnBP was confirmed by SDS-PAGE (Figure 3A). Western blot analysis confirmed that the expressed recombinant protein was ZnBP (Figure 3B).

Overexpression of the cotton ZnBP gene

Using the plant expression vector pCAMBIA2300-35S-OCS, we constructed the recombinant plasmid pCAMBIA2300-ZnBP. This plasmid was then transfected into competent *Agrobacterium* GV3101 by the freeze-thaw method. A recombinant *Agrobacterium* strain was selected and used to transform *A. thaliana* plants. Transformed plants were grown to maturity in a growth chamber. Seeds (T_1) were collected from the transformed plants and checked by growing them on kanamycin-resistant ½ MS plates containing 50 μg ml^{-1}. Some seeds failed to germinate and turned yellow 10 days after germinating. Two resistant plant strains were transferred to pots, and DNA was isolated from the leaves of transgenic and WT plants using a plant gDNA isolation mini kit (Watson Biotech). Kanamycin-resistant plants were screened for the presence of the transgene by PCR using the pCAMBIA2300-ZnBP-specific primers 2300F and 2300R. The amplified fragments were analysed on a 1% agarose gel (Figure 4). Total RNA was isolated from the seedlings of two homozygous strains (ZnBP-1 and ZnBP-2) and WT plants using a plant RNA isolation mini kit (Watson Biotech), and analysed on a 1% agarose gel (Figure 5A). Then the total RNA was fractionated on denaturing agarose gels and transferred to a positively charged nylon membrane according to the method described in the DIG-High Prime DNA Labelling and Detection Starter Kit (F. Hoffmann-La Roche, Ltd) (Figure 5B).

Seeds (T_3) were sown on ½ MS agar after disinfection. Seven days later, transgenic and WT *A. thaliana* seedlings were transferred to pots containing vermiculite, perlite, soil (3:1:1 v/v/v), and ½ MS liquid medium. The transgenic and WT *A. thaliana* plants were compared after 21 (Figure 6A) and 30 days (Figure 6B). The differences in the phenotypes of homozygous and WT plants indicated that the ZnBP gene affects the growth

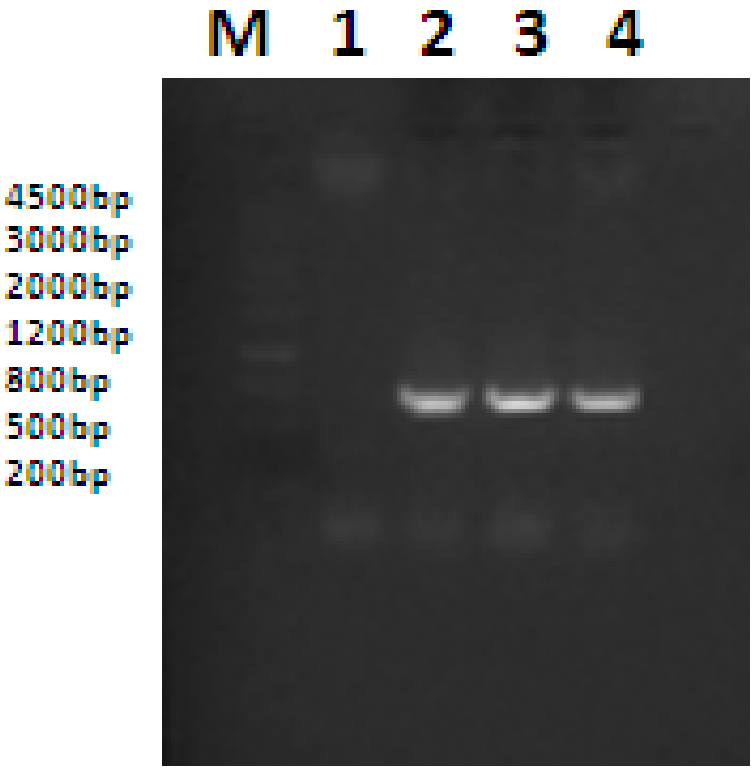

Figure 4. PCR analysis of transgenic *A. thaliana* plants. M, DNA Marker; lane 1, gDNA isolate from wild type *A. thaliana* plants; lane 2, the pCAMBIA2300-ZnBP recombinant plasmid; lanes 3 and 4, gDNA isolate from transgenic *A. thaliana* plants.

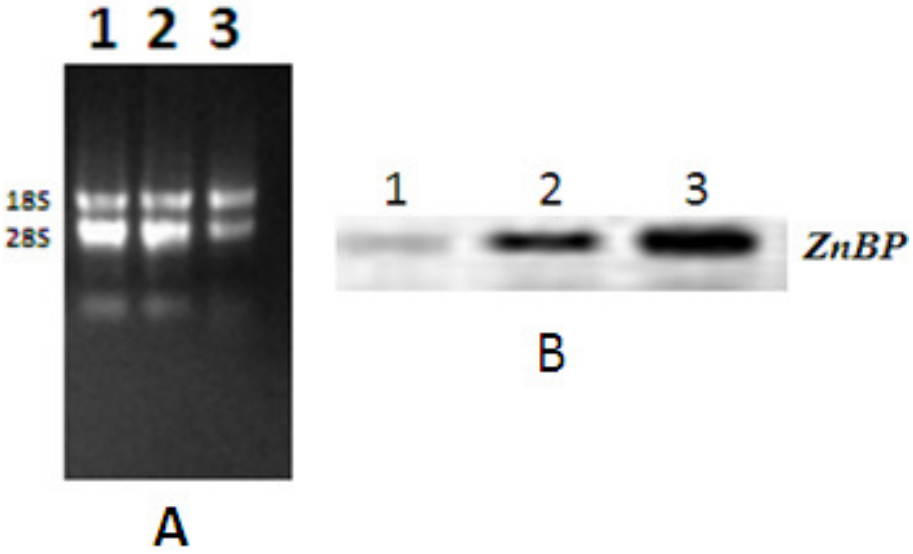

Figure 5. **(A)** Lane 1, Total RNA isolated from the seedling of ZnBP-1 homozygous plants; lane 2, total RNA isolated from the seedling of ZnBP-2 homozygous plants; lane 3, total RNA isolated from the seedling of wild type plants. **(B)** Northern blotting analysis of transgenic *A. thaliana* plants; lane 1, total RNA samples (40 µg) isolated from the seedling of wild type plants; lane 2, total RNA samples (40 µg) isolated from the seedling of ZnBP-1 homozygous plants; lane 3, total RNA samples (40 µg) isolated from the seedling of ZnBP-2 homozygous plants. All of these were tested using the ZnBP cDNA as probe.

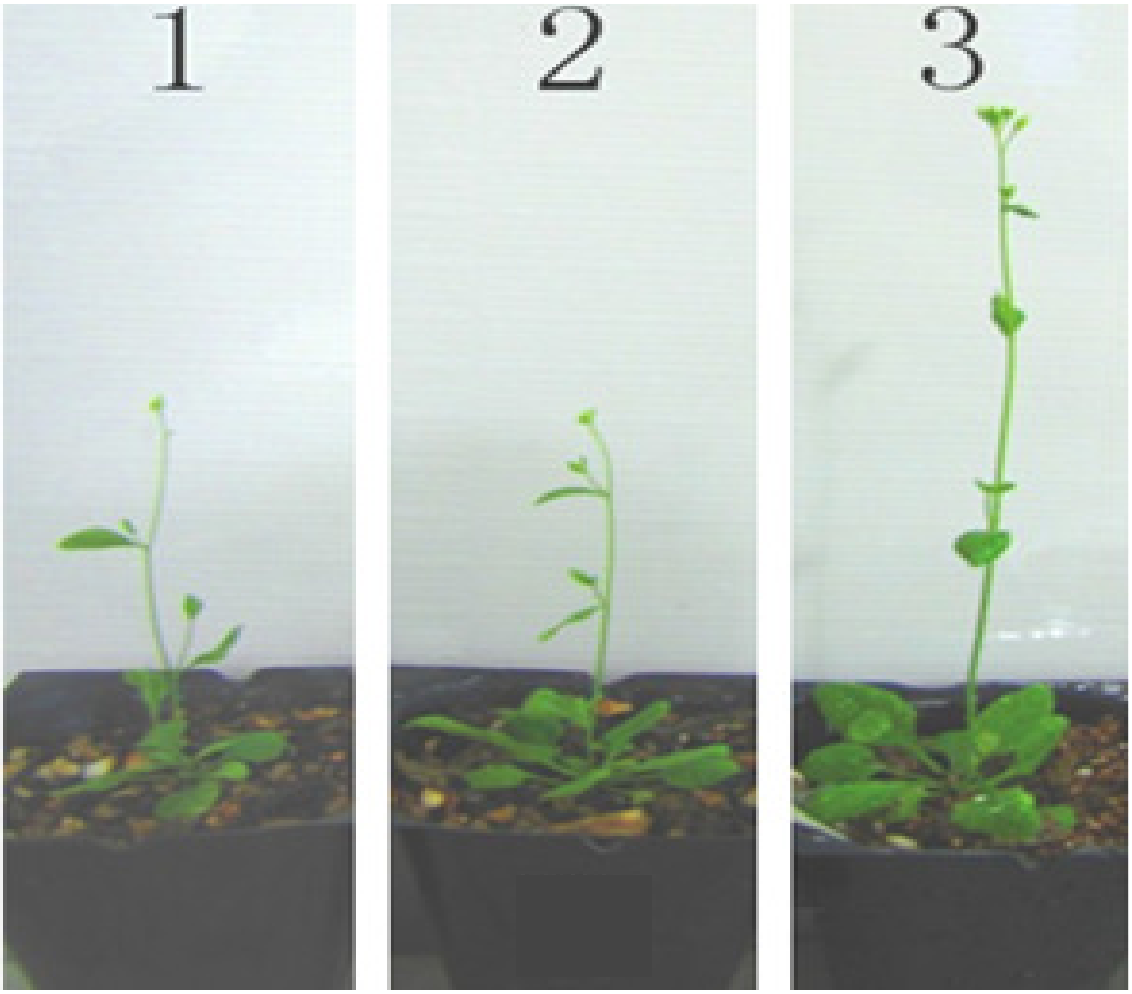

Figure 6. The phenotype comparison between ZnBP transgenic and wild-type *A. thaliana*. 1, 30-day-old seedlings of ZnBP-1 homozygous *A. thaliana*; 2, 30-day-old seedlings of ZnBP-2 homozygous *A. thaliana*; 3, 30-day-old seedlings of wild-type *A. thaliana*.

and development of *A. thaliana*.

DISCUSSION

Zinc ions play important roles in structural stability and complex formation (Wintz et al., 2003; Michel and Berg, 2002), the regulation of gene expression (Wintz et al., 2003; Jensen et al., 2005; Hantke, 2001), DNA processing (Feng, 2004), transport (Hantke, 2001; Harris, 2000), metabolism and control (Wintz et al., 2003; Hantke, 2001; Vallee, 1990), as well as other processes such as cellular respiration and antioxidant defence (Lieu et al., 2001). Most of them are identified as DNA-binding or protein-binding proteins, while others function as RNA-binding proteins. Zinc-binding protein as common transcription factors are important regulators of cellular processes and the complexity of living organisms necessitates a large number of transcription factors. In *Arabidopsis*, zinc-binding protein participates in a new regulatory mechanism governing seed germination and stem growth (Deng, 1992; Ballachanda, 2007).

Given the preliminary study about mechanism of organogenesis genes that regulate widespread changes in gene expression, here we report the characterization of the cotton ZnBP gene and overexpression in the *A. thaliana* transgenic plants expressing the ZnBP cDNA under the control of the constitutive CaMV 35S promoter. The phenotype comparison between ZnBP transgenic and wild-type *A. thaliana* is that the growth of transgenic was slower than wild-type *A. thaliana*. ZAT6 is very closely related and share an identical DNA binding domain. Overexpression of ZAT6 resulted in retarded root and seedling growth during early stages of seedling development. The mechanism of overexpression of ZAT6 suppressed the expression of several Pi stress-responsive genes, suggesting its influence on multiple facets of Pi homeostasis. These results indicate that

ZAT6 is a repressor of primary root growth that regulates Pi homeostasis by controlling the root architecture independent of the Pi status of the plant (Vicente-Carbajosa et al., 1997; Ballachanda, 2007). Also transgenic plants overexpressing AZF under the control of a glucocorticoid-inducible promoter showed severe growth retardation with morphological defects. This dwarf phenotype is consistent with the results obtained from the overexpression of ZAT6 (Sakamoto et al., 2004; Mittler et al., 2006; Ciftci-Yilmaz et al., 2007).

Although, the difference phenotype between ZnBP transgenic and wild-type *A. thaliana* is obvious, the molecular mechanisms controlling plant growth are yet unknown. In future studies, we hope to transform the ZnBP overexpression vector and construct a ZnBP-specific RNAi vector, and transfect into cotton to gain further insights into the function of the ZnBP gene.

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