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Full Length Research Paper

Molecular cloning and characterization of a novel Cys_2/His_2-type zinc finger protein gene from chrysanthemum

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A novel member of the Cys_2/His_2-type zinc finger protein gene family, designated *DgZFP3*, was isolated from chrysanthemum by rapid amplification of cDNA ends (RACE). The *DgZFP3* encodes a protein of 248 amino acids, including two conserved Cys_2/His_2-type zinc finger motifs with a plant-specific QALGGH motif in each zinc finger domain, a B-box (KXKRSKRXR) domain in the N-terminal region as a putative nuclear localization signal (NLS), a L-box (EXEXXAXCLXXL) and an EAR-box (DLNL) at C-terminus. Subcellular localization showed the presence of *DgZFP3* in the nucleus. The transcript of *DgZFP3* was enriched in roots and leaves than in stems and flowers of the adult chrysanthemum plants. Expression patterns revealed that *DgZFP3* was strongly induced by NaCl, drought, cold and abscisic acid (ABA) treatment in the seedlings. We argued that *DgZFP3* is a new member of the Cys_2/His_2-type zinc finger protein gene family, and it may be involved in the plant responses to various stresses.

Key words: Chrysanthemum, *DgZFP3*, gene expression, Cys_2/His_2-type zinc finger protein.

INTRODUCTION

Drought, high salinity and temperature stress including low or high temperature are adverse environmental conditions that limit plant growth and development. In response to these adversities, plants can increase tolerance or adaptation to stress conditions via a series of physiological, cellular and molecular processes culminating in stress tolerance. Multiple signal pathways regulate the various abiotic stress responses of plants (Shinozaki and Yamaguchi-Shinozaki, 2007; Nakashima et al., 2009). Based on stress signal transduction, transcription factors such as AP2/EREBP, bZIP, NAC, MYB, MYC, WRKY and Cys_2/His_2-type zinc finger proteins, activate the expression of many stress-related downstream genes, and finally increase tolerance or adaptation to stress conditions in plants (Agarwal et al., 2006; Chinnusamy et al., 2006; Umezawa et al., 2006). Among them, the Cys_2/His_2-type zinc finger proteins received much attention in the past decade. Since *ZPT2-1* was isolated from petunia(*Petunia hybrida*), the Cys_2/His_2-type zinc finger proteins genes have been isolated from a wide variety of plants such as *Arabidopsis*, rice (*Oryza sativa*), maize (*Zea mays*), soybean (*Glycine max*), wheat (*Triticum aestivum*), pepper (*Capsicum annuum*), etc (Baltz et al., 1992; Sakamoto et al., 2000; Kim et al., 2001, 2004; Huang et al., 2007; Kam et al., 2008). Several Cys_2/His_2-type zinc

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Abbreviations: ABA, Abscisic acid; **CaMV,** the cauliflower mosaic virus; **NLS,** nuclear localization signal; **GFP,** green fluorescent protein; **RACE,** rapid amplification of **cDNA** ends; **qRT-PCR,** quantitative real-time reverse transcriptase-polymerase chain reaction.

finger proteins genes such as *STZ*, *SCOF-1*, *ZPT2-3*, *DgZFP*, etc, have been implicated in the regulation of stress responses (Sakamoto et al., 2000; Kim et al., 2001; Sugano et al., 2003; Mittler et al., 2006, Liu et al., 2010a). However, the Cys_2/His_2-type zinc finger proteins genes have been studied in only a limited number of plant species. Therefore, it is necessary that more Cys_2/His_2-type zinc finger proteins genes be identified and characterized to assess more comprehensively, an overall picture of Cys_2/His_2-type zinc finger proteins genes regulation.

Chrysanthemum is an important ornamental plant in the world. However, chrysanthemum-growing areas are subject to extreme drought, high salinity and low temperature, each of which can affect chrysanthemum growth and production. Now, isolation and characterization of novel stress-responsive Cys_2/His_2-type zinc finger protein genes in chrysanthemum is critical to further our understanding of the molecular mechanisms governing chrysanthemum stress response and tolerance, ultimately leading to enhancement of stress tolerance in chrysanthemum through genetic manipulation. To date, there are few reports on the characterization of the Cys_2/His_2-type zinc finger protein genes from chrysanthemum (Liu et al., 2010a, b). In this study, we isolated a Cys_2/His_2-type zinc finger protein gene from chrysanthemum, designated *DgZFP3*. Furthermore, the expression of *DgZFP3* was induced by salt, drought, cold and abscisic acid (ABA) stresses.

MATERIALS AND METHODS

Plant materials and stress treatments

Chrysanthemum (*Dendronthema grandiform*) cv. Jinba seedlings growing in greenhouse were exposed to air on filter paper for dehydration, or subjected to 4°C cold stress. For salt and ABA treatments, seedlings were put in 200 mM NaCl, and 0.1 mM ABA solution, respectively. Each experiment contained three biological replicate. All excised leaf samples of controlled and treated plants were taken out for treatment for 0, 1, 3, 6, 12 and 24 h, respectively, and then frozen immediately in liquid nitrogen, and stored at -80°C for RNA extraction.

Isolation of the *DgZFP3* gene

For 3' rapid amplification of cDNA ends (RACE), one primer was designed GSP1 (5'-CA(A/G)GCI(T/C)TIGGIGGICA(C/T)-3') corresponding to conversed regions of the amino acid QALGGH. Primers for 5' RACE were: GSP2, 5'-CCGCTAGCTGATGAGGTCGTTGT-3' and GSP3, 5'-TACAAATTGTTGGTGTTGTAGTTGC-3'. The RACE reactions were performed according to the manufacturer's protocol (Takara RACE cDNA amplification kit, Japan). A single full-length cDNA sequence by combining the 5'-RACE fragment and 3'-RACE fragment was obtained. Finally, a pair of primers (F1, 5'-CTCTTAAATTAATAATAATACTCTTA-3' and F2, 5'-CATACAAATTAAATTCACGAAATAC-3') was then designed from the putative 5' and 3' untranslated region (UTR) of the full-length cDNA sequence. The resultant DNA fragments and RACE products were purified by agarose gel and cloned into pMD18-T Vector (Takara) and sequenced (Invitrogen, Beijing).

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR) assay

Total RNA from various chrysanthemum tissues was extracted by Trizol reagent (Mylab, Beijing) underlying with the manufacturer's instructions; putative genomic DNA contamination was removed by DNaseI. The first strand cDNA was synthesized with 1 µg total RNA and 1 µl superscript II enzyme (Invitrogen, USA) according to the manufacturer's protocol. Quantitative real-time PCR assay was performed using SYBR Green I (TOYOBO, Japan) by a Bio-RAD iCycler iQ5 Machine. The primers were designed to amplify a 130 bp fragment of the *DgZFP3* sequence (forward 5'-TTGTTACAG-TAGTAAAGATCCGTTT-3' and reverse 5'-CTACACAAACGGA-TCTTTACTACTG-3'). The chrysanthemum actin gene (GenBank accession number: AB205087) was used as a reference (forward 5'-CCAGTGGTCGTACAACTGGCATT-3' and reverse 5'-CAGTCA-GATCACGACCAGCAAGATC-3'.). A 25 µl PCR amplification mixture contained 10 µl SYBR Green PCR master mix, 0.2 µM of each primer and 12 ng cDNA. The PCR was performed as follows: an initial denaturation of 95°C for 3 min, followed by 40 cycles of 10 s at 94°C, 20 s at 58°C, 50 s at 72°C, followed by a final elongation of 10 min at 72°C. The resulting data were represented by means ± standard deviation (SD) of three replicates. Relative expression levels were calculated by the $2^{-\geq CT}$ method, where $\geq CT$ = (CT, target-CT, actin gene) the indicated time treatment-(CT, target - CT, actin gene) 0 h treatment (Livak and Schmittgen, 2001). The data were scaled by setting the expression of *DgZFP3* in untreated leaves at 0 h as 1.

Subcellular localization

The *DgZFP3* ORF were cloned into the *Sac*I and *Eco*RI sites of the pSAT6-GFP-N1 vector. This vector contains a modified red-shifted green fluorescent protein (GFP) at *Eco*RI-*Nco*I sites. The *DgZFP3*-GFP construct was transformed into onion epidermal cells by particle bombardment as described earlier (Wang and Fang, 2002). The transient expression of the *DgZFP3*-GFP fusion protein was observed using confocal microscopy.

Sequence alignment and phylogenetic tree analysis

The sequence alignment of *DgZFP3* and other plant Cys_2/His_2-type zinc finger proteins amino acid sequences were compared by DNAMAN (ver 6.0) and the phylogenetic tree was constructed by neighbor-joining method with MEGA program (ver 4.1).

RESULTS

Isolation of the *DgZFP3* gene from chrysanthemum

Based on the conserved regions of *Arabidopsis ZAT10* and chrysanthemum *DgZFP*, degenerate primers to conduct the 3'-RACE were proposed to obtain a 643 bp fragment from leaves of chrysanthemum. The full-length cDNAs was obtained by 5'-RACE, and were designated as *DgZFP3* (Genbank accession no. JQ040514). Sequence analysis showed that the *DgZFP3* cDNA was986 bp in length, including a complete open reading

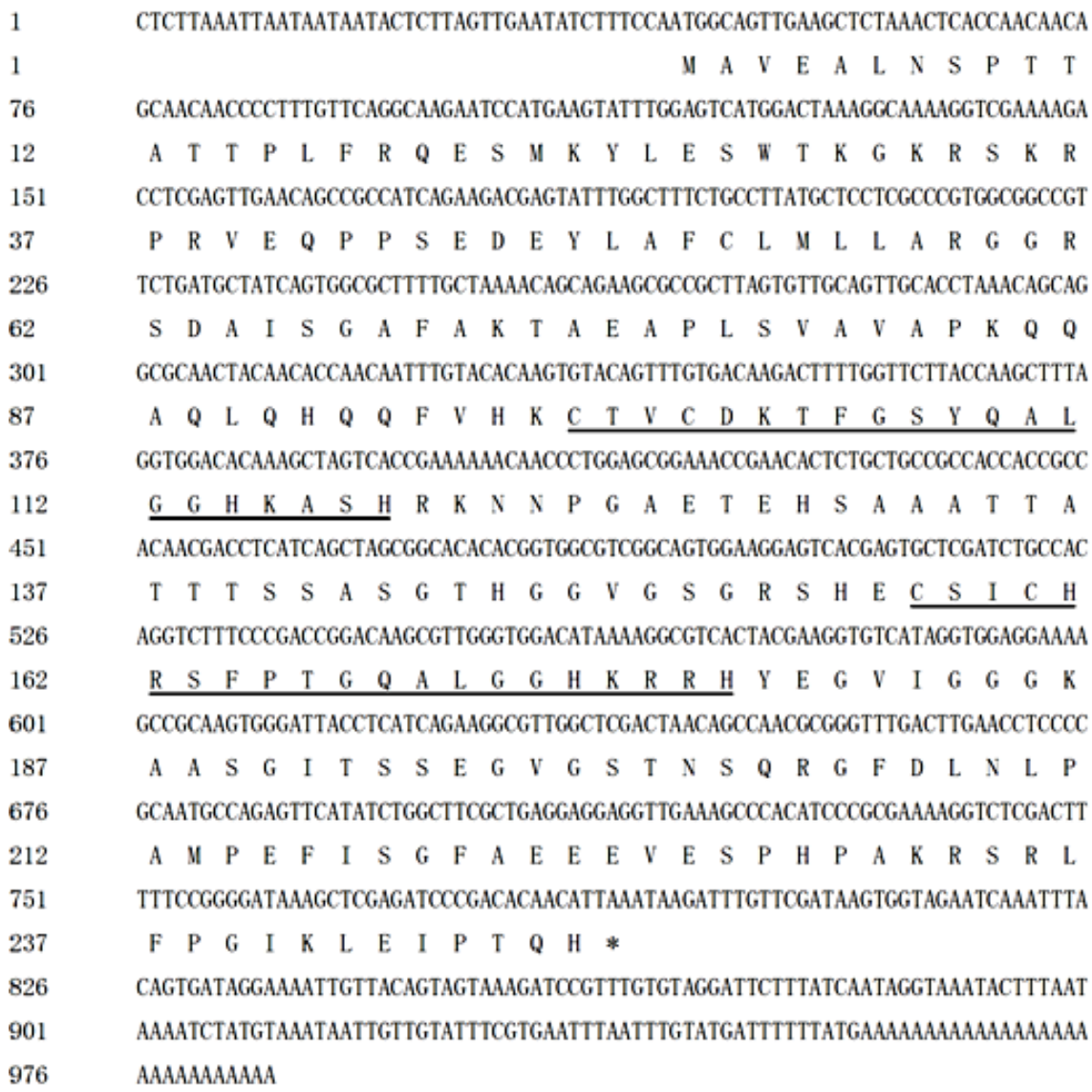

Figure 1. Nucleotide and deduced amino acid sequences of *DgZFP3* (GenBank accession no. JQ040514). The Cys_2/His_2-type zinc finger motifs are underlined.

frame of 747 bp flanking with a 5'-UTR of 42 bp and a 3'-UTR of 197 bp (Figure 1). The predicted protein of *DgZFP3* was composed of 248 amino acids with a calculated molecular mass of 26.36 kDa and its theoretical isoelectric point was 9.27.

The predicted amino acid sequence of the *DgZFP3* protein was compared with other plant Cys_2/His_2-type zinc finger proteins from rice, soybean, chrysanthemum, petunia, pepper and *Arabidopsis* by DNAMAN (Version 6.0) (Figure 2). *DgZFP3* contains two conserved Cys_2/His_2-type zinc finger motifs with a plant-specific QALGGH motif in each zinc finger domain, a B-box (KXKRSKRXR) domain in the N-terminal region as a putative nuclear localization signal (NLS), a L-box (EXEXXAXCLXXL) and an EAR-box (DLNL) at C-terminus. The plant Cys_2/His_2-type zinc finger proteins were then retrieved for construction of a neighbor-joining phylogenetic by MEGA 4.1 (Figure 3). Phylogenetic analysis revealed that *DgZFP3* was clustered with *ZAT10* and *DgZFP*, and more closely related to *DgZFP*.

Expression analysis of *DgZFP3*

The spatial-specific expression of *DgZFP3* in different tissues at the adult stage was determined by real-time RT-PCR. The results show that *DgZFP3* transcripts is more abundant in roots and leaves than in stems and flowers (Figure 4A).

To investigate the expression patterns of *DgZFP3* gene

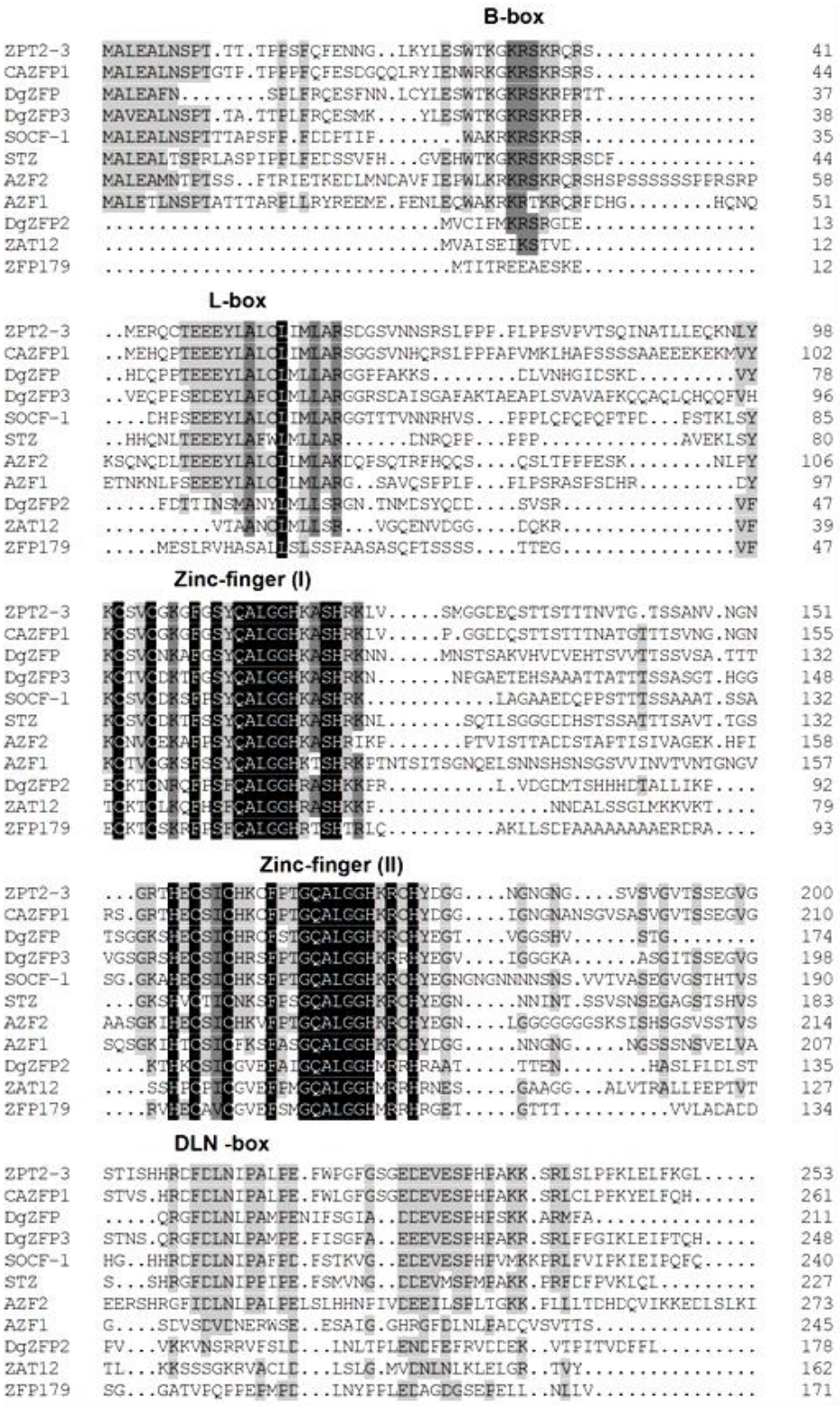

Figure 2. Comparison of the deduced amino acid sequence of *DgZFP3* and other plant Cys_2/His_2-type zinc finger proteins. The comparison was conducted by DNAMAN (version 6.0). The Cys_2/His_2-type zinc finger motif, B-box, L-box and EAR-box are indicated. *Arabidopsis thaliana* (*ZAT10*, AF250336; ZAT12, CCA67232.1; AZF1, BBA85108.1; AZF2, AAG10143); *Capsium annuunm* (CAZFP1, AAQ10954); *Glycine max* (SCOF1, AAB39368) and *Petunia hybrida* (ZPT2-3, BAA05079); *Oryza sativa* (ZFP179, AAL76091.1); chrysanthemum (*DgZFP*, GQ392036; *DgZFP*2, JQ031154).

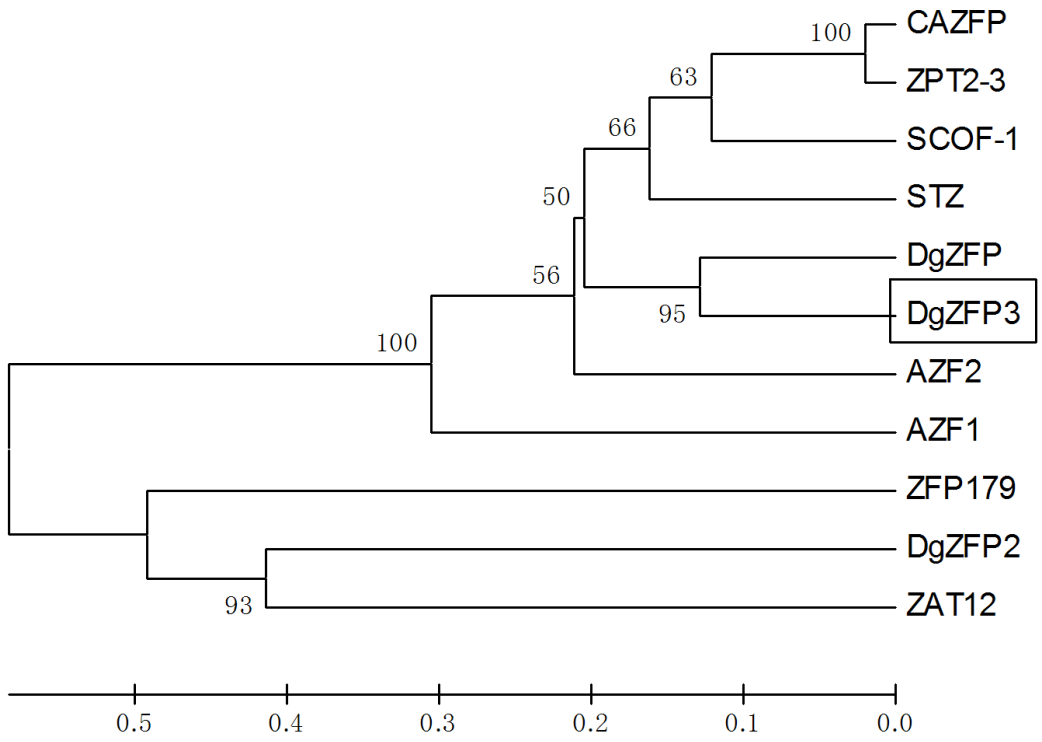

Figure 3. Phylogenetic tree analysis of *DgZFP3* and other plant Cys_2/His_2-type zinc finger proteins. The tree was constructed by neighbor-joining method with MEGA program (ver 4.1). Branch numbers represent percentage of bootstrap values in 1000 sampling replicates and scale indicates branch lengths. The accession numbers were as follows: *ZAT10* (AF250336), ZAT12 (CCA67232.1), AZF1 (BBA85108.1), AZF2 (AAG10143), CAZFP1 (AAQ10954), SCOF1 (AAB39368), ZPT2-3 (BAA05079), ZFP179 (AAL76091.1), *DgZFP* (GQ392036) and *DgZFP*2 (JQ031154).

under stress such as high salinity, drought, low temperature and exposure to ABA, the analysis with real-time RT-PCR was performed, respectively. The expression of *DgZFP3* was kept at low affected level in normal conditions (Figures 4B to E). For salt stress, the concentration of *DgZFP3* mRNA was up-regulated 1 h after 200 mM NaCl treatment and was maintained constant up to 12 h by 200 mM NaCl treatment (Figure 4B). By drought treatment, the expression level of *DgZFP3* began to increase after 3 h and gradually accumulated up to 24 h (Figure 4C). The expression of *DgZFP3* peaked within 6 h and gradually decreased in response to cold treatment (Figure 4D). The expression of *DgZFP3* peaked within 3 h after the beginning of the ABA treatment and gradually decreased by 3 h post imposition of ABA treatment (Figure 4E). Real-time RT-PCR analysis revealed that the expression of *DgZFP3* could be induced by salt, drought, cold and ABA.

Localization of *DgZFP3* in the nucleus

The deduced amino acid sequence contained a B-box (KXKRSKRXR) domain in the N-terminal region as a putative NLS, suggesting that *DgZFP3* might interact with the cell nuclear system. To examine the subcellular localization of *DgZFP3* in living cells, a construct containing *DgZFP3* fused in frame with the GFP (*DgZFP3-GFP*) driven by CaMV 35S promoter was transiently expressing in onion epidermal cells. As shown in Figure 5, confocal microscopic examination shows that the *DgZFP3*-GFP fusion protein was targeted into the nuclear 4',6-diamidino-2-phenylindole (DAPI) staining, whereas the control GFP alone was distributed in the entire cytoplasm.

DISCUSSION

Some Cys_2/His_2-type zinc finger protein genes in plants usually play critical roles in response to abiotic stresses (Ciftci-Yilmaz and Mittler, 2008). A plant Cys_2/His_2-type zinc finger protein gene termed *DgZFP3* from chrysanthemum was isolated and characterized in the present work. Sequence analysis shows that it contained two conserved Cys_2/His_2-type zinc finger motifs with a plant-specific QALGGH motif in each zinc finger domain, a B-box (KXKRSKRXR) domain in the N-terminal region as a putative nuclear NLS, a L-box (EXEXXAXCLXXL) and an EAR-box (DLNL) at C-terminus. The *DgZFP3* was

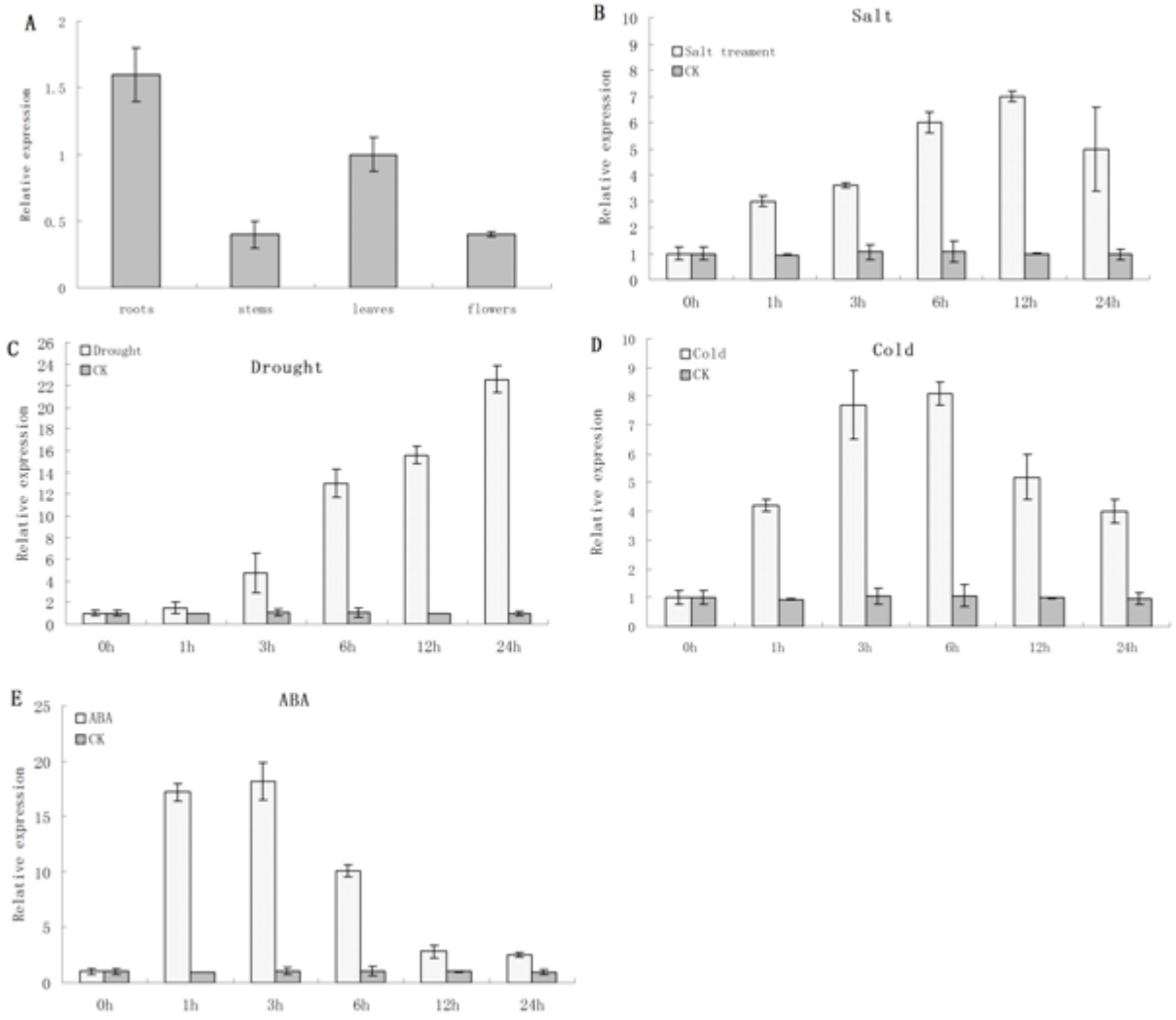

Figure 4. Expression patterns of *DgZFP3* in different organs and in response to various treatments. Mean values and standard deviation calculated from triplicated assays. The relative expression of *DgZFP3* in untreated leaves was used as CK. (A) Expression patterns of *DgZFP3* in roots, stems, leaves and flowers under normal conditions. (B) Salt treatment; (C) drought treatment; (D) cold treatment; (E) ABA treatment.

structurally similar to *DgZFP* which was isolated from chrysanthemum under high-salt, drought and cold stresses (Liu et al., 2010a). Phylogenetic analysis revealed that *DgZFP3* was clustered with *ZAT10* and *DgZFP*, and more closely related to the *DgZFP*. These results indicate that *DgZFP3* is a novel member of the plant Cys_2/His_2-type zinc finger protein genes family. The subcellular localization of the *DgZFP3*-GFP fusion protein in the nuclear DAPI staining implied the role of *DgZFP3* as a transcription factor.

The mRNA expression analysis shows that *DgZFP3* substantially induced by the treatment of NaCl, drought, cold, ABA and *DgZFP3* may be involved in the abiotic-stress response via the ABA-dependent pathway. The expression patterns of *DgZFP3* were similar to *ZPT2-3*, *ZAT10*, *ThZF1* and *ZFP179* during several different stresses (Sugano et al., 2003; Mittler et al., 2006; Xu et al., 2007; Sun et al., 2010). Petunia ZPT2-3 and *Arabidopsis* STZ/*ZAT10* containing an EAR-box (DLNL) have exhibited transcription repressive activities by transient analysis in plants (Sugano et al., 2003; Sakamoto et al., 2004). However, several zinc finger proteins containing the EAR-box (DLNL) have exhibited transcriptional activation activity in yeast cells, such as rice ZFP179 and *Thellungiella halophila* ThZF1 (Xu et al., 2007; Sun et al., 2010). Previous report showed that the EAR-box (DLNL) is directly involved in transcriptional regulatory networks in response to abiotic stresses in plants, and *DgZFP3* also contains an EAR-box (DLNL) at its C-terminus. Thus, further experiments are required to identify the possible function of *DgZFP3* as a transcription activator or repressor.

To our knowledge, this work is the first report on the cloning and expression of a novel Cys_2/His_2-type zinc

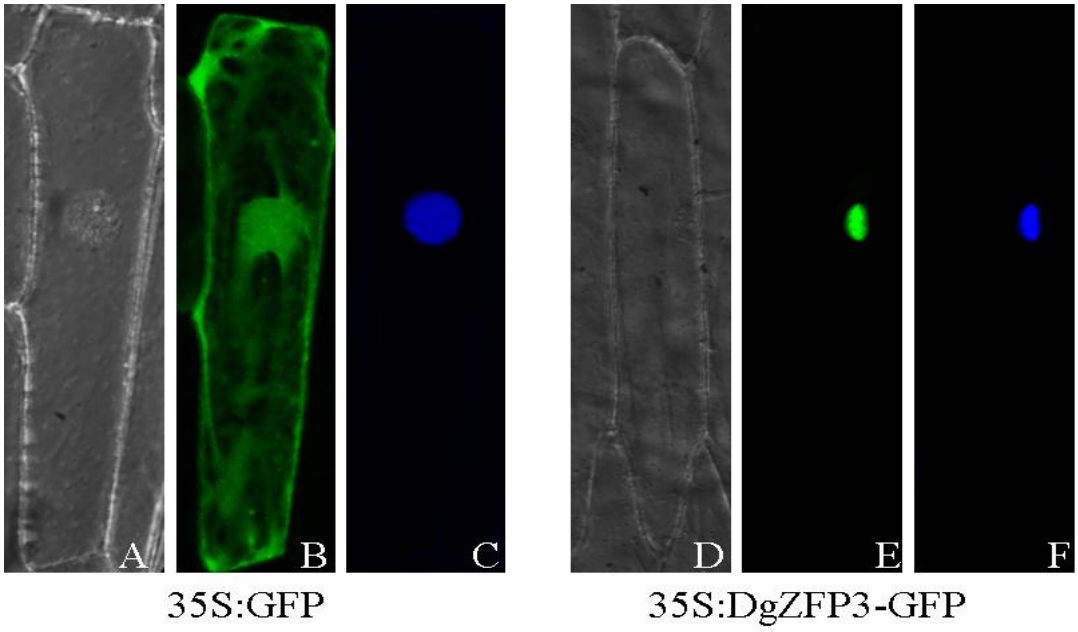

Figure 5. Subcellular localization of *DgZFP3*. Transient expression in onion epidermal cells of 35S-GFP and 35S-*DgZFP3*-GFP translational product was visualized by fluorescence microscopy. The transient vector harboring 35S-GFP and 35S-*DgZFP3*-GFP cassettes were transformed into onion epidermal cells by particle bombardment. The photos were taken in bright light (A and D), in the dark for GFP images (B and E) and DAPI-stained images (C and F) after incubation for 20 h.

finger protein gene termed *DgZFP3* from chrysanthemum. Clarifying the possible functions of *DgZFP3* under various stresses will be helpful for the enhancement of stress tolerance in chrysanthemum through genetic manipulation. The important functions of *DgZFP3* responding to environmental stimuli in chrysanthemum needs further investigation.

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