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NOVEL DROUGHT-INDUCIBLE Cys2/His2-TYPE ZINC FINGER PROTEIN STF-2 FROM SOYBEAN (*GLYCINE MAX*) ENHANCES DROUGHT TOLERANCE IN TRANSGENIC PLANTS

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Abstract

The C_2H_2-type (Cys2/His2-type) zinc finger proteins are transcription factors which play important roles in growth, development and tolerance ability to adverse stresses. We characterized STF-2 from Glycine max to determine its molecular structure and biological function. The results showed that STF-2 was a novel plant C_2H_2-type zinc finger protein gene and encoded a 26.7-kDa protein comprising 249 amino acids. STF-2 is strongly expressed in flowers and leaves but only weakly expressed in stems and roots. We constructed a plant expression vector with STF-2 gene and transformed it into tobacco. We found that overexpression of STF-2 enhanced drought tolerance in transgenic tobacco. Our results indicate the STF-2 plays an important role in soybean response to drought stress; furthermore, plant resistance to adversity can be improved by exogenous expression of this gene.

Key words: C_2H_2-type zinc finger protein; Drought stress; Soybean (*Glycine max*); Transgenic tobacco.

Introduction

Low temperature, drought and high salt are three major abiotic stress factors, which seriously affect plant growth and output (Shinozaki *et al*., 2003). The plant zinc finger proteins are transcription factors related to growth, development and tolerance to adverse stresses in plants. Many zinc finger proteins have been found in plants, and some of the C_2H_2-type proteins have been explicitly described (Takatsuji, 1998). Previous studies have demonstrated that plant C_2H_2-type zinc finger proteins contain between one and four zinc finger domains, and each zinc finger typically has a conserved sequence (QALGGH) in the α-helix domain. External to the zinc finger, most plant C_2H_2-type zinc finger proteins have three other conserved elements, including a nuclear localization signal region (NLS), a leucine-rich region (L-box), and a transcriptional repression domain (DLN-box) (Takatsuji *et al*., 1992). Many such proteins confer tolerance to adverse stresses in *Arabidopsis thaliana*, *Petunia* hybrid and other plants; most of them have been investigated in detail and have been found to contain two zinc-finger motifs (Takatsuji, 1998). Appropriate examples include *ZPT2-3*, which confers drought resistance to transgenic Petunia (Sugano *et al*., 2003), and *STZ*, which bestows very strong salt resistance on transgenic Arabidopsis (Sakamoto *et al*., 2000). Others proteins containing a single zinc-finger motif, such as *SUPERMAN* and *KNUCKLES*, related to floral development (Nakagawa *et al*., 2004; Payne *et al*., 2004; Sakai *et al*., 1995), have important functions in growth and development. Although C_2H_2-type zinc finger proteins are very important for plant growth, development and abiotic stress response, only a small number of such proteins have been reported in soybean (*Glycine max*).

The *SCOF-1* gene isolated from soybean encodes a protein having the typical structure of a C_2H_2-type zinc finger protein, and it has been found to enhance the low-temperature tolerance of transgenic tobacco (Kim *et al*., 2001). The GmZFP1 with a single zinc-finger motif has important functions in soybean growth and development (Huang *et al*., 2006).

In this study, we isolated a novel C_2H_2-type zinc finger protein gene *STF-2* from soybean, and we demonstrated that overexpression of *STF-2* enhanced drought tolerance in transgenic tobacco.

Materials and Methods

Plant material and treatments: The test seeds of 'Jinong 18' soybean were provided by the Agricultural College of Jilin Agricultural University, China and germinated in the Jilin Agricultural University experimental field. Mature (fully expanded) leaves, roots, and stems were collected separately at the third euphyll expansion stage, flowers (including sepals, petals, stamens, and pistils) were collected at blooming, pods were harvested at maturity, and all were placed immediately in an ultra-low temperature freezer at −80°C for storage. After growing control and transgenic plants on germination media (GM) for 3 weeks, one-half was transplanted into soil and grown for 1 week, then watering was stopped for 10 days; the other half was maintained in a culture dish with 10% (w/v) PEG6000 for 12–36 h (Xu *et al*., 2008).

RNA isolation and reverse transcription: Total RNA from different soybean tissues was extracted using Plant Total RNA Miniprep Purification Kit (GeneMark, Taizhong, China). The total RNA was treated by DNase I (GeneMark, Taizhong, Taiwan, China) at 37°C for 15 min. Then, the first-strand cDNA was synthesized by reverse transcription system (Promega, Beijing, China) to yield 20 µl cDNA. The reverse transcription product was then used as a template to perform RT-PCR and real-time PCR.

***STF-2* cloning:** The amino acid sequence of SCOF-1 was used as a target to search for the predicted translated nucleotide sequence, which had a conserved domain of zinc finger protein gene, with tblastn programme (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=tblastn & PAGE_TYPE=Blast Search & LINK_LOC=blasthome). Oligodeoxyribonucleotide primers covering the complete *STF-2* open reading frame (ORF) (sense: 5′-ATGGCTTTGGAGGCTTTGA-3′; antisense: 5′-AAACAAACGCGGCCTCT-3′) were synthesized by Sanboyuanzhi company (Beijing, China). The PCR reaction volume was 25 µl, including 4 µl first-strand cDNA as a template and 5.0 µl of 10× PCR buffer (with $MgCl_2$), 1.0 µl of 20 mM dNTPs, 13.5 µl ddH_2O, 0.5 µl *Taq* polymerase (Takara, Dalian, China), and 0.5 µl of each specific 25 mM primer. The PCR was performed on a Biometra UNO PCR instrument (Germany) using the following protocol: pre-denaturation at 94°C for 5 min followed by 32 cycles of 94°C for 50 s, 56.8°C for 50 s, and 72°C for 1 min and a final extension at 72°C for 10 min followed by a store step at 4℃. Then, the PCR product was detected and separated on 1% agarose gels and purified using a DNA gel extraction kit (Sanboyuanzhi, Beijing, China). Then, purified product was cloned into a pMD18-T vector (Takara, Dalian, China). The recombinant plasmid (pMD18-T-STF-2) was then transformed into *Escherichia coli*, plated onto a Luria-Bertani agar plate, and incubated overnight. The inserted sequence was confirmed by DNA sequencing (Sanboyuanzhi, Beijing, China).

RT-PCR and real-time PCR assay: The *ef-1α* was constitutively expressed in various soybean tissues; therefore, this gene was used as a control, and a 500-bp PCR fragment was amplified from various soybean tissues using ef-1α sense (5′-TGTTGCTGTTAAGGATTTGAAGCG-3′) and ef-1α anti-sense (5′-AGACACAGAGTACAGAAAAG AAAC-3′) primers. The PCRs were performed on a DNA amplification machine using the same protocol, except the annealing temperature was 54℃. The relative expression level of a target gene was normalised to the *ef-1α* gene band intensity.

The transgenic tobacco plants were subjected to drought stress, and the leaves were collected for real-time PCR (qRT-PCR) analysis. The qRT-PCR was based on the primers (QSTF-2S: 5'-TTCGAAGGCAGAGTAGGTGGCT-3'; QSTF-2AS: 5'-GAAGCTTTGGTAGCAATCGGAG-3'). The qRT-PCR assay was performed using SYBR Premix ExTaq system (Takara, Dalian, China) by a fluorescent quantitative PCR machine (Agilent MX3000P, California, USA). The tobacco actin gene 18S rRNA (18S rRNA-S: 5'-GAAGGGCTCACTGTAGATG-3'; 18S rRNA-AS: 5'-GAATGGCTCAACAACTGC-3') was used for the normalization of qRT-PCR analysis (Liu *et al*., 2013).

Plant transformations: To construct transgenic tobacco (*Nicotiana tabacum* 'Xanthi'), the *STF-2* cDNA fragment was digested with restriction enzymes (*XbaI*-*ScaI*) and ligated into a plant binary vector pBI121. The *CaMV*35S promoter controlled the expression of *STF-2* gene. Then, the recombinant plasmid pBI121-*STF-2* was introduced into *Agrobacterium tumefaciens* EHA101, and transgenic tobacco were obtained by leaf disc transformation (Augustine *et al*., 2015; Horsch *et al*., 1986). The gene expression was detected and analysed in transgenic plants (T_2 progenies).

Sequence and structure analyses: The GenBank BLAST programme (http://www.ncbi.nlm.nih.gov/blast) was used to search the non-redundant (NR) and dbEST databases. Protein prediction and sequence alignments were performed in MEGA v5.0. The online PHYRE^2 software (Protein Homology/analogy Recognition Engine V2.0, http://www.sbg.bio.ic.ac.uk/phyre/) was used to derive the 3D structure of STF-2, with a C_2H_2-type zinc finger protein from *Homo sapiens* (PDB code 2YT9) chosen as a template. Model qualities were checked with PROCHECK.

Transgenic tobacco plants under drought stress: To investigate the role of *STF-2* in plants under abiotic stresses, we used transgenic tobacco constitutively overexpressing *STF-2* (Hiratsu *et al*., 2004; Sakamoto *et al*., 2004). Thirty transgenic plant lines overexpressing *STF-2* were selected from 40 transformant lines by RT-PCR analysis. After 2-5 weeks, we compared the growth and development of the *STF-2* transgenic plants on GM agar plates or in soil, respectively. The observed wild-type plants and transgenic lines on either substrate exhibited growth retardation and dwarfed heights.

To investigate whether *STF-2* overexpression affects tolerance to drought stress, we cultivated the wild-type and transgenic plants in GM agar for 3 weeks. Half of the plants were then grown in soil for 1 week and then left unwatered for 10 days; the other half was maintained in a culture dish with 10% (w/v) PEG6000 (Sakamoto *et al*., 2004; Sugano *et al*., 2003; Xu *et al*., 2008).

Results

Molecular cloning and characterization of the *STF-2*: To isolate a novel soybean C_2H_2-type zinc finger protein gene involved in resistance to abiotic stress and tissue development, we first used the SCOF-1 as a target probe to search the EST-database in GenBank using the tblastn alignment systems. Matching expressed sequence tags (ESTs) were downloaded and assembled manually. These sequences were predicted and analysed by software, then we selected 4 ESTs, which were mainly derived under abiotic stress conditions and had zinc finger motifs, for further analysis (data not shown). A complete ORF of 747 bp encoded a product showing similarity to soybean SCOF-1 and containing two zinc finger motifs was selected from these ESTs. We then carried out RT-PCR to isolate this gene from soybean leaves and designated this fragment as *STF-2*.

Available evidence suggests that most plant C_2H_2-type zinc finger genes are intronless (Hiratsu *et al*., 2004; Huang *et al*., 2005; Kubo *et al*., 1998). To analyse the structure of STF-2, we performed PCR amplifications using cDNA (after removal of genomic DNA by DNase I) and genomic DNA from soybean leaves as templates. The products of two different amplifications were cloned into pMD18-T vectors. After sequencing, we found that the

STF-2 had no introns, which was similar to the results of previous studies (Hiratsu *et al*., 2004; Sakamoto *et al*., 2004; Sugano *et al*., 2003; Xu *et al*., 2008).

Protein structural analysis of STF-2: The predicted zinc finger protein product of *STF-2* was composed of 249 amino acids with an Mw (molecular weight) of 26.7 kDa and a pI (isoelectric point) of 8.23. *STF-2* was located on the long arm of chromosome 10(O), with genetic distances of 117.2 cM on chromosome 10(O) (Supplementary Fig. S1). There were two single zinc finger motifs that included the plant-specific sequence QALGGH in the deduced amino acid sequence of STF-2 (Takatsuji, 1998) (Fig. 1A). The sequence homology analysis was carried out by blast aligning programme, and the results revealed that STF-2 and soybean C_2H_2-type zinc finger ZAT10-like protein were close homologues. A detailed comparison of similar zinc finger proteins showed that the zinc finger motif in STF-2 resembled that in many plant C_2H_2-type two-zinc finger proteins, including soybean SCOF-1, Arabidopsis STZ, and Petunia ZAT2-3. STF-2 also has a conserved C-terminally located DLN-box/EAR-motif, originally discovered in SCOF-1 as a transcriptional repressor domain (Hiratsu *et al*., 2004; Kim *et al*., 2001). This finding suggests that *STF-2* functions as a transcriptional repressor. Furthermore, we found that STF-2 had especially high identity to MsZFP1, a protein that had two zinc fingers, a leucine-rich L-box for protein interaction, and an N-terminal NLS (B-box) containing the conserved sequence KRSKR related to subcellular localization. *MsZFP1*, isolated from *Medicago sativa*, related to osmotic stress and nodule development. The close phylogenetic relationship (Fig. 1B) of soybean and *Medicago*, both in the Fabaceae - Faboidae family, is at least partly responsible for the strong similarity between STF-2 and MsZFP1.

A model of STF-2 structure was constructed using the Phyre[2] with a C_2H_2-type zinc finger protein from *Homo sapiens* (PDB code 2YT9) as a template. STF-2 has two typical zinc finger motifs (Fig. 2A), and each of them has a signature structure $CX_{2-4}CX_3FX_5LX_2HX_{3-5}H$ and consists of 21 amino acids, two pairs of conserved cysteine and histidine residues tetrahedrally bound to a zinc ion (Pabo *et al*., 2001). Each zinc finger motif is composed of one α-helix and two antiparallel β-sheets (Fig. 2B). In Fig. 2C, the blue region of the zinc finger corresponds to the positively charged DNA binding domain. In the Ramachandran plot generated by the PROCHECK analysis, 91.0% of residues were in the most favoured regions; the remaining 9.0% were located in the additional allowed region (Supplementary Fig. S2).

STF-2 is highly expressed in various soybean tissues: *STF-2* gene expression profiles in various soybean tissues were detected by RT-PCR (Fig. 3), which revealed that *STF-2* was expressed in leaves, roots, flowers, and stems, suggesting that *STF-2* played diverse roles in soybean. *STF-2* had the highest expression in leaves and flowers followed by stems, with the lowest levels was observed in roots. A study of *MsZFP1*, the closest homologue of *STF-2*, revealed a similar expression pattern in various tissues of *Medicago sativa* (Frugier *et al*., 2000). Both proteins may regulate plant defence against stress via overexpression.

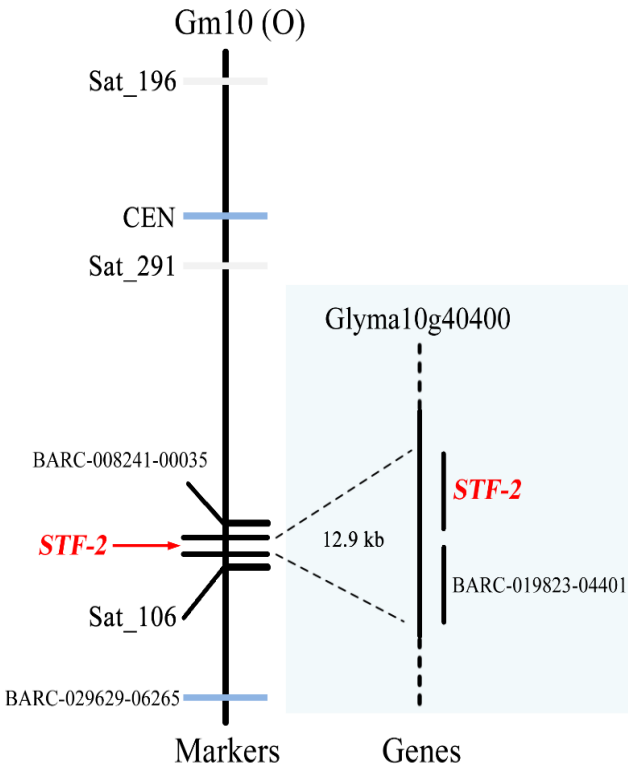

Fig. S1. *STF-2* is located on the long arm of chromosome 10(O), with genetic distances of 117.2 cM on chromosome 10(O).

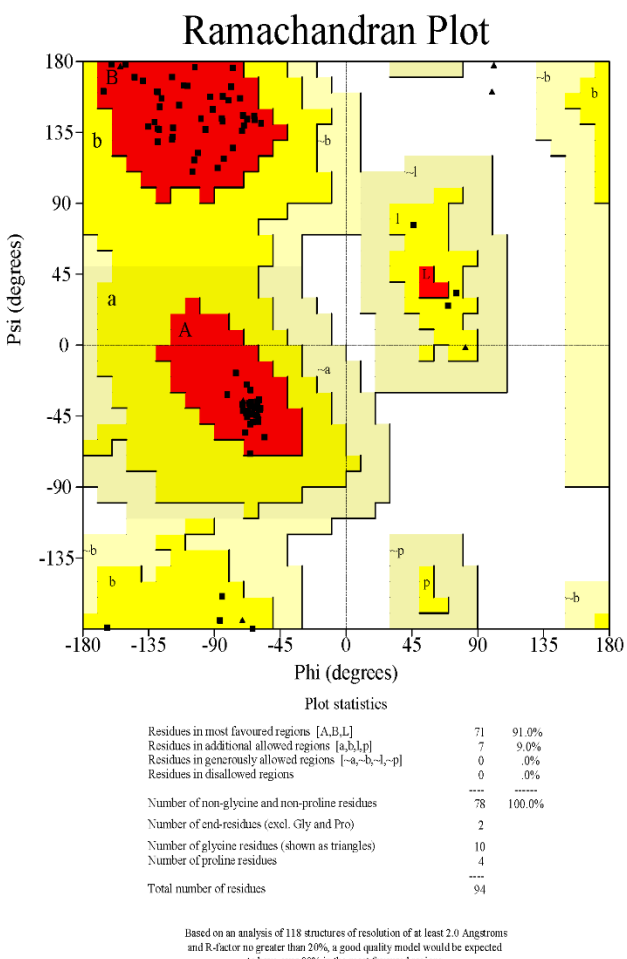

Fig. S2. Quality evaluation of the STF-2 structure model.

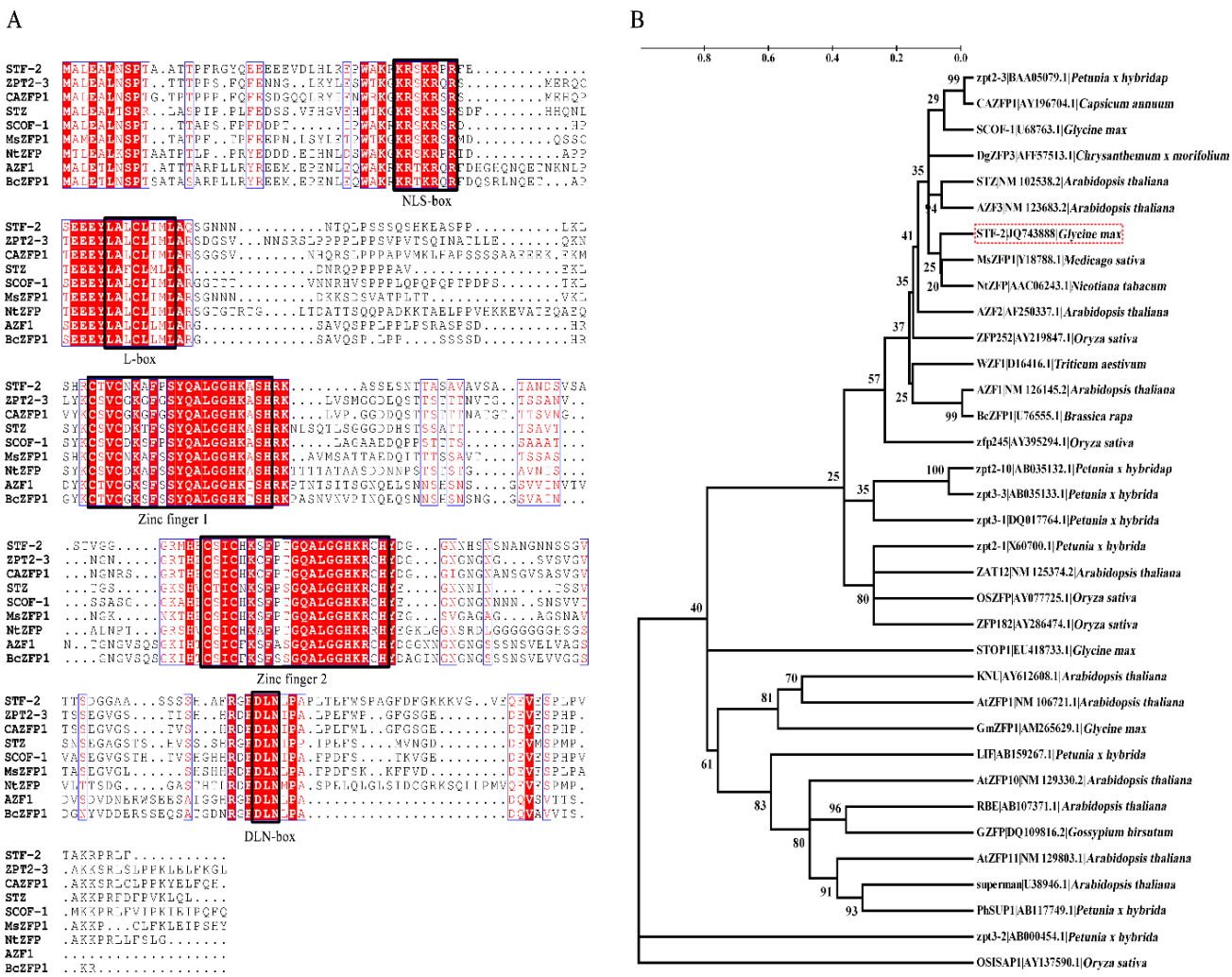

Fig. 1. Protein sequence analysis of STF-2. (A) The deduced amino acid sequence of STF-2. (B) Phylogenetic tree of STF-2 and other C_2H_2-type zinc finger proteins, constructed by neighbour-joining in MEGA v5.0.

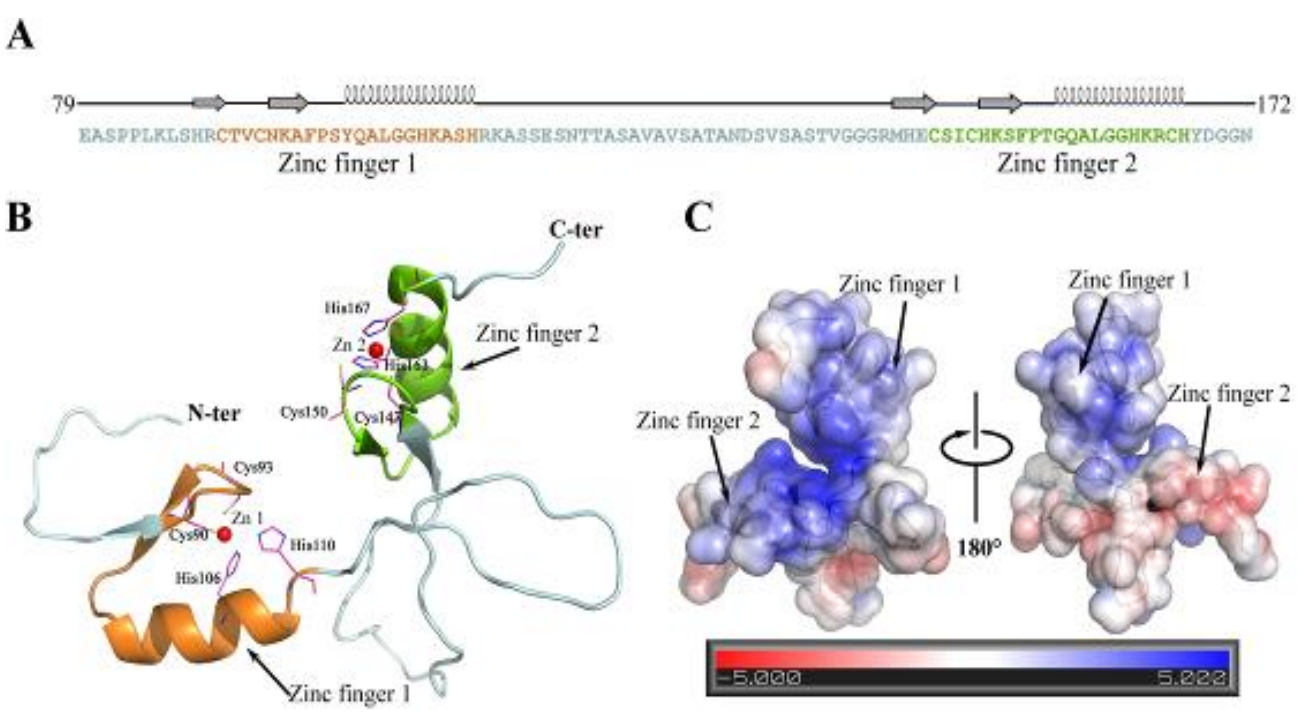

Fig. 2. Homology modelling of STF-2. (A) Secondary structure topology diagram of STF-2. (B) The modelled structure of STF-2. (C) The charge distribution of STF-2.

Overexpression of STF-2 enhanced tolerance to drought stress: Using the transgenic and wild-type plants grown in the presence of 10% (w/v) PEG6000, we analysed *STF-2* expression levels under drought stress by RT-PCR and quantitative qPCR. As shown in Fig. 4A, *STF-2* expression increased over time in 10% (w/v) PEG6000, with the highest expression observed during 12–36 h. The expression analysis (Fig. 4B) demonstrated that *STF-2* was induced by drought stress. The control and transgenic plants grown in GM for 21 days were placed in water for 48 h and then in a culture dish with 10% (w/v) PEG6000 for 24 h.

The growth performance of plants cultivated in soil was analysed. Although 4-week-old *STF-2* transgenic plants showed growth retardation, this reduced growth was not substantially different from the controls. We measured soil water contents of transgenic and control plants during the drought treatment and found little difference in the two types of plants (data not shown). After watering was halted, nearly all wild-type tobacco died in 10 days; in contrast, nearly all *STF-2* transgenic tobacco survived under this level of drought stress and continued to grow after rewatering (Figs. 5A, 5B, 5C), and the survival rate of transgenic tobacco was 90%. Furthermore, nearly all wild-type plants died within 36 h under 10% (w/v) PEG 6000 stress, whereas most *STF-2* transgenic plants survived and continued to grow when rewatered (Fig. 5D), and the survival rate of transgenic tobacco was 86.7%. These results demonstrated that the *STF-2* enhanced drought tolerance in transgenic tobacco.

Discussion

In a previous study, the soybean zinc finger protein gene *SCOF-1* enhanced the low-temperature tolerance of transgenic plants (Kim *et al*., 2001). By analysis of the functional mechanism, we found another transcription factor SGBF-1 (soybean G-box binding bZIP transcription factor), which could bind to DNA. SGBF-1 could enhance the affinity of DNA binding to ABRE (ABA-responsive element) by the *SCOF-1* expressed under the low-temperature stress and then regulated the expression of the response genes to tolerate low-temperature stress. Based on a yeast two-hybrid screen test, we validated the SGBF-1 interaction with SCOF-1. The expression of β-glucuronidase reporter gene showing SGBF-1 transactivation was driven by the ABRE element in *Arabidopsis*. In addition, the SCOF-1 enhanced downstream ABRE-dependent gene expression, which was mediated by SGBF-1, and the plants enhanced the tolerance to cold stress.

The yeast two-hybrid system results also showed that SCOF-1 regulated the expression of a downstream gene *COR*, which enhanced the low-temperature tolerance of transgenic plants. Although STF-2 has protein domains that are similar to those of SCOF-1, its functional mechanism has not been studied (Kim *et al*., 2001). We hypothesize that the expression of drought tolerance-related genes may be directly regulated by *STF-2* or, similar to *SCOF-1*, *STF-2* may interact with some binding proteins to regulate the expression of its related downstream genes to enhance the drought tolerance of transgenic plants.

The leaves of *STF-2* transgenic plants were essentially normal after drought stress, and transgenic plants had a strong root system, but the wild-type plants did not survive. Because the *STF-2* was expressed in roots, leaves, flowers, and stems, the *STF-2* perhaps enhances drought tolerance of roots and leaves in transgenic tobacco (Figs. 3 and 5C). Even though the role of *STF-2* in response to drought stress needs to be studied in more detail, this gene is a potential tool for biotechnological breeding of drought-tolerant plants.

In conclusion, the intronless STF-2 gene encodes a soybean SCOF-1-like zinc finger protein, which has two fingers and similar protein domains to SCOF-1, contains a DLN-box/EAR-motif near the C-terminus, a NLS-box and a Leu-box. The *STF-2* gene is located on the long arm of chromosome 10(O), with genetic distances of 117.2 cM on chromosome 10(O). In tested organs, *STF-2* is expressed in flowers and leaves at a high level and in stems and roots at a low level. Overexpression of *STF-2* enhances drought tolerance in transgenic plants. Future intensive studies are needed to investigate the function of *STF-2* in order to provide better understanding of *STF-2* roles in plant stress tolerance.

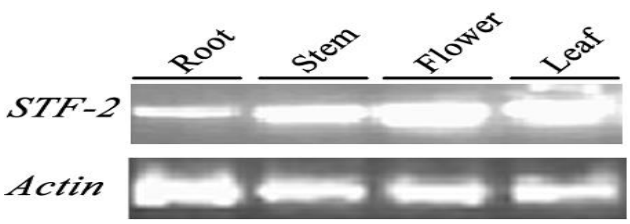

Fig. 3. Expression levels of STF-2 in various soybean tissues by reverse-transcription PCR (RT-PCR).

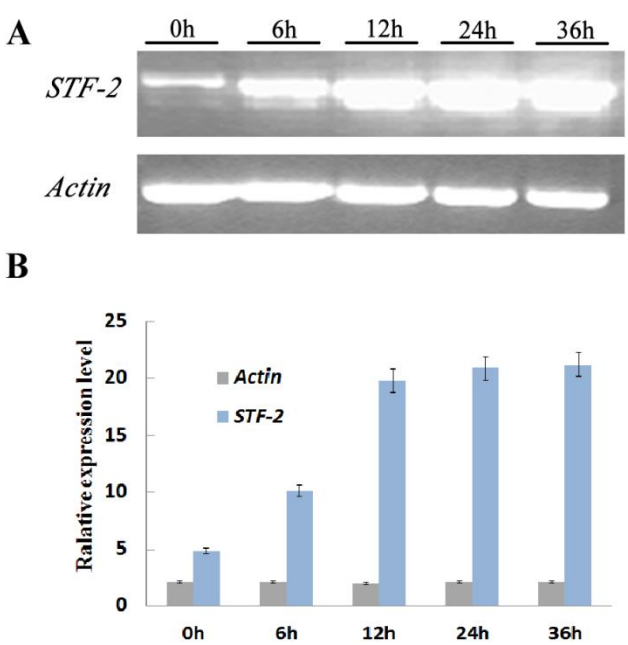

Fig. 4. Expression levels of *STF-2* under drought stress. (A) Expression levels of *STF-2* measured by reverse-transcription PCR under drought stress. (B) Expression of *STF-2* in response to environmental factors measured by quantitative real-time RT-PCR analysis.

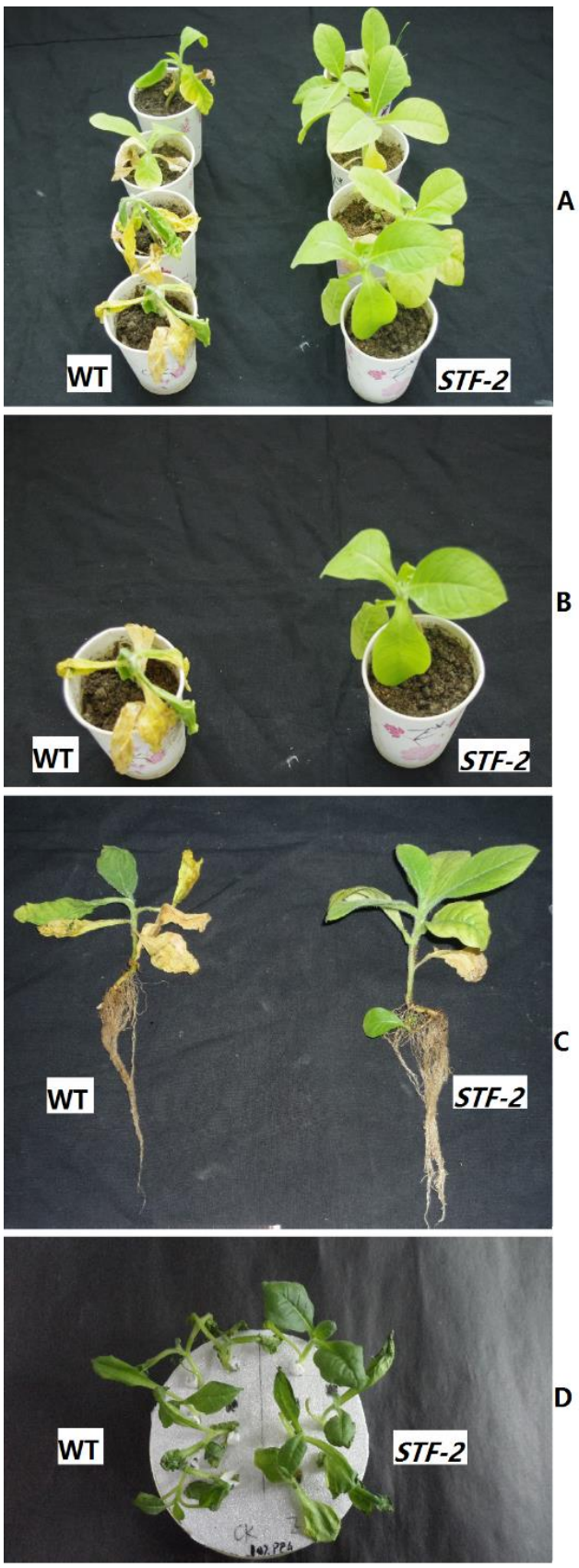

Fig. 5. Drought tolerance of *STF-2* transgenic plants. (A and B) Wild-type and *STF-2* transgenic T_2 seedlings were subjected to drought stress by withholding water for 10 days. (C) A single plant after 10 days of drought stress. (D) A plant subjected to drought stress induced by 10% PEG6000 after 12 h of water culture.

GenBank accession code

The *STF-2* gene sequence has been deposited in NCBI GenBank under the accession code JQ743888.1.

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