INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
21–0429/2021/26–4–536–545
DOI: 10.17957/IJAB/15.1865
http://www.fspublishers.org

Full Length Article

Heterologous Expression of Soybean *GmC$_2$H$_2$4-Like* Gene Confers Cold Tolerance in Transgenic *Arabidopsis*

Yang Xu†, Fan Yan†, Yajing Liu, Ying Wang, Qingyu Wang* and Jingwen Li
Jilin Key Laboratory for Crop Genetic Engineering, College of Plant Science, Jilin University, Changchun 130062, China
*For correspondence: qywang@jlu.edu.cn
†Contributed equally to this work and are co-first authors
Received 05 April 2021; Accepted 12 August 2021; Published 28 September 2021

Abstract

Zinc finger protein is a class of essential transcription factor that exists widely in prokaryotes and eukaryotes. These proteins show importance functions in all kinds of biological resistance and growth of plants, however, are scarcely studied in soybean. In this study, a new *Cys2/His2 (C_2H_2)* gene with the accession No.MK045991 in NCBI database, denoted *GmC$_2$H$_2$4-like*, was isolated from soybean, and the related functions were identified. The cDNA of *GmC$_2$H$_2$4-like* is 756 bp with 251 amino acids encoded. The results of bioinformatics and yeast single hybrid analysis revealed that *GmC$_2$H$_2$4-like* contains a single conserved C_2H_2 domain and is a special zinc finger protein with transactivated activity. Subcellular localization analysis with the *GmC$_2$H$_2$4-like-GFP* fusion gene in *Arabidopsis* protoplast cells indicated that *GmC$_2$H$_2$4-like* protein is mainly localized in the nucleus. *GmC$_2$H$_2$4-like* was transferred into wild type *Arabidopsis*. Four T_3 transgenic *Arabidopsis* lines were obtained as confirmed by glufosinate ammonium, PCR, RT-PCR and bar strip detection. *GmC$_2$H$_2$4-like* transgenic *Arabidopsis* showed better phenotypic characteristic including the root length, plant size and development speed than that of the WT in a normal environment. Quantitative Real Time PCR (qRT-PCR) analyses demonstrated that the expression of *GmC$_2$H$_2$4-like* gene increased a lot under cold stress induction. Under cold treatments, the physiological and biochemical indexes showed that *GmC$_2$H$_2$4-like* transgenic *Arabidopsis* was more tolerant to cold stress. These results suggested that the cold resistance of transgenic *Arabidopsis* was significantly enhanced by the heterologous expression of *GmC$_2$H$_2$4-like*.

Keywords: Soybean; *GmC$_2$H$_2$4-like*; Transgenic *Arabidopsis*; Cold tolerance; Zinc finger proteins
Abbreviations: ABA, Abscisic acid; GFP, Green fluorescent protein; MDA, Malondialdehyde; MS, Murashige and Skoog; NCBI, National Center for Biotechnology Information; ORF, Open reading frame; POD, Peroxidase; qRT-PCR, Quantitative real time PCR; SD, Standard Deviation; WT, Wild type

Introduction

Plants frequently face adverse environmental conditions, such as salt, disease and chilling injury, which may have serious effects on their growth and the crop yields. As the main grain and oil crop, soybean [*Glycine max* (L.) Merr.] often suffers from cold damage in many cold growing areas, which seriously affects the crop productivity and quality. Therefore, it is very important to increase the crop yield and keep stable yields in cold conditions in order to ensure food supplies for an increasing global population. In recent years, many typical, cold related transcription factors (bZIP, MYB, WRKY and C_2H_2) have been cloned and characterized (Luo *et al*. 2012; Yu *et al*. 2014), to confirm a critical relationship with the response of plants to cold stress *via* transcriptional regulation (Ahuja *et al*. 2010; Xu *et al*. 2011).

Zinc finger proteins are a kind of transcription factors with finger shape domain (Tian *et al*. 2010) and were first discovered in *Xenopus* by Miller *et al*. (1985). They are divided into nine categories: C_4, C_6, C_8, CCCH, C_2HC, C_2HC_5, C_2H_2, C_3HC_4 and C_4HC_3 (Michael and Chrisopher 2003). The C_2H_2 zinc finger proteins were studied most and are composed of about thirty amino acids. The conservative sequence is: $CX_{2\sim4}CX_3PX_5LX_2HX_3H$ (Pabo *et al*. 2001). The zinc finger proteins in plants are associated with the processes of morphological changes during growth, pollen and embryo development. And also plays a key part in the regulation of abiotic stress (Luo *et al*. 2012; Zhai *et al*. 2013).

The C_2H_2 zinc finger proteins which contained special conserved motifs which was QALGGH located in the special α helix portion of each protein, are common transcription factors which were characterized (Isernia *et al*. 2010; Fedotova *et al*. 2017). Sugano *et al*. (2010) found that *zpt2-3* gene transferred into petunia was also induced by desiccation stress. However, there are relatively few studies on soybean zinc finger proteins; only *SCOF-1* has been studied in depth (Kim *et al*. 2010). Over expression of *SCOF-1* can increase the expression of *COR* gene, which

enhances the cold resistance of transgenic tobacco and *Arabidopsis*. Besides, Luo *et al*. (2012) confirmed that *GsZFP1* enhanced the cold and drought tolerance significantly.

This study was conducted to clone a novel *GmC$_2$H$_2$4-like* gene and characterize its functions in growth, development and its responses to cold stress. The technology of soybean genetic transformation is quite difficult. Therefore, we verified the functions of *GmC$_2$H$_2$4-like* gene in *Arabidopsis thaliana*, a model plant with easy genetic transformation. This will provide the basis for the further functional study of *GmC$_2$H$_2$4-like* gene in soybean and the cultivation of high-quality transgenic materials in the future.

Materials and Methods

Plant materials

Total RNA of Jilin32 soybean seedlings was extracted for *GmC$_2$H$_2$4-like* gene cloning and the total RNA of various Jilin32 soybean tissues (root, stem, leaf and seed) at 24°C and 4°C was extracted for expression analyses of *GmC$_2$H$_2$4-like*. This Jilin32 soybean variety has strong tolerance to stress and is disease resistant. We constructed the library expression profile of immature Jilin32 embryos for further research. Five hundred plants were planted under natural conditions. Fifty Jilin32 seeds were sowed in the plant incubator at 25°C under 16 h lightness and 23°C under 8 h darkness with 55% relative humidity. The soybean strains are used for gene expression analyses. 10-day-old seedlings were cultivated under cold treatment at 4°C for 24 h before tissues were sampled for qRT-PCR analysis and further investigations. Tissue samples included roots, stems, leaves and mature seeds.

Arabidopsis (Colombia-0) was the basic model material for the transformation of target genes, the extraction of protoplast cells, the observation of phenotype in seedling stage and the cold resistance analysis of *GmC$_2$H$_2$4-like*. After the sterilization and vernalization of *Arabidopsis* seeds, the seeds of WT and transgenic *Arabidopsis* plants were simultaneously planted and cultivated in MS solid medium. After half a month, all the *Arabidopsis* seeds were transplanted to mixed soil with peat and vermiculite for further studies. The roots were used for investigation of root length and development. After one month of growing, the transgenic *Arabidopsis* and the control (WT) were simultaneously subjected to cold treatment at 4°C for 24 h. After cold treatment, *Arabidopsis* leaves were immediately sampled into liquid nitrogen for the next experiments. The leaves were sampled from the same *Arabidopsis* lines at 22°C and 4°C to make sure the experiment rigorous. The leaves were used for qRT-PCR analyses and determination of various physiological and biochemical indexes. All samples were collected randomly in three repeats and saved at -80°C after freezing in liquid nitrogen.

Bioinformatics analysis and cloning of *GmC$_2$H$_2$4-like* in soybean

The whole sequence of *GmC$_2$H$_2$4-like* was obtained in NCBI Blast database (http://www.ncbi.nlm.nih.gov/). The single zinc protein conserved domain was predicted in NCBI with the accession number NM_001255238. Alignment of the cDNA sequence was demonstrated in Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html) and Soybase database (https://www.soybase.org/sbt/). The theoretical pI and molecular mass were calculated on the ExPASy (https://web.expasy.org/protparam/) SIB Bioinformatics Resource Portal. The subcellular location of *GmC$_2$H$_2$4-like* protein was preliminarily predicted using Cell-PLoc online software (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/). The tertiary structure were predicted using Phyre^2 online software (http://www.sbg.bio.ic.ac.uk/servers/phyre2/html/page.).

Total RNA of Jilin32 soybean seedlings were extracted and the cDNA was synthesized using cDNA Synthesis Kit (TaKaRa, Beijing, China). The whole cDNA sequence was obtained with RT-PCR according to forward primers (5′-TAGCTTGAAAACTTAGCACAG-3′) and reverse primers(5′-TAACAGCACATACAGAGCAAA-3′). The RT-PCR experiment system included 1 μL cDNA, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 2.5 μL 10x *Ex-Taq* Reaction buffer (20 mM), 2 μL dNTP (2.5 mM), 0.5 μL extaq DNA polymerase (5 U/μL) and 17 μL distilled water. The PCR product was recycled using Gel Recycling Kit (Takara, China), and sequenced by Biological sequence Company (Sangon Biotech, China). The nine genes which were most similar to *GmC$_2$H$_2$4-like* and containing one single conserved C_2H_2 domain were analyzed by the online SMART software (Letunic *et al*. 2006). The accession numbers of nine genes are ATZFP11, NP_181770; Hypothetical petunia hybrid, BAD11142; RABBIT EARS *A. thaliana*, BAC98433; SUPERMAN, Q38895; LSIF petunia hybrid, BAB58897; AtZFP10, NP_181310; KNUCKLES *A. thaliana*, AAT27472; Cys2-His2 GmZFP1, NP_001341929 and ATZFP1, NP_178188.

Expression analysis by qRT-PCR

Total RNA of different parts including roots, stems, leaves and seeds in soybean was extracted using the same method as cloning experiments before. The qRT-PCR was performed using *TransStart* Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China) and analyzed using ABI PRISM 7900 software according to the QIAGEN Supplementary Protocal (QIAGEN, Germany). Each hole up to 20 μL in the 96 holes testboard was composed of 10 μL 2 x *TransStart* Tip Green qPCR SuperMix, 1 μL Template (cDNA, 1 μg), 0.4 μL Passive Reference Dye (50x), 0.4 μL forward primer, 0.4 μL reverse primer and 6.8 μL DNAase free water. All the primers of *GmC$_2$H$_2$4-like*, *β-Tubulin* (GMU12286), soybean and *Arabidopsis Actin*

(J01298 and NM_112764, respectively) were showed in Table 1. These two kinds of internal genes were used to standardize the experiment data (Jian *et al*. 2008; Hu *et al*. 2009; Le *et al*. 2012). The qRT-PCR systems were divided into two steps: 94°C for 30 s, 55°C for 5 s (45 cycles) and 60°C for 30 s. The ΔCT value method was used as a standard to validate the gene expression (Riedel *et al*. 2014).

Subcellular localization of *GmC$_2$H$_2$4-like* gene

The stop codon of *GmC$_2$H$_2$4-like* was got rid from coding region and the sequence was inserted in the pBI121 vector with *Xba*I restriction enzymes. The recombinant construct was then introduced into *Agrobacterium tumefaciens* strain EHA105 and transformed to the protoplast of *Arabidopsis* cells. Specific experimental methods referred to the paper of Confraria and Baena-González (2016) and Su *et al.* (2014). Finally, the GFP fluorescence was found using a confocal microscope (Olympus, Tokyo). The nucleus was dyed with DAPI. The location was preliminarily predicted using Cell-PLoc online software according to http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/ (Chou and Shen 2010a, b).

Transactivation analysis in yeast

To study the transactivation activity in yeast, the full-length open reading frame of *GmC$_2$H$_2$4-like* was inserted into the GAL4 BD binding domain of pGBKT7 vector (Invitrogen, Carlsbad, CA, USA), which had been digested in advance with the *Eco*RI and *Sal*I restriction enzymes. The pGBKT7-*GmC$_2$H$_2$4-like* was then transformed into the AH109 yeast as the positive control (Zhao *et al.* 2017). The transformant yeast was cultivated on medium without Trp at 35°C for 3 days and subsequently, the yeast was shifted to mediun without Trp, His and Ade including 3-amino-1, 2, 4-triazole. A *β-galactosidase* assay was also conducted to examine the transactivation ability within 10 h. Specific *β-galactosidase* assay method referred to Zhao *et al*. (2017). Finally, the result showed according to color reaction which was monitored and photographed.

***Arabidopsis* transformation**

Gateway technology was used to clone and construct an expression vector (Su *et al.* 2014). The *GmC$_2$H$_2$4-like* gene was inserted into the pDONR221 vector. Through two steps BP and LR recombination reaction, the resultant pCB35S-GFP-GmC$_2$H$_2$4-like was obtained and then transformed into *A. tumefaciens* EHA105, the transgenic *Arabidopsis* was obtained using the floral dip method. The transgenic *Arabidopsis* was screened with 5mg/L glufosinate-ammonium.

Detection and screening of transgenic plants

The *GmC$_2$H$_2$4-like* transgenic *Arabidopsis* were identified using a series of detection methods. The positivity of the transgenic plants for the target gene was examined by RT-PCR. The forward primer (5′-ACCCACGTCATGCCAGTT-3′ and reverse primer 5′-CTAGGGGGATCTACCATG-3′) were used to amplify the 501 bp bar gene. The forward primer pairs (5′-TAGCTTGAAAACTTAGCACAG-3′ and the reverse primer pairs (5′-TAACAGCACATACAGAGCAAA-3′) were used to amplify the 982 bp target gene. The bar strip (A07-13-413, Beijing) is an easy and accurate way to detect the PAT protein in transgenic plants.

Measurement of the cold resistance-related indicators in WT and transgenic *Arabidopsis*

Fresh *Arabidopsis* leaves of WT and four transgenic *Arabidopsis* lines were sampled in 3 biological replicates at the end of the 4°C treatment for a day for further biochemical analyses. In general, under the stress of adversity, the permeability of plant cell membrane will change. Malondialdehyde (MDA) is used commonly as index of cell membrane peroxidation, which can reflect the degree of membrane peroxidation and the response to stress. Malondialdehyde (MDA) content was calculated by the chromogenic reaction of thiobarbituric acid and MDA under acidic conditions. After 4°C cold treatment, *Arabidopsis* leaves (1g) were grinded with quartz sand and 10% trichloroacetic acid. The homogenate was centrifuged at 5000 rpm for 10 min and the supernatant was the extract of malondialdehyde. Then the samples were measured the absorbance values in 532 nm, 450 nm and 600 nm wavelength. The results were calculated referring to the formula in the paper of Tirani and Haghjou (2019). Peroxidase (POD) can measure the resistance of plants to adversity. The stronger the resistance was, the higher the POD value was (Weng *et al.* 2015; Shekaari *et al.* 2019). Peroxidase (POD) activity was measured by the guaiacol method (Wang *et al*. 2019). The enzyme of *Arabidopsis* leaves (0.1 g) were extracted, then the enzyme activity was determined in spectrophotometer in OD_{470}. The results were recorded each 30 s. The relative electric conductivity of the leaves was measured with a conductometer (Chen and Han 2010; Zhang *et al*. 2018). The soluble sugar in the leaves (0.5–1.0 g) was tested by the modified phenol sulfuric acid method. The samples were measured the absorbance values in 485 nm wavelength. The results were calculated by the standard curve (Klotke *et al.* 2010). The proline content in WT and transgenic *Arabidopsis* (0.5–1 g) was determined by the sulfosalicylic acid method. The samples were measured the absorbance values in 520 nm wavelength. The results were still calculated referring to the formula in the paper of Xu *et al*. (2013).

Results

Isolation and characterization of *GmC$_2$H$_2$4-like*

According to the expression of *GmC$_2$H$_2$4-like* in different immature pods, we found four unknown cDNA clones

Table 1: Gene specific primers for qRT-PCR

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
GmC2H24-like	5′-AAAGAACAATAGCGAAGAG-3′	5′-GAGGGAACCTGATGGTAG-3′
β-Tubulin	5′-GGAAGGCTTTCTTGCATTGGTA-3′	5′-AGTGGCATCCTGGTACTGC-3′
soybean *Actin*	5′-GTCCTTTCAGGAGGTACAACC-3′	5′-CCACATCTGCTGGAAGGTGC-3′
Arabidopsis Actin	5′-CCTTGAAGTATCCTATTGAGC-3′	5′-GGTCTTTGAGGTTTCCATCT-3′

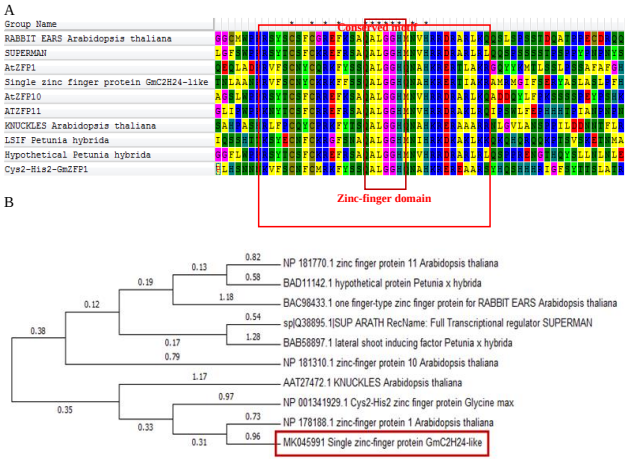

Fig. 1: Amino acid sequence analyses of *GmC$_2$H$_2$4-like* (**A**) Alignment of amino acids of *GmC$_2$H$_2$4-like*. Partial sequences of *GmC$_2$H$_2$4-like* with other nine single zinc finger proteins in plants. Sequences were aligned using ClustalW. Positions containing identical residues are marked with an asterisk (*). The thin-lined red frame contains a special zinc-finger domain. QALGGH is a conserved motif. The GenBank accession numbers are: ATZFP11, NP_181770; Hypothetical petunia hybrid, BAD11142; RABBIT EARS *A. thaliana*, BAC98433; SUPERMAN, Q38895; LSIF petunia hybrid, BAB58897; AtZFP10, NP_181310; KNUCKLES *A. thaliana*, AAT27472; Cys2-His2 GmZFP1, NP_001341929; ATZFP1, NP_178188. (**B**) Phylogenetic tree of *GmC$_2$H$_2$4-like* and other TFIIIA single zinc finger proteins. The tree was constructed using the neighbor-joining method with the program MEGA 5.1 (Tamura *et al*. 2011). Branch numbers represent bootstrap values from 1000 sampling replicates and branch lengths are proportional

which showed much homology with other differentially expressed C_2H_2 genes in plants. The four predicted translation products of the clones contained one or two C_2H_2 domains. QRT-PCR experiments were conducted to analyze the expression of *GmC$_2$H$_2$s* in response to the growth and development of the plant and their tolerance to cold. One gene designated *GmC$_2$H$_2$4-like* showed significant changes compared to the WT in four candidate genes. Based on this discovery, *GmC$_2$H$_2$4-like* was selected for further functional analyses. The open reading frame of *GmC$_2$H$_2$4-like* was 756 bp and 251 amino acids were encoded in the protein with a calculated mass of 27.64 kDa and a pI of 6.30. According to the results of alignment of the cDNA sequences in the Soybase and Phytozome database, *GmC$_2$H$_2$4-like* contained one intron and was located on No.18 Chromosome. The *GmC$_2$H$_2$4-like* protein contained one single conserved C_2H_2 domain, including a conserved QALGGH motif according to the SMART analyses (Fig. 1A). Phylogenetic analysis revealed that the novel single zinc finger gene *GmC2H24-like* from soybean clustered with *Arabidopsis AtZFP1*, which belongs to the C_2H_2 protein family (Fig. 1B). *GmC$_2$H$_2$4*-like protein was preliminarily predicted to be localized in the nucleus. Besides, the results of secondary structure and tertiary structure of *GmC$_2$H$_2$4-like* in Phrye[2] software; the results of gene and protein BLASTs in NCBI were shown in Fig. S1.

Expression analyses of *GmC$_2$H$_2$4-like* in different soybean tissues at 24°C and 4°C

The expression levels of *GmC$_2$H$_2$4-like* in roots, stems, leaves and mature seeds of soybean (Jilin 32) at normal and cold temperatures were examined by qRT-PCR (Fig. 2). The expression of *GmC$_2$H$_2$4-like* was significantly higher in the roots and seeds, however, relatively lower in the stems and leaves at different temperatures. Data showed that the expression of *GmC$_2$H$_2$4-like* in different tissues after cold treatment was even higher compared to normal temperature. The results were consistent with the predictions obtained using online software.

Subcellular localization of the *GmC$_2$H$_2$4-like* protein

The online prediction tool Cell-PLoc, predicted that the *GmC$_2$H$_2$4-like* protein was localized in the cell nucleus. To further identify localization of *GmC$_2$H$_2$4-like*, the stop codon of *GmC$_2$H$_2$4-like* was deleted and the full length was fused to the *GFP* reporter gene with the CaMV 35S promoter. The results showed that the *GmC$_2$H$_2$4-like*: GFP fusion protein was distributed throughout the plant cell, mainly in the nucleus (Fig. 3B). 35S-GFP was a control that showed a distributed fluorescence through all the protoplast cells (Fig. 3A). The result was consistent with the predictions obtained using Cell-PLoc online software.

Transactivation activity of *GmC$_2$H$_2$4-like* in yeast

The transactivation activity of *GmC$_2$H$_2$4-like* was detected in the pGBKT7 vector which expresses special proteins fused to the GAL4 domain from Alcohol Dehydrogenase1 promoter in the yeast system. C_2H_2 proteins usually function as transcriptional activators or repressors. The results showed that all transformants grew normally on SD without Trp medium (Fig. 4A). The transformant of pGAL4 served as positive control. As a result, transformants of pGAL4 and

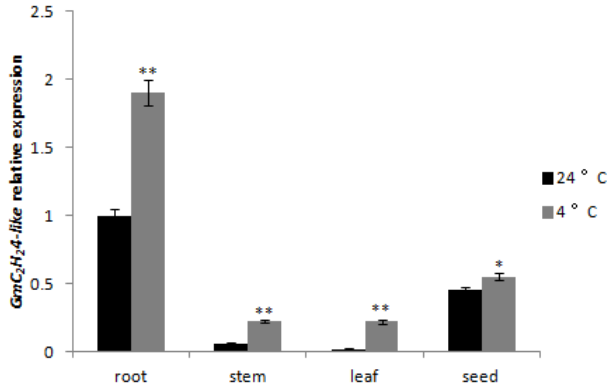

Fig. 2: *GmC₂H₂4-like* expressions in different soybean tissues and different temperatures. Expression analysis of *GmC₂H₂4-like* in different temperatures (24°C and 4°C) and tissues (root, stem, leaf, and seed) of soybean Jilin32 is determined by qRT-PCR. The data represent the average of three independent experiments ± SD. Results were normalized against for *β*-tubulin and soybean *Actin*. Statistical significance was determined by independent-sample *t*-test (*$P < 0.05$, **$P < 0.01$)

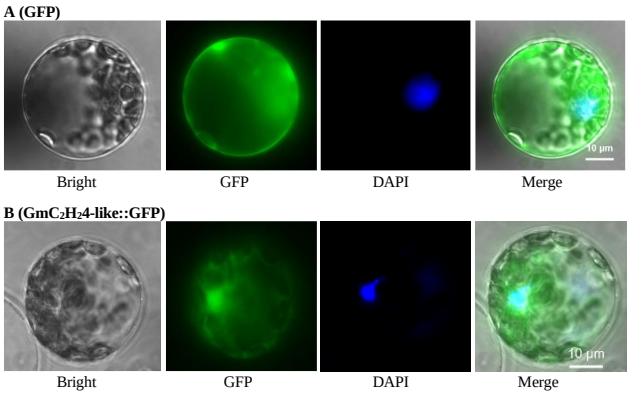

Fig. 3: Subcellular localization of the *GmC₂H₂4-like* protein in the protoplast of *Arabidopsis thaliana* cells. (**A**) Images expressing the GFP control (p35S-GFP); (**B**) Images of expression in the *GmC₂H₂4-like*-GFP fusion protein. The cells were visualized mainly localized in the nucleus in bright and fluorescent light fields. The cell nucleus was dyed with DAPI, and the pictures were merged. Scale bars = 10 μm

pGBKT7 fused with *GmC₂H₂4-like* could grow on the selective SD medium without Trp, Ade and His. They exhibited *β-galactosidase* activity in the filter paper with X-gal (Fig. 4B–C). These results showed that *GmC₂H₂4-like* gene was a transcriptional activator.

Generation and screening of *GmC₂H₂4-like* transgenic *Arabidopsis*

The 756 bp *GmC₂H₂4-like* open reading frame was cloned into the pCB35S-GFP vector. The recombinant plasmid pCB35S-GFP-*GmC₂H₂4-like* was transformed into the *A. tumefaciens* EHA105. All T_0 transgenic *Arabidopsis* seeds

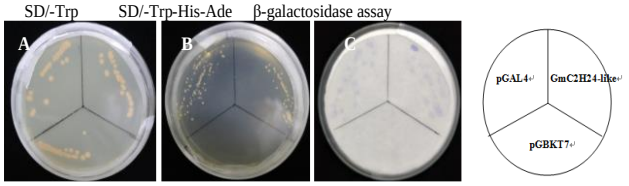

Fig. 4: Transcription activation analysis of the *GmC₂H₂4-like* protein. (**A**) Yeasts containing pGAL4, pGBKT7 and pGBKT7-*GmC2H24-like* grown on SD solid medium without Trp. (**B**) Yeasts containing pGAL4, pGBKT7, and pGBKT7-*GmC₂H₂4-like* grown on SD solid medium SD without Trp, His and Ade with 3-amino-1, 2, 4-triazole. (**C**) *β*-galactosidase activity assay

were sowed and cultivated at 23°C in MS solid medium that contained 5 mg/L glufosinate-ammonium (Fig. 5A). Finally, four regenerated T3 transgenic *Arabidopsis* were confirmed positive by PCR and bar strip analysis (Fig. 5B–D). The different tissues (roots, stems, and leaves) of transgenic *Arabidopsis* and CK (WT) were sampled before and after the 4°C treatment and used in qRT-PCR (Fig. 5E–F) to verify the heterologous expression of *GmC₂H₂4-like*. *GmC₂H₂4-like* transgenic *Arabidopsis* was more tolerant to cold stress than WT.

The key role of *GmC₂H₂4-like* in plant development

Compared to the WT, the roots of *GmC₂H₂4-like* transgenic *Arabidopsis* were much longer (Fig. 6A). Moreover, after being transplanted into soil, transgenic plants grew faster than that of the WT (Fig. 6B). This kind of growth and development state was mainly obvious in the early stage of growth. 30 roots from each *GmC₂H₂4-like* transgenic *Arabidopsis* line and the WT, totally 120 roots from four transgenic lines were sampled for statistical analyses. The root lengths of transgenic *Arabidopsis* were significantly longer than the WT (Fig. 6C).

The heterologous expression of *GmC₂H₂4-like* enhances the cold tolerance

After the 4°C treatment for 24 h, five physiological and biochemical indicators, namely, electrical conductivity, POD activity and MDA, soluble sugar and proline contents were measured. The WT and four different transgenic *Arabidopsis* lines presented significant differences. Under normal temperature conditions, there was no much difference between the leaves of transgenic and WT *Arabidopsis* in relative electrical conductivity. After the 4°C cold treatment, the relative conductivity of the transformed *Arabidopsis* was significantly lower than the WT (Table 2), indicating that cell membrane of the transgenic lines was less damaged at low temperatures. At 22°C, POD activity in transgenic plants was not significantly different compared to the WT plants. After the cold treatment, the POD activity of transgenic *Arabidopsis* was significantly higher compared to

Table 2: Physiological and biochemical indexes (relative conductivity, POD activity, and MDA, soluble sugar, and proline content) of WT and four different transgenic lines (1 to 4) at 22°C and 4°C

	Relative conductivity		POD activity (ΔA470/g. min)		Malondialdehyde (MDA) (μmol•g^{-1})		Soluble sugar (mmol•g^{-1})		Proline content (ng•mg^{-1})	
	22°C	4°C	22°C	4°C	22°C	4°C	22°C	4°C	22°C	4°C
WT	88.86 ±0.11^a	95.31±0.22^a	1.83 ± 0.22^a	15.66 ± 0.12^b	0.013 ± 0.24^a	0.073 ± 0.21^a	0.5387±0.25^a	0.7413±0.33^c	128.54±0.25^b	232.57±0.26^c
1	89.38 ±0.14^a	92.03±0.16^b	1.83 ± 0.24^a	20.15 ± 0.18^a	0.028 ± 0.28^a	0.059 ± 0.29^b	0.6210±0.31^a	0.8061±0.28^a	141.05±0.31^a	291.5±0.21^b
2	90.39 ±0.21^a	92.78±0.12^b	1.76 ± 0.15^a	20.05 ± 0.26^a	0.019 ± 0.21^a	0.048 ± 0.31^c	0.5944±0.24^a	0.7804±0.27^b	139.28±0.28^a	282.6±0.18^b
3	90.77 ±0.17^a	91.68±0.17^b	1.85 ± 0.21^a	21.48 ± 0.24^a	0.025 ± 0.17^a	0.044 ± 0.28^c	0.6306±0.22^a	0.8422±0.19^a	144.71±0.28^a	309.7±0.19^a
4	90.42 ±0.15^a	92.18±0.21^b	1.88 ± 0.12^a	22.13 ± 0.19^a	0.022 ± 0.23^a	0.041 ± 0.17^c	0.6022±0.27^a	0.8140±0.33^a	140.53±0.31^a	290.6±0.23^b

Data are the average values of three biological replicates at 22°C and 4°C. Significantly different results are indicated by different letters (a, b, c)

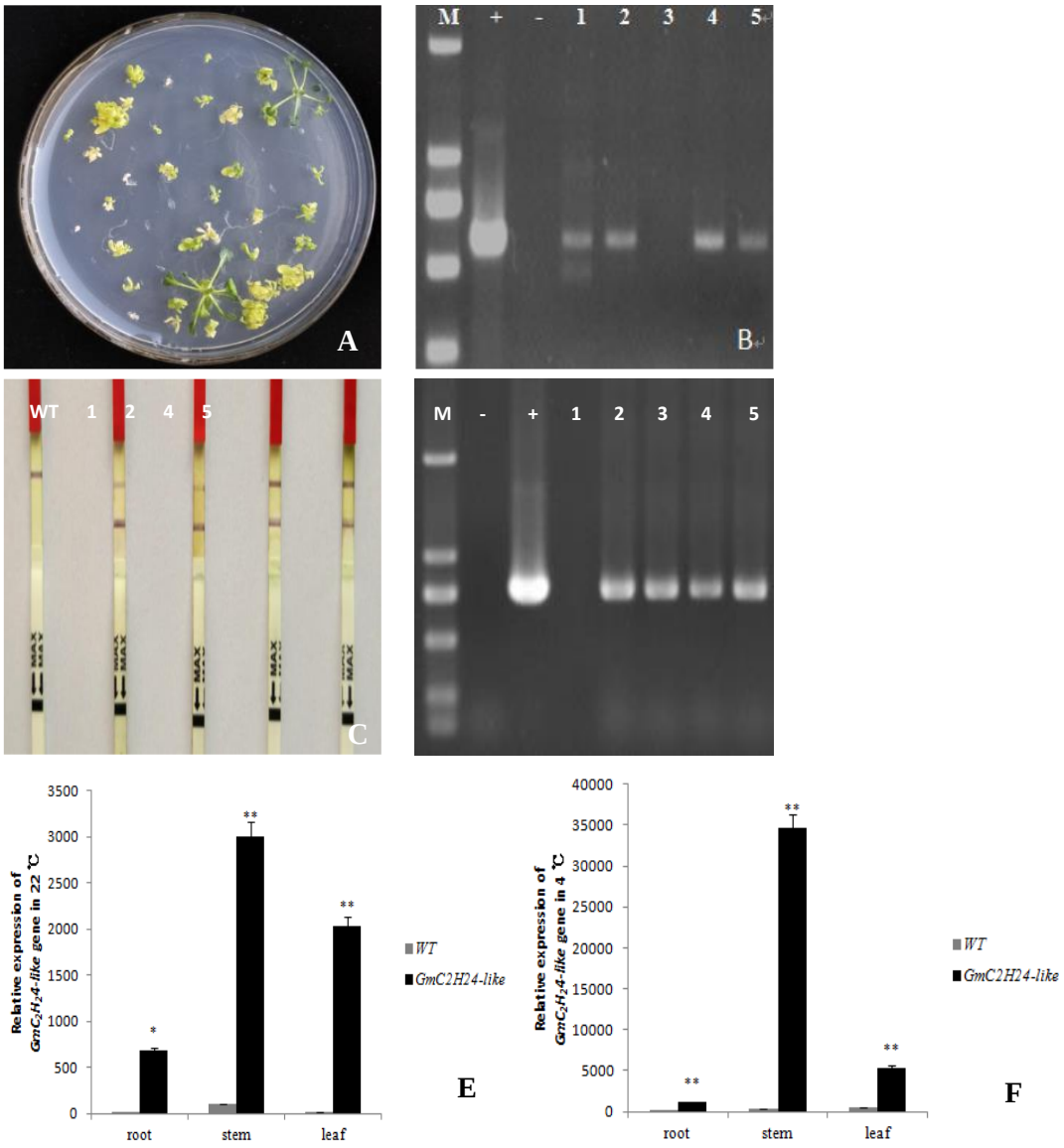

Fig. 5: Molecular characteristics of *GmC$_2$H$_2$4-like* transgenic plants. (**A**) T$_0$ transgenic *Arabidopsis* seeds were sowed in MS solid medium containing 5 mg/L glufosinate-ammonium. (**B**) Electrophoresis images of four regenerated T3 transgenic lines were confirmed positive by PCR. (**C**) Bar strip detection of four transgenic *Arabidopsis* lines. (**D**) Electrophoresis images of four regenerated T3 transgenic lines were confirmed positive by RT-PCR. (**E**, **F**) Expression analysis of *GmC$_2$H$_2$4-like* in different tissues of WT and transgenic *Arabidopsis* at 22°C and 4°C by qRT-PCR. The data represent the average of three independent experiments ± SD. Values were normalized of *β*-tubulin and *Arabidopsis Actin*. Two asterisks (**) indicates that the differences between the transgenic lines and WT are highly significant ($P < 0.01$).

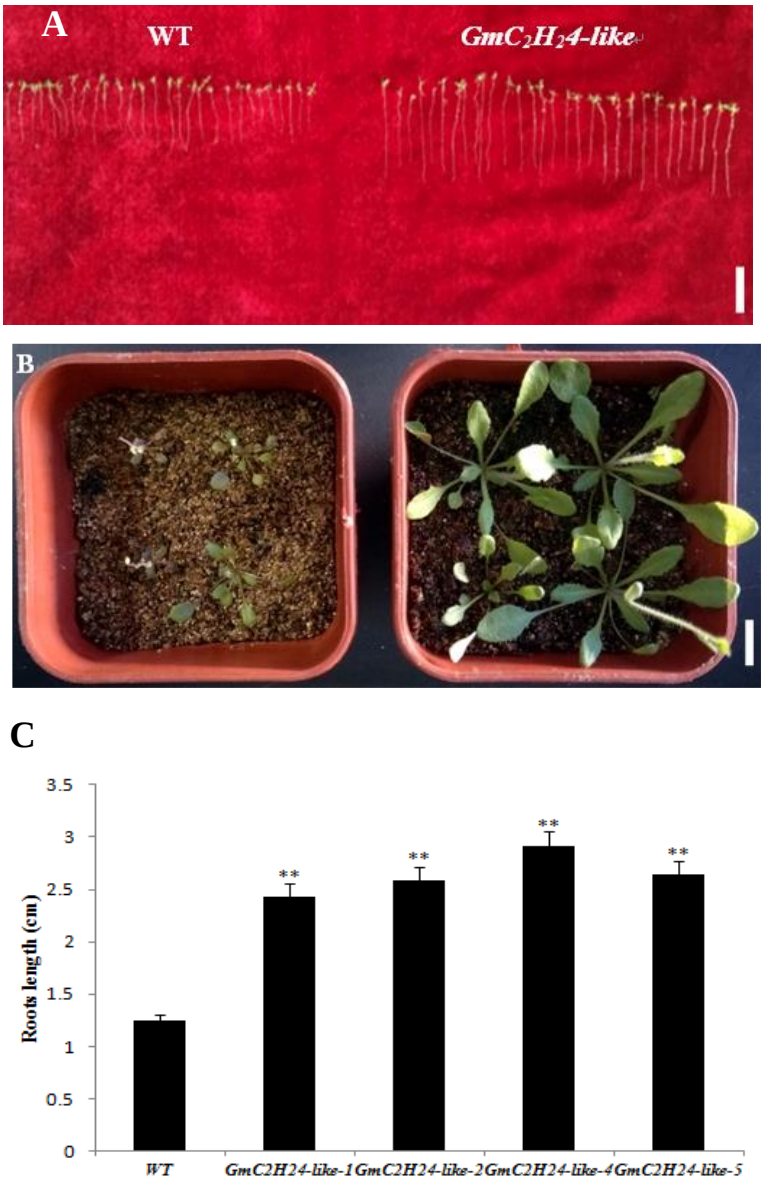

Fig. 6: Comparison of WT and transgenic *Arabidopsis* at various developmental periods. (**A**) Comparison of length of 30 roots of the WT and transgenic *Arabidopsis*. (**B**) Growth and development of the WT and transgenic *Arabidopsis* in soil. (**C**) The roots length of ten-day-old *Arabidopsis* lines at 22°C. WT means wild type *Arabidopsis*; *GmC2H24-like* -1, 2, 4 and 5 are four transgenic lines. Scale bars = 1.0 cm in A and B

the WT, (Table 2), indicating that the reactive oxygen species (ROS) scavenging capacity of the antioxidant systems of transgenic *Arabidopsis* was much higher than the non-transgenic ones. At 22°C, the content of MDA showed no significant difference. After 4°C treatment, the content of MDA in the transgenic *Arabidopsis* was significantly lower than the WT, indicating that transgenic leaves suffered less adverse effects (Table 2). Besides, the contents of soluble sugar and proline in transgenic *Arabidopsis* after the 4°C treatment improved significantly compared to that of WT *Arabidopsis* (Table 2), indicating that the content of soluble sugars facilitated osmoregulation. All these experiments implied that the heterologous expression of *GmC2H24-like* enhanced the cold tolerance of the transgenic *Arabidopsis* plants.

Discussion

It is reported that C_2H_2 zinc finger proteins are involved with plant growth and various adaptive responses to all kinds of stress (Zhang *et al.* 2016; Wang *et al.* 2018). Although C_2H_2 zinc finger proteins are reported that they

were connected with various stress responses and growth and developmental processes, the detailed functions of single zinc finger proteins involved in cold response of soybean are rarely reported (Luo *et al*. 2012).

In our study, a typical single zinc finger protein was cloned from soybean and transferred into *Arabidopsis*. We observed the phenotype including root length, plant size and development speed of the WT and transgenic *Arabidopsis* lines at various stages of growth and development, especially in the early stage. We found that heterologous expression of *GmC$_2$H$_2$4-like* can promote the plant development which was consistent with reported papers (Pomeranz *et al*. 2011). Sendon *et al*. (2014) found the *Arabidopsis* thaliana dwarf1 (Atdwa1) mutant displayed severe dwarfism and loss of apical dominance, as well as other pleiotropic defects, such as earlier flowering, fewer leaves, and shorter sliliques than those of the wild-type plant. They indicated that the zinc finger proteins may play a role in regulation of plant growth and development. Therefore, we speculated that the *GmC$_2$H$_2$4-like* gene affected plant growth and development in transgenic *Arabidopsis*, consistent with its reported functions (Xu *et al*. 2020).

There were not many effects that have been proposed or reported for soybean single zinc finger proteins on cold tolerance. The double zinc finger protein plays an essential role in resistance to many stresses, and the single zinc finger protein controls plant development. Zhang *et al*. (2016) found that *GmZFP3* in soybean belonging to C_2H_2 zinc finger protein contained a special conserved motif, and negatively regulates drought responses by transgenic *Arabidopsis*. Yu *et al*. (2014) reported that *GmZF1* in soybean enhances cold tolerance in transgenic *Arabidopsis* because of the cold gene regulated. Therefore, the expression and many physiological and biochemical indicators of WT and transgenic *GmC$_2$H$_2$4-like Arabidopsis* were measured under normal and cold conditions to verify its functions. Transgenic *Arabidopsis* showed higher cold resistance than the WT. This corroborates previously reported functions of double zinc finger proteins. Kielbowicz-Matuk (2012) conducted a detailed study and analyzed the transcription factors of a double C_2H_2 protein involved in stress responses. Heterologous expression of *GmC$_2$H$_2$4-like* improves much of the proline and soluble sugar contents in transgenic *Arabidopsis* in cold stress, indicating that *GmC$_2$H$_2$4-like* transgenic *Arabidopsis* have adaptive physiological mechanisms to cold. The research of Yu *et al*. (2014) showed that over-expression of *GmZF1* increases the expression level of cold-regulated cor6.6. *GmZF1* could interact with cold regulation genes to improve cold tolerance. A double zinc finger protein, ZAT12 was identified as a negative regulator downregulating the expression of the *CBF* genes. Compared to WT, *ZAT12* over-expressing plants exhibit cold tolerance under freezing stress. The results of transcriptome profiling and mutagenesis experiments indicated that additional cold response pathways exist and may have important roles in life at low temperature (Vogel *et al*. 2010). Besides, Yang *et al*. (2016) identified 118 members of the tobacco C_2H_2 zinc finger protein transcription factor family from the N. tabacum genome database by using Pfam, SMART and Blastp. The analyses of phylogenetic tree, physical and chemical properties, chromosomal mapping, gene structures, protein three-dimensional structures and tissue expression patterns were performed. Therefore, there were still many difficult problems that need to be studied in depth In our study, qRT-PCR analyses showed that *GmC$_2$H$_2$4-like* expression was connected with cold stress. Under cold treatments, the physiological and biochemical indexes showed that transgenic *Arabidopsis* were more tolerant to cold stress compared to the WT. Furthermore, we speculated that the *GmC$_2$H$_2$4-like* transcription factor with single zinc finger protein positively regulated the cold stress response in *Arabidopsis*. However, the function of this *GmC$_2$H$_2$4-like* gene in soybean has not been confirmed. Gene editing and silencing are possible methods to verify the functions in soybean. Further research on regulatory mechanism in our study needs a more step.

Recent studies have shown that the zinc finger proteins can interact with themselves and similar kinds of zinc finger proteins, as well as with some other types of proteins, to regulate their corresponding expressions. The interaction between different zinc finger proteins can allow the recognition of different DNAs or prevent the zinc finger proteins from binding with the corresponding DNA to regulate gene transcription and expression. Therefore, the interaction of *GmC$_2$H$_2$4-like* zinc finger protein and other key proteins needs to be further studied.

In our study, a new single zinc finger gene, *GmC$_2$H$_2$4-like*, was cloned in soybean and transfected into *Arabidopsis*. *GmC$_2$H$_2$4-like* encodes a protein localized in the nucleus and has a transcription activation. The results of our phenotypic observations and physiological and biochemical analyses suggested that transgenic *Arabidopsis* was superior to the WT in growth development, and cold tolerance. However, the specific mechanism of cold resistance needs additional clarification to ascertain its functional importance.

Conclusion

In conclusion, our study reported a new *C$_2$H$_2$* gene, *GmC$_2$H$_2$4-like*, which contains a single C_2H_2 domain and belongs to the zinc finger proteins. *GmC$_2$H$_2$4-like* protein is mainly localized in the nucleus and activates the transcription of the reporter genes. Heterologous expression of *GmC$_2$H$_2$4-like* in *Arabidopsis* can improve the cold tolerance and promote the plant growth and development.

Acknowledgments

Thanks to the Project of the National Natural Science Foundation of China (No.32001572), the Major Science and Technology Sponsored Program for Transgenic Biological

Breeding (No. 2016ZX08004-003) and the National Key Research and Development Program of China (No. 2017YFD0101304).

Author Contributions

YX and FY conducted the experiments; YL, YW and QW and conceived the idea; YX, FY analyzed the data and results. YX, FY and QW designed and conducted the study. YX and FY finished the manuscript. QW, JL, YL and FY critically commented on the manuscript.

Conflict of Interest

There are no conflicts of interest.

Data Availability

Primary and supplementary data reported in this article are available with the corresponding authors

Ethics Approval

Not applicable

Funding Source

This study was supported by the National Key Research and Development Program of China (No. 2017YFD0101304), the Major Science and Technology Sponsored Program for Transgenic Biological Breeding (No. 2016ZX08004-003) and the National Natural Science Foundation of China (No. 32001572).

References

Ahuja I, RCHD Vos, AM Bones, RD Hall (2010). Plant molecular stress responses face climate change. *Trends Plant Sci* 15:664–674

Chen AK, RH Han (2010). Comparative study on determination methods of relative electrical conductivity of plant leaves. *J Guangdong Educ Coll* 30:88–91

Chou K-C, HB Shen (2010a). Cell-PLoc 2.0: An improved package of web-servers for predicting subcellular localization of proteins in various organisms. *Nat Protoc* 3:153–162

Chou KC, HB Shen (2010b). Plant mPLoc: A top-down strategy to augment the power for predicting plant protein subcellular localization. *PLoS One* 5; Article e11335

Confraria A, E Baena-González (2016). Using *Arabidopsis* Protoplasts to Study Cellular Responses to Environmental Stress. *Environmental Responses in Plants* Methods in Molecular Biology, Vol. 1398. Humana Press, New York, New York, USA

Fedotova AA, AN Bonchuk, VA Mogila, PG Georgiev (2017). C_2H_2 zinc finger proteins: The largest but poorly explored family of higher eukaryotic transcription factors. *Acta Nat* 9:47–58

Hu RB, CM Fan, HY Li, QZ Zhang (2009). Evaluation of putative reference genes for gene expression normalization in soybean by quantitative real-time RT-PCR. *BMC Mol Biol* 10; Article 93

Isernia C, EM Bucci, M Leone, L Zaccaro, R Fattorusso (2010). NMR structure of the single QALGGH zinc finger domain from the *Arabidopsis thaliana* SUPERMAN protein. *Chembiochem* 4:171-180

Jian B, B Liu, YR Bi, WS Hou, CX Wu (2008). Validation of internal control for gene expression study in soybean by quantitative real-time PCR. *BMC Mol Biol* 9; Article 59

Kielbowicz-Matuk A (2012). Involvement of plant C_2H_2-type zinc finger transcription factors in stress responses. *Plant Sci* 185–186:78–85

Kim JC, HL Sang, HC Yong, C Yoo, MJ Cho (2010). A novel cold-inducible zinc finger protein from soybean, SCOF-1, enhances cold tolerance in transgenic plants. *Plant J Cell Mol Biol* 25:247–259

Klotke J, J Kopka, N Gatzke, AG Heyer (2010). Impact of soluble sugar concentrations on the acquisition of freezing tolerance in accessions of *Arabidopsis thaliana* with contrasting cold adaptation evidence for a role of raffinose in cold acclimation. *Plant Cell Environ* 27:1395–1404

Le DT, DL Aldrich, B Valliyodan, Y Watanabe, CV Ha, R Nishiyama, SK Guttikonda, TN Quach, JJ Gutierrez-Gonzalez, LSP Tran, HT Nguyen (2012). Evaluation of candidate reference genes for normalization of quantitative RT-PCR in soybean tissues under various abiotic stress conditions. *PLoS One* 7; Article e46487

Letunic I, RR Copley, B Pils, S Pinkert, J Schultz, P Bork (2006). SMART 5: Domains in the context of genomes and networks. *Nucl Acids Res* 34:257–260

Luo X, X Bai, D Zhu, Y Li, W Ji (2012). GsZFP1, a new Cys2/His2-type zinc-finger protein, is a positive regulator of plant tolerance to cold and drought stress. *Planta* 235:1141–1155

Miller J, AD Mclachlan, A Klug (1985). Repetitive zinc-binding domains in the protein transcription factor IIIA from Xenopus oocytes. *EMBO J* 4:1609–1614

Michael M, U Chrisopher (2003). Recent developments in the engineering of zinc finger proteins. *Brief Funct Genomics Proteomics* 1:342–355

Pabo CO, E Peisach, RA Grant (2001). Design and selection of novel Cys2His2 zinc finger proteins. *Annu Rev Biochem* 70:313–340

Pomeranz M, J Finer, JC Jang (2011). Putative molecular mechanisms underlying tandem CCCH zinc finger protein mediated plant growth, stress, and gene expression responses. *Plant Signal Behav* 6:647–651

Riedel G, U Rüdrich, N Fekete-Drimusz, MP Manns, M Bock (2014). An extended ΔCT-method facilitating normalisation with multiple reference genes suited for quantitative RT-PCR analyses of human hepatocyte-like cells. *PLoS One* 3; Article e93031

Sendon PM, MM Oo, JB Park, BH Lee, JH Kim, HS Seo, SK Park, JT Song (2014). Activation of *C_2H_2-type* zinc finger genes induces dwarfism in *Arabidopsis thaliana*. *J Kor Soc Appl Biol Chem* 57:35–41

Shekaari H, SN Mirheydari, MT Zafarani-Moattar (2019). Electrical conductivity studies of 1-Buytl-3-methylimidazolium salicylate as an active pharmaceutical ingredient lonic liquid (API-IL) in aqueous amino acids solutions. *Phys Chem Res* 7:217–234

Su LT, JW Li, DQ Liu, Y Zhai, HJ Zhang, XW Li, QL Zhang, Y Wang, QY Wang (2014). A novel MYB transcription factor, GmMYBJ1, from soybean confers drought and cold tolerance in *Arabidopsis thaliana*. *Gene* 538:46–55

Sugano S, H Takatsuji, H Kaminaka (2010). *Use of Zinc Finger Transcription Factor zpt2-3 to Generate Plants with Increased Desiccation Tolerance*, pp:1–12. US Patent Application 20060272059

Tamura K, D Peterson, N Peterson, G Stecher, M Nei, S Kumar (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28:2731–2739

Tian ZD, Y Zhang, J Liu, CH Xie (2010). Novel potato C_2H_2-type zinc finger protein gene, *StZFP1*, which responds to biotic and abiotic stress, plays a role in salt tolerance. *Plant Biol* 12:689–697

Tirani MM, MM Haghjou (2019). Reactive oxygen species (ROS), Total antioxidant capacity (AOC) and Malondialdehyde (MDA) make a triangle in evaluation of Zinc stress extension. *J Anim Plant Sci* 29:1110–1111

Vogel JT, DG Zarka, HAV Buskirk, SG Fowler, MF Thomashow (2010). Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. *Plant J* 41:195–211

Wang HY, Q Ding, HB Shao (2019). Overexpression of KvP5CS1 increases salt tolerance in transgenic tobacco. *Pak J Bot* 51:831–836

Wang K, Y Ding, C Cai, Z Chen, C Zhu (2018). The role of C_2H_2 zinc finger proteins in plant responses to abiotic stresses. *Physiol Plantarum* 165:690–700

Weng MY, LR Cui, FL Liu, Z Min, SS Yang, XP Deng (2015). Effects of drought stress on antioxidant enzymes in seedlings of different wheat genotypes. *Pak J Bot* 47:49–56

Xu JY, CC Xu, D Xu, JM Zhang, JY Gao, N Gao, H Xu (2013). Proline content and quantitative real-time PCR analyses of relative expressions of AtP5CS1 in transgenic *Arabidopsis* plants. Accessed at: https://journals.plos.org/plosone/article/figures?id=10.1371/journal.pone.0069810

Xu Q, H Yu, S Xia, Y Cui, D Ren (2020). The C_2H_2 zinc-finger protein lacking rudimentary GLUME 1 regulates spikelet development in rice. *Sci Bull* 9:753–764

Xu ZS, M Chen, LC Li, YZ Ma (2011). Functions and application of the AP2/ERF transcription factor family in crop improvement. *J Integr Plant Biol* 53:570–585

Yang M, J Chao, D Wang, J Hu, H Wu, D Gong, G Liu (2016). Genome-wide identification and expression profiling of the C_2H_2-type zinc finger protein transcription factor family in tobacco. *Hereditas* 38:337–349

Yu GH, LL Jiang, XF Ma, ZS Xu, MM Liu, SG Shan, XG Cheng, C Fan (2014). A soybean C_2H_2-type zinc finger gene *GmZF1* enhanced cold tolerance in transgenic *Arabidopsis*. *PLoS One* 9; Article e109399

Zhai Y, JW Li, XW Li, TT Lei, F Yan, Y Zhao, YJ Li, LT Su, Y Wang, QY Wang (2013). Isolation and characterization of a novel transcriptional repressor GmERF6 from soybean. *Biol Plantarum* 57:26–32

Zhang D, J Tong, Z Xu, P Wei, L Xu, Q Wan, Y Huang, X He, J Yang, H Shao, H Ma (2016). Soybean C_2H_2-type zinc finger protein GmZFP3 with conserved QALGGH motif negatively regulates drought responses in transgenic *Arabidopsis*. *Front Plant Sci* 7; Article 325

Zhang ZQ, S Wang, FS Li, Y Tang (2018). Effects of different treatments on the relative electric conductivity of jujube fruits peel and the jujube cracking. *Nonwood For Res* 36:107–114

Zhao MZ, TL Wang, P Wu, WY Guo, LT Su, Y Wang, YJ Liu, F Yan, QY Wang (2017). Isolation and characterization of *GmMYBJ3*, an R2R3-MYB transcription factor that affects isoflavonoids biosynthesis in soybean. *PLoS One* 12; Article e0179990