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# *CsWRKY2*, a novel *WRKY* gene from *Camellia sinensis*, is involved in cold and drought stress responses

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**Abstract**

One of the largest families of transcriptional regulators contains WRKY proteins. They play important roles in plant defense responses. In this study, a novel WRKY gene, *CsWRKY2*, was isolated from the tea [*Camellia sinensis* (L.) O. Kuntze] plant. The full-length cDNA of *CsWRKY2* was 2 050 bp in length and encoded a 522-amino acid peptide chain containing two typical WRKY domains and two zinc finger motifs, suggesting that CsWRKY2 was member of the WRKY group I family. A subcellular localization assay shows that CsWRKY2 was localized to the nucleus. Real time qPCR analysis shows that *CsWRKY2* expression was higher in leaves than in other organs and was induced by cold (4 °C), drought stress, and exogenous abscisic acid (ABA). Additionally, ABA content was enhanced after the cold or drought stress and the effects were relieved by an ABA biosynthesis inhibitor. Furthermore, the expression of *CsWRKY2* was up-regulated by exogenous ABA under the cold and drought stresses and down-regulated by an ABA biosynthesis inhibitor. Our findings indicate that *CsWRKY2* played an important role in plant defense responses to the cold and drought stresses by participating in the ABA signaling pathway, downstream to ABA.

*Additional key words*: abscisic acid, expression analysis, polyethylene glycol, transgenic plants.

## Introduction

Plants manage various biotic and abiotic stresses during their life cycle and have developed a series of elaborate mechanisms for adaptation to stress conditions. To date, many transcription factors, including those belonging to the WRKY, MYB, ERF, and bZIP families, have been implicated in responses to various types of stresses by regulating the expression profiles of different genes (Singh *et al*. 2002). The members of the WRKY transcription factor family, one of the largest transcription factor families in plants, are induced in response to abiotic stresses and have emerged as essential candidates for participation in stress tolerance (Agarwal *et al*. 2011).

The members of the WRKY family contain a highly conserved 60-amino-acid WRKY domain having a conserved WRKYGQK amino acid sequence motif at its *N* terminus and a zinc finger motif (C-$X_{4-5}$-C-$X_{22-23}$-H-$X_1$-H or C-$X_7$ -C-$X_{23}$-H-$X_1$-C) at the *C* terminus (Eulgem *et al*. 2000). According to the number of the WRKY domains and the type of the zinc finger motif, WRKY proteins can be categorized into three distinct groups (Eulgem *et al*. 2000). The members of group I contain two WRKY domains, whereas groups II and III contain a single WRKY domain; the latter two groups are distinguished by their zinc finger motif. The members of groups I and II contain a $C_2H_2$-type zinc finger motif (C-$X_{4-5}$-C-$X_{22-23}$-H-$X_1$-H) and group III contains a $C_2HC$ zinc finger motif (C-$X_7$-C-$X_{23}$-H-$X_1$-C). Recently, many *WRKY* genes have been identified in plant species including *Arabidopsis*, rice, soybean, papaya, poplar, sorghum, *Physcomitrella patens*, *Selaginella moellendorffii*, and *Pinus* sp. (Agarwal *et al*. 2011). The functions of many *WRKY* genes have been elucidated using genetic and molecular approaches. For example, Northern blotting analysis revealed that 10 *OsWRKY* genes respond differentially to NaCl, polyethylene glycol (PEG), cold, or heat treatments in rice (Qiu *et al*. 2004).

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*Abbreviations*: ABA - abscisic acid; GFP - green fluorescent protein; NLS - nuclear localization signal; ORF - open reading frame; PEG - polyethylene glycol; RT-PCR - reverse transcriptase PCR; qPCR - quantitative PCR; TS - tungstate; UTR - untranslated region.

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Moreover, 8 out of 15 wheat *WRKY* genes were also found to be responsive to low or high temperature or to treatment with NaCl or PEG (Wu *et al*. 2008). Furthermore, many recent reports indicate that expression of *WRKY* genes is regulated by various phytohormones, including abscisic acid (ABA, Yang *et al*. 2009). Moreover, some *WRKY* genes participate in crosstalk among multiple signaling pathways, especially the ABA signaling pathway, which is one of the most important signaling pathways for plant abiotic stress responses. For instance, *OsWRKY51* and *OsWRKY71* expression is induced by ABA but repressed by gibberellic acid in the embryo and aleuronic cells (Jiang and Deyholos 2006, Mangelsen *et al*. 2008). Chen *et al*. (2010) reported that *Arabidopsis AtWRKY18* and *AtWRKY40* are rapidly induced by ABA, whereas induction of *WRKY60* by ABA is delayed; these authors also found that the *AtWRKY60* gene is a direct target of AtWRKY18 and AtWRKY40 in the ABA signaling pathway. However, the details of these complex mechanisms and their crosstalk during defense responses to abiotic stress remain to be elucidated.

The tea plant (*Camelia sinensis*), one of the most economically important crops, endures various abiotic stresses during its lifecycle, including salinity, heavy metals, drought, and low temperatures (Wang *et al*. 2014). However, WRKY transcription factors have not previously been considered important in stress responses in *C. sinensis*. Here, we isolated a novel *WRKY* gene, *CsWRKY2*, from *C. sinensis*; it encodes a putative WRKY protein of group I. In addition, we investigated the subcellular localization of this putative CsWRKY2 protein. Furthermore, the organ-specific expression of this protein was analyzed, and the expression of *CsWRKY2* in response to cold and drought stresses was monitored to explore the role of this gene during stress response. Moreover, ABA content was measured after exposure to the cold and drought stresses, and the expression profile of *CsWRKY2* after treatment with the stress and ABA were measured to investigate possible mechanisms by which *CsWRKY2* participates in defense responses in *C. sinensis*.

## Materials and methods

**Plants, growth conditions, and treatments:** Two-year-old tea [*Camellia sinensis* (L.) O. Kuntze cv. Longjing 43] plants were pre-incubated in a control nutrient solution (Wan *et al*. 2012) in a climate chamber under a 16-h photoperiod, an irradiance of 300 μmol $m^{-2}$ $s^{-1}$, day/night temperatures of 25 ± 1/23 ± 1 °C, and an air humidity of 75 % for two weeks. Some pre-incubated plants were then cultured in a nutrient solution containing 10 % (m/v) PEG 6000 (osmotic potential of -0.15 MPa) or 50 μM ABA; some other were transferred to a second climate chamber and maintained at 4 °C. The first or second tender leaves from the control and treated plants were collected at different times (0, 1, 2, 4, 6, 8, 12, and 24 h), immediately frozen in liquid nitrogen, and then stored at -80 °C for further analysis. Additionally, 50 μM ABA and 0.3 mM tungstate (TS, an ABA biosynthesis inhibitor) were used.

To obtain organ-specific expression profiles, roots, stems, leaves, buds, flowers, and fruits from tea plants grown in soil under the normal conditions were collected,

Table 1 Primers used in this study (R = A/G, Y = C/T, N = A/C/T/G, I = A/C/T/G, the enzyme restriction sites of *Hin*dIII and *Xba*I are underlined). RT - reverse transcriptase, RACE - rapid amplification of cDNA ends.

| Primer name | Sequence(5′-3′) | Description |
|---|---|---|
| WRKY-JB- F | TGGAGRAARTAYGGNCARAA | RT-PCR |
| WRKY-JB- R | TTRTGYTYICCTTCRTAIGT | |
| 3 ′ GSP1 | AGTGGTCAAGGGCAATCCTCATC | 3′RACE |
| 3 ′ GSP2 | ACATCTGAAGTAACCTCGTCGCA | |
| 5 ′ GSP1 | ATTACGCTCAACCTTCTTCT | 5′RACE |
| 5 ′GSP2 | TGGAAGCAAAGGATTGATGGTCG | |
| CsWRKY2-Full - F | CCATACATTCACACACACTGACGAT | RT-PCR |
| CsWRKY2-Full - R | TGTTCAAACATTATATCCATGCCTC | |
| CsWRKY2-*Hin*dIII- F | CCCAAGCTTATGGCTAAGACCGACAAGG | subcellular localization |
| CsWRKY2-*Xba*I- R | GCTCTAGATGCTATGATTTGCTCTTCTT | |
| CsWRKY2- qF | GAGACAGAAATGAGCAGGGAAAA | real time PCR |
| CsWRKY2- qR | TGTATCGGTGTCAGTTGGGTAGA | |
| Csβ-actin- qF | GCCATCTTTGATTGGAATGG | |
| Csβ-actin- qR | GGTGCCACAACCTTGATCTT | |
| CsGAPDH- qF | GGCAGCACCTTACCAACAGC | |
| CsGAPDH- qR | GTTTGGCGTCGTTGAGGGTC | |

immediately frozen in liquid nitrogen, and stored at -80 °C for further analysis.

**Cloning and sequence analysis:** Total RNA extraction and cDNA synthesis were executed according to the methods described by Wang *et al.* (2015). To acquire internal conserved fragments, degenerate primers (WRKY-JB-F and WRKY-JB-R) were designed and synthesized according to the conserved amino acids of WRKY proteins from *Arabidopsis*, bean, grape, apple, and cotton. Then, the conserved sequence of *CsWRKY2* was amplified using the polymerase chain reaction (PCR). Subsequently, the 5' and 3' untranslated region (UTR) sequences were identified using specific primers 5'GSP1/5'GSP2 and 3'GSP1/3'GSP2 and rapid amplification of cDNA ends (Guo *et al*. 2011). Finally, a pair of gene-specific primers (CsWRKY2-Full-F and CsWRKY2-Full-R) was designed and used to amplify the full-length sequence of *CsWRKY2*; the product was then cloned into the *pMD®18-T Vector* (*TaKaRa*, Dalian, China) for sequencing (*GenScript*, Nanjing, China). The primers used in this study are listed in Table 1.

The *DNAMAN* software *v. 5.2.2 (Lynnon Biosoft*, St. Louis, Canada) and the *BLAST* online software (http://www. ncbi.nlm.gov/blast) were used to analyze the DNA and protein sequences. CsWRKY2 and other WRKY proteins were subjected to phylogenetic analysis using *MEGA 5.05*. The online tool *WoLF PSORT* (http:// wolfpsort.org/) and *Softberry ProComp v. 9.0* (http:// linux1.softberry.com/berry) were used to predict CsWRKY2 protein localization.

**Subcellular localization analysis:** The open reading frame (ORF) of *CsWRKY2* without the termination codon was obtained using PCR amplification with specific primers CsWRKY2-*Hin*dIII-F and CsWRKY2-*Xba*I-R; the PCR product was cloned into the *pJIT166-GFP* vector to generate a CsWRKY2::GFP in-frame fusion protein. Subsequently, vectors encoding *CsWRKY2::GFP* and *green fluorescent protein* (*GFP*) alone were transiently introduced into onion (*Allium cepa*) epidermal cells using the biolistic transformation system (*PDS-1000/He*, *Bio-Rad*, Hercules, USA). The onion tissue was incubated according to Wang *et al*. (2014), and fluorescence was observed using a laser scanning confocal microscope (LSCM, *TCS SP2*, *Leica*, Wetzlar, Germany).

**Real time quantitative PCR** assays were performed using *SYBR® Premix Ex Taq™* (*TaKaRa*) and an *iQ5 Multicolor* real time PCR detection system (*Bio-Rad*) according to the manufacturer's instructions. Specific primers CsWRKY2-qF and CsWRKY2-qR were used to amplify an 89-bp *CsWRKY2* fragment, and *Cs glyceraldehyde phosphate dehydrogenase* and *Csβ-actin* were selected as internal controls. The primers used are listed in Table 1. Real time quantitative qPCR conditions were: pre-denaturation at 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 59 °C for 30 s. Relative gene expression was estimated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

**Abscisic acid extraction, purification, and quantification:** *C. sinensis* leaves were collected after incubating the plants for 0, 1, 4, 8, 12, or 24 h under the cold or drought stress, immediately frozen in liquid nitrogen after fresh mass determination, and then stored at -70 °C until use. Abscisic acid was extracted and purified as described by Yang *et al*. (2006) and Frankowski *et al.* (2015), and ABA content was determined using an enzyme-linked immunosorbent assay according to Yang *et al*. (2001) and Teng *et al*. (2006). In addition, 50 μM exogenous ABA and 0.3 mM TS were used to treat the tea plants to further verify the effects of the cold and drought stresses on endogenous ABA content.

**Statistical analyses:** All data are expressed as arithmetic means ± standard deviations (SDs) obtained from at least three independent replicates. Statistical significance was calculated by one-way *ANOVA* following Duncan's test. Analyses were performed with the IBM *SPSS Statistics v. 20* software.

## Results

The full-length cDNA sequence of *CsWRKY2* (accession No. JQ739460) consisted of 2 050 nucleotides containing a 109-bp 5'-UTR, a 1 569-bp ORF, and a 372-bp 3'-UTR. *CsWRKY2* encoded a polypeptide containing 522 amino acid residues with a predicted molecular mass of 57.3 kDa and an isoelectric point of 6.97. The sequence analysis shows that the CsWRKY2 protein included two typical WRKY domains that contained a highly conserved amino acid sequence WRKYGQK and two putative $C_2H_2$ zinc finger motifs (C-X4-C-X22-23-H-X1-H) (Fig. 1), suggesting that CsWRKY2 belongs to group I of the WRKY family. The phylogenetic analysis also shows that CsWRKY2 shared a high degree of homology with other WRKY proteins of group I (Fig. 2). In addition, a putative nuclear localization signal (NLS) PKRR was found at residue positions 372 - 376 (Fig. 1).

The CsWRKY2 protein was predicted to localize to the nucleus according to the online tools *WoLF PSORT* and *Softberry ProComp v. 9.0*. To confirm the predicted localization, the ORF of *CsWRKY2* was fused to GFP and transiently expressed in onion epidermal cells. As shown in Fig. 3*A*-*C*, a green fluorescent signal from GFP (blank control) was observed in all regions of the onion epidermal cells, whereas a green fluorescent signal from

the CsWRKY2::GFP fusion protein was detected exclusively in the nucleus (Fig. 3*D-F*), suggesting that *CsWRKY2* encoded a protein that was localized in the nucleus.

To determine the pattern of *CsWRKY2* expression in various organs of *C. sinensis*, real time qPCR assays were performed. The results show that the amount of *CsWRKY2* transcripts in roots, stems, leaves, buds, flowers, and fruits differed and that the amount of mRNA was highest in leaves and lowest in stems and flowers (Fig. 4*A*). To investigate whether *CsWRKY2* expression wass induced by the abiotic stress, the patterns of *CsWRKY2* expression under the cold (4 °C) and drought (PEG 6000) stresses were examined. Expression of *CsWRKY2* increased rapidly during the first hour of the cold treatment and then decreased gradually during 2 - 6 h. Subsequently, *CsWRKY2* expression increased strongly and reached a maximum at 12 h; thereafter, *CsWRKY2* expression decreased significantly (Fig. 4*B*). Similarly, *CsWRKY2* expression increased and then decreased within 4 h during the drought stress and then increased two-fold between 6 and 8 h; after 8 h, expression decreased (Fig. 4*C*). In addition, *CsWRKY2* expression gradually increased after the fluctuation similarly to the cold treatment during 2 - 12 h under the exogenous ABA treatment, and expression rapidly decreased after 24 h (Fig. 4*D*).

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ACGAAGAATCCATACATTCACACACACTGACGATCGAACCAAAACCCAATTCCCAAAAAACTTCCGTGTAAAACCTCCACAGATCTCTTCCTCCCCGTGTAGAGAGAGAA
ATGGCTAAGACCGACAAGGTCTCAGCGCCTTCACGGCCTACGATCATACTCCCACCTCGCACCTCCTTCGACTCCATCTTCGCCGGTTTGAGCCCTGGTCCGATGACCCTCGTCTCGAGT
 M  A  K  T  D  K  V  S  A  P  S  R  P  T  I  I  L  P  P  R  T  S  F  D  S  I  F  A  G  L  S  P  G  P  M  T  L  V  S  S
TTCTTCTCCGACAGCTACTCCGACTCCGACTGCCCTTCCTTCTCTCAGCTCCTGGCCGGAGCCATGGCTTCCCCTGTTGCTGCCGATGGTGGAATCCCAACTCTGCCCAGCTTTTTAGCC
 F  F  S  D  S  Y  S  D  S  D  C  P  S  F  S  Q  L  L  A  G  A  M  A  S  P  V  A  A  D  G  G  I  P  T  L  P  S  F  L  A
AGTAGTTTGTCGAACGAAGTGGATTCTGAAGATGCGTGTGAACAGAAATTAGGGTTTAAGCAGAATCGGCCAGTGAATTTGGTGGTTGCTCATTCTCCCCTGTTTATGGTTCCTCCTGGG
 S  S  L  S  N  E  V  D  S  E  D  A  C  E  Q  K  L  G  F  K  Q  N  R  P  V  N  L  V  V  A  H  S  P  L  F  M  V  P  P  G
TTGAGCCCTTCTGGGTTGCTTAATTCGCCCGGTTTCTTCTCTCCACTTCAGAGCCCCTTTGGAATGACCCACCAGCAGGCCCTAGCACAAGTTACAGCGCAGGCTGCATTATCTCAATGT
 L  S  P  S  G  L  L  N  S  P  G  F  F  S  P  L  Q  S  P  F  G  M  T  H  Q  Q  A  L  A  Q  V  T  A  Q  A  A  L  S  Q  C
CATTTGCATGTCCAAGCCGAATCTCAACCTTCTTCACTAGTAGCTTCTGAAGAATCTTTGAACGACCATCAATCCTTTGCTTCCAACACAACCATACAACAACAGGTGCCCACTGTGATA
 H  L  H  V  Q  A  E  S  Q  P  S  S  L  V  A  S  E  E  S  L  N  D  H  Q  S  F  A  S  N  T  T  I  Q  Q  Q  V  P  T  V  I
TCAGAACCTGAAAGTTCCGTAATTGAATCATCAGAAGTTTCTCAACCCAATAGGAAATCACTACCTCCTTGTGCTGCTGTTGACAAACCTGGGAATGATGGCTACAACTGGCGAAAATAT
 S  E  P  E  S  S  V  I  E  S  S  E  V  S  Q  P  N  R  K  S  L  P  P  C  A  A  V  D  K  P  G  N  D  G  Y  N  W  R  K  Y
GGGCAGAAACAGGTCAAGACAAGTGATCATCCTCGAAGCTACTATAAATGTACACATCCCAATTGTCCAGTTAAGAAGAAGGTTGAGCGTAATTTTGATGGGCAGATAACTGAGATTATC
 G  Q  K  Q  V  K  T  S  D  H  P  R  S  Y  Y  K  C  T  H  P  N  C  P  V  K  K  K  V  E  R  N  F  D  G  Q  I  T  E  I  I
TACAAAGGCCAGCACAATCACGAACTTCCGCAATCTAATAAACGTGCAAAAGATGGTATTGATAAGAACTCAAATACAAACTCTCAGGTAAGGCGTGAACTTGGTGTGCAGGGTGAGACA
 Y  K  G  Q  H  N  H  E  L  P  Q  S  N  K  R  A  K  D  G  I  D  K  N  S  N  T  N  S  Q  V  R  R  E  L  G  V  Q  G  E  T
GAAATGAGCAGGGAAAATGGAACTTTTCATTCAGTTCCTAGGAGGGTCCAGGCGTCTACCCAACTGACACCGATACAGTTATCAGGATCAAGTGATCATGTTGAAATGGGTGACATTGAA
 E  M  S  R  E  N  G  T  F  H  S  V  P  R  R  V  Q  A  S  T  Q  L  T  P  I  Q  L  S  G  S  S  D  H  V  E  M  G  D  I  E
ATGAGACTAAATCAAGCCGACAATGATGAACCAAATCCCAAGAGAAGGAACACAGAGGTCGGGACATCTGAAGTAACCTCGTCGCATAATACAGTCACAGAACCCAGAATTGTTGTGCAG
 M  R  L  N  Q  A  D  N  D  E  P  N  P  K  R  R  N  T  E  V  G  T  S  E  V  T  S  S  H  N  T  V  T  E  P  R  I  V  V  Q
ACAAGGAGTGAAGTTGATCTCTTGGATGATGGGTACAAGTGGCGAAAGTATGGGCAGAAAGTGGTCAAGGGCAATCCTCATCCAAGGAGTTACTACAAATGCACTTATGCAGGATGCAAT
 T  R  S  E  V  D  L  L  D  D  G  Y  K  W  R  K  Y  G  Q  K  V  V  K  G  N  P  H  P  R  S  Y  Y  K  C  T  Y  A  G  C  N
GTTCGTAAGCATGTCGAGAGGGCTTCAACTGACCCCAAAGCTGTCGTAACCACATACGAAGGGAAACACAACCATGATGTCCCTGGAGGTAGAAAGAGTGGTTCCAACACAGCAAATAGT
 V  R  K  H  V  E  R  A  S  T  D  P  K  A  V  V  T  T  Y  E  G  K  H  N  H  D  V  P  G  G  R  K  S  G  S  N  T  A  N  S
AACACATTGCAGTTAAAATCACATAAGGTGGTGACTAAGAAGCCTGCTCTACTTGAAGAAATGGATTTTGGAAACAAAGATGAAAGGCCGGTTCTTTTACAGCTAAAAGAAGAGCAAATC
 N  T  L  Q  L  K  S  H  K  V  V  T  K  K  P  A  L  L  E  E  M  D  F  G  N  K  D  E  R  P  V  L  L  Q  L  K  E  E  Q  I
ATAGCATAA
 I  A  *
GCTTGTTGTGAAGCCCACAAGAATGGTTTGCTGCGTGTATAGTAAATGCATTGGCTGCTATATGTATCTGCACTGTATTGTGTTATGTATATGTGAAATCTGAGCAGGGGTTGCTGTATG
TATAGTAAATGCGTCGCCTGCAATATGTATCTGCACCTTAATTTCTTCTGTATGTGGGATATTAGGCCTAGTGTGTGTGAGTATTTGTTCAGACTTTTGCAGAGGCATGGATATAATGTT
TGAACAAGATGTTAAGTTTCTCTCAAAAAAAAAAAAAAAAA
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Fig. 1. The nucleotide and deduced amino acid sequences of *CsWRKY2* cDNA. WRKY domains containing an invariant WRKYGQK sequence are indicated using the *squares*, and putative zinc-finger motifs are *circled*. A nuclear localization signal (PKRR) at amino acid positions 372 - 376 is marked *grey*.

The content of ABA in tea plant leaves increased significantly and remained high after the cold stress treatment; thereafter, the ABA content decreased slightly at 24 h (Fig. 5*A*). Similarly, the content of ABA increased gradually from 1 to 12 h and reached a maximum at 12 h after the PEG 6000 treatment, later decreased slightly at 24 h (Fig. 5*B*). In addition, the exogenous ABA further increased ABA accumulation induced by the cold or drought stress, but in contrast, ABA accumulation was significantly inhibited by the ABA synthesis inhibitor TS (Fig. 5*C-D*).

To examine whether the changes in *CsWRKY2* expression were related to the endogenous ABA content under the cold and drought stresses, we analyzed *CsWRKY2* expression after the treatment with the cold and exogenous ABA/TS for 12 h and after the treatment with PEG 6000 and ABA/TS for 6 h, respectively. Expression of *CsWRKY2* was significantly induced by the cold stress or exogenous ABA and inhibited by TS, and a similar change of *CsWRKY2* expression was detected under the drought stress (Fig. 6*B*). In addition, exogenous ABA increased induction of *CsWRKY2* expression by the cold or drought stress, whereas the treatment with TS significantly decreased expression of *CsWRKY2* induced

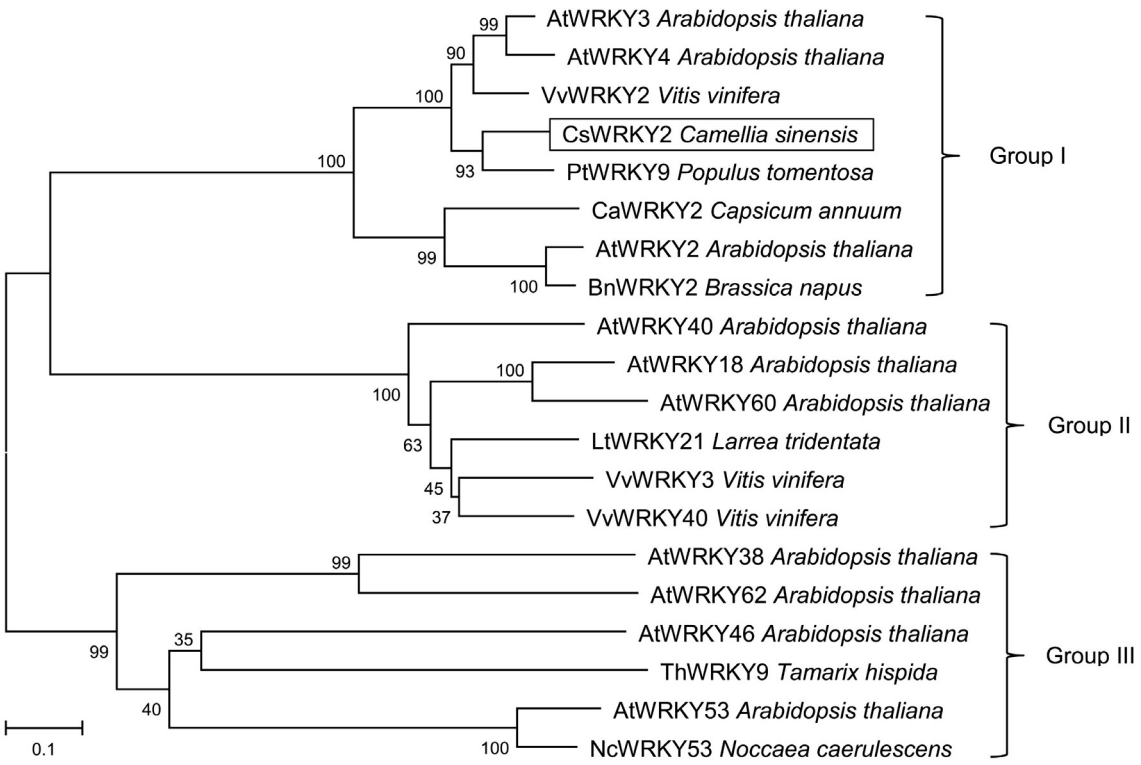


Fig. 2. The phylogenetic analysis of plant WRKY proteins. Comparison with other plant WRKY proteins reveals that CsWRKY2 was a group I WRKY protein. A phylogenetic tree was constructed using *MEGA 6.05* and the neighbor-joining method with the following parameters: Poisson model, the pairwise deletion and bootstrap method (1 000 replicates). The *numbers* shown at the branches are bootstrap values.

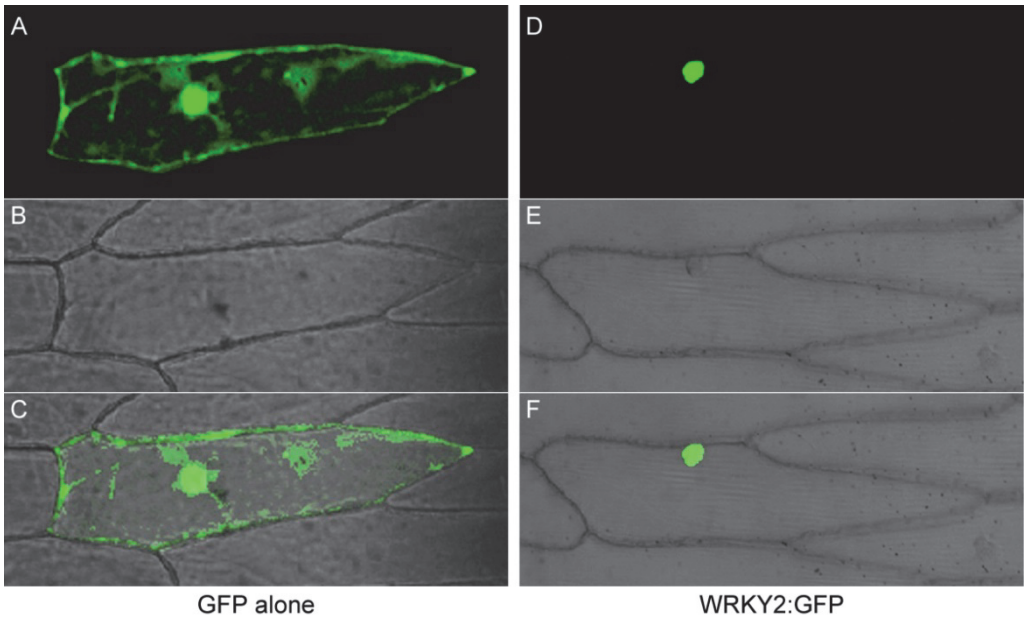


Fig. 3. Subcellular localization of the CsWRKY protein in onion epidermal cells. An onion epidermal cell expressing green fluorescent protein (GFP) alone (*A*), its differential interference contrast (DIC) image (*B*), and the merged image (*C*) showing green fluorescence in the nucleus, plasma membrane, and cytoplasm. An onion epidermal cell expressing a CsWRKY2::GFP fusion (*D*), its DIC image (*E*), and the merged image (*F*) showing green fluorescence only in the nucleus.

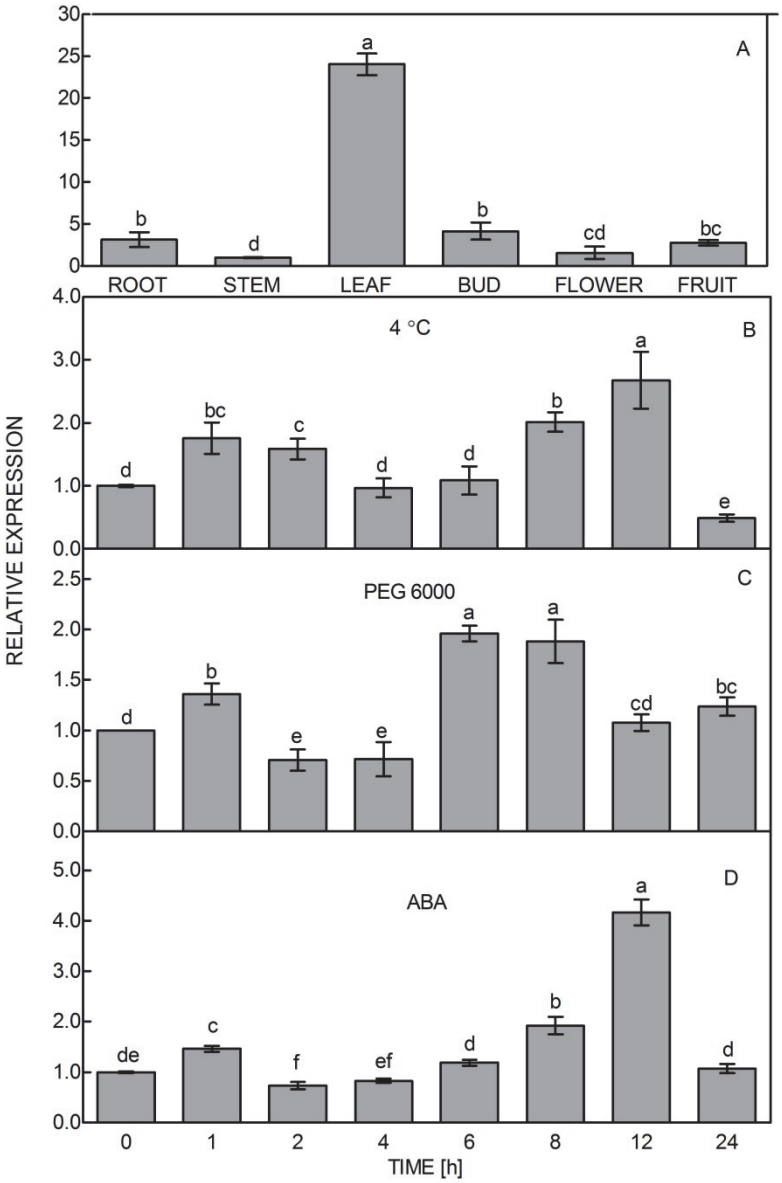


Fig. 4. The expression profile of *CsWRKY2* in *C. sinensis* was detected using real time qPCR. *C.* sinensis *β-Actin* and *C. sinensis glyceraldehyde phosphate dehydrogenase* were used as internal controls. Data represent three replicates. *A* - The expression of *CsWRKY2* in various organs of *C. sinensis*; *B* - the time course of *CsWRKY2* expression following exposure to a cold stress; *C* - the time course of *CsWRKY2* expression following exposure to a drought stress; *D* - the time course of *CsWRKY2* expression following exposure to exogenous ABA. The expression at 0 h was set to 1.

by the cold or drought stress (Fig. 6*A,B*), which is consistent with the changes of endogenous ABA accumulated by the cold and drought stresses after the treatment with exogenous ABA/TS (Fig. 5*C-D*), implying an increase of *CsWRKY2* expression induced by the cold and drought stresses partly depended on endogenous ABA accumulation in *C. sinensis* leaves.

## Discussion

WRKY proteins are among the main transcription factors that regulate plant responses to various stresses and phytohormones (Chen *et al*. 2012, Tripathi *et al*. 2014), and their orthologs have been found in various plant species. Although these orthologs exhibit a high conservation in sequence and function, WRKY family members in different plant species differ a little bit (Agarwal *et al*. 2011). Furthermore, the functions of

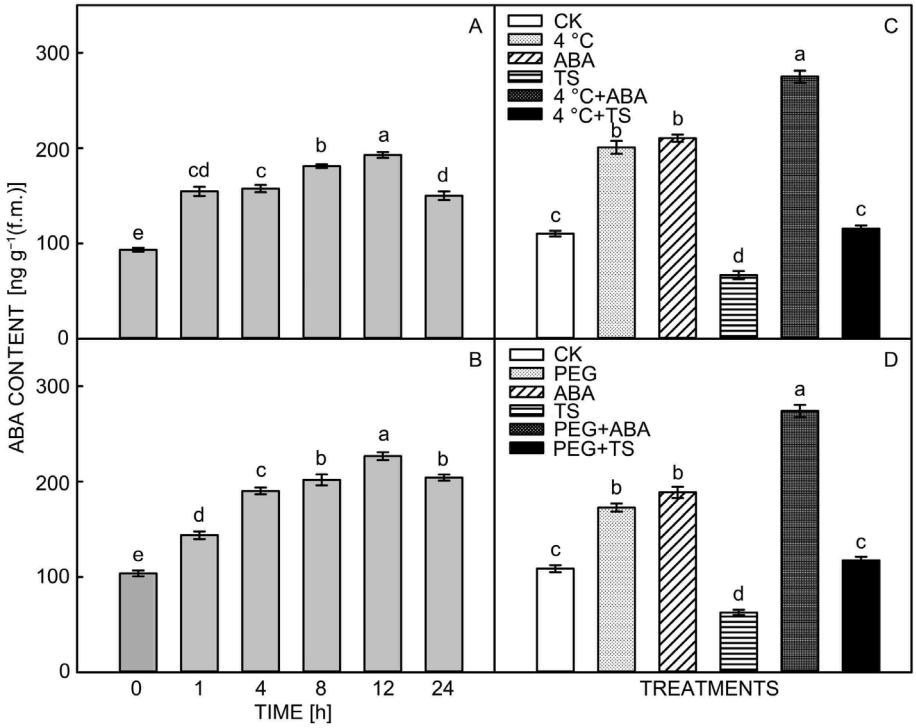


Fig. 5. The content of abscisic acid (ABA) in leaves of *C. sinensis* after cold stress (4 ºC) (*A*) and polyethylene glycol (PEG) 6000 treatment (*B*), the combination of the cold stress (*C*) or the PEG 6000 treatment (*D*) with application of ABA or tungstate (TS). Means ± SEs, $n = 3$.

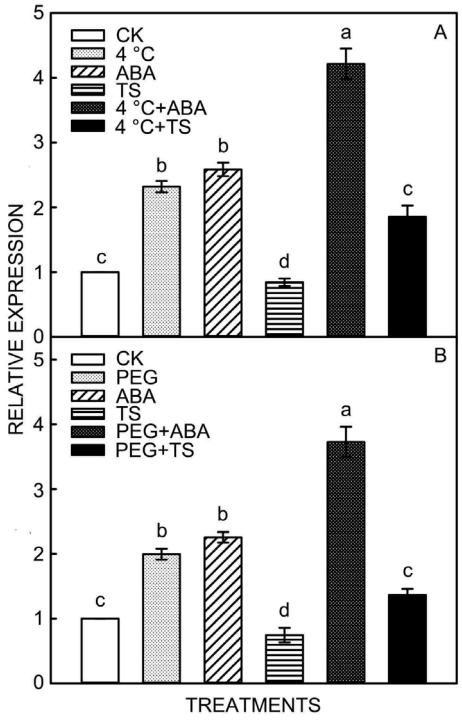


Fig. 6. Changes in *CsWRKY2* expression in response to combination of cold stress (4 ºC) (*A*) or polyethylene glycol (PEG) 6000 treatment (*B*) with application of abscisic acid (ABA) or tungstate (TS). Means ± SEs, $n = 3$.

*WRKY* genes during abiotic stresses have been extensively studied in various plant species (Sun *et al*. 2015, Tripathi *et al*. 2014). However, almost no studies have examined the role of *C. sinensis WRKY* genes in regulating defense responses. In the present study, a novel *WRKY* transcription factor gene, *CsWRKY2*, was isolated from *C. sinensis*. The deduced CsWRKY2 protein contained two typical WRKY domains and two zinc finger motifs; thus, CsWRKY2 is a group I WRKY transcription factor as reported by Eulgem *et al*. (2000). The phylogenetic analysis of the plant WRKY proteins reveals that CsWRKY2 shared a high degree of homology with other group I WRKY proteins, further confirming that CsWRKY2 belongs to group I. In addition, previous studies have shown that most WRKY proteins contain a basic NLS (Agarwal *et al*. 2011). In our investigation, we found that CsWRKY2 contained a putative NLS sequence PKRR and the subcellular localization assay confirmed that CsWRKY2 localized to the nucleus, indicating that CsWRKY2 might function in the nucleus of *C. sinensis*.

Many studies have shown that *WRKY* expression is organ-specific; for example, *LcWRKY5* is expressed only in sheepgrass leaves and roots (Ma *et al*. 2014) and *AtWRKY25* is mainly expressed in *Arabidopsis* roots (Jiang and Deyholos 2009). In our study, we found that *CsWRKY2* was mainly expressed in leaves and the lowest *CsWRKY2* expression was detected in stems and flowers.

A relatively high expression of *CsWRKY2* in leaves might be related to its participation in defense responses (Zheng *et al*. 2011). Therefore, the expression profiles of *CsWRKY2* in the leaves of *C. sinensis* were determined under the cold and drought stresses. The results indicate that *CsWRKY2* was induced by both the cold and the drought; this finding is consistent with the results of Marè *et al*. (2004); they indicated that *CsWRKY2* is involved in cold and drought stress responses in *C. sinensis*. In addition, previous studies have shown that the stress hormone ABA plays essential roles in plant responses to abiotic stresses and that WRKY proteins might act as activators or repressors in ABA signaling (Chi *et al*. 2013). For example in creosote bush, *LtWRKY21* has been shown to function as activator to control ABA-regulated gene expression (Zou *et al*. 2004), however in rice, *OsWRKY24* and *OsWRKY45* act as repressors, and *OsWRKY72* and *OsWRKY77* act as activators on the same ABA-inducible promoter (Xie *et al*. 2005). Recently, two research groups reported that a series of structurally related WRKY proteins *AtWRKY18*, *AtWRKY40*, and *AtWRKY60* are involved in the ABA signaling pathway under abiotic stress in *Arabidopsis* (Shang *et al*. 2010, Liu *et al*. 2012). These results indicate that WRKY proteins function as key components in the ABA signaling pathway. Therefore, we investigated ABA content under the cold and drought stresses and found that endogenous ABA was induced by these abiotic stresses, indicating that endogenous ABA also participated in *C. sinensis* defense responses. Furthermore, the expression profiles of *CsWRKY2* were determined after the combined treatment of the abiotic stress and ABA/TS, and the results indicate that exogenous ABA increased expression of *CsWRKY2* under both the cold stress and the drought stress. In contrast, the treatment with the ABA biosynthesis inhibitor TS decreased *CsWRKY2* expression under both the cold stress and the drought stress, implying that *CsWRKY2* might act downstream to ABA in response to the cold and drought stresses. Thus, the results reveal that *CsWRKY2* played important roles in *C. sinensis* responses to the cold and drought stresses, possibly by participating in the ABA signaling pathway downstream to ABA.

In summary, a novel *WRKY* transcription factor gene, *CsWRKY2*, was isolated from *C. sinensis*. The bioinformatics analysis indicates that the CsWRKY2 protein was a group I WRKY protein, and the subcellular localization assay shows that CsWRKY2 localized to the nucleus, suggesting that CsWRKY2 might function in the nucleus of *C. sinensis*. The *CsWRKY2* was highly expressed in the leaves of *C. sinensis* and was induced by the cold and drought stresses; these findings indicate that *CsWRKY2* participates in plant responses to cold and drought stresses. In addition, *CsWRKY2* expression was enhanced by ABA under the cold and drought stresses; in contrast, the treatment with the ABA biosynthesis inhibitor reduced *CsWRKY2* expression. Taken together, these findings reveal that *CsWRKY2* acted downstream to ABA during responses to the cold and drought stresses in *C. sinensis*.

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