

The tea plant *CsWRKY26* promotes drought tolerance in transgenic *Arabidopsis* plants

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Abstract

WRKY proteins participate in various plant physiological processes, especially in response to stress. However, limited information is available for WRKY proteins in tea plants. *Camellia sinensis* WRKY26 (*CsWRKY26*), a group I WRKY member, was characterized in this study. Tissue specificity of *CsWRKY26* expression revealed that its transcripts are abundant in leaves and roots. Moreover, *CsWRKY26* transcripts were markedly induced by treatments with sodium chloride (NaCl), polyethylene glycol (PEG), drought and different hormones. Ectopic expression of *CsWRKY26* conferred ABA insensitivity and tolerance to transgenic *Arabidopsis* plants subjected to PEG treatments. When exposed to 15% PEG, the expression levels of some stress-tolerant genes, such as *responsive to dehydration 22 (RD22)*, *9-cis-epoxycarotenoid dioxygenase 3 (NCED3)*, and *Δ¹-pyrroline-5-carboxylate synthetase 1 (P5CS1)*, were up-regulated in transgenic plants compared to wild-type (WT) plants. Under natural drought stress, the growth performance of transgenic lines was better than those of WT plants, consistent with their significantly lower relative electrolyte leakage (REL) and higher proline content. These findings indicate that *CsWRKY26* could enhance drought tolerance in *Arabidopsis*. This study provides a potential strategy for future crop improvement research.

Citation: Chen W, Zheng C, Yao M, Chen L. 2021. The tea plant *CsWRKY26* promotes drought tolerance in transgenic *Arabidopsis* plants. *Beverage Plant Research* 1: 3 <https://doi.org/10.48130/BPR-2021-0003>

INTRODUCTION

Tea plants (*Camellia sinensis*) are one of the most economical woody plants^[1]. The cultivation of tea originated in southwest China. However, unfavorable conditions and extreme climate, such as heavy metal toxicity, drought, high salinity, ABA and temperature stress, affect the quality and yield of tea during the growth of tea plants^[2,3].

Transcription factors (TFs) play vital regulatory roles in plant response to diverse stresses^[4]. Among them, members of the WRKY family, which are designated by the WRKYGQK signature domain, widely exist in plants. WRKY proteins usually contain a highly conserved WRKYGQK sequence and a zinc finger motif (either C2H2 or C2HC) at their C-terminus. Based on the number of WRKY domains and the pattern of the zinc finger motif, the WRKY family is further divided into groups I-III^[5-7]. The first identified WRKY protein, SPF1, was isolated from sweet potato^[8]. Subsequently, numerous WRKY proteins have been identified in different plant species^[7,9-11]. Moreover, the majority of relevant studies have suggested that WRKY plays a vital role in plant growth and development. For example, *Arabidopsis* AtWRKY44 regulates epidermal color and root hairs^[12,13]. AtWRKY53 and AtWRKY75 act as positive regulators of plant senescence^[14,15].

Furthermore, WRKY proteins have been discovered to respond to abiotic stresses. *Arabidopsis* AtWRKY57 positively regulates drought stress^[16]. AtWRKY63 plays an important role in plant response to ABA and drought stress^[17]. In rice, the

homolog OsWRKY45 also acts in ABA signaling and drought tolerance^[18].

Growing evidence suggests that group I WRKY proteins are involved in responses to abiotic stress. *Gossypium hirsutum* WRKY25 positively regulates the response to salinity stress in transgenic tobacco^[19]. Similarly, wheat TaWRKY2 and TaWRKY19 regulate salt, drought and/or freezing stress responses in transgenic *Arabidopsis* plants^[20]. *Arabidopsis* AtWRKY3 provides resistance to herbivory stresses, while AtWRKY25, AtWRKY26, and AtWRKY33 act synergistically in thermotolerance^[21]. Jiang and Deyholos showed that *Arabidopsis* WRKY25 and WRKY33 function redundantly as positive regulators of the NaCl stress response^[22]. Some recent studies have focused on WRKY factors in tea plants^[23-26]. However, the physiological functions of *CsWRKY* proteins in abiotic stress are still poorly understood. Here, we aimed to provide a functional characterization of *CsWRKY26*, a member of the group I WRKY family in tea plants. *CsWRKY26*, was a close homolog of *Arabidopsis* AtWRKY26, AtWRKY25 and AtWRKY33, three well-characterized Group I WRKY family proteins with important roles in plant thermotolerance, osmotic stress responses, biotic stresses and camalexin biosynthesis^[21,22,27-29]. In the present study, we cloned and characterized *CsWRKY26* from tea plant cultivar 'Longjing 43'. *CsWRKY26* was significantly induced by multiple abiotic stresses. Overexpressing *CsWRKY26* in transgenic *Arabidopsis* increased resistance to drought stress. These findings may

provide new insights into the abiotic stress tolerance mechanism of *CsWRKY26*, which may serve as a target for molecular breeding for crop improvement and enhancement of stress tolerance.

RESULTS

Isolation and bioinformation analysis of *CsWRKY26*

The full-length *WRKY26* was obtained by high fidelity PCR. Owing to its high sequence identity with *A. thaliana* *WRKY26*, the *WRKY* protein was named *CsWRKY26*. The gene contains a 1,674 bp open reading frame encoding a 557 amino acid protein, with a predicted isoelectric point of 8.77 and a molecular mass of 61.41 kDa.

CsWRKY26 shared high amino acid sequence similarity with *AtWRKY26*, *AtWRKY25*, and *AtWRKY33* (49%, 46% and 48% respectively). Multiple sequence alignment showed that *CsWRKY26* shared two typical *WRKY* DNA-binding domains, i.e., *WRKYGQK* and one *CX₁₋₅CX₂₂₋₂₃HXH* zinc finger motif (Fig. 1a), which categorized it as a group I *WRKY* superfamily member^[5]. And the sequence of *CsWRKY26* contained an extended C-terminal domain (CTD) compared to those of *AtWRKY33*.

To analyze the evolutionary relationship of *CsWRKY26* within group I *WRKY* subfamily members from *A. thaliana*, a phylogenetic tree was constructed using MEGA 6.0 software (Fig. 1b). Among these proteins, *AtWRKY25*, *AtWRKY26* and *AtWRKY33* contribute to plant defense stress and plant thermotolerance^[21], these findings imply that *CsWRKY26* is involved in stress response.

Expression patterns of *CsWRKY26* under multiple stress treatments

Quantitative RT-PCR was performed to detect *CsWRKY26* transcript abundance in leaves and roots under osmotic stress

(Fig. 2). In leaves, *CsWRKY26* was dramatically induced by NaCl and PEG treatments, the transcript levels rapidly peaked nearly 30 and 70-fold at 24 h post treatment, respectively. Additionally, *CsWRKY26* was also induced both in sucrose and mannitol treatments. In roots, *CsWRKY26* was significantly induced by osmotic stresses at the initial stages (1–3 h). Simultaneously, *CsWRKY26* displayed a sustained increase during natural drought stress and rehydration processes. These results suggest that *CsWRKY26* may play important roles in stress signaling, especially in response to osmotic stress.

To investigate the association between *CsWRKY26* and some signaling molecules, the expression of *CsWRKY26* under salicylic acid (SA), abscisic acid (ABA), Gibberellic acid (GA₃) and Methyl Jasmonate (MeJA) treatments was analyzed by qRT-PCR. As shown in Fig. 3, *CsWRKY26* displayed upregulation to some extent under various hormone treatments, with results that could be divided into three expression profiles: (A) sustained upregulation, peaking at 6 h (GA₃); (B) sharply increasing at the initial stage and then decreasing (ABA and MeJA); (C) rapidly increasing in the late stage (SA). It is worth noting that *CsWRKY26* was significantly induced by ABA and MeJA treatments, with more than a 5-fold induction compared with that under normal conditions.

CsWRKY26 localized in the nucleus

Subcellular localization of *CsWRKY26* was investigated. The full-length ORF of *WRKY26* was fused to the N-terminal of enhanced green fluorescent protein (eGFP) and then transiently expressed in *Nicotiana benthamiana* epidermal cells (Fig. 4a). The control 35S::GFP showed green fluorescence throughout the whole cell. Confocal microscopy demonstrated that green fluorescence was detected in the nucleus when the *CsWRKY26*-fusion protein was transformed in tobacco. In addition, targeting ability was also determined in

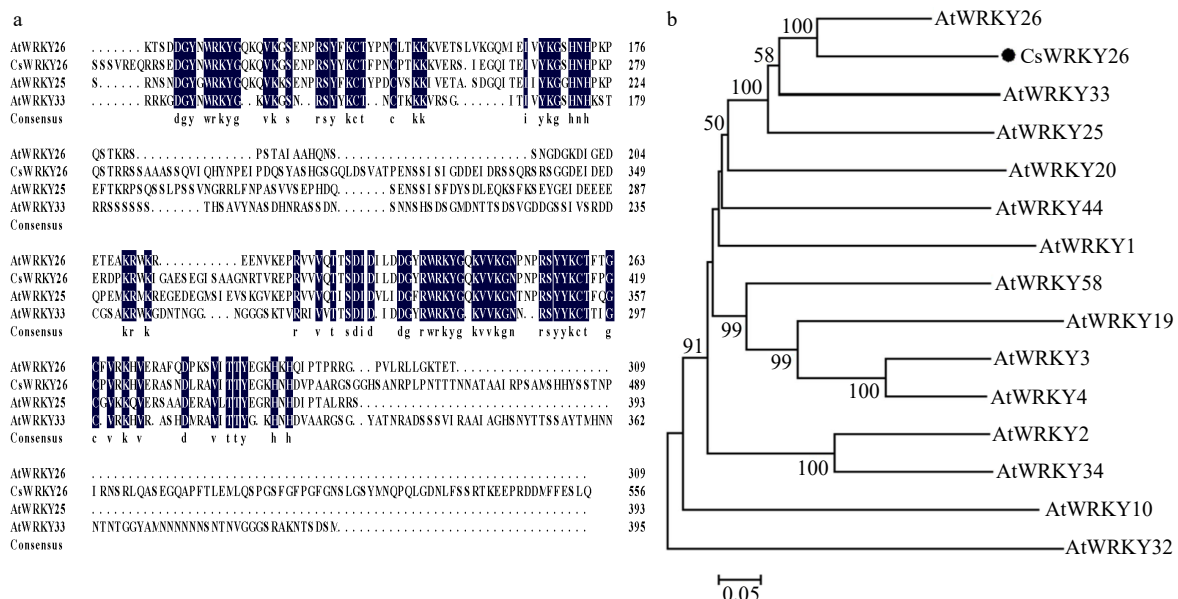


Fig. 1 Sequence alignment and phylogenetic tree analysis of *CsWRKY26*. (a) Multiple sequence alignment of amino acid sequences of *CsWRKY26* with homologous *WRKY*s from Arabidopsis. Sequences were aligned by the DNAMAN program. (b) Phylogenetic analysis of *CsWRKY26* and Arabidopsis group I *WRKY* proteins, constructed using MEGA 6.0.

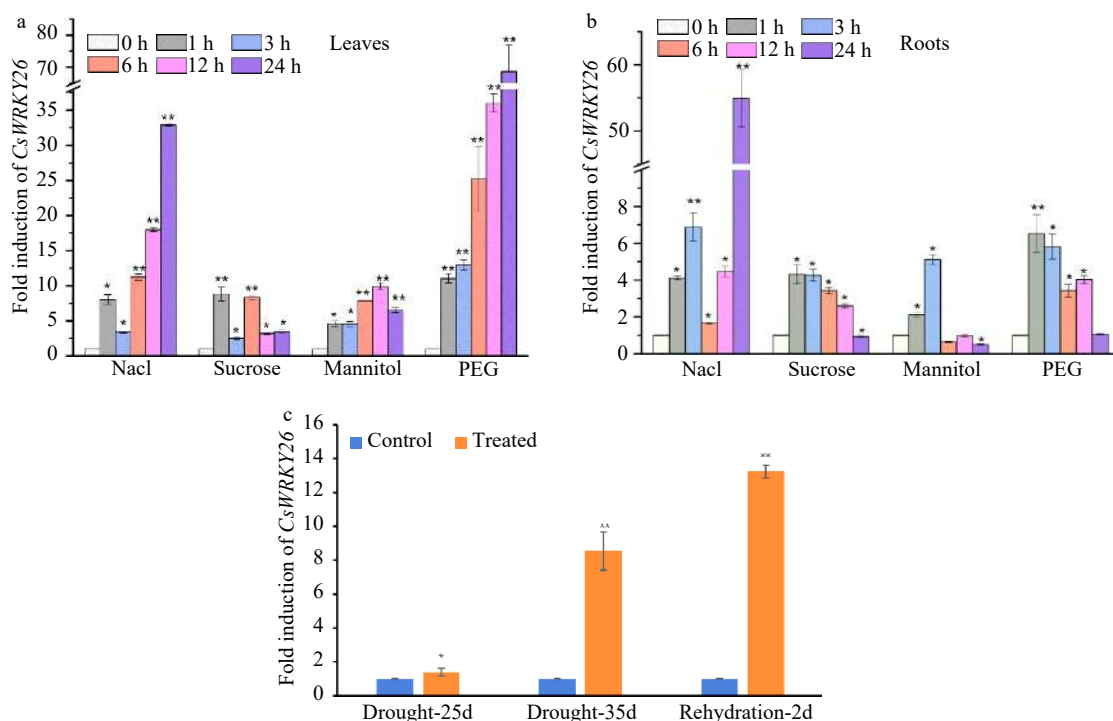


Fig. 2 Expression patterns of *CsWRKY26* gene in tea plants under different abiotic stress. (a) *CsWRKY26* transcripts accumulate in leaves under 150 mM NaCl, 90 mM sucrose, 10% (W/V) PEG4000, 90 mM mannitol stresses. (b) *CsWRKY26* transcripts accumulate in roots under the same stress as in (a). (c) The fold change of *CsWRKY26* in leaves under natural drought stress. The relative expression levels of the *CsWRKY26* gene were detected using a qRT-PCR approach. Asterisks indicate that the expression level is significantly different from the value of the control (* $p < 0.05$, ** $p < 0.01$).

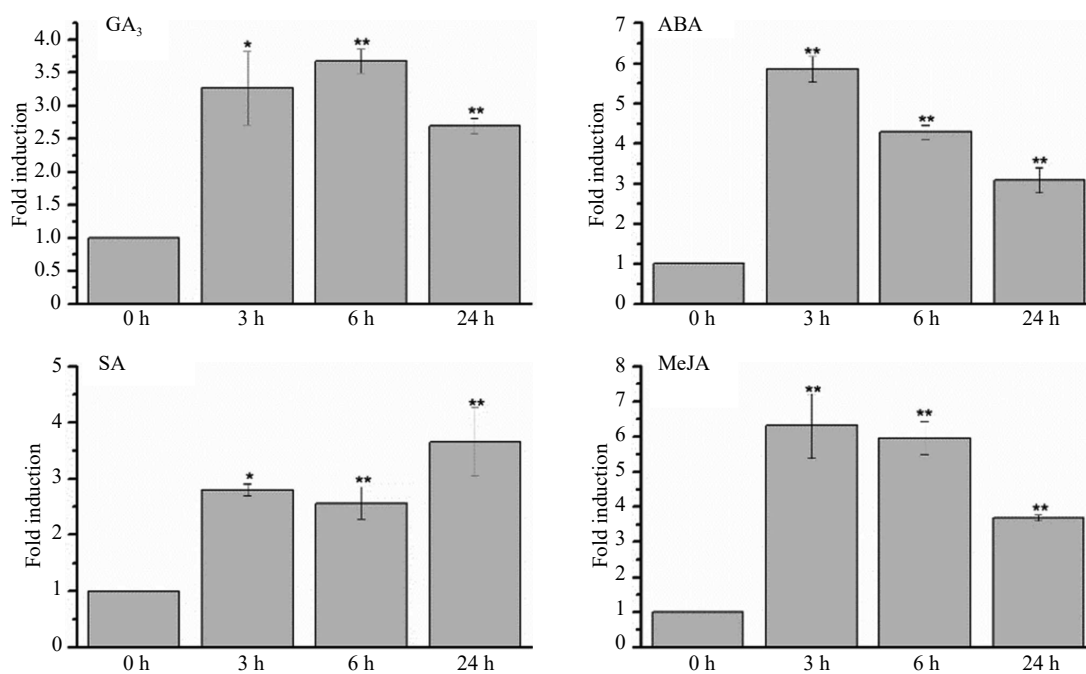


Fig. 3 *CsWRKY26* expression in leaves under various hormones treatments. The seedlings were treated with different hormones (100 μ M GA₃, 100 μ M ABA, 5 mM SA and 50 μ M MeJA), and leaves were harvested for expression analysis after 24 h of treatment. The level of *CsWRKY26* expression were normalized to *GAPDH* and compared with control conditions. Data shown are averages of three biological replicates, with error bars representing SD. The asterisks indicate that the expression level is significantly different from the value of the control (* $p < 0.05$, ** $p < 0.01$).

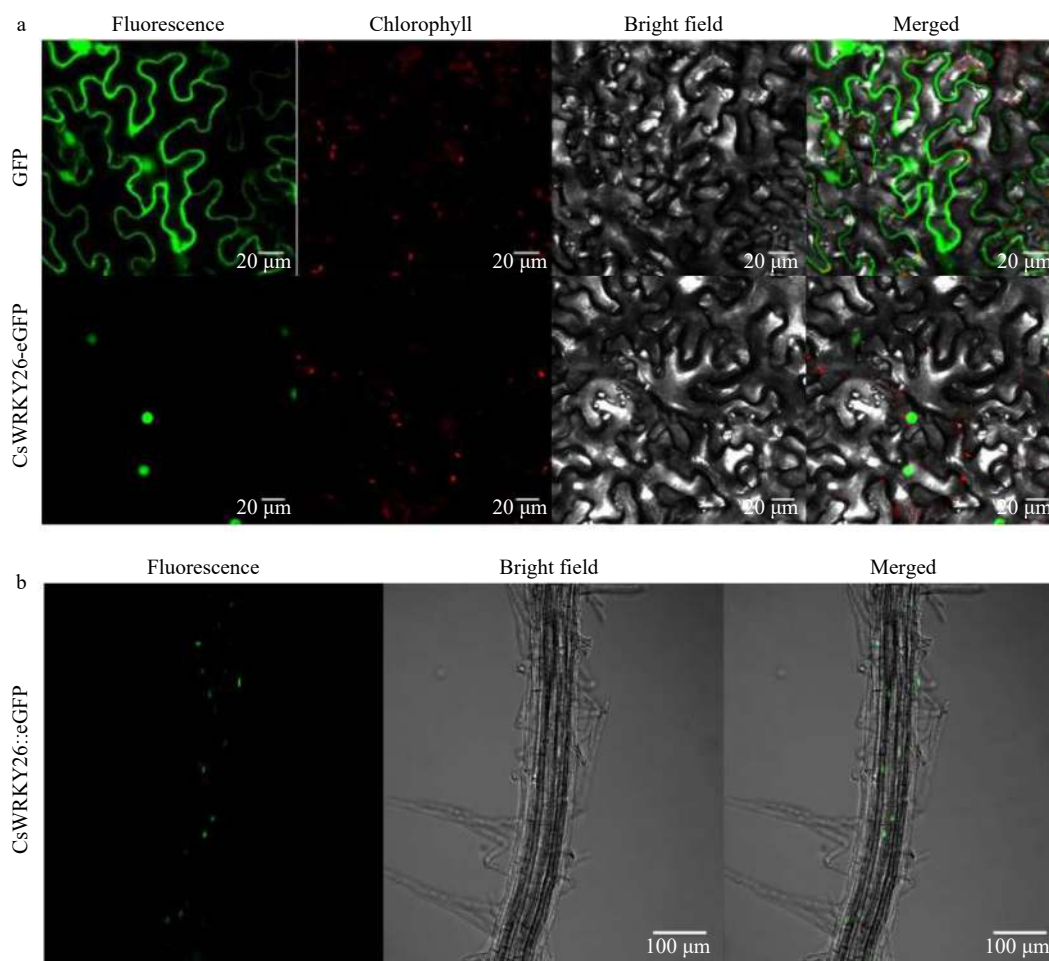


Fig. 4 Subcellular localization analysis of *CsWRKY26* protein. (a) GFP alone (upper panel) and *CsWRKY26*-eGFP (middle panel) were transiently expressed in tobacco epidermal cells. Representative images from left to right in each panel were taken under fluorescence, chlorophyll, transmitted light and an overlay of both channels. Scale bar = 20 μm . (b) Subcellular localization of *CsWRKY26*-eGFP in *Arabidopsis* roots. Representative images from left to right were taken under fluorescence, bright field and an overlay of both channels. Scale bar = 100 μm .

CsWRKY26 overexpression seedling roots (Fig. 4b), and confocal microscopy showed that *CsWRKY26* is localized in the nucleus.

Tissue-specific expression analysis

The expression profiles of *CsWRKY26* was assayed by qRT-PCR or β -glucuronidase (*GUS*) transcript under control of the *CsWRKY26* promoter fragments (the 1.25 kb fragment upstream of the ATG start codon was fused to the *GUS* open reading frame).

CsWRKY26 exhibited high expression levels in leaves and roots, especially in older leaves, which were approximately 17 times higher than that in buds (Fig. 5a). Meanwhile, pro*CsWRKY26*::*GUS* reporter gene fusion constructs were investigated by histochemical staining. Under normal conditions, we detected strong expression in the whole 7-d-old seedlings and in the roots and veins of leaves in both 10 and 20-d-old seedlings. Among inflorescence tissues, moderate to strong *GUS* expression was observed in filament, petal, stigma, ovary, sepal, and silique tissues (Fig. 5b). However, these mRNA accumulation patterns were inconsistent with the *GUS* expression patterns, indicating that regulatory elements beyond the promoter region may have

affected this result or that the sensitivity of the reporter system may be insufficient to detect more moderate changes.

Overexpression of *CsWRKY26* in *Arabidopsis* enhances tolerance to osmotic stresses

CsWRKY26 was induced by salinity and PEG treatments (Fig. 1). Thus, to investigate whether *CsWRKY26* mediates the responses to osmotic stress, three independent transgenic *Arabidopsis* lines (OE1-3) and WT seedlings were subject to a series of abiotic stress. The root length of WT plants was slightly longer than those of *CsWRKY26* transgenic plants under normal conditions (Fig. 6). There was no difference between WT and transgenic plants under salt stress. However, two lines showed longer root lengths than WT plants both in the presence of 0.3 μM ABA and 15% PEG6000. Thus, we speculate that *CsWRKY26* may be involved in ABA-dependent drought-responsive regulatory pathways during early growth.

Transgenic *Arabidopsis* plants expressing *CsWRKY26* showed improved drought tolerance

Under normal conditions and salt treatment, the growth vigor of *CsWRKY26*-overexpression plants was weaker than that of WT plants (Fig. 7). However, WT plants exposed to the 15% PEG6000 treatment for 7 d showed leaf curling and

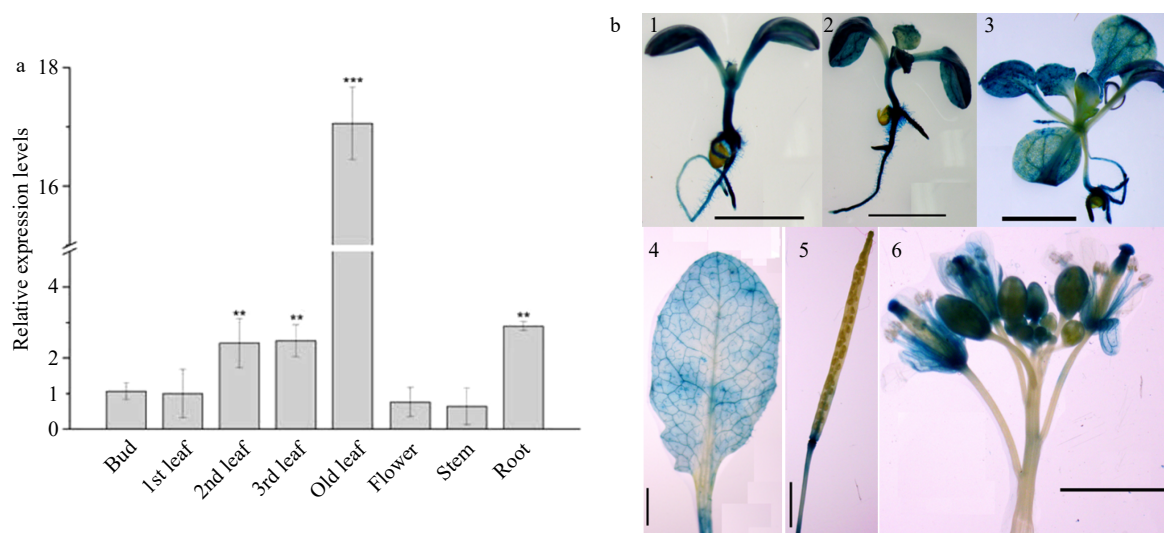


Fig. 5 Tissue-specific expression patterns of *CsWRKY26* gene. (a) Quantitative RT-PCR of *CsWRKY26* expression in various tissues: bud, 1st leaf, 2nd leaf, 3rd leaf, old leaf, flower, stem and root. The transcript levels of *CsWRKY26* gene in various tissues were related to *GAPDH* and compared with that of bud. Asterisks indicate that the expression level is significantly different from the value of the control (* $p < 0.05$, ** $p < 0.01$). (b) Histochemical staining of transgenic Arabidopsis with *ProCsWRKY26::GUS*, (1) 7-d-old seedling, (2) 10-d-old seedling, (3) 20-d-old seedling, (4) mature rosette leaf, (5) silique, (6) flowers. Scale bars = 2 mm.

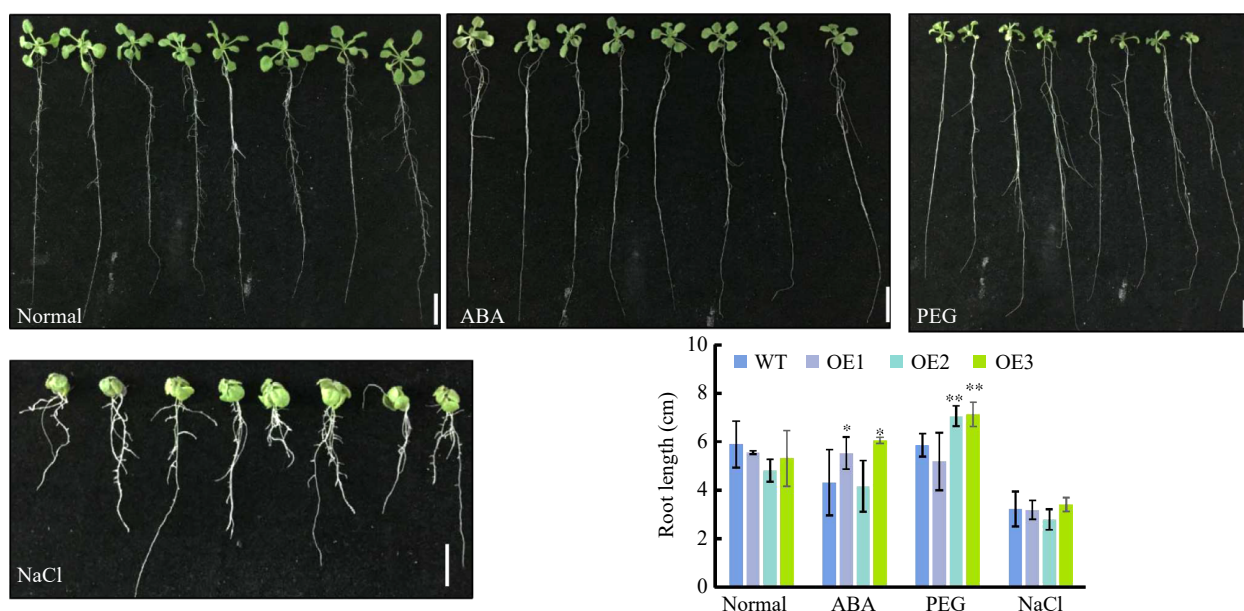


Fig. 6 Root elongation of *CsWRKY26*-overexpressing Arabidopsis lines under various stress conditions. Four-d-old seedlings were transferred onto plates with or without 0.3 μ M ABA, 15% PEG6000, 150 mM NaCl for 10 d, and numbers of root length were determined after 10 d of vertical growth ($n > 12$). Bars and error bars represent mean \pm SE, scale bars = 1 cm.

atrophy, while most of the transgenic lines showed undamaged leaves. Under drought stress for 15 d, leaves from WT lines were purple with severe wilting compared to those in the overexpression lines.

Electrical leakage was closely correlated with the degree of damage to cell membranes under various stresses. The relative electrical conductivity (REL) in transgenic plants under salt treatment was higher than that of WT plants, but there was no significant difference. Compared with WT plants, the transgenic plants had lower REL under PEG6000 and natural drought treatment. The chlorophyll content of

WT and transgenic leaves under drought treatment were assayed. Both *CsWRKY26*-overexpression and WT plants exhibited increases in chlorophyll content, whereas chlorophyll content did not significantly differ between most transgenic lines and WT plants. Meanwhile, proline content varied among transgenic plants after salt treatment. The proline content in OE1 and OE2 lines were lower than that in WT plants, but these differences were not statistically significant. However, OE1 and OE3 lines showed significantly higher proline content compared with that of WT plants under drought stress (Fig. 7). Collectively, our results indicate

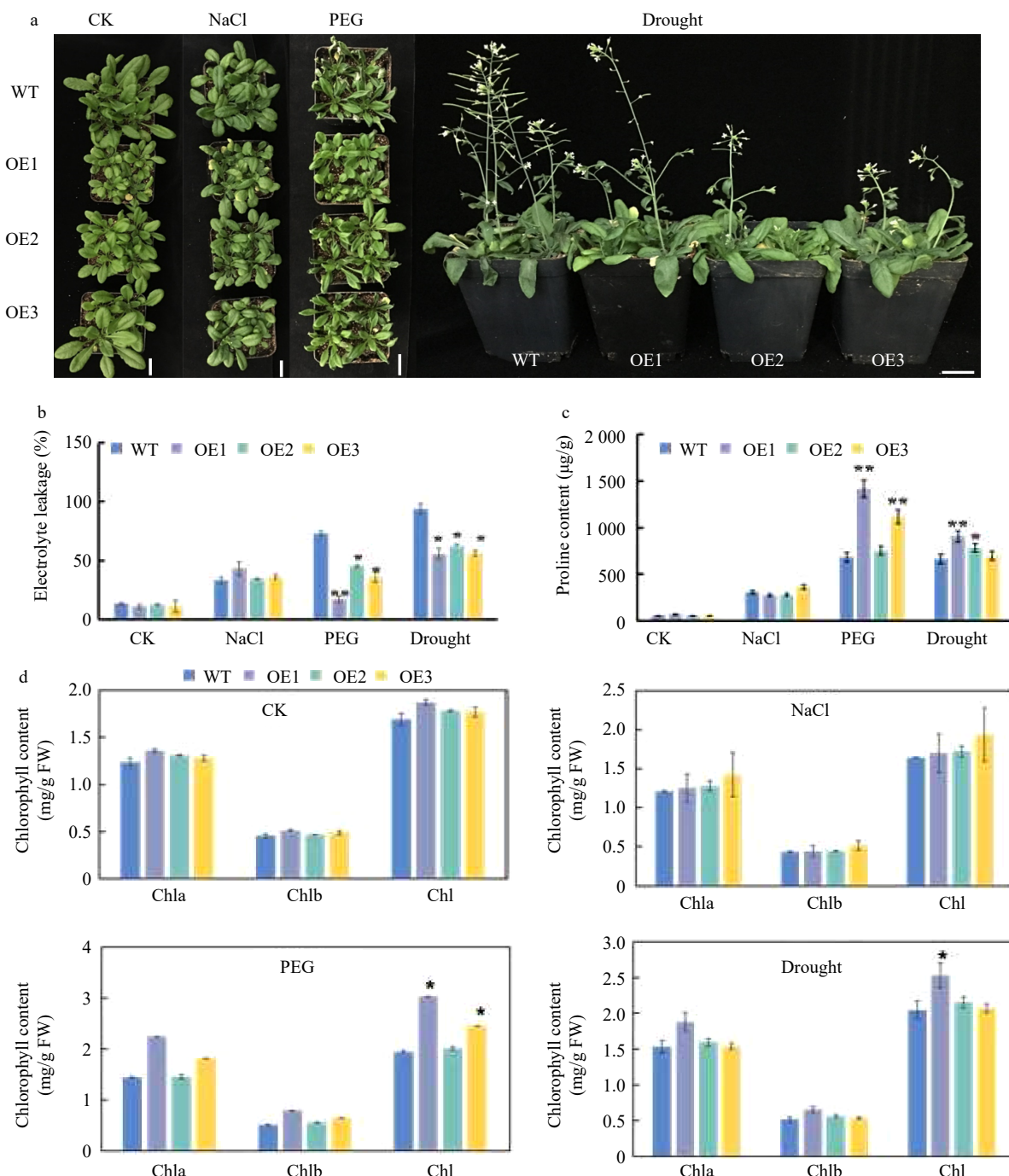


Fig. 7 Phenotype of *CsWRKY26*-overexpressing transgenic plants and WT plants under different stress conditions. (a) Phenotype of WT and of *CsWRKY26*-overexpression Arabidopsis lines grown with or without exposure to salt treatment for 7 d, 15% PEG6000 treatment for 7 d, and a natural 15-d drought. (b–d) Determination of physiological indicators: relative electrolyte leakage, proline content, and chlorophyll content. Scale bars = 2 cm.

that *CsWRKY26* overexpression enhanced drought stress in transgenic Arabidopsis.

Expression of stress-related genes in *CsWRKY26* transgenic Arabidopsis

To elucidate the regulation mechanisms underlying drought resistance in *CsWRKY26* overexpression lines, we further detected the expression profiles of stress-responsive

genes (namely *DREB2A*, *RD29A*, *P5CS1*, *RD22*, *NCED3*, *ABI1*, and *ABI5*) in 7-d-old vertically grown seedlings cultivated under drought. The expression levels of *RD29A*, *NCED3*, and *ABI1/5* were constitutively increased in transgenic plants under normal conditions compared to that of WT plants (Fig. 8a). Furthermore, the expression levels of *NCED3*, *RD22*, and *P5CS1* were significantly elevated in OE1 and OE3 lines, which is consistent with the proline content increases in transgenic

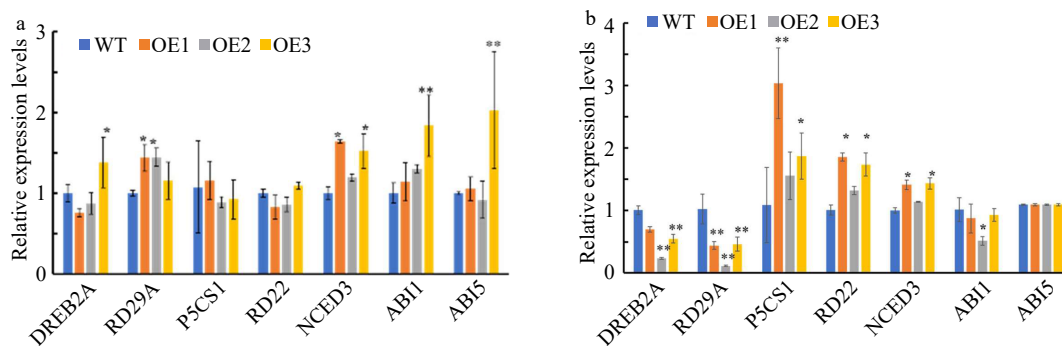


Fig. 8 Analysis of expression levels for stress-related genes in *CsWRKY26*-overexpressing Arabidopsis lines and WT plants under normal and 15% PEG6000 conditions. Transcript levels of these genes in the transgenic lines and WT plants were determined by quantitative RT-PCR using *Actin2* as a quantification control. Asterisks indicated that the expression level is significantly different from that of the control (* $p < 0.05$, ** $p < 0.01$). (a) Normal conditions. (b) Simulated drought conditions induced by 15% PEG6000.

lines (Fig. 8b). In addition, the stress-related genes *DREB2A* and *RD29A* were significantly down-regulated, indicating that *CsWRKY26* may be involved in either ABA-dependent or ABA-independent stress signal transduction pathways.

DISCUSSION

WRKY transcription factors can act as transcriptional activators or inhibitors in response to stress stimuli^[30]. Members of this family have been reported to participate in responses to multiple abiotic stresses, for example, Arabidopsis AtWRKY57/-63^[16,17] and sunflower HaWRKY76 participate in drought stress responses^[31], while Arabidopsis AtWRKY18/-25/-30/-60 participate in osmotic stress responses^[22,32,33]. However, the functions of WRKY proteins in tea plants under abiotic stress have remained unclear.

In the present study, *CsWRKY26* was cloned from tea plants. Two typical WRKY domains and a group I CX₁₋₅CX₂₂₋₂₃HXH zinc finger motif (Fig. 3a) were observed in the *CsWRKY26* sequence^[5]. *CsWRKY26* showed high amino acid sequence identities with AtWRKY25, -26, and -33. Notably, the sequence alignment revealed an extended C-terminal domain (CTD) compared to those of AtWRKY33. Previous studies have shown that the CTD in AtWRKY33 plays a vital role in plant stress response^[34]. Arabidopsis AtWRKY25 or AtWRKY33 overexpression increases the salt tolerance and sensitivity to ABA of transgenic plants^[22]. Thus, it is likely that the CTD of *CsWRKY26* plays vital roles in plant response to stress.

Here, *CsWRKY26* was significantly expressed in salt- and drought-induced tea leaves (Fig. 2). This induction is similar to that of soybean *GmWRKY16*^[35], indicating that *CsWRKY26* in tea plants may participate in coping with drought stress. Previous studies have shown that many WRKY TFs are involved in diverse hormone signaling pathways. For example, OsWRKY24/-53/-70 inhibited ABA and GA signal transduction in rice aleurone cells^[36], while banana MaWRKY31/-33/-60/-71 positively regulated drought tolerance in its ABA-dependent pathway^[37]; similarly, Arabidopsis AtWRKY28/-46 activated the transcription levels of related genes in the SA signaling pathway^[38]. *CsWRKY26* was significantly induced by ABA and MeJA hormones (Fig. 2), indicating that it may participate in one or more phytohormone signaling cascades.

Drought is a major abiotic factor limiting growth, adversely

affecting both plant growth and economic benefits. The role of WRKYs in drought stress responses have been reported in many plants. *Boea hygrometrica* BhWRKY1 regulated the transcription level of galactosidase *BhGalSI* (a key rate-limiting enzyme in the oligosaccharide synthesis pathway) in the ABA-dependent signaling in response to drought stress; thus, the content of osmotic substances was increased^[39]. In tobacco, expression of cotton *GhWRKY41* or tomato *SpWRKY1* conferred tolerance to drought by regulating stomatal conductance and reactive oxygen level^[40,41]. Overexpression of cotton *GhWRKY17* increased sensitivity to drought in tobacco by impairing ABA-induced stomatal closure and reducing the level of ABA^[42]. Arabidopsis AtWRKY40/-18/-60 proteins act as negative regulators of *ABI4* and *ABI5* genes in the ABA signaling pathway^[32]. Rice OsWRKY45 confers resistance to drought stress^[18]. In the present study, *CsWRKY26* overexpression promoted root elongation in transgenic Arabidopsis treated with 0.3 μ M ABA or 15% PEG6000 (Fig. 6). Moreover, *NCED3* (*9-cis epoxy-carotenoid dioxygenase 3*), a key enzyme in the ABA synthesis pathway that is involved in drought stress responses^[43], was induced in *CsWRKY26* transgenic plants under PEG-simulated drought conditions (Fig. 8). Similar functions were also found in other WRKY genes. For example, overexpressing AtWRKY57 in Arabidopsis increased tolerance to drought by enhancing *NCED3* expression and increasing accumulation of ABA content^[16].

Studies have reported that osmotic stress imposed by drought are transmitted through at least two pathways: ABA-dependent and ABA-independent signaling pathways^[6,16,18,32,42-45]. Notably, in our research, some related genes involved in those two pathways, such as *RD29A*, *RD22*, and *DREB2A*, were found to be significantly induced by drought stress, showing that *CsWRKY26* may be involved in the abscisic acid signaling pathway (Fig. 8). Dehydration response element binding (DRE) TFs play important roles in plant stress response. Dubouzet et al.^[46] showed that OsDREB1A and OsDREB2A, which specifically bound to DRE, were significantly up-regulated in transgenic rice lines, and conferred drought tolerance to rice. However, the expression levels of *DREB2A* in *CsWRKY26*-overexpressing transgenic lines were significantly reduced compared to that of WT plants, which may relate to sampling time (Fig. 8). Miao et al.^[47]

showed that the expression of *DREB2A* was significantly up-regulated in the early stage during drought stress but later down-regulated in *MaASR1* transgenic *Arabidopsis* lines. Proline plays a critical role in plant osmotic stress tolerance by stabilizing subcellular structures and maintaining cellular functions^[48]. Here, Proline content significantly increased in WT and transgenic plants under drought stress. Meanwhile, *P5CS1* expression was significantly elevated, consistent with the proline accumulation observed in transgenic plants (Fig. 7). Similar regulatory mechanisms have been found in other studies, which have confirmed that the adaptability of similar transgenic plants to drought stress is related to the dehydration avoidance mechanism of proline metabolism regulation^[49].

In addition, WRKY TFs form a complex network with other TFs to regulate plant stress. For example, *Glycine max* GmWRKY27 interacts with GmMYB174 to suppress *GmNAC29* expression and enhance stress tolerance in soybean plants^[50]. AtWRKY33 acts downstream of the mitogen activated protein kinase genes *MPK3* and *MPK6* in reprogramming camalexin biosynthesis genes^[51]. Andreasson et al.^[52] reported that MPK4 was found to interact with MKS1, a VQ motif protein substrate of MPK4 substrate, that in turn interacts with *Arabidopsis* WRKY25 and WRKY33. Zhou et al. (2015)^[34] reported that WRKY33 is an evolutionarily conserved WRKY transcription factor with a broad and critical role in plant stress responses. However, most reports indicate that WRKY25 and WRKY33 may be involved in diverse defense mechanisms against most stresses^[22,27–29]. Notably, the MBS motif was found in the *CsWRKY26* region, implying that *CsWRKY26* may interact with MYB TF to participate in drought stimuli (unpublished). Therefore, further research should focus on the components related to WRKY26 during drought stress in tea plants.

CONCLUSIONS

WRKY proteins play a vital role in response to abiotic stress. This study has identified a *CsWRKY26* gene from tea plant cultivar 'Longjing 43'. *CsWRKY26* was induced by sodium chloride (NaCl), polyethylene glycol (PEG), drought and different hormones treatments. *CsWRKY26*-overexpression transgenic *Arabidopsis* lines exhibited tolerance to drought stress. The findings reported here shed new light on the abiotic stress tolerance mechanism of *CsWRKY26*, which may serve as a target for molecular breeding of drought resistance tea cultivar.

MATERIALS AND METHODS

Plant materials and growth conditions

'Longjing 43' (LJ43), a very popular and famous green tea (in particular for Longjing tea) cultivar bred by the Tea Research Institute of the Chinese Academy of Agricultural Sciences (TRICAAS), was used in this study. The buds, leaves, stems, flowers and roots were collected for tissue-specific analysis from seven-year-old tea plants, grown in China National Germplasm Hangzhou Tea Repository of the TRICAAS.

Two-year-old rooted cuttings, similar in both size and

growth stage, were grown in pots inside the greenhouse of TRICAAS. These cuttings were subject to various abiotic stresses, such as natural drought, 150 mM NaCl, 10% (W/V) PEG4000, 90 mM sucrose or mannitol, and different hormones including 100 μ M abscisic acid (ABA), 100 μ M gibberellic acid (GA_3), 50 μ M Methyl Jasmonate (MeJA) and 5 mM salicylic acid (SA) treatment. Different treatments were performed as previously described^[53]. For various osmotic treatments, the functional leaves (the third leaves from two-year-old rooted cuttings of tea plants) and roots were sampled at 0, 1, 3, 6, 12, or 24 h. For phytohormone treatments, the functional leaves were harvested at 0, 3, 6 or 24 h post-treatment. For natural drought stress, the functional leaves were harvested from two-year-old rooted cuttings of tea plants by withholding water for 25 d, 35 d and re-watering after 2 d. All samples were immediately frozen in liquid nitrogen and stored at -80 °C before analysis. All treatments contained at least three biological replicates.

Healthy leaves from tobacco (*Nicotiana benthamiana*) grown for 2–4 weeks were used for protein subcellular localization. The seeds of *Arabidopsis thaliana* were surface sterilized, vernalized for 3 d at 4 °C, and plants were grown on 1/2 Murashige and Skoog (MS) medium. Plants were analyzed on plates or transplanted to a sterilized soil 7 d after germination. *Arabidopsis* plants were grown in growth cabinets at 22 ± 1 °C under long day (LD, 16 h /8 h day/night cycle) photoperiods, while tobacco plants were maintained at 24 ± 2 °C under a photoperiod of 12 h /12 h day/night. The light intensity was at approximately 108 μ mol/m²s.

Cloning of *CsWRKY26* genes and phylogenetic analysis

Total RNA was extracted from different tissues of LJ43 using QIAGEN RNeasy Mini Kit and a reverse transcription reaction was performed using a FastKing gDNA Dispelling RT SuperMix Kit (KR118). The full-length sequence of *CsWRKY26* was cloned using KOD DNA polymerase. The specific primers are listed in Supplemental Table S1. Polymerase chain reaction (PCR) product was purified from agarose gel, and the purified product was cloned into the pEASY-Blunt simple vector (Transgen Biotech Co., Ltd., Beijing, China). Multiple sequence alignments of *CsWRKY26* in *C. sinensis* and Group I WRKY members in *A. thaliana* were performed in MEGA6.0 by the neighbor-joining method with 1000 bootstrap replicates.

Expression profiles analysis of *CsWRKY26*

The expression analysis of *CsWRKY26* were detected by real-time quantitative polymerase chain reaction (qRT-PCR) or histochemical staining of β -glucuronidase (*GUS*). For *GUS* staining, the homozygous promoter-WRKY26::*GUS* (pro*CsWRKY26*::*GUS*) transgenic *Arabidopsis* plants were immersed in 0.5 mg/mL XGluc (5-bromo-4-chloro-3-indolylglucuronide) solution in 100 mM sodium phosphate buffer (pH 7.0), 10 mM EDTA, 1 mM $K_4Fe(CN)_6$, 1 mM $K_3Fe(CN)_6$, 0.5% Triton X-100, and incubated at 37 °C in the dark as previously described^[54]. Tissue was cleaned in 70% ethanol before the visualization.

The *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was used as the housekeeping gene. And the gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ or $2^{-\Delta Ct}$ method^[55,56].

Generation of *CsWRKY26* overexpression lines

For overexpression analysis, the 35S::*CsWRKY26* plasmid was generated, carrying the coding sequence of *WRKY26* in the binary destination vector pH7FWG2.0. Floral dip-transformation of the plasmid into *Arabidopsis* cv. Columbia (Col-0) resulted in at least three independent transgenic *Arabidopsis* lines selected by growth on plates containing 30 $\mu\text{g mL}^{-1}$ hygromycin B^[57].

For subcellular localization prediction of *CsWRKY26* protein, the pH7FWG2.0-*CsWRKY26* was expressed in tobacco leaf epidermal cells. Plants were incubated for 48 h before fluorescence imaging using a Nikon C2-ER confocal laser microscope. In addition, GFP signals were also detected in the young roots of *CsWRKY26* overexpression lines.

For promoter- β -glucuronidase (*GUS*) fusion, the promoter region of *WRKY26* from *C. sinensis* was amplified by high fidelity PCR. The identified fragment was inserted into the pBI101.3-*GUS* plus vector. The recombinant pro*WRKY26*::*GUS* was introduced into the *A. tumefaciens* strain GV3101 using the freeze-thaw method and introduced into *Arabidopsis* using the floral dip method^[57]. Homozygous transgenic lines were used in subsequent experiments. The amplification primers are listed in Supplemental Table S1.

Abiotic stress analysis of *CsWRKY26*-overexpression transgenic plants

The seeds of wild type (WT) and *CsWRKY26* overexpression lines were planted on 1/2 MS medium for 4 d, and then transferred to 1/2 MS or 1/2 MS medium containing 150 mM NaCl, 15% PEG6000 or 0.3 μM ABA for 10 d. For osmotic stress assay, 4-week-old transgenic plants and WT plants were irrigated with a final concentration of 250 mM NaCl for 7 d. To elevate drought tolerance, 4-week-old plants were stopped irrigation for 15 d or subjected to 15% PEG6000 for 7 d.

Measurement of physiological index

The sterilized and vernalized seeds of WT and *CsWRKY26* overexpression plants were germinated on 1/2 MS medium for 7 d, and then transferred to 41 mm Jiffy seedling blocks. Plants were grown in a light incubator for three weeks. After a 7-d period of salinity, 15% PEG6000 treatment, or natural drought stress treatment for 15 d, the rosette leaves were collected for measuring the REL, proline and chlorophyll content. The REL was measured as described by Wang et al.^[58]. Proline accumulation was assayed using the proline test kit cat. A107 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The chlorophyll content from each sample was estimated as previously described by Li et al.^[59].

Analysis of stress related genes by qRT-PCR in transgenic plants

Four-d-old seedlings were placed vertically in 1/2 MS medium with or without 15% PEG6000 for 7 d, and then sampled for RNA isolation. *Actin-2* gene act as the reference control gene, and the $2^{-\Delta\Delta Ct}$ method was used to evaluate the relative expression level. All qRT-PCR experiments contained three biological replicates.

Statistical analysis

Each experiment described in the research contained at least three biological replicates. Statistical analysis was performed using the one-way analysis of variance (ANOVA).

And the Built-in Functions in Excel 2019 was used for statistical analysis.

ACKNOWLEDGMENTS

This work was supported by China Agriculture Research System of MOF and MARA, the Chinese Academy of Agricultural Sciences through the Agricultural Science and Technology Innovation Program (CAAS-ASTIP-2017-TRICAAS) to LC.

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary Information accompanies this paper at (<http://www.maxapress.com/article/doi/10.48130/BPR-2021-0003>)

Dates

Received 8 March 2021; Accepted 18 May 2021; Published online 28 May 2021

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