The *Kandelia obovata* transcription factor *KoWRKY40* enhances cold tolerance in transgenic *Arabidopsis*

Jiao Fei¹,²,³, You-Shao Wang⁴,²,³*, Hao Cheng¹,²,³*, Yu-Bin Su⁴, Yong-Jia Zhong⁵ and Lei Zheng⁵

**Abstract**

**Background:** WRKY transcription factors play key roles in plant development processes and stress response. *Kandelia obovata* is the most cold-resistant species of mangrove plants, which are the important contributors to coastal marine environment. However, there is little known about the WRKY genes in *K. obovata*.

**Results:** In this study, a WRKY transcription factor gene, named *KoWRKY40*, was identified from mangrove plant *K. obovata*. The full-length cDNA of *KoWRKY40* gene was 1420 nucleotide bases, which encoded 318 amino acids. The *KoWRKY40* protein contained a typical WRKY domain and a C2H2 zinc-finger motif, which were common signatures to group II of WRKY family. The three-dimensional (3D) model of *KoWRKY40* was formed by one α-helix and five β-strands. Evolutionary analysis revealed that *KoWRKY40* has the closest homology with a WRKY protein from another mangrove plant *Bruguiera gymnorrhiza*. The *KoWRKY40* protein was verified to be exclusively located in nucleus of tobacco epidermis cells. Gene expression analysis demonstrated that *KoWRKY40* was induced highly in the roots and leaves, but lowly in stems in *K. obovata* under cold stress. Overexpression of *KoWRKY40* in *Arabidopsis* significantly enhanced the fresh weight, root length, and lateral root number of the transgenic lines under cold stress. *KoWRKY40* transgenic *Arabidopsis* exhibited higher proline content, SOD, POD, and CAT activities, and lower MDA content, and H₂O₂ content than wild-type *Arabidopsis* under cold stress condition. Cold stress affected the expression of genes related to proline biosynthesis, antioxidant system, and the ICE-CBF-COR signaling pathway, including *AtP5CS1*, *AtPRODH1*, *AtMnSOD*, *AtPOD*, *AtCAT1*, *AtCBF1*, *AtCBF2*, *AtICE1*, *AtCOR47* in *KoWRKY40* transgenic *Arabidopsis* plants.

**Conclusion:** These results demonstrated that *KoWRKY40* conferred cold tolerance in transgenic *Arabidopsis* by regulating plant growth, osmotic balance, the antioxidant system, and ICE-CBF-COR signaling pathway. The study indicates that *KoWRKY40* is an important regulator involved in the cold stress response in plants.

**Keywords:** Mangrove plant, *Kandelia obovata*, WRKY gene, Transgenic, Cold stress

---

**Background**

Mangrove plants are tropical/subtropical communities of xyllophyta that grow in the intertidal zones of coastlines [1]. As an important marine wetland ecosystem, mangrove plants play important ecological and economic roles, such as keeping coastlines and beaches away from storm tide and tsunami, remediating contaminated environment, and providing food and shelters for marine organisms [2, 3]. As a dominant community, mangrove plants have evolved to adapt and survive in such extreme habitat (high ultraviolet radiation, high salinity, submerged and hypoxia soil) [4]. Generally, plants have developed complex mechanisms to adapt to stress conditions. Among them, some important transcription

---

¹State Key Laboratory of Tropical Oceanography, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China

*Correspondence: yswang@scsio.ac.cn, chenghao@scsio.ac.cn

Full list of author information is available at the end of the article
The WRKY TFs, one of the largest transcription factor families, involved in various biological processes in plants [7]. WRKY family members have a highly conserved 60 amino acids of DNA-binding regions, called WRKY domain. The WRKY domain contains the conserved WRKYGQK sequence at N-terminal and zinc-finger structure at C-terminal [8]. The DNA binding domain of WRKY is mostly invariant WRKYGQK, but it still has differences from other domain, such as WKKYGQK, WRKYGMK, WSKYGQK, WKRYGQK, but it still has differences from other domain, such as WKKYGQK, WRKYGMK, WSKYGQK, WKRYGQK, WRKY proteins was analyzed. The expression patterns of WRKY proteins, indicating that they belong to the Group II WRKY subfamily (Fig. 1).

KoWRKY40, a novel WRKY gene isolated from K. obovata, had high homology with those known WRKY genes. According to RACE technology, the full-length cDNA of this WRKY gene was obtained by sequence assembly and re-amplification. Sequence analysis revealed that the cDNA fragment is 1420bp in length, containing a 127-bp 5'-untranslated region (UTR), a 336-bp 3'-UTR and a 957-bp complete open reading frame (ORF). The gene encoded a protein with 318 amino acid residues with an estimated molecular mass (MW) of 33.59kDa and isoelectric point (pI) of 8.76. This gene has been deposited in GenBank (GenBank accession No. KP267757.1) and was designated as KoWRKY40 in this study. According to EXPASy Molecular Biology Server, the Ser (10.1%), Lys (7.9%), Thr (7.2%), Val (7.2%), Ala (6.9%), Asn (6.9%) Glu (6.9%) and Leu (6.6%) contents were high, but the Trp occupied the lowest (0.6%) portion in KoWRKY40 amino acid sequence. In the secondary structure of KoWRKY40, α-helix accounted for 41.19%, β-sheet for 16.35%, β-turn for 5.97%, and random coil for 36.48%. Bioinformatics analysis showed that KoWRKY40 contained two transmembrane regions (residue positions 45–60 and 299–314), but had no predicted signal peptide. A putative nuclear localization signal (NLS) RKRK is existed on the residue positions 99–102 (Fig. 1), showing that KoWRKY40 is located in the nucleus. The sequence alignment indicated that KoWRKY40 protein had high homology with other eight WRKY TFs (Fig. 1). These nine WRKY TFs all contain a WRKYGQK sequence and a C2H2 zinc-finger structure, which are the typical features of Group II WRKY proteins, indicating that they belong to the Group II WRKY subfamily (Fig. 1).

**Results**

**Characterization and sequence analysis of the KoWRKY40**

Initially, a fragment with 334bp was obtained from our previous study, which has the homology with those known WRKY genes. According to RACE technology, the full-length cDNA of this WRKY gene was obtained by sequence assembly and re-amplification. Sequence analysis revealed that the cDNA fragment is 1420bp in length, containing a 127-bp 5'-untranslated region (UTR), a 336-bp 3'-UTR and a 957-bp complete open reading frame (ORF). The gene encoded a protein with 318 amino acid residues with an estimated molecular mass (MW) of 33.59kDa and isoelectric point (pI) of 8.76. This gene has been deposited in GenBank (GenBank accession No. KP267757.1) and was designated as KoWRKY40 in this study. According to EXPASy Molecular Biology Server, the Ser (10.1%), Lys (7.9%), Thr (7.2%), Val (7.2%), Ala (6.9%), Asn (6.9%) Glu (6.9%) and Leu (6.6%) contents were high, but the Trp occupied the lowest (0.6%) portion in KoWRKY40 amino acid sequence. In the secondary structure of KoWRKY40, α-helix accounted for 41.19%, β-sheet for 16.35%, β-turn for 5.97%, and random coil for 36.48%. Bioinformatics analysis showed that KoWRKY40 contained two transmembrane regions (residue positions 45–60 and 299–314), but had no predicted signal peptide. A putative nuclear localization signal (NLS) RKRK is existed on the residue positions 99–102 (Fig. 1), showing that KoWRKY40 is located in the nucleus. The sequence alignment indicated that KoWRKY40 protein had high homology with other eight WRKY TFs (Fig. 1). These nine WRKY TFs all contain a WRKYGQK sequence and a C2H2 zinc-finger structure, which are the typical features of Group II WRKY proteins, indicating that they belong to the Group II WRKY subfamily (Fig. 1).

**Three-dimensional model of KoWRKY40**

Based on the deduced amino acid sequence, the predicted three-dimensional (3D) model of KoWRKY40 was constructed using SWISS MODEL software (Fig. 2). There is a 50% similarity between the amino acid sequences that need for the formation of the 3D model [24]. Modeling results showed that the sequence similarity was 51.35% between the KoWRKY40 and
demonstrating the 3D model of KoWRKY40 was reasonable and credible. According to the surface charge distribution of 3D structure, positive charges occupied more area than negative charges in KoWRKY40 protein (Fig. 2B), indicating KoWRKY40 was a positive charge protein. Sequence analysis indicated that KoWRKY40 contained 38 negatively charged residues (Glu and Asp) and 43 positively charged residues (Lys and Arg), which means that KoWRKY40 was positively charged. In general, a positive charge indicates the hydrophobicity of the protein. Sequence analysis reconfirmed that the protein is indeed hydrophobic. The structure of KoWRKY40 consisted of a five-strand anti-parallel β-sheet (β1, 149–153; β2, 168–174; β3, 183–188; β4, 197–202; β5, 210–215. see Fig. 2A). The 147 residues at the N-terminus (including the NLS motif) and the 94 residues at the C-terminus are not included in the structure (see Fig. 2D). Due to the α-helix (Leu154-Asp157) and the long bridging loop (Thr158-Gln167) between β1 and β2, the structure of KoWRKY40 looked spherical and stable (Fig. 2A, B). The zinc ion existed as an independent ligand and zinc coordination residues interact with amino acid residues at Cys188, Cys199, His218, His220, which are the core residues of the C2H2 zinc finger structure of KoWRKY40 (Fig. 2A). The C2H2 structure was located at one end of the β sheet (β5), between strands β3 and β4. The presence of zinc ion was essential for the DNA-binding activity, indicating the importance of the zinc-binding motif [14, 26]. In Fig. 1, there were more than 17 well-conserved residues in the area between β2 and β3, including WRKYGQK sequence. Since the WRKY motif is responsible for binding to W-box [14], and the ‘WRKYGQK’ sequence of WRKY motif spanned the entire β2 strand, indicating the importance of β2 of KoWRKY40 in the DNA-binding activity. Therefore, we infer that the β2 and β3 strands are
likely to participate in DNA binding, and the loop between β2 and β3 may participate in conformational changes of DNA binding [25].

**Phylogenetic analysis of KoWRKY40**

We have downloaded all available *Arabidopsis* WRKYs by NCBI blast. The phylogenetic analysis of KoWRKY40 protein with *Arabidopsis* WRKYs was performed as shown in Supplementary Fig. S1. In comparing with the 58 *Arabidopsis* WRKYs, KoWRKY40 showed the closest phylogenetic relationship to WRKY transcription factor 40 of *A. thaliana* that belongs to Group II WRKY family. In order to further investigate the evolutionary relationship of KoWRKY40, 26 WRKY TF proteins that showed close homology with KoWRKY40 sequence by NCBI blast were downloaded and a phylogenetic tree was constructed (Fig. 3). These representative WRKY TFs were classified into three groups, and the clusters and groups were well-supported by the bootstrap values. The phylogenetic tree indicated that KoWRKY40 was more closely related to the Group II of WRKY family. Besides, the KoWRKY40 protein showed the closest phylogenetic relationship to BgWRKY, which was a putative WRKY protein from mangrove plant *Bruguiera gymnorrhiza* (GenBank accession No. BAG15874.1) [27].
As a transcription factor, WRKY proteins always possess NLS motif and were located in nucleus [28]. In order to verify the subcellular localization of KoWRKY40, the control vector 35S-GFP and the fusion expression vector 35S-KoWRKY40-GFP were transiently transformed into the epidermal leaf cells of tobacco *Nicotiana benthamiana* (Fig. 4), respectively. DAPI fluorescent dye was used as a nuclear marker. In leaves transformed with 35S-GFP

---

**Fig. 3** The phylogenetic analysis of KoWRKY40 protein. The phylogenetic tree consisted of 27 amino acid sequences from the NCBI database and marked with accession numbers. The red triangle represented KoWRKY40 protein. The scale indicates the length of the branch.

**Fig. 4** Subcellular localization of KoWRKY40. The fusion protein KoWRKY40-GFP (pCAMBIA2301-35S-KoWRKY40-GFP) and GFP (pCAMBIA2301-35S-GFP) were transiently expressed in *N. benthamiana* epidermal cells. Photographs were taken in bright and fluorescence fields after DAPI staining. Scale bar = 16 μm.
vector, the green fluorescent signal of GFP was distributed through the cell. However, the green fluorescent signal was targeted specifically to the nucleus in leaves transformed with 35S-KoWRKY40-GFP (Fig. 4). These results demonstrated that KoWRKY40 is a nuclear-localized protein, and provided direct evidence for the nuclear localization of KoWRKY40.

Expression patterns of KoWRKY40 in response to cold stress
Cold stress usually affects plant growth. The leaves of K. obovata gradually withered with time extension under cold stress (Fig. 5A). Compared with normal condition (CK), the expressions of KoWRKY40 were obviously induced in different tissues under cold stress (Fig. 5B). In leaves, the expressions of KoWRKY40 were significantly higher under cold stress than that under normal condition during the whole tested-period \((p^* < 0.05)\). In stems and roots, the expressions of KoWRKY40 were significantly increased under cold stress in comparison with normal condition \((p^* < 0.05)\) at 4 d, 7 d, 15 d and 20 d. In addition, the gene expressions of KoWRKY40 were increased highly in the roots and leaves, but lowly in the stems under cold stress (Fig. 5B). During the tested time period, the expressions of KoWRKY40 were all increased at first, and then decreased in leaves, stems and roots, respectively in cold-treatments. The difference was that the time points of the highest expression levels were different, at 2 d (15.28-fold), 4 d (5.47-fold) and 15 d (106.82-fold) in leaves, stems and roots, respectively. These data indicated that leaves were the first to respond to cold stress, followed by stems and roots. After 4-days cold treatment, the expression levels of KoWRKY40 were higher in roots (17.65-fold) than that in leaves and stems under cold stress. These results suggested that KoWRKY40 mainly reacted in the leaves at early phases (before 2 d), and principally played roles in roots at late phases (after 4 d) under cold stress. After 20-days cold treatment, the expressions of KoWRKY40 in different tissues were decreased to low levels, probably because the plants withered at this time point (Fig. 5A). These results may suggest that the 20-days of cold treatment may have exceeded cold tolerance limit time of K. obovata. It was indicated that KoWRKY40 may play important roles in the signaling network of K. obovata in response to cold stress.

Overexpression of KoWRKY40 enhanced the tolerance to cold stress in transgenic Arabidopsis
To investigate the role of KoWRKY40 in the response to cold stress, transgenic Arabidopsis plants that overexpressed KoWRKY40 were generated. The wild-type (WT) Arabidopsis which has no expression of KoWRKY40 transcript, and three KoWRKY40 transgenic Arabidopsis lines

---

Fig. 5  Morphological characters and expression of KoWRKY40 of K. obovata under cold stress. A The morphological characters of K. obovata at different times under cold stress. B The relative expression levels of KoWRKY40 in leaves, stems and roots under cold stress and normal condition. The relative expression of KoWRKY40 were standardized using the expression of reference gene Ko18S. The data represented the average values of three biological repetitions. The error bars showed the standard deviations \((p\) values was calculated by Student’s t test. \(*p < 0.05; **p < 0.01)\)
(Line 1, Line 3 and Line 6) which demonstrated relatively high expression of KoWRKY40 (Fig. 6), were selected and analyzed for stress tolerance. As shown in Fig. 7, both transgenic and wild-type (WT) seedlings grew well and showed no significant difference in phenotype, fresh weight, root length, and lateral root number under normal growth condition (CK). Nevertheless, the transgenic lines showed more green leaves, less black or yellow leaves than the wild type under cold stress (Fig. 7B). Besides, the fresh weight (Fig. 7C), root length (Fig. 7D), and lateral root number (Fig. 7E) of KoWRKY40 transgenic Arabidopsis plants were significantly higher than that of WT plants under cold stress condition. The results showed that KoWRKY40 transgenic lines grew better than WT plants under cold stress condition.

To explore the involvement of KoWRKY40 in osmoregulation in Arabidopsis under cold stress condition, the proline content was measured in WT and transgenic plants. As shown in Fig. 8A, the proline content was significantly higher in transgenic lines than in WT lines under cold stress condition, but did not differ significantly between the two types of plants under normal growth condition. These results suggested that overexpression
of KoWRKY40 enhanced the osmoregulatory capacity of Arabidopsis plants by increasing the proline content of plant cells, conferring cold tolerance to transgenic plants. To confirm the involvement of KoWRKY40 in the antioxidant function of Arabidopsis under cold stress condition, the malondialdehyde (MDA) content, hydrogen peroxide (H$_2$O$_2$) content, and the activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), were examined in WT and KoWRKY40 transgenic lines. As shown in Fig. 8B-F, MDA content and H$_2$O$_2$ content were significantly lower, whereas SOD, POD, and CAT activities were higher in KoWRKY40 transgenic lines than in WT plants under cold stress condition. These results indicated that overexpression of KoWRKY40 decreased membrane damage, ROS (reactive oxygen species) level, and enhanced the efficiency of antioxidant systems Arabidopsis under cold stress condition.

To further investigate the regulatory role of KoWRKY40 in response to cold stress, the expression levels of important genes involved in osmotic adjustment (AtP5CS1 and AtPRODH1), ROS scavenging (AtMnSOD, AtPOD, and AtCAT1) and ICE-CBF-COR signaling pathway (AtCBF1, AtCBF2, AtICE1 and AtCOR47) were examined in KoWRKY40 transgenic and WT plants. The expression levels of AtP5CS1, AtMnSOD, AtPOD, AtCAT1, AtCBF1, AtCBF2, AtICE1 and AtCOR47 were significantly higher in KoWRKY40 transgenic lines than in WT plants (as shown in Fig. 9). However, the expression level of proline dehydrogenase gene, AtPRODH1, was remarkably lower in KoWRKY40 transgenic lines than in WT plants (Fig. 9B). These results suggested that overexpression of KoWRKY40 in Arabidopsis regulated the expression of genes related to osmolytes, antioxidant biosynthesis, and ICE-CBF-COR signaling pathway under cold stress condition, generating transgenic Arabidopsis plants with improved osmoregulation, antioxidant defenses, and cold-related genes activation, thereby conferred to cold tolerance.

**Discussion**

Many researches have reported that the WRKY proteins play important roles in response to abiotic stresses [29–31]. Although there have been many studies on WRKY gene in other plants [8, 13, 14], little was known about WRKY genes in mangrove plants [6]. In this paper, based on our previous study [23], we cloned the
full-length sequence of *KoWRKY40* gene from *K. obovata*. The deduced amino acid sequence showed that KoWRKY40 was a group II WRKY protein.

In general, WRKY proteins are characterized by their domains, which are typically about 60 residues in length and has a conserved WRKYGQK motif. The WRKYGQK motif is a W-box element (C/T) TGAC (C/T) located in the promoter region of the target genes and is essential for DNA binding activity [14]. The 3D structure of WRKY4 exactly showed that conserved WRKYGQK residues can participate in DNA binding [32]. However, the core WRKYGQK sequence of WRKY protein is sometimes replaced by WRKYGKK, which is common variant in canola [33], tomato [9], and pepper [34]. It has been found that each amino acid residue of WRKYGQK sequence was replaced, the binding affinity with DNA was significantly decreased [26]. In our study, the KoWRKY40 possessed the WRKYGQK motif. Therefore, we speculated that the KoWRKY40 may recognize the W-box based on its typical WRKYGQK motif.
The transmembrane helix is required for membrane-associated proteins related to cell signal transduction and substrate transport [35]. Sequence analysis showed that the KoWRKY40 protein contained two transmembrane helixes, indicating the KoWRKY40 might actively participate in signal transduction. Previous researches had reported that most WRKY proteins contained a basic NLS [36], and GFP-WRKY fusion protein have demonstrated that diverse WRKY TFs were located in nucleus [20, 36, 37]. In this study, KoWRKY40 protein contained the NLS sequence RKRK and was predicted to be localized in the nucleus. Some studies have reported that merged fluorescence signals of GFP and DAPI were visualized for subcellular localization [38, 39], the DAPI staining was used to detect the florescent signals of nuclei for further confirmed the nuclear localization of KoWRKY40. Subcellular localization analysis further confirmed that KoWRKY40 was nuclear-localized protein in this study. These data indicated that our results were consistent with previous studies.

Some studies have reported that the expression of WRKY was tissue specific under abiotic stresses. For example, the LcWRKY5 gene was expressed only in leaves and roots in sheepgrass [40]. In Arabidopsis, the AtWRKY25 gene was mainly expressed in roots [41]. The expression of CsWRKY2 in leaves was higher than in other organs in defense response in tea plant [20]. In our study, the KoWRKY40 gene was highly induced in the roots and leaves under cold stress in K. obovata. Thus, we speculated that KoWRKY40 may play an important role in K. obovata under cold stress.

It was worth mentioning that the expression level of KoWRKY40 was increased to the highest at 15 d (106.82-fold) in roots under cold stress, but that was dramatically decreased at 20 d (2.64-fold). This variation tendency of KoWRKY40 expression levels was usually agreement with morphological changes. Seedlings that had been cold-treated (5℃) for 15 days were still alive after being cultured for 20 d at the recovery temperature (25℃). However, seedlings that had been cold-treated (5℃) for 20 days have withered and fallen (data not shown here) under the same recovery condition. These results suggested that the expression of KoWRKY40 may be closely related to the survival of K. obovata, and play important roles in protecting K. obovata from cold stress. Based on the high consistency between expression of KoWRKY40 at the molecular level and recovery survival at morphological level, we speculated that 15–20 days may be the upper limit of survival time of K. obovata seedlings at low temperature (5℃).

WRKY proteins have involved in the response of plants to various stresses, and their homologs have been found in various plant species [29]. Several WRKY proteins were found to be induced in response to cold stress in maize [42], cotton [43] and barley [44]. However, there was an evidence reported that one tobacco WRKY gene was specifically induced during collaborative pressure of drought and heat, but not by drought or heat stress alone, indicating this WRKY genes were induced only by a combination of different stresses [45]. In this study, the KoWRKY40 expression was induced during the entire process under cold stress, indicating the KoWRKY40 gene could be induced by cold stress individually. Since mangrove plants generally grow in the intertidal zones and were subjected to various stresses [1–3], whether other WRKY genes of mangrove plants were induced by individual or combined stresses remains to be studied.

WRKY protein can activate or inhibit the transcription of downstream by combining with W-box cis-acting elements of target genes [8, 46]. Since W-box elements existed in most WRKYs, the WRKYs can bind with their own promoters to achieve self-regulation or cross-regulation networks [47]. CaWRKY6 transcriptionally activated CaWRKY40, and conferred the plant more tolerant to high temperature and humidity in pepper [48]. AtWRKY34 had a negative regulatory effect on cold response pathway, inducing enhanced resistance to cold stress [49]. WRKY proteins could be quickly and differentially expressed, regulating the expression of downstream genes and promoting signal transduction [41]. For example, the antioxidant enzyme genes AtSOD, AtCAT, and AtAPX1, which can scavenge reactive oxygen species (ROS) to protect plants from oxidative damage [50], are important downstream genes regulated by WRKY TFs. In our study, the expression levels of AtMnSOD, AtPOD, and AtCAT1 were up-regulated in KoWRK40 transgenic Arabidopsis plants under cold stress, acceleration of which could increase tolerance to cold stress in transgenic Arabidopsis plants.

The plant response to cold stress is rather complex. A frequent plant response to abiotic stress is to accumulate certain osmolects, particularly proline [51–53], which then function as osmoprotectants. In Arabidopsis, the genes encode P5CS isoforms involved in proline biosynthesis, AtP5CS1 (AT2G39800), and the genes encode ProDH isoforms involved in the degradation of proline, AtPRODH1 (AT3G30775) [54–56]. In this study, the proline biosynthesis gene AtP5CS1 was up-regulated, and the proline degradation gene AtPRODH1 was down-regulated in KoWRKY40 transgenic Arabidopsis, compared with their expression in WT plants (Fig. 9E, F). This suggested that KoWRKY40 increased the proline content to enhance the osmoregulatory capacity of transgenic Arabidopsis in response to cold stress by inducing proline biosynthesis genes and inhibiting proline degradation genes. Cold stress also can induce the rapid generation
and accumulation of ROS, resulting in secondary oxidative stress in plants [57]. Antioxidant enzymes (e.g., SOD, POD and CAT) can scavenge ROS to protect plants from oxidative damage [50]. The increase of cold tolerance in plants may due to the high expression of these genes [58–60]. In the present study, KoWRKY40 transgenic plants exhibited higher activities of SOD, CAT, POD, and lower contents of MDA and \( \text{H}_2\text{O}_2 \) than WT Arabidopsis under cold stress conditions (Fig. 8B–F). Three genes related to ROS scavenging, \( \text{AtMnSOD} \), \( \text{AtCAT1} \), and \( \text{AtPOD} \), were up-regulated in \( \text{KoWRKY40} \) transgenic Arabidopsis (Fig. 9C–E). Thus, our data suggested that \( \text{KoWRKY40} \) increased the activities of antioxidant enzymes, and decreased system damage in transgenic Arabidopsis plants under cold stress condition. In \( \text{A. thaliana} \), \( \text{AtCBF1} \), \( \text{AtCBF2} \), \( \text{AtICE1} \), and \( \text{AtCOR47} \) genes involved in ICE-CBF-COR signaling pathway have been proved to play important roles in cold tolerance [61–65].

In our study, overexpression of \( \text{KoWRKY40} \) enhanced cold tolerance in transgenic Arabidopsis plants and these cold-resistance genes were all significantly increased in \( \text{KoWRKY40} \) transgenic Arabidopsis plants. Thus, we suggested that accumulation of which may help to develop cold acclimation and protect transgenic plants from cold damage.

**Conclusions**

To summarize, \( \text{KoWRKY40} \), a new WRKY transcription factor gene from mangrove plant \( \text{K. obovata} \) was reported in this study. The KoWRKY40 protein was a nuclear-localized protein and a member of group II WRKY family. The expression of \( \text{KoWRKY40} \) was highly induced in the roots and leaves in \( \text{K. obovata} \) under cold stress. In addition, \( \text{KoWRKY40} \) enhanced cold tolerance of transgenic Arabidopsis lines by activating different stress responsive genes involved in osmotic adjustment (\( \text{AtP5CS1} \) and \( \text{AtPRODH1} \)), ROS scavenging (\( \text{AtMnSOD} \), \( \text{AtPOD} \) and \( \text{AtCAT1} \)) and ICE-CBF pathway (\( \text{AtCBF1} \), \( \text{AtCBF2} \), \( \text{AtICE1} \), \( \text{AtCOR47} \)). These results provide key insight into the role of this gene in \( \text{K. obovata} \) that \( \text{KoWRKY40} \) acts as a positive regulator of cold stress tolerance. This study suggested that \( \text{KoWRKY40} \) may be a valuable genetic resource in molecular breeding programs of plants. Future studies are needed to elucidate the functional mechanism of \( \text{KoWRKY40} \) against abiotic stresses.

**Methods**

**Plant material, growth conditions and treatments**

The hypocotyls of \( \text{K. obovata} \) were provided by Guangdong Mangrove Ecological Technology Co. Ltd. (China). The hypocotyls were surface-disinfected and sown in clean sand at room temperature. The 3-months seedlings were transferred into growth chamber with normal condition (25 °C, relative humidity 75%, 14 h light/10 h dark cycle). After 7 days, the seedlings were treated under cold condition (5 °C) for 0 h, 6 h, 12 h, 1 d, 2 d, 4 d, 7 d, 15 d and 20 d. Seedlings treated at 5 °C for 0 h were used as the control. All treatments contained at least three seedlings. The harvested leaves, stems and roots were immediately frozen in liquid nitrogen, and then transferred to −80 °C refrigerator until use.

**RNA extraction and reverse transcription**

Total RNA was extracted from leaves, stems and roots of \( \text{K. obovata} \) using the Tiangen RNA plant Plus Reagent (Tiangen Biotech, Beijing) according to the method as described [66, 67]. The 1.0% agarose gel was used to analyze the integrity and purity of total RNA. The quality and content of RNA were determined by Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). The RNase-free DNase I (Promega, USA) was used to remove potential genomic DNA contamination of total RNA. The RNA samples was then subjected to synthesize the first strand cDNA by SMART™ reverse transcription Kit (Clontech, USA) following the manufacturer’s protocol. The cDNA samples were used for cloning the full-length of \( \text{KoWRKY40} \) gene and RT-qPCR analysis.

**Cloning the complete sequence of \( \text{KoWRKY40} \) gene**

The rapid amplification of cDNA ends (RACE) PCRs was performed from the first cDNA strand of \( \text{K. obovata} \) leaves. A complete cDNA sequence was assembled by combining the 5′-RACE and the 3′-RACE fragments. Based on the partial sequences (GenBank accession number: JZ585678.1) from our previous study [23], the gene-specific primers (GSP1, 5′-GCTACTAGGACTGAAACATCCTCCGCAC-3′, GSP2, 5′-GGTGGCATACGAAACCAGCTGTAGCGA-3′) were used as primary PCR to obtain 5′ and 3′ end sequences, respectively. The nested PCR reactions were performed by nested primers (NGSP1, 5′-ATCCTCCGCAGGCTTCTCCTGACCGGCCTCTTC-3’, NGSP2, 5′-GAAACCAGCTGTAGCGACGAGGTCCTCAAA-3′). All steps of RACE were carried out using SMART™ RACE cDNA Amplification Kit (Clontech, USA) on the basis of the manufacturer’s protocol. The RACE products were purified by agarose gel and then were cloned into pMD19-T Vector for sequencing. The 5′- and 3′- nucleotide sequences were assembled by DNAMAN software through overlap to acquire the full-length \( \text{KoWRKY40} \) sequence. The assembled sequence was used to design the primers, and the full-length sequence of \( \text{KoWRKY40} \) was cloned and sent to the company (BGI, China) for sequencing. Finally, the complete
cDNA sequence of KoWRKY40 was confirmed, and was submitted to GenBank to obtain the accession number KP267757.1.

Bioinformatic analysis
The DNAMAN software was used to obtain full-length cDNA sequence through overlap fragments by linking sequences. NCBI BLAST tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied for sequence analyses and comparisons. The deduced amino acid sequence of KoWRKY40 (GenBank accession number: KP267757.1) was inferred by ORF Finder (http://www.ncbi.nlm.nih.gov/orffinder). Compute pi/MW tool (http://web.expasy.org/compute_pi/) was used to analyze the molecular mass and isoelectric point of KoWRKY40. The transmembrane domain prediction was analyzed by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). The Motif Scan (http://myhits.isb-sib.ch/cgi-bin/motif_scan) was used to detect motif sequences. The subcellular localization was predicted by PSORT (http://www.psort.org/) and Softberry ProComp v. 9.0 (http://linux1.softberry.com/all.htm). The signal peptides of KoWRKY40 were predicted using SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). Multiple alignments of the diverse WRKY proteins were generated by the BioEdit software. The phylogenetic tree of WRKY proteins was constructed by Clustal X software and MEGA 5.0 software with the neighbor-joining algorithm. The reliability of the phylogenetic tree was tested by bootstrap analysis with 1000 replicates. The SOPMA algorithm. The reliability of the phylogenetic tree was tested with GraphPad Prism 7.0 (GraphPad Software, San Diego, California). One-way ANOVA was selected to quantify the significant differences among expressions of KoWRKY under cold stress and normal condition by SPSS statistics 25.

Subcellular localization analysis
To determine the subcellular localization of KoWRKY40, the ORF of KoWRKY40 termination-codon free was cloned into vector pCAMBIA2301-35S-GFP. The Nicotiana benthamiana leaf preparation and transformation were performed as described [39]. The GFP fusion expression vector pCAMBIA2301-35S-KoWRKY40-GFP was analyzed and sequenced, and the successful fusion was confirmed. The recombinant plasmid was introduced into Agrobacterium tumefaciens strain EHA105, and transformed transiently into the leaf epidermal of N. benthamiana by Agrobacterium infection. The 35S-GFP vector was taken as the control. To locate the fluorescent proteins in nuclei, the N. benthamiana leaves were infiltrated with PBS containing 4',6-diamidino-2-phenylindole (DAPI) for 20 min, and the fluorescence microscopic image was observed by Zeiss LSM710 laser scanning confocal microscope to determine the subcellular location of KoWRKY40-GFP.

Expression analysis by RT-qPCR
To analyze KoWRKY40 expression in K. obovata under cold stress, the real-time quantitative PCR (RT-qPCR) was performed to determine the transcription levels in leaves, stems and roots of K. obovata. The samples were collected from different tissues of K. obovata at 0 h, 6 h, 12 h, 1 d, 2 d, 4 d, 7 d, 15 d and 20 d, respectively, under cold condition (5°C). For comparison, the samples were also collected at same time points under normal condition (25°C). The samples collected at 0 h were considered as the control. Total RNA was extracted and reverse-transcribed into cDNA in the above experiment. The internal reference gene was selected with the 18S (Ko18S) rRNA of K. obovata. The RT-qPCR reactions were carried out by iCycler iQ5 real time PCR detection system (Bio-Rad, CA, USA) according to SYBR Premix Ex Taq™ II (Takara, Dalian, China) manufacturer’s instructions. The RT-qPCR reaction parameters were 95°C for 1 min, 40 cycles at 95°C for 10 s, 55°C for 30 s, and 72°C for 40 s. The 2−ΔΔCT method was used to calculate the relative expression levels of gene [68, 69] comparing with 0 h. Sequences of primers used for RT-qPCR analysis are listed in Supplementary Table S1. Three biological replicates and three technical replicates were performed for each sample. The data was expressed as mean ± standard deviation (x ± SD). Statistical analyses were carried out by Student t-test, and diagramming was performed with GraphPad Prism 7.0 (GraphPad Software, San Diego, California). One-way ANOVA was selected to quantify the significant differences among expressions of KoWRKY under cold stress and normal condition by SPSS statistics 25.

Generation of KoWRKY40 transgenic Arabidopsis plants
For KoWRKY40 overexpression in wild-type Arabidopsis thaliana (WT; Columbia), the full-length KoWRKY40 sequence without the stop codon was cloned into pCAMBIA2301 and driven by CaMV 35S promoter. The coding sequence of KoWRKY40 (with KpnI site added to its 5′ and 3′ ends, respectively) was amplified from pMD19-T-KoWRKY40 using gene-specific primers F1 (5′-GGCGGTCCGACGGTACCATGGGATCATTGAGGTGAAC-3′) and R1 (5′-TAGACATATGGTGATCCGAAATGTGTTGTCCTGAAGG-3′). The digested amplicon was inserted into pCAMBIA1301 driven by the CaMV 35S promoter, and confirmed by sequencing. This recombinant vector, named pCAMBIA2301-35S:KoWRKY40, was transformed into A. thaliana EHA105 by the freeze-thaw method and then transformed into Arabidopsis plants by the floral dip method [70]. Positive Arabidopsis
transgenic lines were harvested and selected by culturing on MS medium agar plates containing 50 mg/L kanamycin, and these were advanced by self-pollinated until obtaining T₃ transgenic plants. Transgenic plants were validated further by RT-PCR (reverse transcription-PCR) analysis with gene-specific primers F2 (5′-GAGGCACAA TCCCACTATCC-3′) and R2 (5′-GAATGTTGTTCC TGAAGG-3′). Finally, the T3 or T4 homozygous lines were used for all the subsequent experiments.

Physiological analysis of transgenic A. thaliana lines under cold stress

Seeds of WT Arabidopsis and KoWRKY40 transgenic Arabidopsis lines (Lines 1, 3 and 6) were surface sterilized by soaking in 70% ethanol (v/v) for 5 min, and then rinsed four to five times with sterile distilled water. The sterilized seeds were grown on solidified MS medium for 6 days and transplanted onto new square plates with MS medium under normal conditions for 10 days before treatments. For cold tolerance evaluation, some wild-type and KoWRKY40 transgenic plants were cultured under normal conditions as the control and others were treated under cold stress (5°C) for 10 days, and their fresh weight, root length, lateral root number and other physiological parameters were measured. The contents of proline, MDA, and H₂O₂, and the activities of SOD, POD, and CAT, were measured using the corresponding assay kits (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. The data was expressed as mean ± standard deviation (x ± SD). All the experiments were carried out in triplicate for biological replicates. Statistical analysis was performed with GraphPad Prism 7.0 (GraphPad Software, San Diego, California). One-way ANOVA followed by Duncan’s test was selected to quantify the significant differences by SPSS statistics 25.

Expression analysis of stress-related genes in transgenic A. thaliana lines

To examine the expression of stress-related genes, cDNA was synthesized from RNA extracted from the leaves of WT Arabidopsis and KoWRKY40 transgenic lines (Lines 1, 3 and 6). The expression levels of cold-related genes were measured by qRT-PCR as described above. The stress-related genes monitored were AtP5CS1 (AT2G39800), AtPRODH1 (AT3G0775), AtMnSOD (AT3G56350), AtPOD (AT3G49120), AtCAT1 (AT1G20630), AtCBF1 (AT4G25490), AtCFB2 (AT4G25470), AtICE (AT3G26744) and AtCOR47 (AT1G20440). The expression level of KoWRKY40 in transgenic lines was also measured. Arabidopsis AtACTIN2 (AT3G18780) was used as a reference gene in the RT-qPCR reactions. The gene primer sequences used are listed in Supplemental Table S1. Relative gene expression values were calculated using the 2^ΔΔCt Method as described above. Three biological replicates and three technical replicates were performed for each sample. The data was expressed as mean ± standard deviation (x ± SD). All the experiments were carried out in triplicate for biological replicates. Statistical analysis was performed with GraphPad Prism 7.0 (GraphPad Software, San Diego, California). One-way ANOVA followed by Duncan’s test was selected to quantify the significant differences by SPSS statistics 25.

Abbreviations

TF: Transcription factor; GFP: Green fluorescent protein; RT-qPCR: Real-time quantitative PCR; DAPI: 4′,6-diamidino-2-phenylindole.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-022-03661-2.

Additional file 1: Supplementary Figure S1. The phylogenetic analysis of KoWRKY40 with Arabidopsis WRKYs. The phylogenetic tree contained 58 Arabidopsis thaliana WRKYs, which were downloaded from the NCBI database and marked with accession numbers. The red triangle represented KoWRKY40 protein. The blue triangle represented AtWRKY1 protein, which was the template of KoWRKY40 for building 3D model. The scale indicates the length of the branch.

Additional file 2: Supplementary Table S1 Gene primers used in real-time RT-PCR.

Additional file 3: Supplementary Figure S2. Original gel of Figure 6. RT-qPCR identification of KoWRKY40 expression in WT and KoWRKY40 transgenic lines. The AtACTIN2 gene was amplified as a control.

Acknowledgements

We specially thank Xiao-Mei Li, Bo-Yu Su, Shu-Min Wang and Dong-Xi Liu of University of Chinese Academy of Sciences for their assistance in the analyses of physiological parameters and stress-related genes expression of Arabidopsis plants. We are also very grateful to all our colleagues in our laboratory for their useful technical assistance.

Authors’ contributions

J.F. designed and undertook the experiments and wrote the manuscript. Y.S.W. conceptualized and supervised the manuscript. H.C. supervised the research, and revised and developed this manuscript. Y.B.S. participated the preparation and analysis of subcellular localization and contributed to improve this manuscript. All authors reviewed the manuscript.

Funding

This work was supported by the National Natural Science Foundation of China (No. 41706118, No. 41676086, No. U19101211, No. 41430966 and No. 41876126), the International Partnership Program of Chinese Academy of Sciences (No. 133244KYSB20180012), the National Key Research and Development Plan (No. 2017FY100700) and the Strategic Priority Research Program of the Chinese Academy of Sciences (No. XDA23050200, No. XDA13010500 and No. XDA13020503), the Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou) (GML2019ZD0305). The funding body was not involved in the design of the study, the collection, analysis and interpretation of data, and the writing of the manuscript.
References

1. Woodroffe CD, Rogers K, McKee KL, Lovelock CE, Mendelsohn I, Saintilan N. Mangrove sedimentation and response to relative sea-level rise. Annu Rev Mar Sci. 2016;8:243–66.
2. Muller C, Strydom NA. Evidence for habitat residency and isotopic niche partitioning in a marine-estuarine-dependent species associated with mangrove habitats from the east coast of South Africa. Estuar Coast. 2017;40:1642–52.
3. Theuerkauf D, Rivera-Ingraham GA, Lambert S, Merczy Y, Lejeune M, Lignot JH, et al. Wastewater bioremediation by mangrove ecosystems impacts crab ecology: in-situ caging experiment. Aquat Tox. 2020;28:105358.
4. Kathiresan K, Bingham BL. Biology of mangroves and mangrove ecosystems. Adv Mar Biol. 2001;40:81–251.
5. Chen J, Nolan T, Ye H, Zhang M, Tong H, Xin P, et al. Arabidopsis WRKY46, WRKY54, and WRKY70 transcription factors are involved in brassinosteroid-regulated plant growth and drought responses. Plant Cell. 2017;29:1425–39.
6. Su WY, Ye CT, Zhang YH, Hao SQ, Li QS. Identification of putative key genes for coastal environments and cold adaptation in mangrove Kandelia obovata through transcriptome analysis. Sci Total Environ. 2019;681:191–201.
7. Baillo EH, Hanif MS, Guo Y, Zhang Z, Xu P, Algam SA. Genome-wide identification of WRKY transcription factor family members in sorghum (sorghum bicolor (L.) moench). Plos One. 2020;15:e0236651.
8. Phukan UJ, Jeena GS, Shukla RK. WRKY transcription factors: molecular regulation and stress responses in plants. Front Plant Sci. 2016;7:760.
9. Huang S, Gao Y, Liu J, Peng X, Niu X, Fei Z, et al. WRKY transcription factors in Solanum lycopersicum. Mol Genet Genomics. 2012;287:495–513.
10. Xie Z, Zhang ZL, Zou X, Huang J, Ruas P, Thompson D, et al. Annotation and functional analyses of the rice WRKY gene superfamily reveal positive and negative regulators of abscisic acid signaling in aleurone cells. Plant Physiol. 2004;137:176–89.
11. Van Verk MC, Pappaoaainoud N, Neelam L, Bol DF, Lintohort HJM. A novel WRKY transcription factor is required for induction of PR-1a gene expression by salicylic acid and bacterial elicitors. Plant Physiol. 2008;146:1983–95.
12. Li FF, Zhang L, Ji HK, Xu ZY, Zhou Y, Yang SS. The specific W-boxes of AP2/ERF promoter bound by TaWRKY are involved in drought stress response in wheat. Plant Sci. 2020;296:110460.
13. Eulgem T, Rushton PJ, Robatzek S, Somssich IE. The WRKY superfamily of plant transcription factors. Trends Plant Sci. 2000;5:199–206.
14. Rushton PJ, Somssich IE, Ringler P, Shen QJ. WRKY transcription factors. Trends Plant Sci. 2010;15:247–58.
15. Mehtap SC, Gloria AM. Identification of a drought- and cold-stress inducible WRKY gene in the cold-hardy Citrus relative Poncirus trifoliata. New Zeal J Crop Hort. 2013;41:57–68.
16. Wang LN, Zhu W, Fang LC, Sun XM, Su LY, Liang QZ, et al. Genome-wide identification of WRKY family genes and their response to cold stress in Vitis vinifera. BMC Plant Biol. 2014;14:103.
17. Wei ZP, Ye JF, Zhou ZQ, Chen F, Meng FJ, Li JF. Isolation and characterization of PoWRKY, an abiotic stress-related WRKY transcription factor from Polygonatum odoratum. Physiol Mol Biol Pla. 2021;27:1–9.
18. Zhu H, Jiang YN, Guo Y, Huang JB, Zhou MH, Tang YY, et al. A novel salt inducible WRKY transcription factor gene, AWHRKY75, confers salt tolerance in transgenic peanut. Plant Physiol Bioch. 2021;160:175–83.
19. Wang YJ, Li JH, Chen JQ, Tao L, An Y, Cai HS, et al. Overexpression of the alfalfa WRKY1 gene enhances salt tolerance in soybean. Plos One. 2018;13:e0193382.
20. Wang Y, Shu Z, Wang W, Jiang X, Li D, Pan J, et al. CsWRKY2, a novel WRKY gene from Camellia sinensis, is involved in cold and drought stress responses. Biol Plantarum. 2016;60:1–9.
21. Zhou QY, Tian AG, Zou HF, Xie ZM, Lei G, Huang J, et al. Soybean WRKY-type transcription factor genes, GmWRKY13, GmWRKY21, and GmWRKY54, confer differential tolerance to abiotic stresses in transgenic Arabidopsis plants. Plant Biotechnol J. 2008;6:486–503.
22. Peng YL, Wang YS, Fei J, Sun CC. Isolation and expression analysis of two novel C-repeat binding factor (CBF) genes involved in plant growth and abiotic stress response in mangrove Kandelia obovata. Ecotoxicology. 2020;29:718–25.
23. Fei J, Wang YS, Jiang ZY, Cheng H, Zhang JD. Identification of cold tolerance genes from leaves of mangrove plant Kandelia obovata by suppression subtractive hybridization. Ecotoxicology. 2015;24:1686–96.
24. Dobson CM, Sali A, Karplus M. Protein folding: a perspective from theory and experiment. Angew Chem Int Edit. 1998;37:868–93.
25. Duan MR, Nan J, Liang YH, Mao P, Lu L, Li LF, et al. Arabidopsis thaliana DNA binding mechanism revealed by high resolution crystal structure of Arabidopsis thaliana WRKY1 protein. Nucleic Acids Res. 2007;35:1145–54.
26. Mace K, Hayashi S, Kojima-Suzuki H, Morikami A, Nakamura K. Role of conserved residues of the WRKY domain in the DNA-binding of tobacco WRKY family proteins. Biosci Biotechnol Biochem. 2001;65:2428–36.
27. Yamanaka T, Miyama M, Tada Y. Transcription profiling of the mangrove plant Bruguiera gymnorhiza and identification of salt tolerance genes by Agrobacterium functional screening. Biosci Biotechnol Biochem. 2009;73:304–10.
28. Eulgem T, Rushton PJ, Schmelzer E, Hahlbrock K, Somssich IE. Early nuclear events in plant defence signalling: rapid gene activation by WRKY transcription factors. EMBO J. 1999;18:4689–99.
29. Chen LG, Song Y, Li SJ, Zhang LP, Zou CS, Yu DQ. The role of WRKY transcription factors in plant abiotic stresses. Biochim Biophys Acta. 2012;1819:120–8.
30. Dong J, Chen C, Chen Z. Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response. Plant Mol Biol. 2003;51:211–37.
31. Ulker B, Somssich IE. WRKY transcription factors: from DNA binding towards biological function.Curr Opin Plant Biol. 2004;7:491–8.
32. Yamazaki K, Kigawa T, Iinoue M, Tateno M, Yamazaki T, Yabuki T, et al. Solution structure of an Arabidopsis WRKY DNA binding domain. Plant Cell. 2005;17:944–56.
33. Yang B, Jiang Y, Rahman MH, Deyholos MK, Kaw NN. Identification and expression analysis of WRKY transcription factor genes in canola (Brassica
Armengaud P, Yao Z, Ruan M, Ye Q, Wang R, Zhou G, et al. Identification and characterization of WRKY genes in poplar (Populus trichocarpa) to enable genetic improvement. BMC Plant Biol. 2016;16:1–12.

Nuruzzaman M, Zhang R, Cao HZ, Luo ZY. Plant pleiotropic drug resistance transporters: transport mechanism, gene expression, and function. J Integ Plant Biol. 2014;56:729–40.

Agarwal P, Reddy M, Chikara J. WRKY: its structure, evolutionary relationship, DNA-binding selectivity, role in stress tolerance and development of plants. Mol Biol Rep. 2011;38:983–96.

Guo RY, Yu FF, Gao Z, An HL, Cao XC, Guo XQ. GhWRKY3, a novel cotton (Gossypium hirsutum) WRKY gene, is involved in diverse stress responses. Mol Biol Rep. 2011;38:49–58.

Yu XY, Pan Y, Dong Y, Yu B, Zhang C, Yang MS, et al. Cloning and overexpression of PeWRKY31 from Populus eurameriana enhances salt and biological tolerance in transgenic Nicotiana. BMC Plant Biol. 2021;21:80.

Zheng LP, Yao JN, Gao FL, Chen L, Zhang C, Liang H, et al. The subcellular localization and functional analysis of Fibrillarin2, a nuclear protein in Nicotiana benthamiana. Biomed Res Int. 2016;2016:2831287.

Ma T, Li M, Zhao A, Xu X, Liu G, Cheng L. LcWRKY5: an unknown function gene from sheepgrass improves drought tolerance in transgenic Arabidopsis. Plant Cell Rep. 2014;33:1507–18.

Jiang Y, Deyholos MK. Functional characterization of Arabidopsis NAC-inducible WRKY25 and WRKY33 transcription factors in abiotic stresses. Plant Mol Biol. 2009;69:91–105.

Varagona MJ, Schmidt RJ, Raikhei NV. Nuclear localization signal(s) required for nuclear targeting of the maize regulatory protein Opache-2. Plant Cell. 1992;4:1213–27.

Yang L, Zhi H, Guo W, Zhang T. Molecular cloning and characterization of five genes encoding pentatricopeptide repeat proteins from upland cotton (Gossypium hirsutum L). Mol Biol Rep. 2010;37:281–92.

Mare C, Mazzucotelli E, Crociati C, Francia E, Stanca AM, Cattivelli L. Hv-WRKY38: a new transcription factor involved in cold- and dehydration-response recovery in barley. Plant Mol Biol. 2004;55:399–416.

Rizhsky L, Jiang H, Mittler R. The combined effect of drought stress and heat shock on gene expression in tobacco. Plant Physiol. 2002;130:143–51.

Ciolkowski I, Wanke D, Birkenbihl RP, Somssich IE. Studies on DNA-binding selectivity of WRKY transcription factors and structural clues into WRKY-domain function. Plant Mol Biol. 2008;68:881–92.

Ezenstafugil U, Laun T, Mao T. The complex regulation of WRKY53 during leaf senescence of Arabidopsis thaliana. Eur J Cell Biol. 2010;90:133–7.

Cai H, Yang S, Yan Y, Xiao Z, Cheng J, Wu J, et al. CaWRKY6 transcriptionally activates CaWRKY40, regulates Rab11-related resistance, and confers high-temperature and high-humidity resistance in pepper. J Exp Bot. 2015;66:5163–74.

Zou C, Jiang W, Yu D. Male gametophyte-specific WRKY34 transcription factor mediates cold sensitivity of mature pollens in Arabidopsis. J Exp Bot. 2016;67:3901–14.

Du C, Zhao PP, Zhang HR, Li NN, Zheng LL, Wang YC. The Reaumuria trigona transcription factor RtWRKY1 confers tolerance to salt stress in transgenic Arabidopsis. J Plant Physiol. 2017;215:48–58.

Liu J, Zhu JK. Proline accumulation and salt-stress induced gene expression in a salt-hypersensitive mutant of Arabidopsis. Plant Physiol. 1999;119:591–8.

Armengaud P, Thiery L, Buhor N, Grenier-De March G, Savoure A. Transcriptional regulation of proline biosynthesis in Medicago truncatula reveals developmental and environmental specific features. Physiol Plant. 2004;119:442–50.

Liu Y, Yao Z, Chen N, Ma Q, Li F, et al. Overexpression of OsCOT1, a putative cold inducible zinc finger protein, increased tolerance to chilling, salt and drought, and enhanced proline level in rice. Plant Physiol. 2007;146:1007–16.

Funck D, Winter G, Baumgarten L, Forlani G. Requirement of proline synthesis during Arabidopsis reproductive development. BMC Plant Biol. 2012;12:191–203.

Gecchini NM, Alvarez ME. Proline dehydrogenase contributes to pathogen defense in Arabidopsis. Plant Physiol. 2011;155:1947–59.

Liu Y, Ji X, Nie X, Qu M, Zheng L, Tan Z, et al. Arabidopsis Ath-HLH12 regulates the expression of genes involved in abiotic stress tolerance by binding to their E-box and GCG-box motifs. New Phytol. 2015;207:692–709.

Zhang YT, Luo MW, Cheng LJ, Lin YX, Chen Q, Sun B, et al. Identification of the cysteolic glucose-6-phosphatase dehydrogenase gene from strawberry involved in cold stress response. Int J Mol Sci. 2020;19:7322.

Dogan M. Investigation of the effect of salt stress on the antioxidant enzymes activities on the young and old leaves of saliola (Seneoptera) and tomato (Lycopersicon esculentum). Afric J Plant Sci. 2012;6:62–72.

Shi W, Liu D, Hao LWC, Guo X, Li H. GhWRKY39, a member of the WRKY transcription factor family in cotton, has a positive role in disease resistance and salt stress tolerance. Plant Cell Tissue Organ. 2014;118:17–32.

Liu Q, Xu K, Pan Y, Jiang B, Liu G, Jia Y, et al. Functional analysis of a novel chrysanthemum WRKY transcription factor gene involved in salt tolerance. Plant Mol Biol Rep. 2014;32:282–9.

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger Q, Thomashow MF. Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science. 1998;280:104–6.

Gilmour SJ, Sebold AM, Salazar MP, Everard JD, Thomashow MF. Overexpression of the Arabidopsis CBF3 transcriptional activator mimics multiple biochemical changes associated with cold acclimation. Plant Physiol. 2000;124:1854–65.

Novillo F, Medina J, Salinas J. Arabidopsis CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon. Proc Natl Acad Sci U S A. 2007;104:21004–7.

Chinnusamy V, Zhu J, Zhu J. Cold stress regulation of gene expression in plants. Trends Plant Sci. 2007;12:444–51.

Miura K, Jin JB, Lee J, Yoo CY, Strm V, Miura T, et al. SIZ1-mediated sumoylation of ICE1 controls CBF3/DREB1A expression and freezing tolerance in Arabidopsis. Plant Cell. 2007;19:1403–14.

Fei J, Wang YS, Zhou Q, Gu JD. Cloning and expression analysis of HSP70 gene from mangrove plant Kandelia obovata under cold stress. Ecotoxicology. 2015;24:1677–85.

Song H, Wang YS, Sun CC, Wang YT, Peng YL, Cheng H. Effects of pyrene on antioxidant systems and lipid peroxidation level in mangrove plants. Bruguiera gymnorrhiza. Ecotoxicology. 2012;21:1625–52.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin Chem. 2009;55:611–22.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using the 2(-Delta Delta C(T)) method. Meth‑ods. 2001;25:402–8.

Bechtold N, Pelletier G. In plants Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration. Methods Mol. Biol. 1998;82:259–66.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Fei et al. BMC Plant Biology  (2022) 22:274