Molecular cloning and characterization of \textit{PtrZPT2-1}, a ZPT2 family gene encoding a Cys2/His2-type zinc finger protein from trifoliate orange (\textit{Poncirus trifoliata} (L.) Raf.) that enhances plant tolerance to multiple abiotic stresses

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\textbf{A B S T R A C T}

In plants, most Cys2/His2 (C2H2) zinc finger proteins with two zinc finger domains (ZPT2) are involved in abiotic stress responses. In this study, a ZPT2 family gene \textit{PtrZPT2-1} was cloned from trifoliate orange (\textit{Poncirus trifoliata} (L.) Raf.). \textit{PtrZPT2-1} is composed of 245 amino acids, has a putative molecular weight of 25.99 kDa and an isoelectric point of 8.41. \textit{PtrZPT2-1} contained two C2H2 zinc finger domains, one nuclear localization signal (B-box), one transcription repression domain (DLN-box), and one protein-protein interaction domain (L-box). \textit{PtrZPT2-1} was localized to the nucleus. The \textit{PtrZPT2-1} expression was strongly induced by cold, drought, salt and ABA stresses. Overexpression of \textit{PtrZPT2-1} increased the survival rates, and the ABA, soluble sugar and proline levels but decreased the ion leakage, the malondialdehyde (MDA) content and reduced the H$_2$O$_2$ accumulation in the transgenic tobacco after cold, drought or salt treatments. Furthermore, the expression levels of 15 abiotic stress-related genes were significantly increased in the transgenic tobacco overexpressing \textit{PtrZPT2-1} after cold, drought or salt stress treatments. Our results indicated that overexpression of \textit{PtrZPT2-1} in the transgenic tobacco could improve the cold, drought and salt resistance of the plants by increasing the levels of osmotic regulatory solutes and decreasing the accumulation of H$_2$O$_2$.

\textbf{Keywords:}
Trifoliate orange
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1. Introduction

Plant development, yield and geographical distribution are typically limited by various abiotic stresses, including cold, drought and salt, with a series of physiological and molecular changes occurring in plants subjected to these stresses. Transcription factors (TFs) can initiate or limit the transcription of many downstream genes involved in stress signal transduction pathways, and they can also improve the stress tolerance in plants. So far, many kinds of stress responsive TFs have been identified in plants, such as WRKY, MYB, MYC, NAC, AP2/EREBP, bZIP and so on [1,2].

In eukaryotes, zinc finger proteins (ZFPs) belong to a TF family that has highly conserved zinc finger (ZF) domains. ZFPs TF family contains 176 members in Arabidopsis (\textit{Arabidopsis thaliana}) and 189 members in rice (\textit{Oryza sativa}) [3,4]. ZFPs can interact with DNA, RNA or other regulatory elements through the ZF domain [5]. Thus, many types of ZFPs, which are classified by the order and number of cysteine (Cys) and histidine (His) residues, have been found in eukaryotes, in which the most abundant and most studied ZFPs are the Cys2/His2-type (C2H2-type) ZFPs [6,7]. All of the C2H2-type ZFPs have a CX$_2$-4CX$_3$FX$_5$LX$_2$HX$_3$-5H motif that contains approximately 30 amino acids. Two Cys and His residues in this motif can bind tetrahedrally to a zinc ion to form a ZF structure that interacts with the target DNA site [8]. Interestingly, a highly conserved and plant-specific motif (QALG-GH) is located in the ZF domain of plant C2H2-type ZFPs. In plants, the adjacent ZFs of C2H2-type ZFPs are isolated via a long spacer of diverse lengths and sequences. In contrast, the C2H2-type ZFs in yeast and animals are mostly clustered and isolated via 6–8 amino acids [9]. These differences indicate that ZFPs in plants and animals may interact with target DNA sites to control the transcription of downstream genes in different ways [10]. In plants, petunia (\textit{Petunia hybrida}) ZPTs were the first reported C2H2-type ZFPs [11,12]. Thus, the C2H2-type ZFPs were also called ZPTs in plants. Plant ZPTs typically have one to four ZFs. The two-fingered protein family, which is called the ZPT2 family in this study, is one of the largest subclasses within the ZPT family. To date, many identified ZPT2 proteins have been shown to play important roles in plant responses to abiotic stress. The expression levels of Arabidopsis \textit{AtZAT10}, \textit{AtAZF1}, \textit{AtAZF2} and \textit{AtAZF3} genes were increased
by desiccation, ABA or salt treatment [13]. Moreover, the overexpression of AtZAT10 in Arabidopsis can suppress plant development and improve plant resistance to heat, drought and salt stress. Interestingly, zat10 knockout lines also exhibit enhanced stress resistance compared with the wild type (WT) Arabidopsis. Thus, AtZAT10 probably has dual functions for regulating plant stress tolerance [14]. The Arabidopsis AtZAT12 gene is related to plant cold and oxidative stress tolerance [15–17]. The expression level of SCOF-1 in soybean (Glycine max) is induced after cold treatment, and GmSCOF-1 overexpression increases the transcription levels of cold responsive genes and improves cold stress tolerance in transgenic plants [18]. The expression of Petunia PhZPT2-3 is induced by multiple stresses, including mechanical wounding, heavy metal ions, drought, and cold stresses. Petunia PhZPT2-3 overexpressing lines exhibit improved resistance to drought stress [19]. MiZFP1 from Medicago truncatula has been shown to be involved in the responses to ABA, jasmonate, and cytokinin treatments [20]. Expression levels of OsZFP182, OsZFP252, OsZFP245 and OsZFP179 in rice increase under various stress treatments, and the overexpression of these genes improves drought, cold, and salt tolerance in transgenic rice [21–24]. Other stress-related ZPT2 genes include ThZF1 in salt (Thellungiella halophila), which may be related to drought and salt responses [25]; PSTZ in Populus euphratica, which improves the tolerance for salt stress in transgenic tobacco [26]; PtzZFP2 in poplar (Populus tremula x Populus alba), which is related to cold, salt, gravitropism and wound stress responses [27,28]; and DgZFP in chrysanthemum (Chrysanthemum x morifolium), which is involved in the salt stress response [29].

Citrus is an important fruit crop with great economic value. However, its development, fruit yield and geographical distribution are severely limited by various environmental stresses. The isolation and characterization of citrus ZPT2 genes involved in environmental stress responses could help provide valuable insights into the citrus response to abiotic stresses at a molecular level and provide novel stress-resistance genes that could be used to breed stress-hardy citrus cultivars; via transgenic technology. Trifoliate orange (Poncirus trifoliata (L.) Raf.) is a stress-hardy citrus relative that is typically used as stock for citrus breeding programs. Until recently, a large number of abiotic stress-related genes, such as PtCBF1 [31], PtaABF [32], GaNAC1 [33], CitERF [34], PtbHLH1 [35], citrus R2R3MYB genes [36], CrNACD1 [37], PrtICE1 [38] and CrHsf [39], were cloned and characterized in citrus. However, to the best of our knowledge, the ZPT2 family genes involved in abiotic stress responses have not been studied in citrus. In this study, a ZPT2 family gene, which is designated PrtZPT2-1, was isolated from trifoliate orange. PrtZPT2-1 is a nuclear-located protein, and PrtZPT2-1 transcription was elevated after cold, drought, salt and ABA treatments. Furthermore, the overexpression of PrtZPT2-1 enhanced the cold, drought and salt tolerance in transgenic tobacco lines. Our study indicated that PrtZPT2-1 is an important gene for controlling abiotic stress responses in citrus.

2. Materials and methods

2.1. Plant materials

After accelerated germination in a plant incubator at 30 °C, seeds of trifoliate orange were transferred to plastic pots filled with nutritional soil. The pots were placed in a culture room under normal growth conditions (25 °C, 16 h light/8 h dark in a day), and the seedlings were grown to a height of 30 ± 2 cm and then used for subsequent gene cloning and gene expression analyses under cold, drought, salt and ABA stress treatments.

2.2. Cloning and bioinformatics analysis of PrtZPT2-1

Expressed sequence tags (ESTs) of citrus were screened using the HarvEST-Citrus database (http://harvest.ucr.edu/) to assemble a PrtZPT2-1 contig. After incubating the leaf samples at 4 °C for 6 h, the leaves of the trifoliate orange seedlings were sampled to extract the total RNA. After the total RNA extraction, trace genomic DNA was removed, the RNA quality was tested and first-strand cDNA synthesis was performed using the same method applied in our previous report [40]. A pair of gene-specific primers P1 (Table S1) were designed for cDNA sequence cloning based on the assembled PrtZPT2-1 contig. RT-PCR was conducted using the protocol from our previous study [41]. The PCR product was ligated with a pMD18T vector (Takara, Otsu, Japan), introduced into Escherichia coli DH5a and sequenced at Sangon Biotech (Shanghai, China). The bioinformatics analysis, including a plant ZPT2 homology analysis, multiple sequence alignment and ZPT2 phylogenetic study, was conducted using the methods of previous report [42].

2.3. Subcellular localization of PrtZPT2-1

For subcellular localization, the PrtZPT2-1 coding sequence with the stop codon deleted was isolated by RT-PCR using the suitable primers P2 (Table S1) with two restriction sites for Xba I and BamH I. The PrtZPT2-1 coding sequence was then cloned into the green fluorescent protein (GFP) in pBI121-GFP to construct a novel fusion vector (pBI121-PrtZPT2-1-GFP). The original vector was used as a control in this analysis. Both the fusion vector and the control were introduced into onion (Allium cepa) epidermal cells via particle bombardment using a Helios™ Gene Gun (BioRad, Hercules, CA, USA) according to the manufacturer’s instructions. After incubation in the dark on MS medium (pH 5.8) at 28 °C for 48 h, the transformed onion cells were observed using a universal fluorescence microscope BX61 (Olympus, Tokyo, Japan).

2.4. Expression analysis of PrtZPT2-1 in trifoliate orange

The leaves, stems, roots, flowers and fruits used for organ-specific expression analysis were sampled from the trifoliate orange trees of 8-year-old. Prior to the stress treatments, trifoliate orange seedlings with a height of 30 ± 2 cm were transferred to a novel growth chamber containing Hoagland’s solution for 5 days to accommodate the seedlings to new circumstances. Then, the seedlings were transferred to 4 °C for 0, 0.5, 1, 3, 6, 12 and 24 h for the cold treatment. For the drought, salt and ABA treatments, we transferred the seedlings to Hoagland’s solution containing 20% PEG 6000, 250 mM NaCl, and 100 μM ABA, respectively, for 0, 0.5, 1, 3, 6, 12 and 24 h. For each treatment, the leaves and roots were sampled from 15 randomly selected seedlings at each time point and mixed as a material pool. Each time point for each treatment was repeated three times. Total RNA was extracted, the trace genomic DNA was removed, the RNA quality was examined and first-strand cDNA synthesis was performed using a method described in our previous report [40].

Real-time quantitative PCR (qRT-PCR) was used for the PrtZPT2-1 transcript analysis with the specific primers P3 (Table S1). Citrus β-ACTIN (Table S1) was amplified as a control gene to normalize the expression between different samples. The details of the qRT-PCR protocol can be found in our previous report [40].

2.5. Generation of tobacco plants overexpressing PrtZPT2-1

The PrtZPT2-1 coding sequence was cloned via RT-PCR using the specific primers P5 (Table S1) with Bgl II and BstE II restriction sites. The product from RT-PCR was digested by Bgl II and BstE II and cloned into a pCAMBIA1301 vector digested by the same restriction enzymes to generate the recombinant plasmid pCAMBIA1301-PrtZPT2-1 under the control of the CaMV 35S promoter. The pCAMBIA1301-PrtZPT2-1 was transformed into an Agrobacterium tumefaciens strain LBA4404 using the freeze–thaw method. Transgenic tobacco (Nicotiana tabacum) lines were obtained using Agrobacterium-mediated leaf disc
transformation as described by previous report [43]. Seeds of T₀ transgenic lines were sown on MS with kanamycin (100 mg L⁻¹) to screen the positive T₁ transgenic lines based on their kanamycin resistance. The positive T₁ transgenic lines were further confirmed via genomic PCR amplification of PtrZPT2-1 and the CaMV 35S promoter using two pairs of primers, P1 and P6 (Table S1). The EasyPure Plant Genomic DNA Kit (Transgen, Beijing, China) was used to extract genomic DNA from the transgenic tobacco leaves according to the manufacturer’s instructions. The seeds of positive T₁ tobacco lines were grown on MS solid medium containing kanamycin (100 mg L⁻¹) to produce T₂ transgenic plants. Two T₂ transgenic lines (Line 2 and Line 7) with high PtrZPT2-1 expression were selected for further characterization. The qRT-PCR was used to investigate the transcript levels of PtrZPT2-1 in leaves, stems and roots of the WT and transgenic lines before and after cold, drought and salt treatments with the specific primers P7 (Table S1). The specific methods of cold, drought and salt treatments were described as “2.6”. Total RNA was extracted from the leaves, stems and roots of the WT and transgenic tobacco plants, the trace genomic DNA was removed, the RNA quality was examined and first-strand cDNA synthesis was performed using a method described in our previous report [40]. Tobacco NtACTIN was used as an endogenous control with specific primers (Table S2). The qRT-PCR reaction was performed according to the protocol from our previous report [40].

2.6. Cold, drought and salt tolerance comparison between WT plants and transgenic lines

Seeds from the WT plants and two transgenic lines (Line 2 and Line 7) were sown in plastic pots filled with nutritional soil. The pots were placed in a culture room under normal growth conditions (25 °C, 16 h light/8 h dark) for 8 weeks. The 8-week-old seedlings were then used to compare the abiotic stress resistance between the WT plants and transgenic lines. For the cold treatment, 30 tobacco seedlings with similar growth states from the two types of tobacco plants were incubated at 4 °C for 1 week, which was followed by 2 days of recovery. For the drought treatment, 30 seedlings from the two types of tobacco plants were cultivated without watering for 3 weeks and then re-watered for 1 week. For the salt treatment, 30 seedlings from the WT and two transgenic lines were treated with 300 mM NaCl for 2 weeks, which was followed by 3 days of recovery. After the stress treatments, the leaves of the WT and two transgenic lines were collected to examine the levels of malondialdehyde (MDA), ABA, proline, soluble sugar, H₂O₂, and ion leakage and the activity of three antioxidant enzymes (SOD, POD, and CAT). Furthermore, 8-week-old seedlings were also used to observe the phenotype and gene expression differences between the WT and two transgenic lines after the stress treatments. In another experiment to assess the survival rate, 3-week-old seedlings directly cultivated in plastic pots with nutritional soil were incubated at 4 °C for 5 days, allowed to recover for 2 days (cold treatment), withheld from watering for 2 weeks, received 2 days of re-watering (drought treatment) and then were irrigated with 300 mM NaCl for 1 week before 2 days of recovery (salt treatment).

2.7. Measurement of physiological indices and antioxidant enzyme activity

The thiobarbituric acid (TBA)-based colorimetric method was used to investigate the MDA level according to the method of previous report [44]. The ninhydrin reaction method was used to determine the proline level [45]. The phenol reaction method was used to detect the soluble sugar content [45]. The enzyme-linked immunosorbent assay (ELISA) method was used to measure the ABA content [46]. The ion leakage was examined using the methods of previous report [32]. The extraction and spectrophotometric measurements of the SOD, POD and CAT activity were also conducted according to the methods of previous report [32]. The test solutions for H₂O₂ analysis were extracted from tobacco leaves according to previous report [47]. In detail, 100 mg of leaves from the WT tobacco plants and two transgenic lines were ground to fine powder respectively in liquid nitrogen. The powder samples were homogenized in 1.5 mL 1 M HClO₄ with 100 mg insoluble polyvinylpyrrolidone (PVP). Homogenates were centrifuged at 12,000 × g for 10 min at 4 °C. Then, the H₂O₂ content in the supernatant was measured by a modified ferrous ammonium sulphate/xylene orange (FOX) method as previously described [48]. In detail, the eFOX agents were prepared by mixing 250 μM ferrous ammonium sulfate, 100 μM sorbitol, 100 μM xylene orange, and 1% ethanol in 25 mM H₂SO₄. Then, 600 μL FOX reagents were mixed with 60 μL leaf test solutions. The absorbance difference between 550 and 800 nm was recorded at least 30 min by the mixed solutions. A standard curve of H₂O₂ was used to determine the H₂O₂ content.

2.8. Expression analysis of the stress-related genes regulated by PtrZPT2-1

After the cold, drought and salt treatments described as above, the transcriptional differences of 16 stress-related genes controlled by PtrZPT2-1 were examined in 8-week-old seedlings of the WT and two transgenic lines using qRT-PCR with specific primers (Table S2). Tobacco NtACTIN was utilized as an internal control to normalize the transcription among different samples. The leaves from the WT and transgenic tobacco lines were collected for total RNA extraction. Subsequently, trace genomic DNA was removed, the RNA quality was examined, and first-strand cDNA synthesis was conducted as previously described [40]. The qRT-PCR analysis was conducted according to the methods of our previous study [40].

2.9. Statistical analysis

Three biological replicates were collected for all of the experiments in this study. All data in this paper are shown as the means ± SE. Statistical analyses were conducted using Student’s t-test (for two independent samples) and Tukey’s multiple range tests (for one-way ANOVA) in SPSS version 22. Statistical significance was considered at the P < 0.01 or P < 0.05 level.

3. Results

3.1. Isolation and sequence characterization of PtrZPT2-1

Based on the in silico PCR and RT-PCR methods, the PtrZPT2-1 gene (GenBank accession number KC820894) was cloned from trifoliate orange. The length of the PtrZPT2-1 cDNA sequence was 1054 bp with an open reading frame (ORF) of 735 bp. The PtrZPT2-1 protein contained 245 amino acids with a putative molecular weight of 25.99 kDa and an isoelectric point of 8.41. A BLAST analysis indicated that PtrZPT2-1 showed high identity with other ZPT2 proteins in plants, including Arabidopsis thaliana AtAZF1 (42%, GenBank accession number BAA85108.1), AtAZF2 (46%, AAG10143.1) and AtAZT10 (49%, CA67228.1), Petunia x hybrida PhZPT2-2 (46%, BAA05077.1) and PhZPT2-3 (60%, BAA05079.1), Glycine max GmSCOF-1 (55%, AAB39638.1), Solanum lycopersicum SIZFP1 (53%, ADK91083.1), Thellungiella halophila ThZF1 (45%, ABI74621.1), Triticum aestivum TaWZF1 (38%, Q42430.1), Medicago sativa MsZFT-2 (54%, CAB77055.1) and Medicago truncatula MtZFP1 (56%, AAP81801.1). A ClustalW alignment analysis indicated that PtrZPT2-1 contained two C2H2-type ZF domains. Each ZF domain contained a plant-specific QALGGH motif. Similar to most C2H2-type ZFPs in plants, PtrZPT2-1 had a putative transcription repression domain, which was the so-called DNL-box (DNL) at the C-terminus. Additionally, PtrZPT2-1 contained a B-box (KRKSKR) at the N-terminus that functioned as a putative nuclear localization signal (NLS) and an L-box (EEYELALCLVML) motif related to protein-protein interactions (Fig. 1A). In this study, we also constructed a phylogenetic tree of plant ZPT2 proteins using MEGA 4 software. The results revealed that PtrZPT2-1 was classified with
SlZFP1, PhZPT2-3, CaZFP1, DgZFP, AtZAT10, AtAZF3, AtAZF6, AtAZF2, ThZF1, PhZPT2-2, MsZPT2-1, MsZCOF-1, MsZFP1, OsZFP252, TaWZF1, AtZAT13, BcZFP1 and AtAZF1, whereas other ZPT2 proteins were categorized in another large branch except AT4G04404 and AT2G26940. These two ZPT2 proteins constituted two branches respectively. Moreover, the phylogenetic tree showed that PtrZPT2-1 was more close to AtZAT6, AtAZF3 and AtZAT10 than other ZPT2 proteins (Fig. 1B).

### 3.2. PtrZPT2-1 is a nucleus-localized protein

In this study, we found a putative NLS motif at the N-terminal region of PtrZPT2-1, which suggested that it may be localized to the nucleus similar to other TFs. To confirm this hypothesis, the subcellular localization of PtrZPT2-1 was investigated using a PtrZPT2-1 and GFP fusion protein that was overexpressed in onion epidermal cells. The original pBI121-GFP was used as a control vector. Microscopic

![Fig. 1.](image-url)
visualization revealed that GFP fluorescence from the fusion protein was only detected in the nucleus of the onion cells, whereas the fluorescence in the control was observed all over the onion cells (Fig. 2). These results suggested that \textit{PtrZPT2-1} is a nucleus-localized protein similar to other TFs.

### 3.3. Expression analysis of \textit{PtrZPT2-1} in trifoliate orange

The spatial-specific expression of \textit{PtrZPT2-1} in different organs of trifoliate orange was determined by qRT-PCR. The results revealed that the highest \textit{PtrZPT2-1} transcript was found in roots, followed by leaves, stems, flowers and fruits. The \textit{PtrZPT2-1} expression in flowers and fruits were much lower than that in roots, leaves and stems (Fig. 3A).

The \textit{PtrZPT2-1} expression patterns in trifoliate orange leaves and roots after cold, drought, salt and ABA treatments were investigated by qRT-PCR. Specifically, the expression of \textit{PtrZPT2-1} in the leaves was strongly induced after 1 h of cold treatment (4 °C), peaked at 6 h and then decreased (Fig. 3B). After a 20% PEG 6000 treatment for drought simulation, a significant increase in \textit{PtrZPT2-1} expression in the leaves was also observed at 1 h. The maximum expression of \textit{PtrZPT2-1} was found 3 h after PEG 6000 treatment, and then the \textit{PtrZPT2-1} expression declined gradually from 6 h to 24 h (Fig. 3C). Under salt stress (250 mM NaCl), the expression of \textit{PtrZPT2-1} in the leaves was slightly increased at 0.5 h, peaked at 1 h and then continuously decreased from 3 h to 24 h (Fig. 3D). Moreover, the expression of \textit{PtrZPT2-1} increased at 0.5 h after the ABA (100 μM) treatment, reached the maximum level at 3 h, and then continuously decreased from 6 h to 24 h (Fig. 3E).

The \textit{PtrZPT2-1} expression in the roots was obviously induced at 1 h after the cold (4 °C) treatment, peaked at 3 h and then declined gradually from 6 h to 24 h (Fig. 3F). After 0.5 h of drought (20% PEG 6000) treatment, the \textit{PtrZPT2-1} expression in the roots was significantly induced in roots, then reached the maximum level at 1 h and decreased gradually afterwards (Fig. 3G). Under salt stress (250 mM NaCl), the \textit{PtrZPT2-1} expression in the roots was strongly induced at 0.5 h, peaked at 3 h and then declined continuously afterwards (Fig. 3H). Similarly, the expression of \textit{PtrZPT2-1} was obviously increased at 0.5 h after the ABA (100 μM) treatment, reached the maximum level at 3 h, and then declined continuously afterwards (Fig. 3I). These results revealed that \textit{PtrZPT2-1} expression levels in trifoliate orange leaves and roots were increased after cold, drought, salt and ABA treatments.

### 3.4. Overexpression of \textit{PtrZPT2-1} in tobacco enhanced cold, drought and salt tolerance

To further study whether \textit{PtrZPT2-1} is involved in the citrus response to abiotic stress, transgenic tobacco plants overexpressing \textit{PtrZPT2-1} were obtained via \textit{Agrobacterium}-mediated transformation. Two \textit{T2} transgenic lines (Line 2 and Line 7) with higher \textit{PtrZPT2-1} expression were selected for further characterization. No \textit{PtrZPT2-1} expression was detected in leaves, stems or roots of the WT plants. The \textit{PtrZPT2-1} expression levels among leaves, stems and roots of the two transgenic lines were similar under normal growth conditions (Fig. 4A). The cold, drought and salt treatments had no significant impact on the expression of \textit{PtrZPT2-1} in leaves, stems or roots of the WT plants. The \textit{PtrZPT2-1} expression levels among leaves, stems and roots of the two transgenic lines were similar under normal growth conditions. However, obvious phenotypic differences were observed between the two types of tobacco plants in response to abiotic stress. For example, after the 4 °C treatment for 1 week and 2 days of recovery, more severe leaf wilting was observed in the WT plants compared with the transgenic lines. Similarly, after 3 weeks of drought treatment and 1 week of re-watering, severe leaf wilting was observed in the WT plants, whereas both transgenic tobacco lines grew well with only a few damaged leaves observed at the bottom. For the salt tolerance analysis, the two types of tobacco plants were treated with 300 mM NaCl for 2 weeks and allowed to recover for 3 days. The damage to the WT plants was more severe compared with that of the transgenic lines after the salt treatment (Fig. 4B). After the cold, drought and salt treatments, the transgenic tobacco lines exhibited much higher survival rates compared with the WT plants (Fig. 4C and D). These results suggest that the transgenic tobacco plants show much higher cold, drought and salt tolerance compared with WT tobacco plants.
3.5. Overexpression of PtrZPT2-1 in tobacco decreases ion leakage and MDA levels but increases ABA, proline and soluble sugar content under cold, drought and salt stress

To further study the mechanisms underlying the increase in abiotic stress tolerance in transgenic tobacco, experiments were performed to investigate the physiological changes caused by PtrZPT2-1 expression. Under normal growth conditions, the leaf ion leakage and MDA levels in lines 2 and 7 were indistinguishable from that in the WT plants. However, after cold, drought and salt treatments, these two physiological indices in the transgenic tobacco lines were significantly decreased compared with that in the WT plants (Fig. 5A and B). The lower ion leakage observed in the transgenic lines indicated less plasma membrane (PM) damage and better osmotic regulation. Proline and soluble sugar are important osmotic-regulatory solutes involved in abiotic stress responses. ABA is an important regulator that activates...
plant resistance to abiotic stresses. Under a normal growth environment, the ABA, proline and soluble sugar content in transgenic lines was indistinguishable from that in the WT plants. However, the ABA, proline and soluble sugar content in the transgenic lines increased significantly compared with that of the WT plants after cold, drought and salt treatments (Fig. 5C–E). These results indicated that the overexpression of *PtrZPT2-1* could increase the tolerance of transgenic tobacco seedlings to abiotic stress probably by improving the osmotic regulation ability.

### 3.6. Overexpression of *PtrZPT2-1* in tobacco elevates antioxidant enzyme activities but reduces H$_2$O$_2$ levels after cold, drought and salt treatments

The observed decrease in ion leakage and MDA levels in the transgenic tobacco suggested that overexpression of *PtrZPT2-1* in tobacco plants probably reduced oxidative membrane damage after cold, drought and salt treatments. H$_2$O$_2$ is a major active oxygen compound that can cause oxidative membrane injury when it accumulates and influence plant abiotic stress tolerance [49,50]. The H$_2$O$_2$ content and the activity of three antioxidant enzymes (SOD, POD, and CAT) in the leaves of the WT and transgenic plants were investigated in this study. The H$_2$O$_2$ content and antioxidant enzyme activities were similar in the WT and transgenic tobacco plants under a normal growth environment. However, after cold, drought and salt treatments, the antioxidant enzyme activities in the transgenic lines were significantly increased compared with that of the WT plants (Fig. 6A–C), and the H$_2$O$_2$ content was obviously decreased in the transgenic tobacco plants (Fig. 6D). These results revealed that the overexpression of *PtrZPT2-1* limited oxidative membrane damage after cold, drought and salt treatments by increasing the antioxidant enzyme activities and decreasing the H$_2$O$_2$ and MDA content.
3.7. PtrZPT2-1 regulates the expression levels of stress-related genes after cold, drought and salt treatments

To further understand the molecular mechanisms of PtrZPT2-1 in response to abiotic stress, the transcript levels of 16 genes, including 7 stress-induced genes (NtDREB1, NtDREB2, NtDREB3, NtDREB4, NtRD22, NtLEA5, NtPS5CS), 3 ROS detoxification genes (NtSOD, NtCAT and NtPOX), 4 ABA biosynthesis genes (NtNCED1, NtZEP, NtABA2, NtAAO), 1 sucrose biosynthesis gene (NtSPSA), and 1 NADPH oxidase gene (NtRBOHD) in the WT and transgenic tobacco lines before and after cold, drought and salt treatments were compared using qRT-PCR. The results showed that the expression levels of most stress-related genes in the two types of tobacco plants were significantly increased after the cold, drought and salt treatments; however, the exception of NtRD22 was only induced by drought and salt stress (Fig. 7). However, the expression levels of NtRBOHD in the transgenic lines were indistinguishable from that in the WT plants after cold, drought and salt treatments. These results reveal that over-expression of PtrZPT2-1 in tobacco improves stress resistance by elevating the transcript levels of stress-related genes.

4. Discussion

A large number of Cys2/His2 ZFPs with two ZF domains (ZPT2 proteins) that respond to abiotic stress have been identified in various plant species [51]. However, the ZPT2 family genes have not been
characterized in citrus. In this study, a ZPT2 family gene called *PtrZPT2-1* was cloned and characterized from trifoliate orange for the first time. Protein sequence analyses showed that *PtrZPT2-1* contained two typical C2H2 ZF domains. Each ZF domain contained a QALGGH motif that is only present in plants (Fig. 1A) and has been shown to be critical for the DNA-binding activity of plant ZPT2 proteins [12,52,53]. Additionally, a B-box and an L-box in the N-terminus and a DLN-box in the C-terminus were also found in *PtrZPT2-1* (Fig. 1A). The B-box is a conserved motif with an amino acid sequence of KXKRSKR, which probably functions as a NLS. This motif was completely conserved in *PtrZPT2-1* (KRKRSKR) (Fig. 1A), which suggests the *PtrZPT2-1* was localized to the nucleus. Additional experiments confirmed that *PtrZPT2-1* was located in the nucleus (Fig. 2), similar to other TFs. The L-box region (EXXXAXCLXXL), which was related to protein-protein interactions, was also found in *PtrZPT2-1* (EEEYLALCLVML) (Fig. 1A). Furthermore, the DLN-box, which is highly conserved in *PtrZPT2-1* (Fig. 1A), is typically found in ZPT2 transcriptional repressors, such as petunia PhZPT2-3 [19], tobacco NtZFT1 [54], Arabidopsis AtAZF1, AtAZF2, AtAZF3, AtZAT7 and AtZAT10 [10,55,56], and other genes. Interestingly, when expressed in yeast cells, many ZFPs with a DLN-box, including rice OsZFP179 and salt cress ThZF1 [24,25], were shown to function as transcriptional activators. Therefore, further studies should be performed to confirm whether *PtrZPT2-1* functioned as a transcriptional repressor or activator in plant responses to abiotic stress. Phylogenetic analysis revealed that *PtrZPT2-1* was clustered with plant ZPT2 proteins involved in stress responses, especially AtZAT6, AtAZF3 and AtZAT10, which were more close to *PtrZPT2-1* than other ZPT2 proteins (Fig. 1B). The structural features and phylogenetic results indicated that *PtrZPT2-1* was a member of the plant ZPT2 gene family.

Based on the phylogenetic analysis, AtZAT6 is the most closely related ZPT2 protein of *PtrZPT2-1* (Fig. 1B). The expression of AtZAT6 was strong in cotyledons, leaves, and roots, but weak in flowers, siliques, and stems [57]. Similarly, we also found that the *PtrZPT2-1* expression in roots and leaves of trifoliate orange were much higher than that in stems, flowers and fruits (Fig. 2A).

Previous studies have revealed that a considerable number of ZPT2 TFs in plants are induced by multiple instances of abiotic stress. For example, the *AtZAT6* expression was obviously induced by salt, dehydration, cold stress treatments and pathogen infection [57]; *ZmZFT1* expression in maize (*Zea mays*) was upregulated after salt, ABA and drought treatments [58]; *SIZFP1* transcription in tomato significantly increased after salt, dehydration and cold treatments [59]; *GsZFP1* expression in soybean was induced in the leaves by salt, cold and ABA stresses and in the roots by drought, ABA and cold stress [60]; *SIZFP2* transcription in tomato was rapidly induced after drought, NaCl and KCl treatments [61]; C2H2 ZF family genes in poplar were involved in the responses to heat, salt and drought stress [62]; and *IbZFP1* expression in sweet potato increased after ABA, polyethylene glycol and NaCl treatments [63]. In this study, we also found that the expression of *PtrZPT2-1* in trifoliate orange leaves and roots strongly increased after cold, drought, salt and ABA treatments (Fig. 3B-E). The strong upregulation of *PtrZPT2-1* expression following the ABA treatment suggested that *PtrZPT2-1* regulates the abiotic stress tolerance of citrus through the ABA-dependent signal transduction pathway.

The strong upregulation of *PtrZPT2-1* expression after abiotic stress treatments inspired us to focus on the role of *PtrZPT2-1* in the regulation of abiotic stress tolerance in citrus. In this research, the function of *PtrZPT2-1* in abiotic stress responses was identified using transgenic tobacco plants overexpressing *PtrZPT2-1*. Under standard growth conditions, the transgenic tobacco lines exhibited no obvious phenotypic differences compared with the WT plants. However, after cold, drought and salt treatments, less plant damage and significantly higher survival
Fig. 7. Comparison of stress-related gene expression levels between the WT and transgenic lines (line 2 and line 7) under cold, drought and salt stresses. After cold, drought and salt treatments, the leaves of 8-week-old WT and transgenic plants were collected to measure the expression levels of stress-related genes in the WT and transgenic lines by real time quantitative PCR. The y-axis records the relative gene expression levels as calculated by the $2^{-\Delta\Delta CT}$ method with NtACTIN as the endogenous reference. Data are shown as the means ± SE calculated from three biological replicates. Asterisks indicate that the value in transgenic tobacco lines is significantly different from that of WT (**P < 0.01, *P < 0.05).
rates were observed in the transgenic tobacco plants (Fig. 4B–D), which indicated that the overexpression of \textit{PtrZPT2-1} enhances plant resistance to cold, drought and salt stress. This result is consistent with that of previous reports on other ZPT2 genes from different plant species [10,14,18,19,22–24,61,63–65].

The physiological and biochemical changes caused by \textit{PtrZPT2-1} overexpression were also studied in this paper. The results revealed that the overexpression of \textit{PtrZPT2-1} reduced the MDA level, \textit{H}_{2}\text{O}_{2} content and ion leakage level but increased the soluble sugar and proline content in transgenic tobacco lines after cold, drought and salt treatments (Figs. 5A–E and 6D). Proline is an important osmolyte involved in the regulation of plant abiotic stress tolerance [66,67]. In addition, soluble sugar functions as both a signaling molecule and metabolite in plants subjected to abiotic stress [68–70]. Thus, soluble sugar and proline accumulation in transgenic tobacco could maintain the osmotic balance between the extracellular and intracellular environments, thereby reducing cellular membrane damage and leading to an increase in stress tolerance in the transgenic lines. This result is similar to that of several previous reports [71–74]. MDA is a soluble product during the process of membrane lipid peroxidation, and MDA content can be used to measure the extent of oxidative stress [75]. Redundant lipid oxidation ultimately leads to cellular membrane injury, and the degree of cellular membrane injury can be measured by ion leakage [76]. \textit{H}_{2}\text{O}_{2} accumulation can also lead to oxidative injury in plant cellular membranes [49,50]. Thus, the decrease in MDA level, \textit{H}_{2}\text{O}_{2} content and ion leakage observed in this study indicated that the transgenic tobacco lines suffered less oxidative injury compared with the WT plants after cold, drought and salt treatments. ROS in plants are overproduced under abiotic stress, which can lead to serious oxidative damage in plant cells. The antioxidant enzymes belonging to ROS scavenging systems can reduce oxidative injury in plant cells [77]. In this study, we also compared the activity of major antioxidant enzymes between the WT and transgenic lines. The results revealed that the SOD, POD and CAT activities in the transgenic plants were much higher than that of the WT plants (Fig. 7). Combined with the decrease in \textit{H}_{2}\text{O}_{2} levels in the transgenic plants, we deduced that the increased antioxidant enzyme activities caused the decrease in ROS content in the transgenic plants after the cold, drought and salt treatments. Several previous studies have shown similar results [32, 50, 78–80]. Taken together, we concluded that the overexpression of \textit{PtrZPT2-1} improved osmotic regulation, limited ROS damage and enhanced the stability of the PM in the transgenic lines after cold, drought and salt treatments by reducing the MDA level and \textit{H}_{2}\text{O}_{2} content and increasing the proline level, soluble sugar content and antioxidant enzyme activity.

Several plant \textit{ZPT2} genes have been reported to respond to abiotic stress via \textit{ABA} signaling transduction [60,63,81]. \textit{ABA} is an important regulator of plant stress responses and activates the transcription of stress-related genes in an \textit{ABA}-dependent pathway [82]. In this study, we also found that the \textit{ABA} level in the transgenic plants was much higher compared with that of the WT plants after cold, drought and salt treatments (Fig. 5C). This result suggests that the overexpression of \textit{PtrZPT2-1} increased the abiotic stress tolerance in the transgenic lines through the \textit{ABA} signaling transduction pathway.

To obtain additional information on the function of \textit{PtrZPT2-1} in the regulation of plant tolerance to abiotic stress at the molecular level, the transcript levels of 16 stress-related genes in the WT and transgenic lines were compared before and after cold, drought, and salt treatments. The dehydration responsive element binding (DREB) \textit{TF} family genes were involved in plant resistance to multiple types of abiotic stress, such as cold, drought and salt stress [83–88]. In addition, the \textit{RD22} and \textit{LEA5} genes also play important roles in plant responses to abiotic stress [89–91]. In this study, the overexpression of \textit{PtrZPT2-1} in tobacco plants significantly increased the expression levels of 3 DREB family genes (\textit{NdDREB1}, \textit{NdDREB2}, \textit{NdDREB3}) after cold treatment. The expression levels of \textit{NdLEA5} and another DREB family gene \textit{NdDREB4} were strongly increased in the \textit{PtrZPT2-1} overexpression lines under cold, drought and salt stress treatments. The \textit{NtRD22} had a much higher transcript level in the \textit{PtrZPT2-1} overexpression lines compared with the WT plants after the drought and salt treatments (Fig. 7). These results suggest that the overexpression of \textit{PtrZPT2-1} likely increased the transcript levels of the stress-related genes, thereby improving the stress resistance in transgenic tobacco lines.

Previous reports have shown that zeaxanthin epoxidase (\textit{ZEP}), 9-cis-epoxycarotenoid dioxygenase (\textit{NCED}), xanthin dehydrogenase (\textit{ABA2}), and aldehyde oxidase (\textit{AAO}) are involved in the \textit{ABA} biosynthesis of plants [37,92–97]. As previously mentioned, the \textit{ABA} content in the \textit{PtrZPT2-1}-overexpressing lines was obviously higher than that of the WT after cold, drought and salt treatments. Consistent with this result, the transcript levels of 4 genes related to \textit{ABA} biosynthesis (\textit{NtNCED1}, \textit{NtZEP}, \textit{NtABA2} and \textit{NtAAO}) were much higher in \textit{PtrZPT2-1}-overexpressing lines compared with the WT plants after the cold, drought and salt stress treatments (Fig. 7). These results revealed that the overexpression of \textit{PtrZPT2-1} could elevate the expression levels of \textit{ABA} biosynthesis genes, which leads to an increase in \textit{ABA} levels and improves the stress resistance of transgenic tobacco. These results are consistent with that of a previous study that found that the overexpression of the \textit{ZPT2} gene (\textit{IbZPT1}) from sweet potato) and an increase in \textit{ABA} levels could enhance the stress resistance of transgenic plants [63].

\textit{P5CS} is an important gene related to proline glutamate biosynthesis. In Arabidopsis, the expression level of \textit{P5CS} is increased after \textit{ABA} treatment [98,99]. The upregulation of \textit{P5CS} expression may elevate the proline level after stress via an \textit{ABA}-dependent pathway [100]. Similar results were obtained in rice [101]. In this study, the \textit{NtP5CS} transcript was increased in the \textit{PtrZPT2-1}-overexpressing tobacco plants subjected to cold, drought and salt stress (Fig. 7), and these treatments also induced significantly higher proline and \textit{ABA} contents in the transgenic lines compared with the WT plants (Fig. 5C and D). Therefore, the increased \textit{ABA} levels in the transgenic lines caused by the enhanced transcription of \textit{ABA} biosynthesis genes after cold, drought and salt treatments promoted an increase in \textit{NtP5CS} expression, which resulted in proline accumulation and led to an increase in stress tolerance in transgenic tobacco plants. Previous studies of other plant species have reported similar results [22,24,63].

As shown above, the overexpression of \textit{PtrZPT2-1} enhanced the activity of antioxidant enzymes and reduced the \textit{H}_{2}\text{O}_{2} levels in transgenic tobacco. Consistent with these results, the transcripts of 3 ROS scavenging genes (\textit{NtSOD}, \textit{NtCAT} and \textit{NtPOX}) were strongly increased in the transgenic lines, which likely induced an increase in the activity of antioxidant enzymes and a decrease in \textit{H}_{2}\text{O}_{2} levels after cold, drought and salt treatments. Thus, the overexpression of \textit{PtrZPT2-1} enhanced the stress resistance of transgenic plants, which was at least partially because of improvements in the ROS scavenging ability. Several studies of other plant species have reported similar results [63,80].

In conclusion, a \textit{ZPT2} family gene, \textit{PtrZPT2-1}, was cloned from trifoliate orange in this study. Our results indicated that \textit{PtrZPT2-1} is a nuclear-located protein. The expression of \textit{PtrZPT2-1} was significantly increased in the leaves of trifoliate orange subjected to cold, drought, salt and \textit{ABA} treatments, and \textit{PtrZPT2-1} overexpression significantly enhanced the cold, drought and salt tolerance in the transgenic tobacco lines. Further research revealed that the overexpression of \textit{PtrZPT2-1} increased the transcript levels of various genes related to proline, \textit{ABA} and sugar biosynthesis, ROS scavenging, and stress responsive pathways, thereby elevating the \textit{ABA}, proline, and soluble sugar content and decreasing MDA levels and ROS accumulation, which resulted in better osmotic regulation, less ROS damage, and finally improved abiotic stress resistance in the transgenic tobacco plants. Our findings suggest that \textit{PtrZPT2-1} likely plays an important role in citrus abiotic stress responses. Furthermore, \textit{PtrZPT2-1} could be used as a candidate gene to enhance the abiotic stress resistance of citrus via genetic engineering techniques.
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References


