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

Dechun Liu, Li Yang, Man Luo, Qi Wu, Shanbei Liu, Yong Liu*

Department of Pomology, College of Agronomy, Jiangxi Agricultural University, Nanchang 330045, China

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ABSTRACT

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 *PtrZPT2-1* was cloned from trifoliate orange (*Poncirus trifoliata* (L.) Raf.). *PtrZPT2-1* is composed of 245 amino acids, has a putative molecular weight of 25.99 kDa and an isoelectric point of 8.41. *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). *PtrZPT2-1* was localized to the nucleus. The *PtrZPT2-1* expression was strongly induced by cold, drought, salt and ABA stresses. Overexpression of *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_2O_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 *PtrZPT2-1* after cold, drought or salt stress treatments. Our results indicated that overexpression of *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_2O_2.

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 (*Arabidopsis thaliana*) and 189 members in rice (*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 CX2-4CX3FX5LX2HX3-5H 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 (QALGGH) 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 (*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 *AtZAT10*, *AtAZF1*, *AtAZF2* and *AtAZF3* genes were increased

* Corresponding author.
E-mail address: liuyongjxau@163.com (Y. Liu).

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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]. *MtZFP1* 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/or salt tolerance in transgenic rice or tobacco lines [21–24]. Other stress-related *ZPT2* genes include *ThZF1* in salt cress (*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]; *PtaZFP2* 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 grafting [30], and it is also typically used to study the molecular mechanisms of citrus stress tolerance. Until recently, a large number of abiotic stress-related genes, such as *PtCBF1* [31], *PtrABF* [32], *CsNAC1* [33], *CitERF* [34], *PtrbHLH* [35], citrus *R2R3MYB* genes [36], *CrNCED1* [37], *PtrICE1* [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 *PtrZPT2-1*, was isolated from trifoliate orange. *PtrZPT2-1* is a nuclear-located protein, and *PtrZPT2-1* transcription was elevated after cold, drought, salt and ABA treatments. Furthermore, the overexpression of *PtrZPT2-1* enhanced the cold, drought and salt tolerance in transgenic tobacco lines. Our study indicated that *PtrZPT2-1* 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 PtrZPT2-1

Expressed sequence tags (ESTs) of citrus were screened using the HarvEST-Citrus database (http://harvest.ucr.edu/) to assemble a *PtrZPT2-1* 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 *PtrZPT2-1* 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* DH5α 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 PtrZPT2-1

For subcellular localization, the *PtrZPT2-1* 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 *PtrZPT2-1* coding sequence was then cloned into the green fluorescent protein (GFP) in pBI121-GFP to construct a novel fusion vector (pBI121-PtrZPT2-1-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 PtrZPT2-1 in trifoliate orange

The leaves, stems, roots, flowers and fruits using 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 *PtrZPT2-1* 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 PtrZPT2-1

The *PtrZPT2-1* 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-PtrZPT2-1 under the control of the CaMV 35S promoter. The pCAMBIA1301-PtrZPT2-1 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