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CLONING AND CHARACTERIZATION OF A PATHOGENESIS-RELATED GENE (*ThPR10*) FROM *TAMARIX HISPIDA*

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A PR10 gene (ThPR10) was cloned from *Tamarix hispida* and characterized. Real-time RT-PCR was employed to evaluate gene expression levels. *ThPR10* was expressed in both leaves and roots of *T. hispida* under normal growth conditions, and can be highly induced in both leaf and root tissues by abiotic stresses including NaCl, PEG, cold, $CdCl_2$, and ABA (abscisic acid) treatments. Our results indicated that *ThPR10* is involved in the abiotic stress response, and regulated by an ABA-dependent signaling pathway. Subsequently, *ThPR10* was localized at the subcellular level. The gene was fused with the GFP N-terminal driven by CaMV35S promoter and transiently expressed in onion epidermal cells. This strategy localized the ThPR10 protein to the nucleus of onion epidermal cells, suggesting that the pathogenesis-related proteins play a functional role in the cell nucleus.

Key words: Abiotic stress, gene expression, pathogenesis-related protein, subcellular location, *Tamarix hispida*.

INTRODUCTION

Pathogenesis-related (PR) proteins are plant proteins induced by pathogens and various abiotic stresses, and are usually considered to be part of multiple defense systems in plants (Odjakova and Hadjiivanova, 2001). PR family proteins have been demonstrated to have diverse functions; they include β-1, 3-glucanase, chitinases, RNase activity, proteinase inhibitors and peroxidases, all of which are related to pathogen defense (Sarowar et al., 2005; Onishi et al., 2006; Yan et al., 2008; El-kereamy et al., 2009). Most *PR* genes are induced by biotic or abiotic stress, and expression of *PR* genes has been found in response to stress from salt (Moons et al., 1997), drought (Dubos and Plomion, 2001), cold (Wisniewski et al., 2004), heavy metals (Koistinen et al., 2002) and treatment with hormones such as ABA (abscisic acid) (Kim et al., 2008). Seo (2008) showed that *PR* genes from Arabidopsis are differentially regulated by plant hormones such as SA (salicylic acid), ABA (abscisic acid), JA (jasmonic acid), ET (ethylene) and BR (brassinosteroid), suggesting that the PR proteins play a role in plant developmental processes other than disease resistance response. Some *PR* genes are also differentially regulated by diverse abiotic stresses and can be induced by high salt and ABA treatment, showing that the *PR* genes in *Arabidopsis* mediate ABA-dependent salt stress signals.

PR proteins are grouped into 17 families (PR1 to PR17) based on sequence homology, serological relationships and biological activity (Van Loon et al., 1994; Hoffmann-Sommergruber et al., 1997; Van Loon et al., 2006; Christensen et al., 2002). Among these PR families, PR10 proteins are small, acidic intracellular proteins and are encoded by multiple genes; they are a class of intracellular proteins, in contrast to the extracellular nature of most PR proteins. *PR10* genes were originally identified in *Pisum sativum* (Riggleman et al., 1985), encoding a number of low molecular weight proteins ranging from 15 to 20 kDa, each with different biochemical characteristics (El-kereamy et al., 2009). Many studies have demonstrated that PR10 proteins are functionally involved in abiotic stress mechanisms and can be induced by various abiotic stresses (Liu and Ekramoddoullah, 2006; El-kereamy et al., 2009). PR10 proteins were able to inhibit the hyphal growth of fungi such as *Phytophthora capsici* (Park et al.,

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TABLE 1. Primers used in real time RT-PCR

Gene name	Accession number	Forward and reverse primer
ThPR10	FJ463256	F:5'GCGACCATACTGCTGTTGG 3'
		R:5'TGCATCTTCTCGAAGCCC 3'
α-tubulin	EH050602	F:5'CACCCACCGTTGTTCCAG 3'
		R:5'ACCGTCGTCATCTTCACC 3'
β-tubulin	EH052343	F:5'GGAAGCCATAGAAAGACC 3'
		R:5'CAACAAATGTGGGATGCT 3'
β-actin	EG971352	F:5'AAACAATGGCTGATGCTG 3'
		R:5'ACAATACCGTGCTCAATAGG 3'
Ubiquitin	EG971639	F:5'TGTAATCGGCCAAAGGTACG 3'
		R:5'GAGTTGCTTACCAGCGAAG 3'
18S rRNA	EH052343	F:5'GGAAGCCATAGAAAGACC 3'
		R:5'CAACAAATGTGGGATGCT 3'

2004) and *Gibberella zeae* (Liu et al., 2006). Some PR10 proteins possess cytokine-specific binding activity in plants (Fujimoto et al. 1998; Gonneau et al. 2001), and constitutive expression of *PR10* cDNA from *Brassica napus* results in an increase of cytokine level (Srivastava et al., 2006). *PR10* genes are also developmentally regulated in different plant tissues, suggesting roles beyond abiotic and biotic responses (Breiteneder et al., 1995; Vanek-Krebitz et al., 1995; Krishnaswamy et al., 2008).

PR genes have been isolated from several plant species and gene expression in response to abiotic stress has been investigated, but few of them from woody plants, particularly from woody halophytes. Physiological differences between woody plants and grasses in response to abiotic stresses may exist but have not been elucidated. Cloning and analysis of genes from woody plants should provide greater insights into abiotic and biotic stress tolerance.

Tamarix species are shrubs or small trees that belong to the family Tamaricaceae. *Tamarix* plants grow well in arid or semi-arid areas, or on saline soil. They are distributed mainly in desert and semi-desert regions, on saline soil in river valley plains or lakeshores from central Asia to China, and usually are the main component of forest communities in desert river valleys. *Tamarix* plants are also invasive weed species in western North America. The ability of *Tamarix* plants to grow and survive in various stress environments indicates that they must have developed a molecular and physiological system to adapt to these stress conditions. This makes them potentially useful as a source of genetic determinants of abiotic stress tolerance and biological invasion control.

In this study we cloned a *PR* gene from *Tamarix hispida* by screening 3 cDNA libraries (Li et al., 2008). Sequence analysis showed the gene to be a member of a subfamily of PR10, designated *ThPR10*. To investigate the role of *ThPR10* in abiotic stress response we analyzed time-course expression of *ThPR10* in response to different abiotic stresses and exogenous ABA. Leaves and roots of *T. hispida* were subjected to real-time quantitative RT-PCR. For learn more about *ThPR10* biochemical characteristics, we performed subcellular localization analysis of ThPR10 protein, using GFP fusion to its C-terminus. The aim is to provide fundamental insight into the role of *ThPR10* in abiotic stress response.

MATERIALS AND METHODS

PLANT MATERIAL AND TREATMENTS

Seeds of *T. hispida* were sown in pots containing a mixture of turf peat and sand (2:1 v/v) under normal growth conditions. Two-month-old *T. hispida* seedlings were grown under greenhouse conditions at 65-75% relative humidity, 14 h light (400 $\mu mol \cdot m^{-2} \cdot s^{-1}$), and average temperature of 24°C. Before initiating stress treatments the seedlings were well watered. The seedlings were treated with 0.4 $mol \cdot L^{-1}$ NaCl, 20% (W/V) PEG6000, 100 μM ABA, 150 μM $CdCl_2$, and 4°C for 0 (normal growth conditions, as control), 6, 24, 48 and 72 h. Leaves and roots from at least 6 seedlings were then harvested and pooled for RNA isolation.

CLONING AND BIOINFORMATICS ANALYSIS OF *THPR10* GENE

The full cDNA length of *ThPR10* was cloned directly from *T. hispida* root cDNA libraries using the EST

analysis method (Li et al. 2008). The *ThPR10* open reading frame (ORF) was determined using the ORF Finder program from NCBI. Sequence alignment and phylogeny reconstruction were carried out using ClustalX (1.83) with the neighbor-joining method. Molecular weight (MW) and isoelectronic point (pI) predictions for every deduced *ThPR10* gene were performed with Compute pI/Mw tool (http://www.expasy.org/tools/protparam.html).

TOTAL RNA EXTRACTION AND CDNA SYNTHESIS

Total RNA of each sample was extracted following the CTAB method of Wang (2003), and further treated with DNase I (Promega) to remove any DNA residue. RNA (0.5 μg) was reverse-transcribed to cDNA with the PrimeScriptTM RT Reagent Kit (TaKaRa), an oligodeoxythymidine primer and 6-mer random primers in a volume of 10 μl. The reverse transcription product was diluted 10-fold and used as the real-time RT-PCR template.

REAL-TIME RT-PCR ASSAY

The specific primers used to perform real-time PCR are given in Table 1. All RT-PCR experiments were conducted in three replicates. To normalize the amount of total RNA present in each reaction, α-actin, α-tubulin, α-tubulin, 18S rRNA and ubiquitin were chosen as internal controls (Tab. 1). The real-time PCR reaction mixture (20 μL) contained 10 μL SYBR Green Realtime PCR Master Mix (Toyobo), 0.5 μM of each forward and reverse primer, and 2 μL cDNA template. PCR was conducted at 94°C for 2 min, followed by 45 cycles at 94°C for 10 s, 58°C for 15 s, 72°C for 30 s, and 81°C for plate. Real-time PCR was performed in triplicate to ensure the reproducibility of results. The clone expression levels were calculated from the threshold cycle according to the delta-delta Ct method (Livak and Schmittgen, 2001). To confirm RNA purity, control PCR experiments were performed using purified RNA from a different sample as template instead of cDNA.

SUBCELLULAR LOCALIZATION ANALYSIS OF THPR10 PROTEIN

The full-length *ThPR10* coding region (without the termination codon) was fused with the GFP gene N-terminal for subcellular localization analysis. The ThPR:GFP fusion gene was driven by a cauliflower mosaic virus (CaMV) 35S promoter, and 35S::GFP was used as a control. The ThPR10:GFP and 35S::GFP constructs were introduced into onion epidermal cells by particle bombardment (Bio-Rad). Transiently transformed cells were analyzed by confocal laser scanning microscopy (CLSM) using LSM410 (Zeiss, Jena, Germany) with the following settings: GFP excitation 488 nm and emission 507 nm long-pass.

RESULTS

ThPR10 GENE CLONING AND BIOINFORMATICS ANALYSIS

The cDNA length of the cloned *PR* gene is 1082 bp, including the 102 bp of the 5' untranslation region (UTR) and the 494 bp of 3' UTR. A 486 bp ORF was detected in *ThPR10*, encoding a putative protein of 161 amino acids, showing high homology with the Bet v I family. The Bet v I family belongs to the PR10 group, indicating that this gene is a member of the PR10 group, and now designated *ThPR10* (GenBank number: FJ463256). The molecular mass of ThPR10 is 18.16 kDa and it is an acidic protein with a PI of 5.70.

Multiple sequence alignments were performed using PR protein sequences from nine different plant species. The results showed that ThPR10 shared more than 46% amino acid identity with the other PR10s analyzed (Fig. 1). Twenty-five amino acids are wholly conserved among these PR proteins, and may be important in PR protein function. In particular, the N-terminals are more highly conserved relative to other PR protein regions. Phylogeny reconstruction showed that the nine PR proteins are classed into three subgroups. One subgroup contained AAC13315 (*Pyrus communis*) and ABW99634 (*Prunus domestica*); another subgroup contained PR proteins CAN79558 (*Vitis vinifera*) and CAD33532 (*Datisca glomerata*). The PR proteins CAA50325 (*Corylus avellana*), AAU00066 (Solanum surattense), ACK38253 (*Tamarix hispida*), ACH63224 (*Rheum*) and AAL50007 (*Pinus monticola*) shared a relatively closer genetic relationship and constituted one subgroup. Among these nine proteins, the ThPR10 protein (ACK38253) exhibited a closer genetic relationship with RaPR10 (ACH63224) and AAU00066 (*Solanum surattense*) (Fig. 2).

PR RELATIVE ABUNDANCE IN ROOTS AND LEAVES

Our results showed that under normal growth conditions the *ThPR10* gene was expressed in roots and leaves of *T. hispida*. The abundance of *ThPR10* relative to the transcription levels of the five internal references in root and leaf tissue was calculated. The mean CT value of the five internal controls was used as the calibrator (designated 1) to calculate the relative expression of *ThPR10*. The results are shown in Figure 3. The relative abundance of *ThPR10* in roots was threefold higher than in leaves.

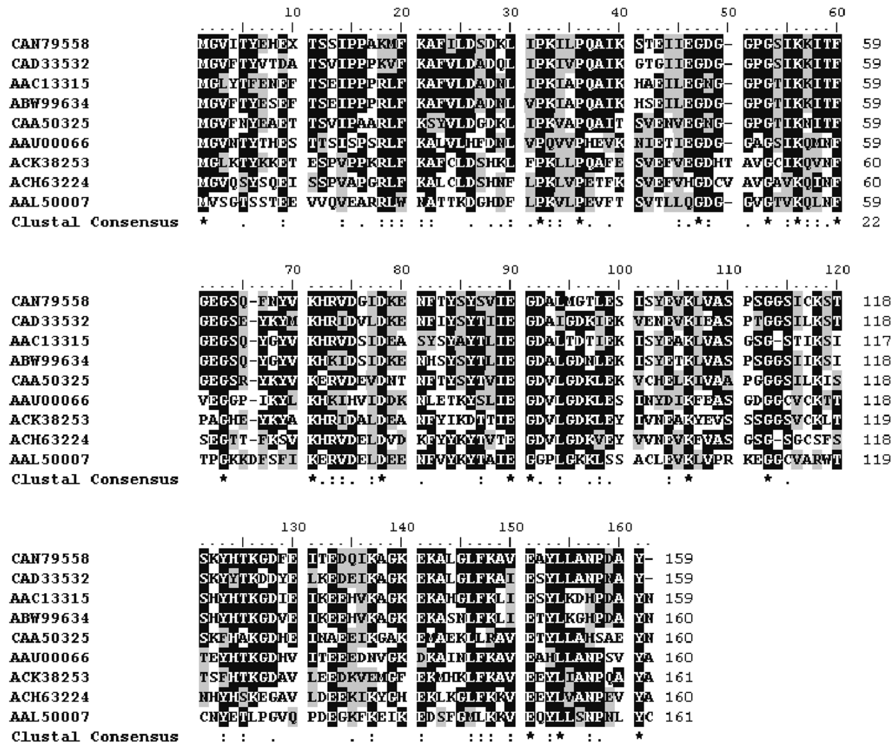

Fig. 1. Multiple-sequence alignment of PR10 proteins from 9 plant species, AAC13315:PR10 from *Pyrus communis*; ABW99634:PR10 from *Prunus domestica*; ACH63224:PR10 from *Rheum australe*; CAN79558:PR10 from *Vitis vinifera*; AAL50007:PR10 from *Pinus monticola*; ACK38253:PR10 from *Tamarix hispida*; CAD33532:PR10 from *Datisca glomerata*; AAU00066:PR10 from *Solanum virginianum*; CAA50325:PR10 from *Corylus avellana*. Identical amino acids are highlighted with a black background.

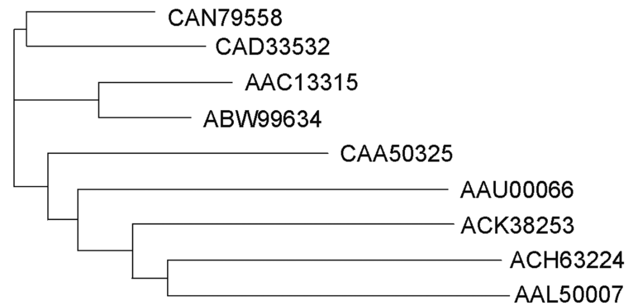

Fig. 2. Phylogenetic tree analyses of PR10 proteins from 9 plant species. AAC13315:PR10 from *Pyrus communis*; ABW99634:PR10 from *Prunus domestica*; ACH63224: PR10 from *Rheum australe*; CAN79558:PR10 from *Vitis vinifera*; AAL50007:PR10 from *Pinus monticola*; ACK38253:PR10 from *Tamarix hispida*; CAD33532: PR10 from *Datisca glomerata*; AAU00066:PR10 from *Solanum virginianum*; CAA50325:PR10 from *Corylus avellana*.

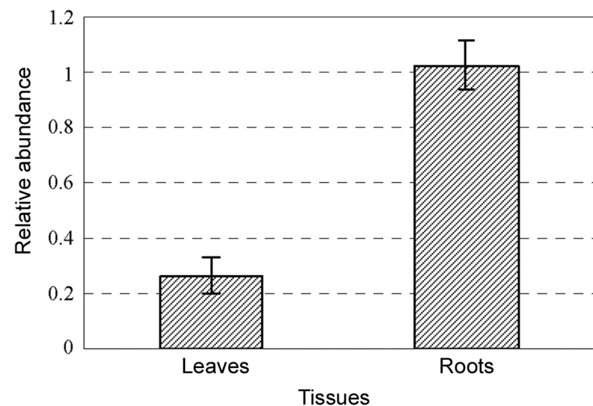

Fig. 3. Abundance of *ThPR10* relative to the transcription levels of the five internal references in root and leaf tissue.

ThPR10 EXPRESSION PATTERNS IN RESPONSE TO ABIOTIC STRESSES

We used real-time RT-PCR to investigate *ThPR10* expression in response to abiotic stresses. No PCR product was found using purified RNA (digested with DNase I) as PCR template, indicating that there was no DNA contamination of the purified RNA. The real-time RT-PCR results showed that abiotic stresses highly up-regulated the *PR10* gene (Fig. 4), sug-

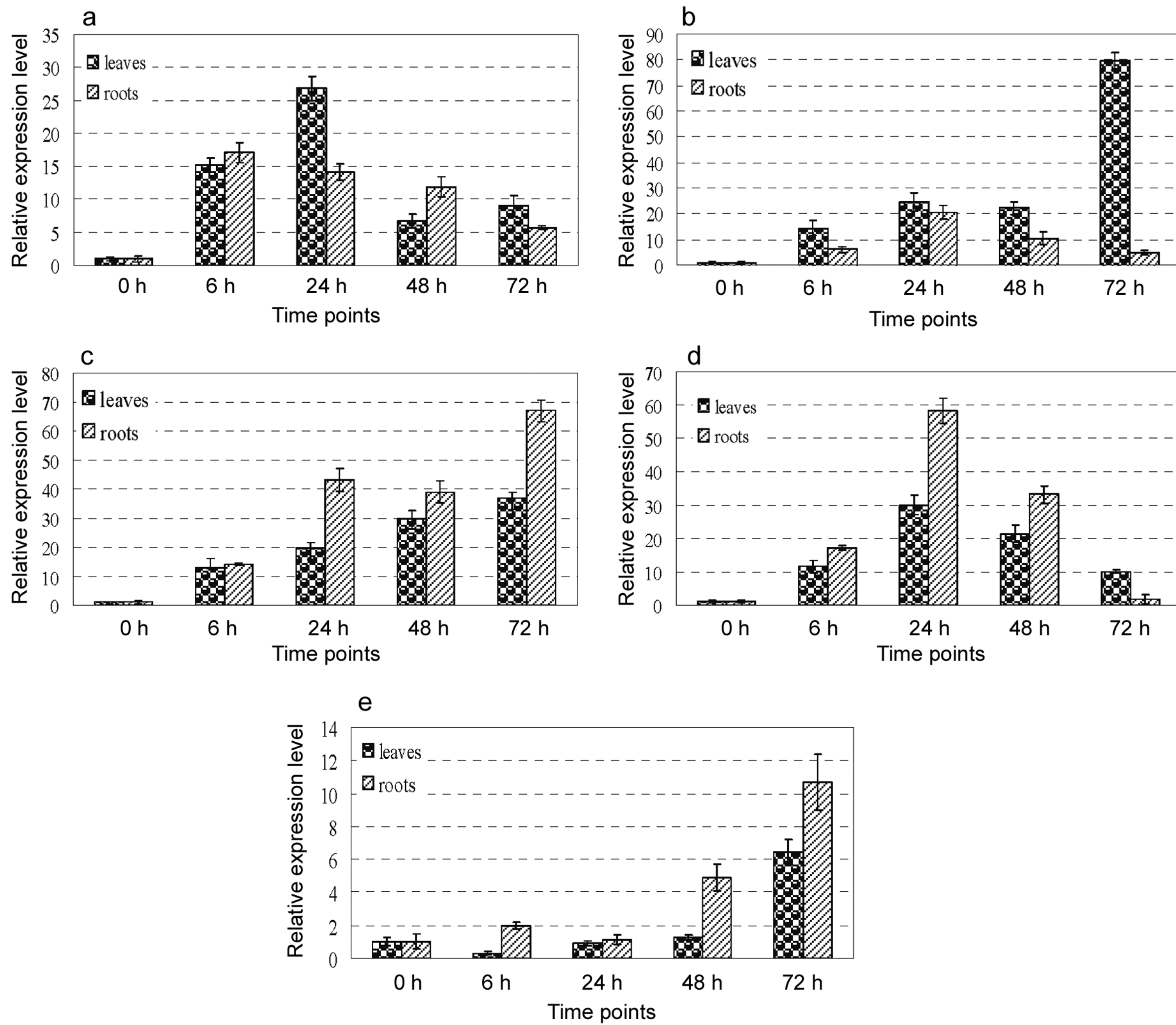

Fig. 4. Expression of ThPR10 in response to different abiotic stresses and ABA exposure. (**a**). NaCl stress; (**b**) PEG6000 treatment; (**c**) low temperature (4°C); (**d**) $CdCl_2$ stress; (**e**) ABA treatment.

gesting a role of *PR10* in abiotic stress responses. The *ThPR10* gene was highly induced in both leaf and root tissue under NaCl stress. The expression profiles were generally similar in roots and leaves; the transcript of the *ThPR10* gene was highly accumulated in roots and leaves before 24 h of stress, and then dropped (Fig. 4a). *ThPR10* displayed distinct expression profiles in root and leaf tissue under PEG stress. In leaves, *ThPR10* transcript was highly accumulated and reached maximum at 72 h stress; in roots the *ThPR10* gene was also highly induced during the stress period, but reached its maximum expression level at 24 h (Fig. 4b). Under low-temperature stress the *ThPR10* gene showed very similar expression profiles in roots and leaves; transcription levels increased throughout the cold treatment (up to 72 h) (Fig. 4c). *ThPR10* expression patterns for root and leaf tissue under $CdCl_2$ stress were similar. *ThPR10* was induced during the CdCl2 stress period, and reached peak expression at 24 h of stress in both root and leaf tissue (Fig. 4d). The transcription level of *ThPR10* under ABA treatment increased consistently in roots during the entire ABA treatment period; in leaves, in contrast, *ThPR10* was slightly down-regulated at 6 h, recovered at 24 h, and continued to increase for 48–72 h under ABA. Expression of the ThPR10 gene was highest at 72 h of ABA stress in both root and leaf tissue (Fig. 4e).

SUBCELLULAR LOCALIZATION OF ThPR10

The subcellular localization of ThPR10 was determined by transient expression assay. Both con-

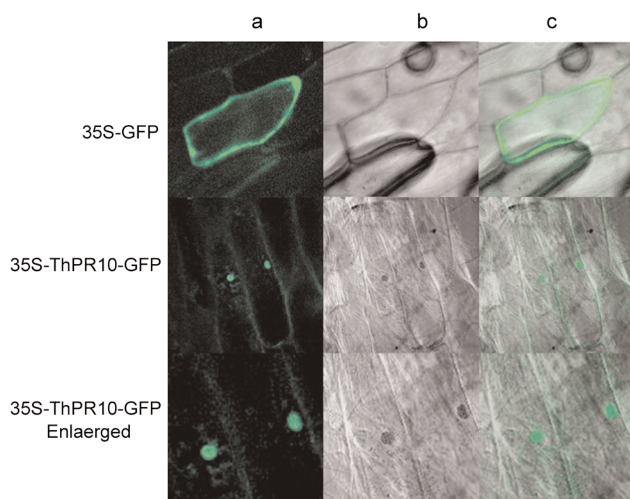

Fig. 5. Subcellular localization of ThPR10. 35S-GFP: 35S promoter driving GFP, as control; 35S-ThPR10-GFP, ThPR10 was fused with GFP and driven by 35S promoter. (a) fluorescent image, (b) bright field, (c) a and b merged image.

structs encoding ThPR10::GFP and GFP protein were introduced into onion epidermal cells by particle bombardment. Figure 5 presents confocal imaging of the ThPR10::GFP fusion protein, showing that the protein was accumulated mainly in the nucleus, while control GFP protein was detected in the cell membrane (Fig. 5), suggesting that ThPR10 is an intranuclearly localized protein.

DISCUSSION

We found that the *ThPR10* gene was expressed in both leaf and root tissue under normal growth conditions, and was induced by different stresses. The level of *ThPR10* expression relative to the five internal control genes varied between roots and leaves, and was much higher than in leaves (Fig. 3), suggesting that *ThPR10* has a relatively stronger physiological reaction in roots than in leaves under the experimental conditions. A previous study showed that a *PR10* from *Solanum surattense* was higher in roots than in other tissues of sterile seedlings cultured in jars (Liu et al. 2006). The high level of *PR10* in root tissue may be part of a constitutive defense mechanism in the plant organ most sensitive to environmental stress (Liu et al., 2006).

Previous work has shown that PR genes are involved in salt tolerance. For example, a soybean PR-5-homologous gene (*GmOLPa*) was highly induced in soybean roots, stems and leaves after initiation of high-salt stress (Onishi et al., 2006). A PR protein was differentially expressed by salt stress in grapevine (*Vitis vinifera*) (Jellouli et al., 2008), and a *PR* gene was induced by salt stress in *Arabidopsis* (Seo et al., 2008), potato (Zhu et al., 1995; Hmida-Sayari et al., 2005), maize (Andjelkovic and Thompson, 2006) and *Mesembryanthemum crystallinum* (Kore-eda et al., 2004). Previous study has shown that overexpression of the *PR* gene conferred salt tolerance in transgenic plants (Srivastava et al., 2006). Consistent with these studies, we found that the *ThPR10* gene was highly up-regulated in both roots and leaves, with the induction level highest in roots, more than tenfold that in leaves (Fig. 4a). The high induction of the *ThPR10* gene in root and leaf tissue suggests that it plays a role in root and leaf salt tolerance in *T. hispida*.

Differential regulation of *PR* genes has been demonstrated in maize (Andjelkovic and Thompson, 2006) and loblolly pine (*Pinus taeda*) (Lorenz et al., 2006). Dubos and Plomion (2001) cloned a PR10 protein from maritime pine drought-stressed seedlings and found that the transcript level of *PR10* displayed transient accumulation in the needles of drought-stressed plants. In other work, however, some *PR*s were found to be down-regulated by drought stress (Colditz et al., 2004). Our present results show that the *ThPR10* gene is highly induced by osmotic stress in both root and leaf tissue, particularly in leaves (nearly 80-fold) (Fig. 4b). This finding suggests that *ThPR10* is closely associated with the osmotic stress response.

PR genes also exhibited a low-temperature response. Some studies have shown that *PR* genes change on the gene expression or protein levels in response to low-temperature stress. For example, Ding (2002) treated tomato fruits with MeSA or MeJA, which increased some PR protein expression levels and enhanced plant resistance to chilling temperatures. Three potato PR type 5 proteins were up-regulated by low temperature in potato cell cultures and plants grown in vitro (Zhu et al., 1995). Yu (2001) showed that some antifreeze proteins similar to pathogenesis-related proteins are accumulated in the apoplast of winter rye leaves during cold acclimation. Consistent with these results, we found that *ThPR10* transcription increased throughout the treatment period in both leaf and root tissue under cold conditions (Fig. 4c), indicating that the *ThPR10* gene is closely related to cold stress. Some studies have noted down-regulation of a *PR* gene by cold stress (Liu et al., 2006), indicating that genes of the *PR* family may play different roles in response to cold stress.

Cadmium (Cd) pollution is a major concern worldwide, and has deleterious effects on plants and other organisms. Kieffer (2008) analyzed proteins in young poplar leaves exposed to $CdCl_2$ using 2-D DIGE, and found that the abundance of pathogenesis-related proteins markedly increased during treatment, suggesting that PR was involved in the

$CdCl_2$ response. Sarowar (2005) introduced the *CABPR1* gene, which encodes a basic pathogenesis-related protein 1 into tobacco, and found that overexpression of the *CABPR1* gene enhanced tolerance to $CdCl_2$ in the transgenic plants. We found that *ThPR10* is highly elevated in both leaves and roots, especially in root tissue with 120-fold induction at 24 h of cadmium stress (Fig. 4d), confirming that *ThPR10* gene expression is highly responsive to $CdCl_2$ stress. The transiently high increase of *ThPR10* expression in both leaves and roots of *T. hispida* may contribute to a rapid response to $CdCl_2$ shock.

ABA is a signal transducer which modulates protective mechanisms against environmental stress and plays a key role in initiation of many environmental stress responses. It is also known that ABA can induce the expression of a variety of different genes, including genes responding to dehydration, salt or cold stress (Tang et al., 2004; Xiong et al., 2001; Huang et al., 2008), antioxidant genes (Hu et al., 2007) and photosynthetic enzyme genes (Ueno, 1998). Previous work has demonstrated that *PR* genes can be up-regulated by ABA exposure, in particular the *PR* genes from *Solanum surattense* (Liu et al., 2006), rice (Randeep et al., 2001; Agrawal et al., 2000), soybean (Onishi et al., 2006), lily (Wang et al., 1999) and potato (Zhu et al., 1995). In the model legume *Medicago truncatula*, Colditz (2004) demonstrated that some PR proteins are induced by exogenous ABA, and among these PR proteins the PR10 proteins play a major role during root response to various stress conditions. We used exogenous ABA to investigate *PR* gene expression and our results were consistent with Colditz (2004): the *ThPR10* gene was highly induced by exogenous ABA in both roots and leaves, and its expression level increased with the duration of stress (Fig. 4e). The high rate of *ThPR10* induction by ABA showed that an ABA-dependent signaling pathway regulated *ThPR10*. This means that *ThPR10* is involved in an ABA-dependent stress response.

Arbuscular mycorrhizal fungi (AMF) have been shown to improve plant salt tolerance by employing various mechanisms such as enhancing nutrient acquisition, defending roots against soil-borne pathogens, and improving rhizospheric and soil conditions (Evelin et al., 2009). Moreover, AMF can improve the water absorption capacity of host plants by adjusting the osmotic balance and composition of carbohydrates and increasing root hydraulic conductivity (Evelin et al., 2009). *Tamarix ramosissima* has been found to be a non-mycotrophic plant (Beauchamp et al., 2005) but it is not known whether *T. hispida* is also a mycorrhizal plant. Whether AMF affects the expression of ThPR10 or not requires further study.

Some PR10 proteins are cytoplasmic proteins (Liu and Ekramoddoullah, 2006). Interestingly, our study indicated that the ThPR10 protein accumulated mainly in the nucleus (Fig. 5). Some other PR10 proteins might also be targeted to the nucleus. This is another area for further study.

We cloned the ThPR10 gene from *T. hispida* and found that it can be induced by different abiotic stresses and ABA exposure in both leaf and root tissue. These results suggest that ThPR may be involved in abiotic stress response and is part of ABA-dependent regulatory networks. *Tamarix* species are invasive plants which tolerate various adverse environments. Cloning and characterizing the stress-related gene *THPR10* may have potential applications in environmentally responsible invasion control.

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