Environmental and Experimental Botany xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Environmental and Experimental Botany

journal homepage: www.elsevier.com/locate/envexpbot

Tamarix hispida aquaporin *ThPIP2;5* confers salt and osmotic stress tolerance to transgenic *Tamarix* and *Arabidopsis*

Liuqiang Wang[a,b], Chunrui Zhang[a], Yanmin Wang[c], Yucheng Wang[a], Chuanping Yang[a], Mengzhu Lu[b,*], Chao Wang[a,*]

[a] State Key Laboratory of Tree Genetics and Breeding (Northeast Forestry University), Harbin 150040, China
[b] State Key Laboratory of Tree Genetics and Breeding, Key Laboratory of Tree Breeding and Cultivation of the State Forestry Administration, Research Institute of Forestry, Chinese Academy of Forestry, Beijing 100091, China
[c] Key Laboratory of Fast-Growing Tree Cultivating of Heilongjiang Province, Forestry Science Research Institute of Heilongjiang Province, Harbin 150040, China

ARTICLE INFO

Keywords:
Abiotic stress
Aquaporin
ROS-scavenging capability
Tamarix hispida

ABSTRACT

Aquaporin (AQP) proteins constitute a large family of protein channels that facilitate the transport of water and small neutral solutes through biological membranes, which is important for plants to combat abiotic stress. However, the precise functions of *AQP* genes involved in abiotic stress are not completely understood in plants. In this study, *ThPIP2;5*, an *AQP* gene of the PIP2 subgroup, was cloned and characterized from *Tamarix hispida*. Cellular localization assays showed that *ThPIP2;5* was localized to the plasma membrane. Transgenic *Arabidopsis* plants overexpressing *ThPIP2;5* displayed improved seed germination rates and increased root growth and fresh weight gain under salt and osmotic stresses, indicating that *ThPIP2;5* can improve abiotic stress tolerance. *ThPIP2;5*-overexpressing and RNAi-silencing *T. hispida* plants were generated using the transient transformation method and selected for gain- and loss-of *ThPIP2;5* analyses, respectively. Overexpression of *ThPIP2;5* in transgenic *Tamarix* and *Arabidopsis* plants increased ROS-scavenging capability, activities of antioxidant enzymes and proline contents; decreased malondialdehyde (MDA) and H_2O_2 contents; and reduced electrolyte leakage rates compared to equivalent controls under salt and osmotic stresses. Together, these data suggested that *ThPIP2;5* plays an important physiological role in abiotic stress tolerance in transgenic plants by reducing ROS accumulation and membrane damage and increasing the activities of antioxidants.

1. Introduction

Aquaporins (AQPs), which are also called water channels, belong to a highly conserved, major intrinsic protein family and interact with the cell membrane system to transport water and a variety of low-molecular-weight solutes. AQPs are present in all living organisms, from bacteria to higher plants and mammals (Danielson and Johanson, 2010; Mitani-Ueno et al., 2011). In higher plants, AQP proteins encoded by multiple genes form a large family that can be divided into five different subfamilies based on amino acid sequence homology and subcellular localization, including PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), NIPs (NOD26-like intrinsic proteins), SIPs (small basic intrinsic proteins) and XIPs (X intrinsic proteins) (Danielson and Johanson, 2008). Thus far, many *AQP* genes have been identified and cloned in different plant species, including 35 genes in *Arabidopsis thaliana* (Johanson et al., 2001), 36 genes in *Zea mays* (Chaumont et al., 2001), 33 genes in *Oryza sativa* (Sakurai et al., 2005), 54 genes in *Populus trichocarpa* (Gupta and Sankararamakrishnan, 2009) and 71 genes in *Gossypium hirsutum* (Park et al., 2010). Compared to these plant species, little is known about the *AQPs* in *Tamarix hispida* because of the unavailability of a complete genome sequence for this species.

Although the PIP, TIP, NIP and SIP subfamilies are conserved in plants, AQPs have diverse functions and play important roles in maintaining water balance, such as regulation of the water transport system in the roots, protection against a variety of environmental stimuli and facilitation of water transport through inner leaf tissues during transpiration (Maurel et al., 2008; Nguyen et al., 2013). Recently, some studies have shown that *AQPs* play several important roles in numerous physiological processes, including plant reproduction, seed germination, cell division and elongation (Kaldenhoff and Fischer, 2006; Okubo-Kurihara et al., 2009; Liu et al., 2013). Moreover, increasing evidence has demonstrated that *AQPs* participate in plant responses to environment stress (Hu et al., 2012; Sreedharan et al., 2013). For example, overexpression of *NtAQP1* in tomato (*Solanum lycopersicum*) plants resulted in higher stomatal conductance, whole-plant

* Corresponding authors.
E-mail addresses: lumz@caf.ac.cn (M. Lu), chaowang811@yahoo.com (C. Wang).

http://dx.doi.org/10.1016/j.envexpbot.2017.05.018
Received 25 March 2017; Received in revised form 26 May 2017; Accepted 27 May 2017

transpiration and leaf net photosynthesis under all conditions tested (Sade et al., 2016). The *TaTIP2;2* gene is down-regulated by salinity and drought stresses. Heterologous expression of the wheat *AQP* gene, *TaTIP2;2*, compromises the drought and salinity tolerance of transgenic *Arabidopsis via* an ABA-independent pathway (Xu et al., 2013). The *TsTIP1;2* gene is expressed highly in the leaves and flowers and is induced by several abiotic and hormonal stresses. Ectopic overexpression of *TsTIP1;2* significantly increases tolerance to drought, salt and oxidative stresses in *Arabidopsis* (Wang et al., 2014c).

Recently, many *AQP*s have been characterized for their diverse functions in model plants. However, *AQP*s from woody halophyte plants have rarely been cloned and characterized. *Tamarix hispida* is highly tolerant to salinity, drought and extreme temperatures and is widely distributed in the saline soils of drought-stricken areas of Central Asia and China. These characteristics make the species suitable for the investigation of stress tolerance mechanisms. Previously, we identified and analysed the expression patterns of 18 *AQP* genes in *T. hispida* under abiotic stress and found that *ThPIP2;5* is significantly induced by NaCl and PEG stresses and ABA treatment. To further study the physiological functions of *ThPIP2;5* in response to salt and osmotic stresses, *ThPIP2;5*-overexpressing and RNAi-silencing *T. hispida* plants were generated to investigate tolerance to salt and osmotic stresses. Furthermore, *Arabidopsis* plants overexpressing *ThPIP2;5* were also generated and used for study to further confirm the results obtained from *T. hispida* plants. Our results showed that the expression of *ThPIP2;5* significantly increased tolerance to salt and osmotic stresses in transgenic *T. hispida* and *Arabidopsis* plants.

2. Materials and methods

2.1. Plant materials and growth conditions

Seedlings of *T. hispida* were cultured in tissue culture bottles containing on half-strength Murashige-Skoog (1/2 MS) solid medium [2% (w/v) agar] in a culture room at an average temperature of 24 °C with 14 h light/10 h darkness photocycle conditions. Seeds of *Arabidopsis* plants (ecotype Columbia) were surface sterilized in 5% (v/v) sodium hypochlorite and germinated on 1/2 MS solid medium plates containing 0.8% agar. One-week-old seedlings were transferred from the plates to pots containing a mixture of soil/vermiculite/perlite (3:1:1) and grown in the greenhouse under the following conditions: 70–75% relative humidity, 24 °C temperature, and 16 h light/8 h dark photoperiod.

2.2. Cloning and sequence analysis of ThPIP2;5

The full-length cDNA of *ThPIP2;5* was cloned using gene-specific primers (Table S1), and the deduced amino acid sequence of the ThPIP2;5 protein, along with other that of PIPs from different plant species, were used for sequence alignment using CLUSTALX1.81 and for phylogenetic analysis using MEGA5.0 software with the neighbour-joining (NJ) method. The isoelectronic point (p*I*) and theoretical molecular weight (M_W) of the deduced ThPIP2;5 protein were predicted with the ExPASy compute p*I*/*M*w tool (http://www.expasy.org/tools/protparam.html). The transmembrane helical domain was predicted using TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

2.3. Real-time quantitative polymerase chain reaction (qRT-PCR) analysis

Total RNA was isolated from the whole *T. hispida* plants according to the CTAB (hexadecyltrimethylammonium bromide) method (Chang et al., 1993), and first-strand cDNA was synthesized using the PrimeScript™ RT Reagent Kit (TaKaRa, China). Real-time qRT-PCR was performed on an Opticon 2 System (Bio-Rad, Hercules, CA, USA) following the protocol as described by Wang et al. (2014b). The relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). All primer sequences used are shown in Table S1. At least three biological replicates with three technical replicates for each sample were performed.

2.4. Subcellular localization analysis of ThPIP2;5 protein

The full-length coding region of *ThPIP2;5* without the termination codon was ligated in frame to the N-terminus of the green fluorescent protein (GFP) in the pBI121 vector driven by the CaMV 35S promoter to generate the 35S:ThPIP2;5-GFP construct. The *GFP* gene under the control of the CaMV 35S promoter (35S:GFP) was used as a control. The constructs were introduced into onion epidermis cells by particle bombardment (Bio-Rad). The transformed cells were then analysed using confocal laser scanning microscopy (LSM410, Zeiss, Jena, Germany).

2.5. Plasmid construction and plant transformation

The full-length coding region of *ThPIP2;5* was cloned into the pROKII vector under the control of the 35S promoter. The recombinant plasmid was transferred into *Agrobacterium tumefaciens* strain EHA105 by the freeze-thaw method and introduced into *A. thaliana* by the floral dip method (Clough and Bent, 1998). Transformants were selected on 1/2 MS agar plates containing 50 mg/L kanamycin and further confirmed by PCR. The resulting T_3 homozygous transgenic lines were selected for further analyses.

A truncated inverted-repeat cDNA of *ThPIP2;5* (203 bp in length) was cloned into the RNAi vector pFGC5941 at the two sides of the CHSA intron to generate the construct pFGC5941:ThPIP2;5 to silence the expression of *ThPIP2;5*. Transient transformation of 6-week-old *T. hispida* seedlings was performed according to the transient transformation method described by Ji et al. (2014) with some modifications. Briefly, the whole seedlings were soaked in the transformation solution [1/2 MS + 150 μM acetosyringone + 3% (w/v) sucrose + 0.01% (w/v) Tween20, pH 5.8] with *Agrobacterium tumefaciens* EHA105 strain at OD_{600} of 0.6 and incubated with shaking at 100 r/min for 4 h at 25 °C. The seedlings were then washed with distilled water twice and whipped with sterile filter paper to remove excessive water. The whole seedlings were grown vertically on 1/2 MS solid medium [150 μM acetosyringone + 2.5% (w/v) sucrose, pH 5.8] in tissue culture bottles. Three groups of transgenic *T. hispida* plants were generated by transient transformation with 35S:ThPIP2;5 to overexpress *ThPIP2;5* (OE), with pFGC5941:ThPIP2;5 to silence the expression of *ThPIP2;5* (RNAi) or with an empty pROKII vector as a control (VC). After growth for 12, 24 and 48 h under normal conditions, the expression of *ThPIP2;5* in these transformed *T. hispida* plants was studied using qRT-PCR.

2.6. Analysis of stress tolerance

Two T_3 generation lines of *ThPIP2;5*-transformed *Arabidopsis* plants were selected for stress tolerance. To examine the germination rates under salt and osmotic stresses, seeds of wild-type (WT) and transgenic *Arabidopsis* plants were sown on 1/2 MS medium containing 80 mM NaCl or 100 mM mannitol and incubated at 22 °C for 7 days. The germination rates of transgenic lines and WT were calculated. The seeds were sown on 1/2 MS medium for 5 days and transferred to 1/2 MS medium or 1/2 MS medium plus 100 mM NaCl or 150 mM mannitol for 7 days. The root length and fresh weight were measured and the photographs of seedling were taken. The experiments were independently repeated at least three times.

DAB and NBT *in situ* staining for the detection of hydrogen peroxide (H_2O_2) and superoxide ($O_2\cdot^-$) were performed. The transformed *T. hispida* seedlings were treated with 120 mM NaCl or 175 mM mannitol for 24 h, and 4-week-old *Arabidopsis* plants were exposed to 100 mM NaCl or 150 mM mannitol for 2 h. Young branches from *T. hispida* and