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Molecular cloning of a plasma membrane aquaporin in *Stipa purpurea*, and exploration of its role in drought stress tolerance

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ABSTRACT

Stipa purpurea is widely distributed on the Tibetan Plateau, and has high drought resistance. Plasma membrane intrinsic proteins are a type of aquaporin. They regulate the movement of water and are associated with plant protective reactions to biotic and abiotic stresses. We characterized a plasma membrane intrinsic protein from *S. purpurea* (SpPIP1) and elucidated its role in molecular aspects of the plant's response to drought stress. The full-length open reading frame of *SpPIP1* was 870 bp and encoded 289 amino acids. The transcript level of *SpPIP1* was higher in the root of *S. purpurea* than in the flower, leaf and stem. The level of *SpPIP1* transcript increased significantly when treated with drought treatment. Subcellular localization result showed that *SpPIP1* was localized in the plasma membrane. Ectopic expression of *SpPIP1* in *Arabidopsis thaliana* resulted in plants with higher tolerance to drought treatment. *SpPIP1* of *S. purpurea* may mediate plant response to arid environments.

1. Introduction

Stipa purpurea Griseb. is a perennial plant that is endemic to the alpine steppes and meadows of the Tibetan Plateau, and dominant within that environment (Li et al., 2016; Yang et al., 2015; Yue et al., 2011). Known as the third pole, the Tibetan Plateau is characterized by arid, cold conditions, and high elevations. It is also strongly affected by global climate change (Carlyle et al., 2014). Many plants on the Tibetan Plateau have evolved survival mechanisms to adapt to stresses such as drought, and changing surroundings (Yang et al., 2014).

Aquaporins (AQPs) play an important role in regulating growth and development in almost all plants and are especially involved in mediating the response of plants to abiotic stresses such as drought, cold, and salinity (Aroca et al., 2007; Ayadi et al., 2011; Li et al., 2015; Yaneff et al., 2016; Yaneff et al., 2015). AQPs include a large family of channels that transport water across cell membranes (Ayadi et al., 2011), including plasma membrane intrinsic proteins (PIPs) (Li et al., 2015), nodulin-26-like intrinsic membrane proteins (NIPs), small basic intrinsic proteins (SIPs), tonoplast intrinsic proteins (TIPs) (Maurel et al., 2008). PIPs are considered representative of protein-mediated hydraulic conductivity in roots and leaves (Ayadi et al., 2011). PIPs have been clustered into two groups, PIP1 and PIP2 (Yaneff et al., 2015). PIP1 has a number of different roles in plants, including water or solute transport, maintenance of root hydraulic conductance, phloem loading and unloading, and stomatal activity (Fraysse et al., 2005; Kelly et al., 2014; Nouri and Komatsu, 2013; Zhou et al., 2014). Some AQPs may be redundant or replaceable by other homolog proteins (Li et al., 2015; Siefritz, 2002).

Expression of *AQP* genes depends on organ location, the presence of hormones, and the amount of abiotic stress (Ayadi et al., 2011; Heinen et al., 2009; Maurel et al., 2008; Sakurai et al., 2005). It has been reported that stem parenchyma cells of *Populus trichocarpa* responded to drought stress with sizable up-regulation of the PIP1 of water channels (Secchi and Zwieniecki, 2010). PIPs are well-studied in the important food plants of maize, wheat or soybean; however, there is no previous research on *S. purpurea* PIPs. We hypothesized that, in transgenic *Arabidopsis thaliana* plants, an *S. purpurea* PIP might function cooperatively with endogenous AQPs and increase the tolerance to abiotic stress.

Abbreviations: AQPs, aquaporins; PIPs, plasma membrane intrinsic proteins; *SpPIP1* and *SpPIP2*, *PIP1* and *PIP2* gene from *S. purpurea*; NIPs, nodulin-26-like intrinsic membrane proteins; *SpNIP1* and *SpNIP3*, *NIP1*, *and NIP3* gene from *S. purpurea*; SIPs, small basic intrinsic proteins; *SpSIP1* and *SpSIP2*, *SIP1*, *and SIP2* gene from *S. purpurea*; TIPs, tonoplast intrinsic proteins; *SpTIP1*, *SpTIP2*, and *SpTIP4*, *TIP1*, *TIP2*, *and TIP4* gene from *S. purpurea*; WT, wild-type; GFP, green fluorescent protein; CaMV, cauliflower mosaic virus; Fv/Fm, variable fluorescence/maximal fluorescence; MDA, malondialdehyde; RWC, relative water content; EL, electrolyte leakage; TW, turgid weight; DW, dry weight

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Similar studies also demonstrated that overexpression of *Brassica napus AQP* gene (*BnPIP1*) in transgenic tobacco plants could increase water stress tolerance at the whole-plant level (Yu et al., 2005). In this study, we describe the cloning and characterization of a novel *PIP1* gene from *S. purpurea*, designated *SpPIP1*. We analyzed the sequence, and measured expression levels of this gene involved in plant response to abiotic stress. We further investigated the relationship between *SpPIP1* and tolerance to drought stress in transgenic *A. thaliana* plants overexpressing full-length forms of *SpPIP1*. *35S:SpPIP1-GFP A. thaliana* plants were generated and drought stress tolerance was determined.

2. Materials and methods

2.1. Plant materials

Mature *S. purpurea* seeds were collected from the Nyima county of Nagqu prefecture, northern Tibet (N32°39′15″, E86°50′54″), China. The seeds were germinated in the dark and were transferred into soil in plastic containers (10 × 10 × 8 cm). Robust, uniformly growing 2-week-old seedlings were selected for testing and treatments. *A. thaliana* (ecotype: Col-0) plants were cultured for ectopic expression experiments on 1/2 solid Murashige and Skoog (MS) medium. After 10 days, seedlings were transferred to soil. All plants were grown in a phytotron (relative humidity of 75–80%, light intensity of 200 μmol photons $m^{-2} s^{-1}$) at 21 °C under photoperiod of 16 h day/8 h night.

2.2. Relative expression level of aquaporin genes of S. purpurea under drought stress

According to the previous drought stress-responsive transcriptome sequencing results (NCBI SRA: SRR825213), we found some aquaporin genes, including *SpNIP1*, *SpNIP3*, *SpPIP1*, *SpPIP2*, *SpSIP1*, *SpSIP2*, *SpTIP1*, *SpTIP2*, and *SpTIP4*. To determine these *aquaporin* gene expression levels under drought stress, *S. purpurea*, seedlings of uniform size were selected for drought stress treatment. Drought conditions were simulated by depriving seedlings of water for 7 and 11 days, and then rewatering as normal for 7 days. Leaves were sampled at the above-indicated time points for qRT-PCR analysis.

2.3. RNA isolation and SpPIP1 gene fragment cloning

Using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), we isolated total RNA from frozen leaves of *S. purpurea* samples according to the instructions. RNAs were treated with RNase-free DNase (Promega, Madison, WI, USA). DNA-free total RNA (3 μg) was reverse-transcribed for first-strand cDNA synthesis using GoScript™ Reverse Transcription System (Promega) according to the manufacturer's protocol. To obtain the full-length *SpPIP1* cDNA, rapid amplification of cDNA ends (RACE) was performed according to the manufacturer's instruction (BD Biosciences Clontech, MountainView, CA, USA). The *SpPIP1* full-length sequence was amplified using primers: *PIP1F* (5′-ATGGAGGGAAAGGAGGAGGAC-3′) and *PIP1R* (5′-CCTTCAAGAGCAAGTCATAA-3′), which were designed based on the expressed sequence tag (EST) fragment from transcriptome data of *S. purpurea* (NCBI SRA: SRR825213).

2.4. Sequence analysis of SpPIP1 gene

Using DNAMAN software, we translated the full-length *SpPIP1* cDNA sequence (Woffelman, 2004). The full-length cDNA sequence was analyzed using the online ExPASy ProtParam tool (http://web.expasy.org/protparam/). A phylogenetic tree was constructed by the neighbor-joining method using MEGA7.0 software (with 1000 replicates) (Kumar et al., 2016). We used the TMHMM 2.0 tool to predict protein transmembrane helices (Krogh et al., 2001).

2.5. SpPIP1 gene expression in different plant organs and under different stresses

Total RNA was extracted from roots, stems, leaves, and flowers of *S. purpurea* plants. For the drought, cold, and salt stress tests, we also extracted total RNA of *S. purpurea* seedlings which had been treated with 30% PEG-6000, cold (4 °C), and NaCl (250 mM) for 1 h, 6 h, and 12 h (0 h as the control). First-strand cDNA was synthesized from total RNA (3 μg) and stored at −80 °C. Quantitative real-time polymerase chain reaction (qRT-PCR) was used to test the expression levels of the *SpPIP1* gene. The primer sequences (Table S1) were used to amplify *SpPIP1*, with *SpACT1* (Table S1) as the internal control. FS Universal SYBR Green Master (Roche, Switzerland) was used for the qRT-PCR assay in ABI Steponeplus real-time PCR system (ABI, USA). The relative expression of genes was calculated using the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001).

2.6. Generation and screening of transgenic A. thaliana plants

The full-length coding sequence of *SpPIP1* was subcloned into a binary vector *pRI101-GFP*, containing the *GFP* gene driven by the cauliflower mosaic virus (CaMV) 35S promoter, forming a *35S:SpPIP1-GFP* fusion construct. The correct SpPIP1-GFP plasmid was transformed into *Agrobacterium tumefaciens* GV3101. Transgenic *A. thaliana* plants were produced via the floral dip method (Clough and Bent, 1998). The seeds of transgenic plants (T0) were selected on 1/2 MS medium containing 50 mg L^{-1} kanamycin. The overexpressing *35S:SpPIP1-GFP* transgenic lines (T3) were examined by PCR with npt II primers (5′-GAGGCTATTCGGCTATGACT-3′ and 5′-AATCTCGTGATGGCAGGTTG-3′) (Sun et al., 2012).

2.7. Subcellular localization of the SpPIP1

Root tissues of transgenic plants were incubated using propidium iodide (10 mg L^{-1}) for 5 min and washed three times with ultrapure water, then observed for expression of green fluorescent protein (GFP) using a laser-scanning confocal microscope (Olympus Optical Co. Ltd., Tokyo, Japan). GFP of the plants was excited with 488 nm and emitted at 505–530 nm, and propidium iodide was observed using 600–640 nm.

2.8. Functional analysis of transgenic A. thaliana plants

Drought tests were firstly performed on 1/2 MS medium with 6% polyethylene glycol 6000 (PEG). Seeds of wild-type and transgenic *A. thaliana* plants (T3) were cleaned and surface-sterilized, and sown on the culture medium. Root lengths were measured after 10 days. For examination of phenotypes of transgenic and wild-type plants under drought and natural growth conditions, the transgenic *A. thaliana* seedlings were cultured in plastic containers (10 × 10 × 8 cm) filled with nutrient soil under a normal watering regime, with four 3-week-old seedlings (three transgenic and one wild-type) per pot, in the phytotron previously mentioned. Seedlings of uniform size were selected for drought stress treatment. Drought conditions were simulated by depriving transgenic and wild-type *A. thaliana* seedlings of water for 5, 10 or 15 days, and then rewatering as normal for 2 days. Plants were sampled at the above-indicated time points for each treatment, and then frozen in liquid nitrogen. Chlorophyll fluorescence was measured using a Pulse-Amplitude-Modulation (PAM) Chlorophyll Fluorometer (Heinz-Walz-GmbH, Effeltrich, Germany). To measure the maximum quantum yield of PS II, plants were dark-adapted for 30 min. Fv/Fm (variable fluorescence/maximal fluorescence) was recorded during a saturating photon pulse (4000 μmol $m^{-2} s^{-1}$) using a whole plant.