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The *Panax ginseng PgTIP1* gene confers enhanced salt and drought tolerance to transgenic soybean plants by maintaining homeostasis of water, salt ions and ROS

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ABSTRACT

The *Panax ginseng PgTIP1* gene has been demonstrated to have high water channel activity by its heterologous expression in *Xenopus laevis* oocytes and in yeast, and it also plays significant roles in enhancing the salt/drought tolerance of *PgTIP1*-transgenic *Arabidopsis* plants and salt adaptation of *PgTIP1*-transformed *Glycine max*. In this work, *PgTIP1* gene was transformed into the hybrid soybean strain 4076 (F_5), which was selected for salt tolerance generation by generation from the cross assembly of *G. max* N23674 cultivar × *G. soja* BB52 accession. The *PgTIP1*-transgenic soybean plants mediated by the pollen tube pathway, represented by lines L19 and L29, were simultaneously analyzed at physiological and molecular levels for enhanced salt and drought tolerance. The results showed that the salt-stressed *PgTIP1*-transgenic lines L19 and L29 acquired better leaf stomatal movement and water-gas exchange capacity; less absorption, transport and accumulation of Na^+, Cl^-; a lower Na^+/K^+ ratio; and enhanced antioxidase activities of SOD, POD, CAT, and APX than WT in the roots and leaves. Under PEG-induced drought stress, the *PgTIP1*-transgenic plants showed greater leaf water-retention capacity and reduced cell membrane damage in roots and leaves. Therefore, the *PgTIP1*-transgenic soybean lines showed both salt and drought tolerance, which are related not only to the primary functions of the *PgTIP1* gene in terms of water status regulation but also to its secondary roles in up-regulating the expression of stress-related genes (*GmNHX1, GmSOS1, GmCLC1, GmCAT1, GmAPX1*, *GmNCED1* and *GmP5CS1*), which are involved in homeostasis of Na^+, K^+, and Cl^-; ROS scavenging; and synthesis of ABA, proline, etc.

1. Introduction

Aquaporins (AQPs), which localize to the plasma and intracellular membranes of plant cells, are membrane-intrinsic channel proteins with a conserved structure that can facilitate the transport of water and many small neutral or uncharged solutes, such as glycerol, urea, boric acid, silicic acid, arsenite, NH_3, CO_2, and H_2O_2 (Maurel et al., 2008; Chaumont and Tyerman, 2014; Srivastava et al., 2016; Tian et al., 2016a,b). Based on sequence similarity, subcellular localization and expression patterns, plant AQPs can be classified into five subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin26-like intrinsic proteins (NIPs), small and basic intrinsic proteins (SIPs), and uncategorized X intrinsic proteins (XIPs Kaldenhoff and Fischer, 2006; An et al., 2017). AQPs (mostly PIPs and TIPs) play direct and vital regulatory roles in plant water regulation and homeostasis during plant growth, development, and even stress adaptation by controlling root water uptake or transport, leaf transpiration, and water loss mediated by root and leaf hydraulic conductivity (Maurel et al., 2008; Chaumont and Tyerman, 2014).

Crop plants suffer from many abiotic (salt, drought, low temperature, heat, etc.) and biotic (disease, insects, weeds, etc.) stresses during their growth and development. Under a variety of adverse environments, AQPs may have positive (such as strengthening root water uptake or reducing leaf water loss) or negative effects depending on plant species, stress type and intensity. Additionally, the mechanisms by which AQPs regulate plant water homeostasis are particularly complex due to the diverse classes and functions of AQPs themselves. As a result, researchers often select one or several interesting aquaporin members in a certain plant to conduct concentrated and systematic studies on their physiological and molecular functions, particularly involving adaptation to adverse environments. Various analytical biological assays, such as *X. laevis* oocytes, yeast expression systems, mesophyll

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protoplasts, and even transgenic plants have been adopted to investigate beneficial functions of AQPs in plant water homeostasis and stress adaptation (Deshmukh et al., 2016). Many investigators have suggested that the differential (down- or up-regulated) transcriptional expression of aquaporin genes, especially for both PIPs and TIPs, and changes in aquaporin density through endomembrane trafficking and activity including posttranslational modifications, protein interaction, and subcellular relocalization are essential for water homeostasis in plant growth, development, and stress adaptation (Lin et al., 2007; Maurel et al., 2008; Wudick et al., 2009; Chaumont and Tyerman, 2014).

The *TIP* gene *PgTIP1*, which is specifically expressed in hormone-autotrophic ginseng cells, was demonstrated to possess high water channel activity by its heterologous expression in *X. laevis* oocytes and in yeast. In comparison to the wild-type (WT), the *PgTIP1* gene was also shown to have beneficial roles in the growth and development of *PgTIP1*-transgenic *Arabidopsis* plants under favorable conditions, including enhanced seed size and mass, and higher fatty acid content (Lin et al., 2007; Li and Cai, 2015). However, Peng et al. (2007) reported that *PgTIP1*-transgenic *Arabidopsis* plants have enhanced tolerance to salt and drought stress but a reduced ability to cold-stress. Further work by Li and Cai (2015) suggested that the conferred faster growth and enhanced salt tolerance in *PgTIP1*-transgenic *Arabidopsis* plants are related to its water channel activity determined by the Ser^{128} residue of PgTIP1.

Cultivated soybean (*G. max*) is one of the important grain and oil crops in the world. Although *G. max* is normally classified as a moderately NaCl-tolerant plant, conventional breeding of soybean cultivars for improved salt tolerance is challenging due to the narrow basis of genetic germplasm and relatively limited salt-tolerance phenotype variation (An et al., 2014; Zhang et al., 2011). Moreover, during soybean planting and production, except salt stress, water deficit or drought stress are often other major abiotic stresses that seriously hinder soybean growth and development, and obviously reduce the yield (Thao and Tran, 2012). In this case, wild soybean (*G. soja*), a relative of *G. max*, is often regarded as an important genetic germplasm resource for *G. max* breeding for good yield and quality (Lam et al., 2010; Zhang et al., 2011). To date, approximately 70 aquaporins have been discovered in the soybean genome, of which 23 are TIPs (Zhang et al., 2013; Song et al., 2016; Deshmukh et al., 2016). Wang et al. (2011) reported that *Arabidopsis* plants overexpressing *GsTIP2;1* from *G. soja* had reduced tolerance to salt and drought stress when the dehydration speed or water loss from the plant was increased. Song et al. (2016) studied the solute transport function of two soybean *TIPs* (*GmTIP1;5* and *GmTIP2;5*) by expression in *X. laevis* oocytes and found that GmTIP1;5 facilitated rapid water movement across the oocyte membrane, whereas GmTIP2;5 facilitated the movement of both water and boric acid. In a previous study from our group, we heterologously expressed *PgTIP1* in *G. max* (cv. Lee68 or Nannong 8831), and enhanced salt tolerance, which is associated with the positive regulation of water status, ion homeostasis, and ROS scavenging, was observed not only in *PgTIP1*-transformed soybean cotyledon hairy roots and composite plants but also in *PgTIP1*-transgenic whole plants (An et al., 2017). However, this previous study only explored the enhanced salt tolerance and related possible physiological mechanisms of *PgTIP1* gene expression in *G. max* cultivars, it is not clear whether heterologous expression of this gene would improve soybean salt tolerance in other species. If *PgTIP1* were expressed in *G. soja* or high-performing hybrids with *G. max*, will the effect on salt tolerance be similar or even greater? In addition to increasing salt tolerance, would it also contribute to soybean drought tolerance? Furthermore, if the *PgTIP1*-transgenic soybean plants are subjected to salt and drought stress, would *PgTIP1* affect the transcription of stress-related genes? Such genes might include *NHX1*, *SOS1*, and *CLC1*, which are directly involved in Na^+ and Cl^- transport (Li et al., 2006; Nie et al., 2015; Zhao et al., 2017; Wei et al., 2016); *SOD, POD, CAT*, and *APX,* which normally encode representative antioxidases that contribute to ROS scavenging (Cao et al., 2016; Zhao et al., 2017); *P5CS*, which encodes the enzyme that synthesizes the osmolyte proline (Porcel et al., 2005); *NCED*, which encoded a key enzyme of ABA synthesis (Yan et al., 2014); and *CIPK* (CBL-interacting protein kinase), which is closely related to Ca^{2+} signaling and transduction (Tsou et al., 2012; Hu et al., 2015).

In this work, the *PgTIP1* gene was transformed into the hybrid strain 4076 (F_5), which was selected for salt tolerance generation by generation from the cross assembly of *G. max* N23674 cultivar × *G. soja* BB52 accession (Du and Yu, 2010; Zhang et al., 2011), and the contributions to salt and/or drought tolerance of the selected *PgTIP1*-transgenic lines (L19 and L29) were investigated. The objective of this study was to further elucidate the physiological and molecular mechanisms of enhanced salt and/or drought tolerance of *PgTIP1*-transgenic soybean plants in relation to aspects of water status, ion uptake and distribution, antioxidase activity or ROS level, cell membrane damage, and plant growth and photosynthesis and to provide a theoretical and practical basis for the further exploration of stress-tolerant gene resources to be used in the cultivation or breeding of stress-tolerant transgenic soybean and other crop germplasms.

2. Materials and methods

2.1. Plant materials

Seeds of the hybrid strain 4076 (F_5), which was selected for salt tolerance generation by generation from the cross assembly *G. max* N23674 cultivar ×*G. soja* BB52 population, were used as WT for *PgTIP1*-transgenic soybean lines (L19 and L29) and subsequent salt and drought tolerance tests.

2.2. Vector construction and plant transformation

Primer sequences designed specifically for amplifying *PgTIP1* ORF (753 bp) were F: CCCAAGCTTATGCCGATTCACGAAATTGC; R: TCCCCCGGGGCTAGTAATCAGCGACAGGCAA where the underlined nucleotides indicate the *Hind* III and *Sma* I sites. cDNA from *Panax ginseng* was used as a template for amplification of the *PgTIP1* coding region. Amplified PCR products were subcloned into a pMD-18 T vector and sequenced (Sangon Biotech., Co. Ltd., China). The ORF of *PgTIP1* anchored in the cloning vector was isolated following digestion with *Hind* III and *Sma* I and cloned into the plant expression vector (pCAMBIA0390) using a double promoter (35S + pUbi) and NOS terminator. Additionally, the reconstructed plasmid pCAMBIA0390-*PgTIP1* from *Escherichia coli* DH5α was transformed into soybean WT (hybrid 4076 strain) using the pollen-tube pathway method (Moore et al., 1996; Li and Wu, 2007) and advanced to transgenic soybean lines (T_3 generation).

2.3. Nested PCR assay

A nested polymerase chain reaction (PCR) method (Ao et al., 2011) was adopted to detect the exogenous *PgTIP1* gene expression in two runs for the transformed soybean cotyledon hairy roots, composite and whole plants. The primer for the first PCR for a target product of 1865 bp was F: 5′-CTTCTTCGCCCGCCGTAAT-3′, R: 5′-ATGGCTCGTGACTG CTGCTGA-3′. The primer for the second PCR for a target product of 469 bp was F: 5′-TCGGTAGGTACGAAGAGTTCCGCC-3′, R: 5′-TCGATGGCG GTTGCGTAAACGG-3′. PCR reactions were performed using a PCR-cycler (ABI 2720, Applied Biosystems China, Inc. Beijing), with the reaction mixture consisting of 10 × rTaq buffer (2.5 μL), 2.5 mM dNTPs (2 μL), 25 mM $MgCl_2$ (2 μL), 10 μM primer (1 μL), 50 ng of prepared cDNA (1 μL), 5U/μL rTaq (0.2 μL), 16.3 μL of ddH_2O in a final volume of 25 μL. The first PCR conditions were as follows: an initial denaturation (3 min, 94 °C) followed by 35 cycles of denaturation (30 s, 94 °C), annealing (50 s, 61 °C) and extension (1 min and 50 s, 72 °C), and an