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A *ginseng PgTIP1* gene whose protein biological activity related to Ser^{128} residue confers faster growth and enhanced salt stress tolerance in *Arabidopsis*

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ABSTRACT

Water movement across cellular membranes is mostly regulated by aquaporins. A tonoplast intrinsic protein PgTIP1 from *Panax ginseng* has been found to play an important role in plant growth and development, and also in the response of plants to abiotic stress. However, the regulation of its function and activity remains unknown. To answer this question, mutated forms of PgTIP1 were made by replacing Ser^{128} with Ala (named S128A) or Asp (named S128D), and also by replacing Thr^{54} with Ala (named T54A) or Asp (named T54D). Then, wild type or mutated PgTIP1 was expressed in yeast and water transport was monitored in protoplasts. The substitution of Ser^{128} abolished the water channel activity of PgTIP1, while the substitution of Thr^{54} did not inhibit its activity. Moreover, the overexpression of PgTIP1 but not S128A or S128D in *Arabidopsis* significantly increased plant growth as determined by biomass production, it also had a beneficial effect on salt stress tolerance. Importantly, the overexpression of PgTIP1 led to the altered expression of stress-related genes, which made the plants more tolerant to salt stress. Our results demonstrated that PgTIP1 conferred faster growth and enhanced tolerance to salt in *Arabidopsis*, and that its biological activity related to Ser^{128} residue.

1. Introduction

Aquaporins (AQPs) are small integral membrane proteins in the range of 25–34 kDa that form channels through which water and/or small neutral solutes can across the membrane. They belong to a highly conserved membrane protein family called major intrinsic proteins (MIPs) [1,2]. In higher plants, AQPs are phylogenetically classified into seven subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), Nodulin 26-like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs), unrecognized X intrinsic proteins (XIPs), GlpF-like intrinsic proteins (GIPs), and hybrid intrinsic proteins (HIPs) [3–7]. TIPs can be further divided into five subgroups: TIP1, TIP2, TIP3, TIP4, and TIP5 [3,5,8,9]. Plant aquaporins have been implicated in physiological processes such as water absorption and nutrient uptake by roots, tissue expansion, transpiration, photosynthesis, and resistance to salt and water stresses [10–12]. These studies are developing our knowledge of the physiological significance of aquaporins in plants.

Many studies have shown that environmental stresses factors such as salt, drought and cold can upregulate AQPs and the transgenic approaches established that overexpression of some AQPs could improve the plant tolerance to abiotic stress [13–16]. In addition, data have indicated that plant AQP activity is regulated by a gating mechanism that might be modulated by different factors, including phosphorylation, heteromerization, pH and Ca^{2+}, pressure, solute gradients, and temperature [17–20]. Several plant aquaporins have been shown to be phosphorylated in vivo and in vitro at serine residues within the N- or C-terminal regions [19,21–27]. Moreover, the mutation of the serine residue has been reported to alter the water channel activity of these AQPs. For example, the Ser^{123} mutation of *Mc*PIP2;1 abolished the water channel activity, and the Ser^{282} mutation of *Mc*PIP2;1 also reduced the water channel activity by 51.9% inhibition [25]. The Ser^{278} mutation of *Rs*PIP2;2 resulted in 64% decrease in water transport activity [27]. The mutation of this serine residue to alanine in PvTIP3;1 resulted in a decrease of the oocyte osmotic water permeability (Pf) by 50% compared with oocytes that expressed the wild-type isoforms [22]. These studies all involved the analysis of mutants and highlighted

Abbreviations: T54A, Thr54 of PgTIP1 replaced by Ala; T54D, Thr54 of PgTIP1 replaced by Asp; S128A, Ser^{128} of PgTIP1 replaced by Ala; S128D, Ser^{128} of PgTIP1 replaced by Asp; *PgTIP1*-OXP, *Arabidopsis* plants overexpressed with *PgTIP1*; *S128A*-OXP, *Arabidopsis* plants overexpressed with *S128A*; *S128D*-OXP, *Arabidopsis* plants overexpressed with *S128D*.

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the importance of specific serine residues in this regulatory mechanism. Although the discovery of aquaporin activity regulation has resulted in a paradigm shift in the understanding of AQP functions, a comprehensive picture of the relationship between activity regulation and physiological functions remains elusive.

Tonoplast intrinsic proteins (TIPs) are the major components of the vacuolar membranes, and are the most abundant aquaporins in organisms [28]. It has been suggested that TIPs function as channels to facilitate the transport of water, urea and NH_3 [29–32]. TIPs have been reported to perform important functions in the responses to diverse abiotic stresses, such as drought, salt and oxidative stresses. For example, the overexpression of *SlTIP2;2* in tomato dramatically altered plant water transport, enhancing transpiration and modifying leaf water potential maintenance under drought conditions. Nevertheless, the transgene also had beneficial effects on plant growth and fruit yield under both control and water stress conditions [8]. The overexpression of *TsTIP1;2* enhanced tolerance to drought, salt and oxidative stresses in transgenic *Arabidopsis* [33]. These studies suggested that TIPs play an important role in controlling plant physiology both under normal and stress conditions.

Panax ginseng is a slowly growing perennial herb from the family of *Araliaceae* and is cultivated for its highly valued root used for medicinal purposes. Ginseng cultivation has its own difficulties, as it is a shade-loving crop and cultivated for long periods (4–5 years). In consequence, ginseng plants are more easily exposed to different environmental stresses, which may seriously affect its growth. Recently studies have shown that the higher cellular levels of spermidine and spermine facilitate the transcription of *PgSPD* and *PgSAMDC* in *Panax ginseng* in response to salt stress [34,35]. However, the detailed mechanisms need to further study. In our earlier study to explore the molecular differences between habituated and non-habituated *Panax ginseng* calli, a tonoplast intrinsic protein gene, *PgTIP1* (highly orthologous to *AtTIP1;1*), was found to be highly expressed in hormone-autotrophic ginseng cells, and also has a significant water channel activity [36]. Lin et al. also demonstrated a significant role for *PgTIP1* in the growth and development of *Arabidopsis*. Further investigations that overexpressed *PgTIP1* in *Arabidopsis* plants suggested that the overexpression of *PgTIP1* has a beneficial effect on salt-stress tolerance [37]. These results indicated that PgTIP1 plays a role in plant growth and development, and also plays a role in plant responses to abiotic stress. However, the mechanisms that regulate its function and activity are unknown to date.

This current study aimed to explore the regulation of PgTIP1 function and activity, and also to investigate how its overexpression affected the response of plants to salt stress. We initially mutated the predicted phosphorylation sites using the KinasePhos tool, whereby the Thr^{54} residue of PgTIP1 was mutated to Ala or Asp, and the Ser^{128} residue was mutated to Ala or Asp. Then, we expressed the WT or mutated PgTIP1 protein in yeast. The results showed that PgTIP1 is a functional water channel protein, and that the substitution of Ser^{128} abolished the water channel activity of PgTIP1 while the substitution of Thr^{54} did not inhibit this activity. In addition, *PgTIP1* but not *S128A* or *S128D* overexpression in *Arabidopsis* significantly increased plant growth and had a beneficial effect on salt stress tolerance. The results from this study demonstrated that *PgTIP1* confers faster growth and enhanced tolerance to salt in *Arabidopsis*, and that Ser^{128} plays an important role in the biological activity of PgTIP1.

2. Materials and methods

2.1. Yeast strains and the plasmid

The *Saccharomyces cerevisiae* strains used in this study were W303-1a (*MATa leu2-3/112 ura3-1 trp1-1 his3-11/15 ade2-1 can1-100 GAL SUC2 mal0*). The coding region of *PgTIP1* was cloned into a pYX212 vector (multicopy 2 μl vector, constitutive TPI promoter, *URA3* marker, HA-tag) using EcoR1and BamH1 restriction sites. The single mutation of *PgTIP1* was made using a site-directed mutagenesis kit (TransGen) by replacing Thr^{54} and Ser^{128} with Ala or Asp. The construct was confirmed by sequencing analysis. Transformation of the plasmids into yeast cells was performed using the lithium one step transformation protocol [38].

2.2. Yeast growth conditions

Yeast cells were grown in 2% peptone, 1% yeast extract, and 2% glucose (YPD). Selection and growth of the transformants was performed in a synthetic medium (yeast nitrogen base, YNB; 2% glucose). The synthetic media was buffered with Na/succinate and pH adjusted with NaOH. For the growth assays, the cells were pregrown for 2 days on YNB plates, and then re-suspended in YNB. The cells were then grown at 30 °C in a standard laboratory shaker with 200 rpm.

2.3. Yeast membrane preparation and western blot analysis

Transformed yeast cells were harvested in mid-logarithmic phase, total yeast membrane proteins were extracted by Yeast Membrane Protein Extraction Kit (Pierce). The proteins were quantified by using Bio-Rad Protein Assay reagent (Bio-Rad). In brief, 10 μg protein was loaded for western blot analysis. Membranes were blocked with PBS-5% milk and probed for 2 h with 1:3000 diluted anti-HA mouse monoclonal antibody (Abcam), washed and incubated for 2 h with second antibody (HRP-conjugated anti-mouse Ig G, Abcam), and diluted 1:3000 in PBS-5% milk. Membranes were incubated with Lumi-Light (Bio-Rad) for detection.

2.4. Water transport assay

Transformed cells were grown to a mid-logarithmic phase, harvested, washed once in water and once in 1 M sorbitol, and then suspended in SCE buffer (1 M Sorbitol, 0.1 M sodium Citrate, 10 mM EDTA, 0.2 mM β-mer-captoethanol; pH 6.8) containing 2000 U of lyticase (Sigma) per milliliter of culture before undergoing shaking incubation for 3 h at 30 °C. After confirming protoplast formation microscopically, protoplasts were harvested, washed twice and re-suspended in STC buffer (1 M Sorbitol, 10 mM Tris–HCl, pH 7.5, 10 mM $CaCl_2$). Protoplasts were diluted to 0.5 M sorbitol at time (t) = 0 and OD_{600nm} values were recorded at subsequent known time points as indicated. The recorded values are an average of three independent transformants and each data point has been normalized to its own starting OD.

2.5. In vitro phosphorylation and immunoprecipitation assays

Isolated yeast membranes (20 μg protein) were incubated in a 100 μl reaction mixture containing 24 mM Tris/MES, pH 6.5, 10 mM $MgCl_2$, 0.45 mM EDTA, 2 mM DTT, 0.5 mM $CaCl_2$ and 1 μl [γ-^{32}p]ATP (10 μCi/μl) for 1 h at 25 °C. Afterward, membrane proteins were solubilized with 400 μl NET-gel buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 0.25% BSA, 0.02% NaN_3) and incubated over-night at 4 °C with anti-HA antibodies (1/500 dilution, Abcam) on a rotating table. Then 20 μl Protein A-Sepharose CL-4B (Pharmacia) was added and incubation continued for 2 h at 4 °C. Membrane proteins were pelleted at 12,000 × g for 30 s. After two washes with NET-gel buffer, the protein pellet was washed with 10 mM Tris/HCl, pH 7.5, and 0.1% Nonidet P-40, and centrifuged at 12,000 × g for 30 s. The pellet was then resuspended with 20 μl 2.5%-Laemmli sample buffer and heated at 95 °C for