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RESEARCH PAPER

Isolation and functional characterization of PgTIP1, a hormone-autotrophic cells-specific tonoplast aquaporin in ginseng*

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Abstract

The suppression subtractive hybridization technique was used to identify differentially expressed genes between hormone-autotrophic and hormone-dependent *Panax ginseng* callus lines. A tonoplast intrinsic protein cDNA (*PgTIP1*) was found to be highly and specifically expressed in hormone-autotrophic ginseng cells, which was slightly up-regulated by cytokinin while significantly down-regulated when treated with auxin. *PgTIP1* encodes a polypeptide of 250 amino acids which shows sequence and structure similarity with tonoplast aquaporins in plants. The water channel activity of PgTIP1 was demonstrated by its expression in *Xenopus laevis* oocytes. When over-expressed in *Arabidopsis thaliana*, *PgTIP1* substantially altered the plant's vegetative and reproductive growth and development. *Arabidopsis* plants over-expressing *PgTIP1* showed significantly enhanced seed size and seed mass plus greatly increased growth rate compared with those of the wild type. Moreover, the seeds from *PgTIP1* over-expressing *Arabidopsis* had 1.85-fold higher fatty acid content than the wild-type control. These results demonstrate a significant function of *PgTIP1* in the growth and development of plant cells.

Key words: *Arabidopsis*, ginseng, habituation PgTIP1, tonoplast intrinsic protein.

Introduction

The growth and development of plant cells depend on tight regulation of cellular water movement and homeostasis. Aquaporins (AQPs), which facilitate and regulate passive exchange of water across membranes (Agre, 1992; Chrispeels and Agre, 1994; Schäffner, 1998), belong to a highly conserved membrane protein family MIP (major intrinsic protein). In higher plants, AQPs are divided into four subfamilies: PIPs, TIPs, NIPs, and SIPs, mainly based on the membrane location and function (Chaumont *et al*., 2001; Johanson *et al*., 2001; Baiges *et al*., 2002; Quigley *et al*., 2002). Aquaporin expression can be regulated at both the RNA and protein levels. Over-expression or antisense/knockout reduction of certain AQPs highlights their important roles in numerous physiological processes in plants (Kaldenhoff *et al*., 1995, 1998; Gerbeau *et al*., 2002; Javot *et al*., 2003; Aharon *et al*., 2003; Uehlein *et al*., 2003; Hanba *et al*., 2004; Hachez *et al*., 2006). Although the discovery of AQPs has resulted in a paradigm shift in the understanding of plant water relations, a comprehensive picture of their physiological role(s) in plant growth and development remains elusive.

It is well established that *in vitro* plant cell/tissue cultures require exogenous supply of plant hormones (auxins and cytokinins) for their sustained growth (Collin and Edwards, 1998). However, certain cell-lines, although originally grown in hormone-based culture medium, may lose this dependency on one or more externally supplied plant

* Data deposition footnote: The GenBank accession numbers for *NtAQP1*, *AtTIP1;1*, *OsTIP1;1*, and *ZmTIP1;2* are Y08161, X72581, XM_470213, and AF326500, respectively.

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hormones for growth and become hormone-autotrophic or habituated/autonomous (Meins, 1989; Gaspar *et al*., 2002). Habituation, a stable heritable competency of plant cells to proliferate without hormonal supply, is a distinct response from the one associated with tumour development: tumours are mediated by pathogens or result from genetic transformation (Gaspar, 1998). Despite the reports on habituation in many cell types (Jäger *et al*., 1997), what controls this phenomenon is not well understood.

Although increasing the levels of auxins and cytokinins would result in cell expansion, the physiological factor that directly drives plant cell expansion is turgor pressure, which is mainly generated by a rapid influx of water into the cells. The uptake of water by expanding plant cells may well involve AQPs. Evidence showed that some AQPs were up-regulated by exogenous auxin during cell growth (Werner *et al*., 2001; Ozga *et al*., 2002). We have on hand habitutated callus line from *Panax ginseng* that exhibits autotrophy for both auxin and cytokinin. No significant difference between these two callus lines was found with respect to the level of active free auxin and cytokinin concentration by ELISA measurement (data not shown). In order to explore the molecular differences between habituated and non-habituated ginseng calli, the differentially expressed genes were screened using the suppression subtractive hybridization (SSH) method. Besides the down-regulation of some cDNAs in habituated cells, for example, early auxin responsive gene *GH3*, periodic tryptophan protein gene *PWP*, aconitase gene *ACO*, retrotransposon-like gene, and many other genes with so far unknown functions, an aquaporin gene (*PgTIP1*) was detected, which belongs to plant TIPs subfamily, and was specifically and highly expressed in hormone-autotrophic ginseng cells. The results from our study, which demonstrated that *PgTIP1* significantly altered the growth and developmental attributes of plants when over-expressed in *Arabidopsis thaliana*, are reported here.

Materials and methods

Plant cell cultures

Hormone-autotrophic (H; habituated) and hormone-dependent (NH; non-habituated) *Panax ginseng* calli were cultured on hormone-free 67V medium and 67V medium, respectively (Veliky and Martin, 1970); the latter supplemented with 1.5 $mg \cdot l^{-1}$ dichlorophenoxyacetic acid (2,4-D), 1 $mg\ l^{-1}$ indole-3-acetic acid (IAA), 0.1 $mg\ l^{-1}$ naphthalene acetic acid (NAA), and 0.25 $mg\ l^{-1}$ kinetin (KT), pH 5.8. Both callus lines were maintained at 24 °C in the dark. Suspension cultures of the ginseng calli were maintained at 24 °C in 250 ml flasks with 50 ml medium; cultures were aerated by shaking at 0.5 *g* on a rotary shaker in the dark, and were subcultured every 28 d.

Suppression subtractive PCR and northern blot analysis

$PolyA^+$ RNA were isolated from hormone-autotrophic and hormone-dependent ginseng cells using mRNA purification kit (Qiagen). The $polyA^+$ samples were used to construct driver- and tester-cDNAs following the protocol of the PCR-Select cDNA subtraction kit provided by the manufacturer (Clontech). Selectively amplified products were inserted into pMD18-T vector using a T/A cloning kit (Takara). Northern blot analysis was performed using the digoxigenin-dUTP system (Roche) and the cDNA fragment screened from SSH library was labelled as a probe for hybridization.

Full-length cDNA cloning of PgTIP1 and bioinformatic analysis

A cDNA library of hormone-autotrophic ginseng cells was constructed and screened according to the instructions in ZAP Express Predigested Vector Kit and ZAP Express Predigested Gigapack Cloning Kit (Stratagene). Nucleotide and deduced amino acid sequences were analysed with Bioedit software. Homology searches were made in all major databases. Alignments of amino acid sequences were generated and edited with DNAStar software. Predicted stereo structure of PgTIP1 and AQP2 and ar/R region simulation were performed at http://swissmodel.expasy.org (Schwede *et al*., 2003).

Hormone treatments and analysis of PgTIP1 *expression*

The phytohormones KT and 2, 4-D or both were added to ginseng suspension cultures at the final concentration of 0.25 $mg\ l^{-1}$ and 1.5 $mg \cdot l^{-1}$, respectively. To determine the transcript levels in different hormone-treated samples, the real-time quantification of RNA target was performed in the Rotor-Gene 3000 real-time thermal cycling system using SYBR Green RT-PCR kit (Toyobo). The PCR primers for *Act* were 5′-GTGTTGCCCCAGAAGAGC-3′ in sense and 5′-CAGAATCCAGCACAATACCT-3′ in antisense orientation, and those for *PgTIP1* were 5′-CTCAGGCTTGGCATTTAG-3′ and 5′-CCCAGTTCTCCCTTCTTT-3′, respectively. The reaction mixture (25 μl) contained 200 ng of total RNA, 0.5 μM of each primer, and appropriate amounts of enzymes and fluorescent dyes as recommended by the manufacturer. The Rotor-Gene 3000 cycler was programmed as follows: 2 min at 95 °C for pre-denature; 40 cycles of 15 s at 94 °C, 15 s at 55 °C, 20 s at 72 °C for *Act* and 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C for *PgTIP1*. The data were collected during the extension step. No detectable fluorescence signal was detected in control samples where H_2O was added to the reaction mixture instead of RNAs. A possible contamination by genomic DNA of the RNA sample was carefully monitored and avoided.

Xenopus oocyte expression

The coding region of *PgTIP1* and *AQP2* (positive control) were cloned into pXBG-ev1 vector (Li *et al*., 2000) using a Bgl II restriction site. After digestion and linearization of the plasmid, the complementary RNAs (cRNA) were synthesized *in vitro* using the mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (Ambion). Oocyte preparation, injection, and expression were performed as described by Daniels *et al*. (1996). Osmotic water permeability of oocytes was determined essentially as described by Weig *et al*. (1997).

Generation of PgTIP1*-overexpressing* Arabidopsis *plants and their phenotypic analysis*

The ORF of *PgTIP1* was cloned into pHB vector (Mao *et al*., 2005) using a *Hin*dIII and a *Xba*I restriction site to generate double 35S:*PgTIP1* transgene. *Arabidopsis* plants (ecotype Columbia-0) were transformed with this transgene using the floral-dipping method (Clough and Bent, 1998). Independent hygromycin-resistant lines (T_0) were isolated and amplified. Experiments were conducted with homozygote T_2 plants.

The root length measurements were made on 1-week-old plants cultured with MS medium on a vertical plate. Leaf histological analysis (fifth-leaf samples, 2-week-old) was performed according to Hu *et al*. (2003).

Average mass of the seeds produced by *PgTIP1* over-expressing *Arabidopsis* plants was determined by weighing mature dry seeds in batches of 1000; at least three sample batches (of 1000 each) were weighed for a given data point and average value was from three independent transgenic lines. Size distributions of WT and transgenic seed populations were analysed by a scanning electron microscope equipped with 'smileview' software (JEDL JSM-6360LV, Japan).

Fatty acid extraction was performed as described by Fiehn *et al*. (2000) using 0.3 g dry seeds. Nonadecanoic acid methyl ester stock solution (2 mg ml^{-1} $CHCL_3$) and ribitol stock solution (0.2 mg ml^{-1} H_2O) were used as internal standards for the lipid phase and the polar phase, respectively. Selected subsamples were injected in a GC-mass spectrometer (6890N GC System/5973 MS Selective Detector) and resultant electron ionization mass spectra were used to identify and quantify individual fatty acid species. The quantity of each fatty acid was determined by comparison with the internal standard and average value of total fatty acid content was from three independent transgenic lines as example. Seeds were collected from *Arabidopsis thaliana* plants that were grown at 22 °C with a 16/8 h day/night cycle in a greenhouse.

Leaf net photosynthetic rates, stomatal conductance, intercellular CO_2 concentration, and transpiration rates were measured by a portable gas analysis system, Li-Cor 6400 with a light-emitting diode light source (Li-Cor Inc. Lincoln, Nebraska, USA).

Results

PgTIP1 *belongs to the MIP super gene family*

SSH employing the mRNAs from autonomous and hormone-dependent ginseng cells allowed the isolation of a cDNA for tonoplast intrinsic protein that is referred to here as *PgTIP1*. The full-length cDNA of *PgTIP1* includes a 750 bp open reading frame (GenBank accession number DQ237285), and encodes a protein of 250 amino acids (Fig. 1). BLASTX and ClustalX analyses (Thompson *et al*., 1997) of this gene indicated high similarities to putative plant aquaporins: *PgTIP1* deduced protein is most similar to NtAQP1 (80.8% identity), a tonoplast aquaporin, and has high homology to AtTIP1;1, OsTIP1;1, and ZmTIP1;2 at 78.8%, 77.2%, and 68.4%, respectively. The hydrophobicity profiles of PgTIP1, as determined by using BioEdit and TMHMM (http://www.cbs.dtu.dk/services/TMHMM), indicated six highly hydrophobic regions (A–F) corresponding to membrane-spanning putative α-helices that are characteristic of AQPs. It also has two NPA domains, which form the water pore within the membrane lipid bilayer. Secondary and stereo structural analyses (see Fig. 1 in the supplementary data at JXB online) revealed the similarity of

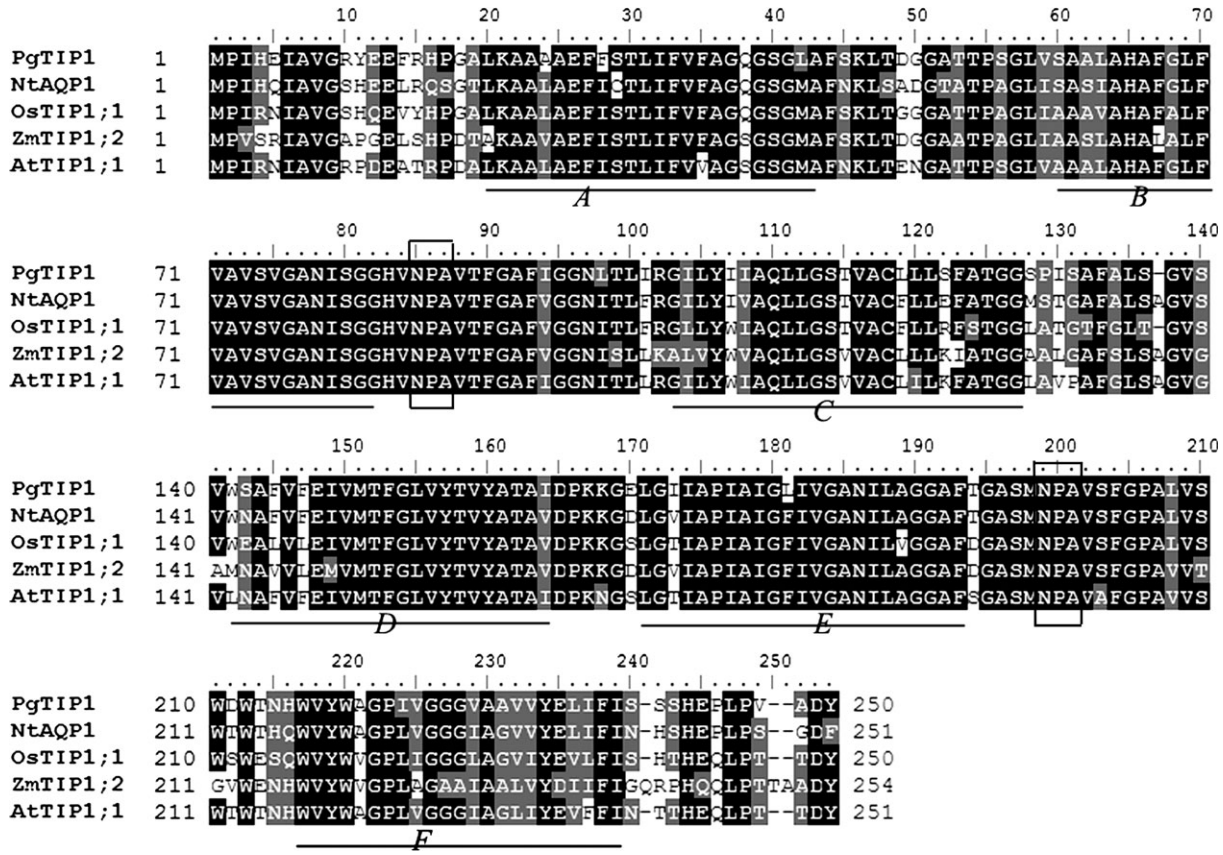

Fig. 1. The sequence alignment of PgTIP1 and other plant TIPs.

three-dimensional structure of PgTIP1 to that of aquaporin 2 (AQP2). The conserved narrow selectivity filter region (the aromatic/Arg [ar/R] filter) of PgTIP1 is formed by H-65(H2), I-186(H5), A-195(LE1), and V-201(LE2) (see Fig. 2 in the supplementary data at JXB online). This ar/R tetrad was identical to that of the Group I TIP in *Arabidopsis* (AtTIP1;1) (Wallace and Roberts, 2004), which has already been shown to have high water channel activity.

PgTIP1 *is a novel gene that is specifically expressed in habituated ginseng cells and inhibited by auxin*

PgTIP1 fragment was strongly expressed in autonomous ginseng cells but was not detected in hormone-dependent cells as indicated by northern blot analysis (Fig. 2A). This is a first report on an aquaporin expression response in context with the phenomenon of autonomous growth. Using the real-time reverse transcriptase (RT)-PCR method, the expression of *PgTIP1* under different hormone treatments was studied in suspension-cultured hormone-autotrophic ginseng cells. Data indicated that the *PgTIP1* was up-regulated by kinetin (KT, 0.25 mg l^{-1}) treatment while significantly down-regulated by 1.5 mg l^{-1} 2, 4-dichlorophenoxyacetic acid (2, 4-D); its expression was also down-regulated when the cells were exposed to both the hormones together (Fig. 2C).

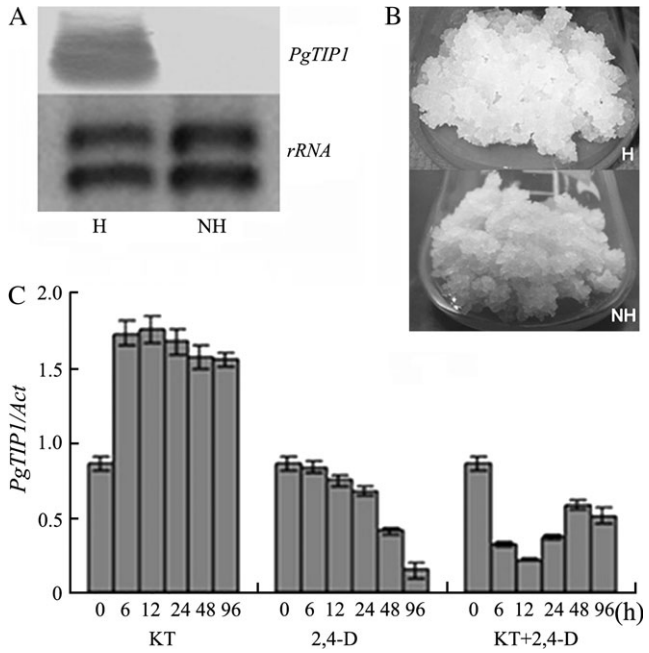

Fig. 2. *PgTIP1* expression in habituated and non-habituated ginseng cells: effect of hormones. (A) Northern blot analysis of *PgTIP1* in habituated (H) and non-habituated (NH) ginseng cells. (B) Habituated ginseng calli (H) and non-habituated ginseng calli (NH). (C) *PgTIP1* transcription in hormone-autotrophic suspension ginseng cells when treated with 0.25 mg l^{-1} KT and/or 1.5 mg l^{-1} 2,4-D for 6, 12, 24, 48, 96 h, respectively; the data are given as the mean ±SE (n=3).

Water-channel activity of PgTIP1

To find out whether PgTIP1 is a functional aquaporin, water-channel activity of PgTIP1 was assayed in the *Xenopus* oocytes system; AQP2 was included as a positive control in these assays. Three days after cRNA or water injection, the rate of cell volume change (Fig. 3A) and the osmotic P_f values (Fig. 3B) were calculated in the presence of an osmotic gradient. The P_f of PgTIP1-expressing, AQP2-expressing, and water-injected oocytes was 3.19×10^{-2} cm s^{-1}, 2.36×10^{-2} cm s^{-1}, and 0.14×10^{-2} cm s^{-1}, respectively. Oocytes expressing *PgTIP1* yielded 23-fold and 1.35-fold higher P_f than that of the water-injected oocytes and the positive control, respectively, suggesting that PgTIP1 is, indeed, a functional aquaporin with high water-channel activity.

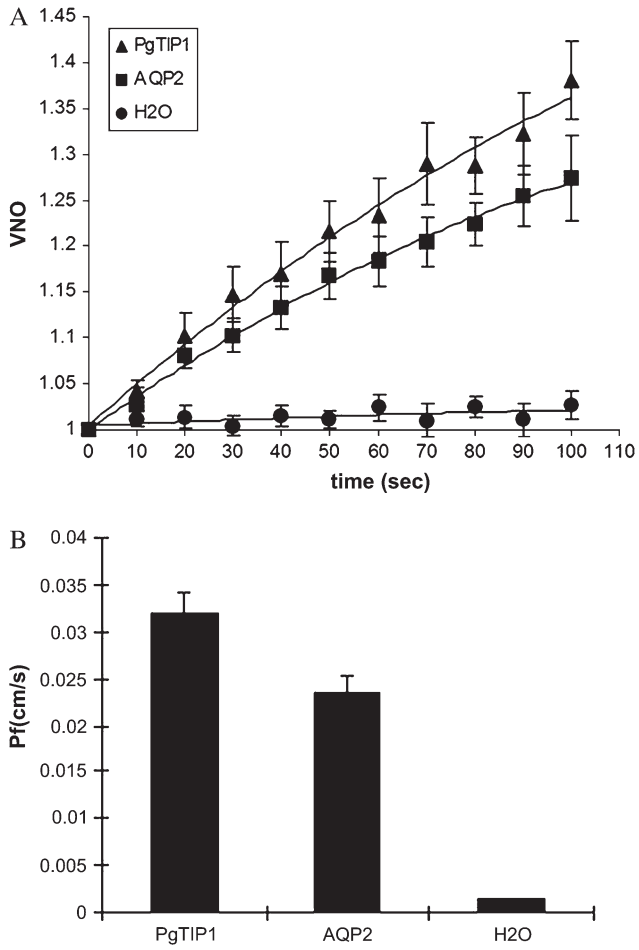

Fig. 3. Water-channel activity PgTIP1. (A) Initial swelling rates of *Xenopus laevis* oocytes injected with cRNA encoding PgTIP1, mammalian AQP2 (as positive control) or water (as negative control). The rate of oocyte swelling upon immersion in hypo-osmotic medium is plotted as V/V_0 versus time, where V is the volume at a given time point and V_0 is the initial volume. (B) Osmotic water permeability coefficient (P_f) of oocytes injected with cRNA encoding PgTIP1, AQP2, or water. The P_f values were calculated from the initial rate of oocyte swelling. Data are given as the mean ±SE (n=30).

Increased growth rate and enhanced seed size, mass, and fatty acid content in Arabidopsis *plants over-expressing* PgTIP1

Specific expression of *PgTIP1* in hormone-autotrophic ginseng cells suggested its potential involvement in cell division and/or growth. A *PgTIP1* over-expression construct was generated in *Arabidopsis* to explore its physiological function, if any. The transcription of *PgTIP1* was observed in roots, stems, leaves, flowers, and siliques of *Arabidopsis* transformants by real-time RT-PCR analysis (data not shown). Among 24 independent transformed lines, 22 (approximately 92%) *PgTIP1* over-expressers exhibited faster growth rate than the wild-type (WT) control as evidenced by the root elongation, leaf expansion, and weight increase of the aerial parts (Fig. 4). Root length of the one-week-old transgenic *Arabidopsis* seedlings was significantly greater than that of the WT control (Fig. 4A, B). The overall leaf-size was bigger in transgenic plants as indicated by the length and width of the leaf-blade and the petiole length of 3-week-old; fifth leaves (Fig. 4C, E, F). Histological observations using 2-week-old seedlings also indicated the size of mesophyll cells in transgenic plants to be bigger than that of the WT leaves (Fig. 4D). The increase in the weight of the aerial parts over time (days after germination) for the WT and *PgTIP1* over-expressors demonstrated faster and stronger growth of transgenic plants (Fig. 4G). *PgTIP1* over-expression also resulted in precocious flowering in *Arabidopsis* plants; transgenic plants flowered at least 3 d earlier than WT (Fig. 4H). The most intriguing phenotype of *Arabidopsis* over-expressing *PgTIP1* was that of the size and mass of mature seeds in that both attributes were significantly higher in transgenic plants compared to the WT (Fig. 4I, J; Table 1).

Differential expression of aquaporin genes is essential to plant growth and stress tolerance but their effect on seed characteristics has never been reported. In order to explore this intriguing transgenic effect further, the protein, sugar and fatty acid content of the seeds from transgenic and WT plants was analysed. Total protein and sugar contents of the seeds showed no significant difference between the transgenic and WT plants (data not shown), while the total fatty acid content per unit weight of the seeds from transgenic plants was ~1.85-fold of that from the WT. Furthermore, compositional analysis of fatty acids showed some differences between the two seed types; with the relative proportion of 18:1 increased and 18:3 decreased in the *PgTIP1* over-expressors (Table 2).

Transpiration and photosynthetic behaviour of Arabidopsis *plants over-expressing* PgTIP1

Leaf transpiration rate (T_r), stomatal conductance (G_s), intercellular CO_2 concentration (C_i), and leaf net photosynthetic rates (P_n) in WT and *PgTIP1* over-expressing plants were measured when leaves were fully expanded. Results indicated that *Arabidopsis* plants over-expressing *PgTIP1* had higher G_s and T_r than the WT plants (Fig. 5A, C), indicating a stronger water absorption and transpiration ability. A higher C_i concentration was also detected in transgenic *Arabidopsis* (Fig. 5B), but the level of P_n in *PgTIP1* over-expressing *Arabidopsis* had no significant change compared to the WT plants (Fig. 5D); higher C_i in *PgTIP1* over-expressing *Arabidopsis* may be a consequence of greater G_s (potentially resulting in greater CO_2 influx) but similar P_n rates of WT and transgenic plants. The explanation for no apparent difference in the P_n rates for the two genotypes, despite the difference in their C_i levels, could not be determined in this study. It is noteworthy, however, that stomatal conductance (G_s) is just one of the factors that would control the CO_2 acquiring capacity and P_n level in transgenic *Arabidopsis*. We still postulate that the transgenic *Arabidopsis* plants may accumulate more assimilation product due to their larger leaf size.

Discussion

Habituation refers to a naturally occurring phenomenon whereby callus cultures, upon continued subculture, lose their requirement for auxin, cytokinin, or both and it is considered to be an *in vitro* epigenetic switch to autotrophy (Meins, 1983; Syono and Fujita, 1994). The physiological basis of habituation is still unknown. General opinion is that habituation results from an enhanced accumulation by cells of the hormone for which they are habituated. But there are reports that auxin and cytokinin are present in both cell types at roughly the same concentration (Meins, 1989). It is noteworthy that no significant difference with respect to the endogenous levels of auxin or cytokinin was observed between the habituated and non-habituated ginseng cells in this study. Based on the latest research on a cytokinin-habituated callus line in *Arabidopsis* (Pischke *et al*., 2006), it also seems less likely that habituation is caused by an over-production of endogenous hormones, and more likely that it may be caused by altered expression of one or more other genes, for example, cytokinin-signalling genes. Cytokinin sensitivity may be modulated through regulation of cytokinin-receptor production. And epigenetic changes, instead of increases in hormone concentration, contribute to the acquisition of auxin/cytokinin-habituation. All these indicate that the endogenous hormone level might not be the key factor in the habituation course and habituation may arise from processes downstream from perception of hormone stimuli, which control cell division and expansion, as in animal cancer cells where activation and expression of genes bypass the requirement for specific growth factors (Hagège *et al*., 1994). Furthermore,

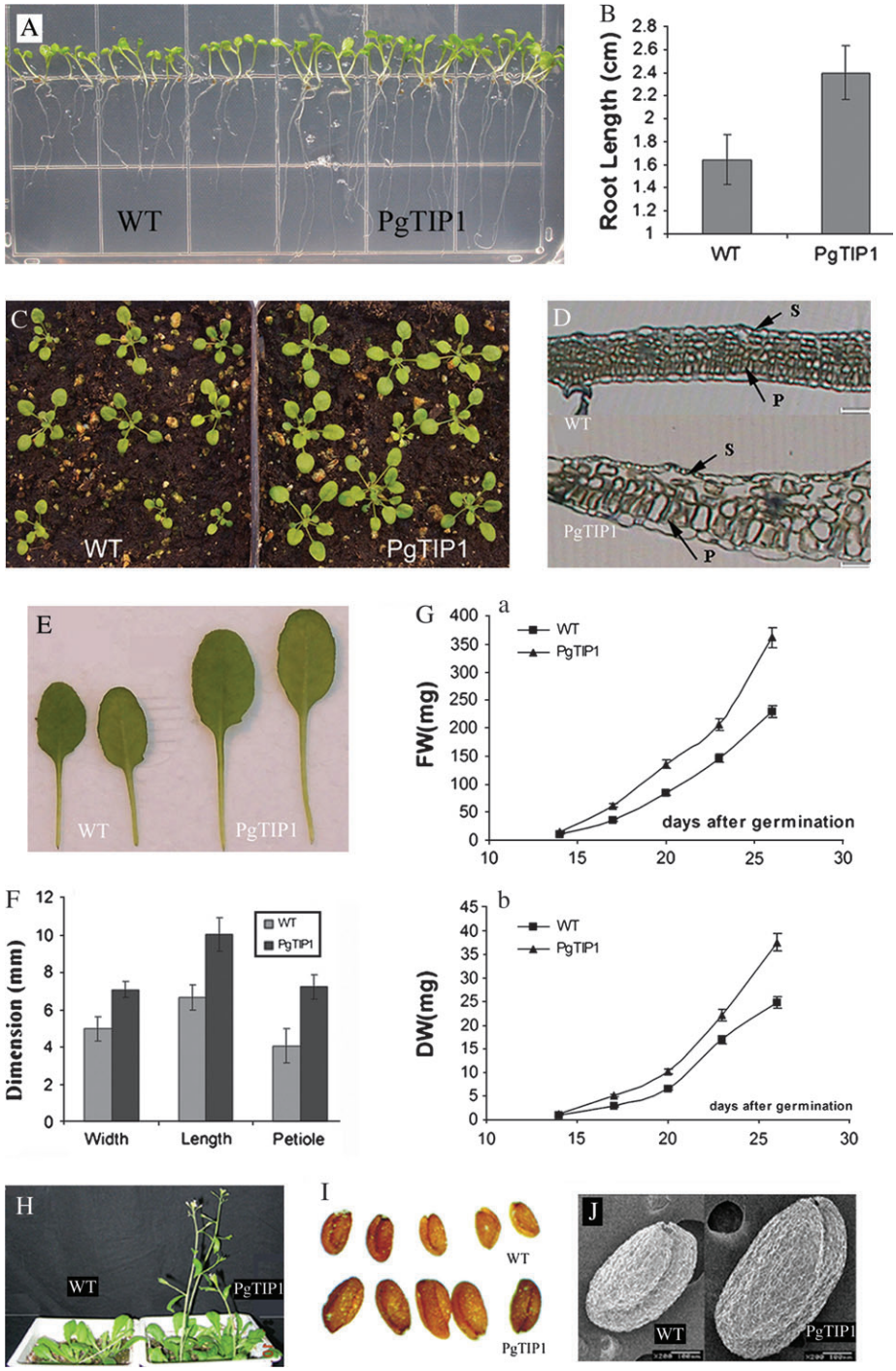

Fig. 4. Phenotypic and molecular characterization of *PgTIP1* over-expressing *Arabidopsis* plants. (A) One-week-old seedlings of WT and transgenic *Arabidopsis* cultured with MS medium on a vertical glass plate. (B) Root length of 1-week-old seedlings from WT and transgenic *Arabidopsis*. Data are given as the mean ±SE (n=50). (C) Three-week-old seedlings of WT and *PgTIP1* over-expressing *Arabidopsis* plants. (D) Transverse sections of the fifth leaves (2-week-old) of WT control and *PgTIP1* over-expressing plants. The palisade (p) and spongy mesophyll (s) cells are indicated. Bars=100 μm. (E) Morphology of 3-week-old fifth leaves. (F) Dimensions of 3-week-old fifth leaves as shown in (E). Data are given as the mean ±SE (n=15). (G) The aerial parts weight over time (days after germination) for the WT and *PgTIP1* over-expressing *Arabidopsis* plants. (a) Fresh weight increases during one month of growth after germination. (b) Dry weight increases during one month of growth after germination. Data are given as the mean ±SE (n=15). (H) Four-week-old WT and *PgTIP1* over-expressing *Arabidopsis* plants, indicating precocious flowering in transgenic plants. (I) Mature dried seeds from WT and *PgTIP1* over-expressing *Arabidopsis* plants. (J) Scanning electron micrographs of mature *Arabidopsis* seeds from WT control and *PgTIP1* over-expressing *Arabidopsis* plants. Bars=100 μm.

over-expression of a specific gene has been shown artificially to confer habituation in callus tissues (Kakimoto, 1996; Hwang and Sheen, 2001; Sakai *et al*., 2001; Osakabe *et al*., 2002). It is speculated that altered hormone signalling routes and hormone sensitivity might lead to this complicated phenomenon. In fact, the proliferation of habituated ginseng callus tissues was inhibited by exogenously applied auxin (the same concentration as in the media for non-habituated cell lines) in our study, which suggested that the sensitivity to auxin in a habituated ginseng cell line might be enhanced during the habituation course. The down-regulation of *PgTIP1* by exogenous auxin might be a side-off effect rather than the result of a directly negative regulation. High level and specific expression of *PgTIP1* in the habituated cell line, as observed in the present study, should result from an acclimation to the environment (exogenous hormones subtracted from the media).

Plant vacuole is a multifunctional organelle with important roles in space filling, osmotic adjustment, storage, and digestion. Vacuole biogenesis and enlargement require transport of osmotically active substances across the tonoplast, followed by a rapid influx of water into the vacuole. This influx generates the turgor pressure that drives cell expansion and maintains the cell shape. Rapid cell expansion may require a high hydraulic permeability of the tonoplast to support water entry into the vacuole. Although vacuole volume increase can never be triggered by the water channel function of aquaporins for the passive process of water transport via them; the uptake of water by expanding vacuoles may well involve tonoplast aquaporins (Chaumont *et al*., 1998; Javot and Maurel, 2002). Reisen *et al*. (2003) reported that the heterologous expression of a cauliflower tonoplast aquaporin (*BobTIP26;1*, orthologous to *AtTIP1;1*) in tobacco suspension cells had no effect on the growth rate, but the cells were larger than in the wild-type. In present study, it is shown that *PgTIP1*, which is highly and specifically expressed in hormone-autotrophic ginseng cells, has a high water-channel activity. In the roots and leaves of two detected transgenic lines, *PgTIP1* has a ~20-fold higher transcription than endogenous *AtTIP1;1* and *AtTIP1;2* (data not shown), both known to be highly expressed in *Arabidopsis* plants. Transgenic *Arabidopsis* plants over-expressing PgTIP1 had faster growth rate, longer roots, and bigger leaves and leaf cells; these results support the interpretation that TIPs might be involved in cell enlargement by modulating the permeability of the tonoplast. The volume increase of leaf cells might be triggered by enlargement of the vacuolar compartment facilitated by accumulating osmotically active substances, and following water influx into the vacuole, which was fine regulated by TIPs.

Whereas, the principal function of vacuoles is the maintenance of cell turgor, they can also accumulate macromolecules and secondary metabolites (Marty, 1999). Plant cells have different types of vacuoles that can coexist in the same cell and the different TIP isoforms may have entirely different or similar functions. While α-TIP alone is a marker for autophagic vacuole, it, coupled with δ- or γ-TIP, is involved in the protein storage function. By contrast, γ-TIP alone marked the lytic vacuoles and it, combined with δ-TIP, has a role in the storage of vegetative storage proteins and pigments (Jauh *et al*., 1999). Takahashi *et al*. (2004) isolated three novel γ-TIP cDNAs in rice, *OsTIP1*, *OsTIP2*, and *OsTIP3*, and demonstrated their specialized function. By contrast with OsTIP2 and OsTIP3, which are expressed specifically in roots and seeds, respectively, OsTIP1 was expressed in mature seed embryos and during early seed germination. These observations indicate that different TIP isoforms, alone or in combination with each other, play complicated physiological functions in plants. Storage vacuole is one of the most important vacuole types in plants, especially in seed cells. A greatly enhanced seed size and mass, and the significantly increased seed fatty acid content of

Table 1. *Morphometric measurements of dimensions and mass of seeds from the WT and* PgTIP1 *over-expressing* Arabidopsis *plants: data are given as means ±SE*

	n	WT	PgTIP1
Seed length (μm)	150	470±40	577±42
Seed width (μm)	150	275±21	320±23
Thousand seeds weight (mg)	3	17±1	34±1

Table 2. *Fatty acid content and composition of seeds from the WT and* PgTIP1 *over-expressing* Arabidopsis *plants: the data are given as the mean ±SE (n=3)*

Genotype	Fatty acid content (mg g^{-1} seed)	Fatty acid composition (mol %)						
		16:0	18:0	18:1	18:2	18:3	20:1	Others[a]
WT	89.6±4.8	8.6±0.6	6.2±0.4	27.4±1.8	28.0±1.2	5.0±0.3	19.4±0.9	5.4±0.4
PgTIP1	166.4±6.8	8.5±0.6	4.5±0.3	35.8±2.2	25.6±1.6	0.7±0.1	20.1±1.1	4.8±0.3

[a] 'Others' are primarily 14:1, 20:0, 20:2, 22:0, and 22:1 fatty acids, the relative composition of which did not change significantly between WT and *PgTIP1* over-expressing *Arabidopsis* seeds.

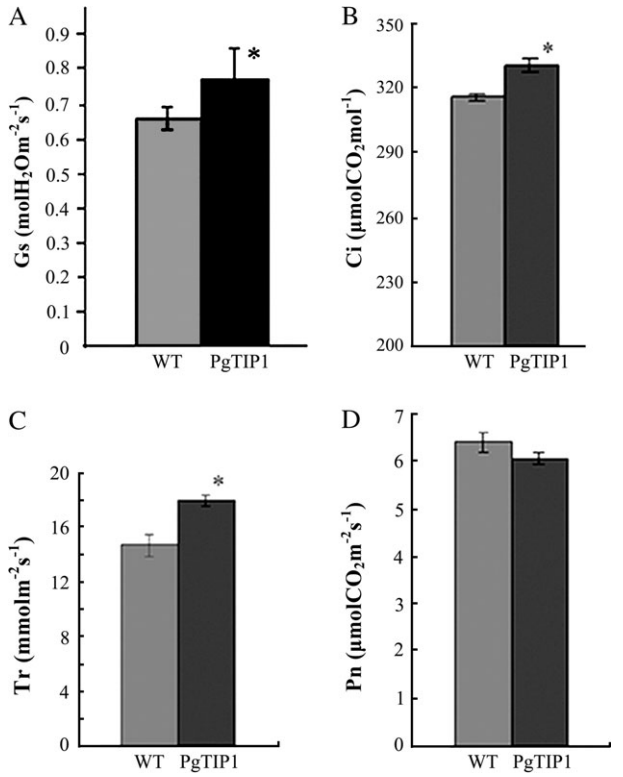

Fig. 5. Leaf stomatal conductance (G_s) (A), intercellular CO_2 concentration (C_i) (B), transpiration rate (T_r) (C), and leaf net photosynthesis rates (P_n) (D) in WT control and *PgTIP1* over-expressing *Arabidopsis*. Values indicate a mean of three measurements with standard deviations, each with a sample size of eight leaves. One of the triplicate trials is shown. Regression analysis confirmed that the G_s, C_i, and T_r values of *PgTIP1* over-expressing plants differ significantly from those of the WT (asterisk, $P \leqslant 0.01$).

Arabidopsis plants over-expressing *PgTIP1* indicate that PgTIP1 might have a role in substance storage and metabolism, in addition to involving in cell expansion. From sequence homology, protein structure to water channel activity, PgTIP1 shares characteristics with *Arabidopsis* tonoplast TIP1;1, which has been classified as a γ-TIP. The physiological role of AtTIP1;1 was investigated in plants using RNA interference (Ma *et al*., 2004). Data indicated that a strong down-regulation of *AtTIP1;1* led to plant death and suggested an essential physiological role of this otherwise highly expressed isoform. Transcript, metabolite profiling, and the cellular localization data suggested a role for AtTIP1;1 in carbon distribution, possibly by regulation of vesicle trafficking towards the central vacuole. We postulate that, similar to AtTIP1;1, PgTIP1 might be involved in substance storage and metabolism in the seeds of *Arabidopsis* plants over-expressing this gene.

As mentioned earlier, AQPs might have physiological functions other than facilitating water movement across cellular membranes. AQP over-expression highlights those physiological processes in which AQPs act as bottlenecks. Transgenic tobacco over-expression of *AtPIP1;2* significantly increased plant growth and transpiration rate, stomatal density, and photosynthetic activity (Aharon *et al*., 2003). Similarly, transgenic rice over-expression of *HvPIP2;1* also resulted in faster growth rate and higher internal CO_2 (by 40%), stomatal conductance (by 27%), and CO_2 assimilation (by 14%) than the wild-type plants (Hanba *et al*., 2004). These studies indicated PIPs may play an important role in regulating plant vigour. However, the mechanism of the involvement of PgTIP1 in this process might be different from that of PIPs, since the photosynthetic activity of transgenic *Arabidopsis* plants over-expressing PgTIP1 was found to be similar to that of wild-type plants. However, it has been shown here that PgTIP1 might be involved in cell enlargement via transport of water across tonoplast, which often represents a limiting factor that regulates plant vigour. Further understanding of the physiological roles of PgTIP1 in plant growth and development should be helpful in understanding AQP functions in plants more comprehensively.

Supplementary data

Supplementary data are available at JXB online. Supplementary Figure 1 shows the predicted stereostructure of PgTIP1 (right panel) and AQP2 (left panel) and Supplementary Fig. 2 shows the ar/R selectivity filter of PgTIP1. Space-filling side-chain residues are also shown.

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