Aquaporin is a water channel that increases water permeability through membranous structures. In plants, vacuoles are essential organelles that undergo dynamic volume changes during cell growth. To understand the contribution of aquaporins to plant cell growth, we developed a transgenic tobacco BY-2 cell line overexpressing the tonoplast intrinsic protein (TIP), γTIP. Vacuolar membranes of isolated vacuoles from γTIP-overexpressing cells showed higher water permeation activities than those from wild-type cells. We then examined the role of γTIP in vacuolar regeneration of evacuolated tobacco BY-2 protoplasts (miniprotoplasts). Vacuolar regeneration from thin to thick tube-network vacuoles and subsequent development of large vacuoles was accelerated in miniprotoplasts of this cell line. A parallel increase in the rate of cell expansion indicated a tight relationship between vacuolar development and cellular volume increases. Interestingly, overexpression of tobacco γTIP also enhanced cell division. Thus, increased vacuolar aquaporin activity may accelerate both cell expansion and cell division by increasing water permeability through the vacuolar membrane.

Keywords: γTIP • Vauolar regeneration • Cell growth • Aquaporin • Tobacco BY-2 cell • Miniprotoplast.

Introduction

Vacuoles are multifunctional organelles that are involved in plant growth, through the storage and lysis of substances, and also in homeostasis of cytoplasmic pH, ions and metabolites (Marty 1999, Martinoia et al. 2007). In addition, as this organelle occupies a large volume in plant cells, vacuolar development leads directly to the enlargement of cells. For example, changes in the vacuolar volume and shape of guard cells modify guard cell shape and regulate stomatal apertures (Fricker and White 1990, Gao et al. 2005, Tanaka et al. 2007). In tobacco suspension cells placed under hyper-osmotic conditions, the vacuolar volume was found to decrease, based on the reduced cell volume, and a complex vacuolar network could be observed (Kutsuna and Hasezawa 2005, Reisen et al. 2005). In contrast, the Arabidopsis mutant, vacuoleless1, which lacks vacuoles, shows defects in cell elongation and in the orientation of cell division of its embryos (Rojo et al. 2001). Therefore, vacuolar development appears to be required for proper cell growth, including cell elongation/expansion and cell division.

During vacuolar development, a number of vacuolar transporters are required for vacuolar enlargement and metabolism, for example proton pump activities, ion transporters/channels and aquaporins that are involved in energy production, osmotic regulation by mineral...
transport and water transport, respectively (Martinoa et al. 2007). Of these, the aquaporins are responsible for the transport of water that directly increases vacuolar volumes. The vacuolar aquaporins, referred to as TIPs (tonoplast intrinsic proteins), are classified according to their localization; those localized in the tonoplast of storage vacuoles are referred to as δTIPs, those in seed-type storage vacuoles as αTIPs, and those in lytic vacuoles as γTIPs. In Arabidopsis, 10 genes have now been found to encode TIPs, with the genes encoding γTIPs, δTIPs and αTIPs being designated in a new nomenclature as TIP1, TIP2 and TIP3, respectively, (Johanson et al. 2001, Maurel 2007).

In this study, to investigate vacuolar development and its contribution to cell growth, we focused on the γTIP that was initially demonstrated to function as a plant aquaporin through its water transport activities in Xenopus oocytes (Maurel et al. 1993). In Arabidopsis, reduced gene expression of AtTIP1;1 by RNA interference produced small plants that died when severely affected (Ma et al. 2004). In contrast, overexpression of the cauliflower γTIP, BobTIP26-1, in tobacco cells increased cell volume (Reisen et al. 2003). In addition, overexpression of the Panax ginseng γTIP, PtγTIP1, in Arabidopsis increased seed size and plant growth, and also enhanced salt and drought stress tolerance as well as cold acclimation ability (Lin et al. 2007, Peng et al. 2007). We isolated the tobacco γTIP (NtTIP1;1) from BY-2 suspension-cultured cells and investigated its role in vacuolar development and cell growth using miniprotoplast culture.

**Results**

**Cloning of tobacco γTIP1;1**

To investigate the role of aquaporins in vacuolar development, we identified a tobacco cDNA homolog of the Nicotiana glauca NgMIP3 (Smart et al. 2001) that was classified as a γTIP and was expected to function as a vacuolar aquaporin. As shown in Fig. 1A, the deduced amino acid sequence encoded by the isolated tobacco cDNA was 97% identical to that of NgMIP3 and 78% to the Arabidopsis TIP1;1 (AtTIP1;1). Furthermore, the putative protein contained two NPA (Asn–Pro–Ala) motifs that are well conserved among aquaporins (Fig. 1A, red boxes), and six hydrophobic segments (p1–p6) as determined by a Kyte and Doolittle hydropathy plot of the amino acid sequence (Fig. 1B). Therefore, we designated the tobacco cDNA as NtTIP1;1.

**Overexpression of NtTIP1;1–GFP in tobacco BY-2 cells**

To investigate the role of NtTIP1;1, as well as its cellular localization, we prepared a transgenic tobacco BY-2 cell line overexpressing NtTIP1;1 tagged with the green fluorescence protein (NtTIP1;1–GFP) and placed under the control of the cauliflower mosaic virus 35S promoter (the cell line being designated BY-TIPG on the basis of tobacco BY-2 cells stably expressing NtTIP1;1–GFP). The GFP fluorescence predominately labeled the vacuolar membrane (VM) as confirmed by vital staining of the VM with FM4-64 (Fig. 2A, Kutsuna and Hasezawa 2002). Gene expression analysis demonstrated that NtTIP1;1 transcript levels were 3-fold higher in the tobacco BY-TIPG cells than in non-transformed BY-2 cells and in tobacco BY-GV cells overexpressing GFP–AtVAM3 (Fig. 2B, Kutsuna et al. 2003). Furthermore, Western blot analysis confirmed the stable expression of NtTIP1;1–GFP (data not shown). AtVAM3 was localized to the VM (Sato et al. 1997), and stable expression of AtVAM3 in tobacco BY-2 cells was found not to affect their growth (Kutsuna et al. 2003). Expression levels of Nt-vag1, encoding a vacuolar ATPase subunit G (Rouquié et al. 1998), were comparable in the BY-TIPG, non-transformed BY-2 and BY-GV cells (Fig. 2B).

**Water permeation activity assay**

To investigate the water transport activities of NtTIP1;1, we monitored vacuolar swelling in prepared isolated vacuoles. Exposure of isolated vacuoles from control BY-GV cells to hypo-osmotic conditions, from 0.70 to 0.45 M mannitol (Fig. 3A), caused them to swell almost immediately, with the volume increases ceasing within 3 min, whereas volumes of the tobacco BY-TIPG vacuoles reached their maximum within 1 min (Fig. 3B). The osmotic water permeability coefficient (P) of the BY-TIPG and BY-GV VMs was calculated to be 14.3 ± 0.9 and 5.7 ± 0.6×10−6 m s−1, respectively. These results demonstrate that NtTIP1;1 possesses water permeation activities within the VM.

**Effects of overexpression of NtTIP1;1 on cell elongation during protoplast and miniprotoplast culture**

To assess the physiological impact of NtTIP1;1 overexpression on the transgenic tobacco BY-2 cells, we induced cell elongation by culturing the cells in a modified FMS medium with low auxin concentrations (Fig. 4A), as previously reported for non-transgenic tobacco BY-2 cells (Hasezawa and Syono 1983). The average diameters of freshly prepared BY-TIPG and BY-GV protoplasts were 37.4 ± 0.9 and 40.9 ± 0.8 µm, respectively, with the BY-TIPG protoplasts being 0.91-fold smaller (Fig. 4B). After a 2 d culture under conditions promoting cell elongation, the BY-TIPG and BY-GV protoplasts elongated to 72.8 ± 7.9 and 63.9 ± 1.8 µm, respectively, with the rate of increase being 1.24-fold higher in the BY-TIPG protoplasts (Fig. 4B). These results suggest that NtTIP1;1 expression promotes cell elongation during protoplast culture.

To confirm the acceleration of cell elongation by NtTIP1;1 expression, we developed a more efficient and synchronized cell elongation system using miniprotoplasts, which are
evacuolated protoplasts generated by removal of the central vacuole by centrifugation (Sonobe 1990). The BY-TIPG and BY-GV miniprotoplasts elongated faster than protoplasts cultured in a modified FMS medium with low auxin concentrations. By 12 h of culture, the miniprotoplasts increased their cell volume but still maintained their spherical shape (Fig. 5A, 0–12 h). From around 12 to 24 h of culture, the cells elongated with a concomitant increase in their cell volume (Fig. 5A, 24 h).

Measurement of cell sectional areas on micrographs revealed that the sectional areas of freshly prepared BY-TIPG miniprotoplasts were 0.8-fold the size of those from BY-GV cells (2,248 ± 175 µm² in BY-GV and 1,885 ± 197 µm² in BY-TIPG), although these became comparable after 24 h culture (4,781 ± 486 and 4,763 ± 412 µm² in BY-GV and BY-TIPG, respectively, Fig. 5A, B). When the relative cell size increases were plotted, the miniprotoplast sectional areas were found to have increased by about 2.5-fold in BY-TIPG and 2.1-fold in BY-GV after 24 h culture (Fig. 5C). Similarly, the major axis lengths of freshly prepared miniprotoplasts were 1.1-fold shorter in BY-TIPG than in BY-GV miniproto- plasts, although these elongated to comparable values after 24 h culture (Fig. 5D, E). However, the rates of miniprotoplast elongation, as evaluated from the ratios of the major to minor axis lengths, were found to be comparable in the BY-TIPG and BY-GV miniprotoplasts (Fig. 5E). These results suggest that NtTIP1;1 overexpression also promotes cell elongation in miniprotoplast culture.

Fig. 1 Multiple amino acid sequence alignment of NtTIP1;1, NgMIP3 and AtTIP1;1. Identical, conserved and semi-conserved amino acid residues are denoted by asterisks (*), colons (:), and periods (.), respectively. Two NPA motifs are boxed in red. GenBank accession numbers of the corresponding proteins are as follows: NtTIP1;1 (AB371711), NgMIP3 (AF290619) and AtTIP1;1 (M84344). (B) Kyte and Doolittle hydropathy plot of NtTIP1;1. The six hydrophobic domains (p1–p6) are indicated.

| A | NtTIP1;1 MPINQIAV08SHR66RQGTOAKALAEPECL1FVIP6AQQGSAANPKNL6VDQTATPSGLI 60 |
|   | NgMIP3 MPINQIAV08SHR66RQGTOAKALAEPECL1FVIP6AQQGSAANPKNL6VDQTATPSGLI 60 |
|   | AtTIP1;1 MPINQIAV08SHR66RQGTOAKALAEPECL1FVIP6AQQGSAANPKNL6VDQTATPSGLI 60 |

| B | Hydrophathy |
|   | Position (a.a.) |
|   | p1 | p2 | p3 | p4 | p5 | p6 |
|   | 0 | 4 | 0 | 4 | 0 | 4 |
|   | 0 | 4 | 0 | 4 | 0 | 4 |

Vacuolar aquaporin and plant cell growth

Effects of overexpression of NtTIP1;1 on vacuolar regeneration during miniprotoplast culture

The rapid increase in cell volume of the miniprotoplasts suggested that vacuoles eliminated by centrifugation were regenerated and then expanded during the culture. Indeed, observation of the VM labeled by GFP–AtVAM3 and NtTIP1;1–GFP showed that only remnants of the VM could be observed in the miniprotoplasts just after centrifugation (Fig. 6A, vacuolar remnant) but that large vacuoles regenerated after 24–36 h culture (Fig. 6A, large vacuoles). Interestingly, the vacuoles formed tube-network-like structures during the

Fig. 2 Vacuolar localization of NtTIP1;1 and its water transport activity. (A) Localization of NtTIP1;1–GFP in tobacco BY-2 cells. Bright field (upper left), NtTIP1;1–GFP (upper right), FM4-64 (lower left) and NtTIP1;1–GFP and FM4-64 merged (lower right) images are shown. FM4-64 predominantly stains the vacuolar membrane. The scale bar represents 10 µm. (B) Gene expression levels of NtTIP1;1 (left) and Nt-vag1 (right) in non-transformed tobacco BY-2 cells, in BY-GV cells that stably express GFP-AtVAM3 and in BY-TIPG cells that stably express NtTIP1;1-GFP. Results were standardized to expression levels of the constitutive tobacco actin gene, Tob103. Values are given as mean values (n = 3, ±SE).

Fig. 3 Swelling of isolated vacuoles from BY-TIPG and BY-GV cells. (A) Confocal images of isolated vacuoles. Vacuoles from BY-GV (upper panels) and BY-TIPG (lower panels) cells are shown in 0.70 M mannitol (left panels) and after exposure to hypo-osmotic conditions from 0.70 to 0.45 M by water application (right panels). Scale bars represent 10 µm. (B) Changes in diameter of isolated vacuoles from BY-TIPG (filled squares) and BY-GV (open squares) cells after the change from 0.70 to 0.45 M mannitol. Data represent mean values (n = 3, ±SE), and 30 vacuoles were measured in each experiment. The original values are shown in Supplementary Table S1.

Fig. 4 Accelerated cell elongation in BY-TIPG protoplasts. (A) Bright field images of BY-GV (upper panels) and BY-TIPG (lower panels) protoplasts. Scale bars represent 10 µm. (B) Changes in major axis lengths of BY-TIPG (filled squares) and BY-GV (open squares) cells during protoplast culture. Data represent mean values (n = 3, ±SE).
regeneration of large vacuoles (data not shown; Fig. 6A, thin and thick tube-network), and the series of vacuolar structures formed during this process could be categorized as follows (data not shown): as thin tube-network vacuoles (Fig. 6A, thin tube-network), from conversion of the remnants of the vacuolar network; as thick tube-network vacuoles (Fig. 6A, thick tube-network), from the isotropic swelling and increase in diameter of the vacuolar network; and as large vacuoles (Fig. 6A, large vacuole), from a subregion of the vacuolar network.

To examine the effects of NtTIP1;1 overexpression on vacuolar regeneration, we time-sequentially compared the frequency of appearance of these vacuolar types in the BY-GV and BY-TIPG cells. The population of miniprotoplasts with vacuolar remnants and thin tube-network vacuoles after 9 h culture was approximately 60% in control BY-GV cells, whereas in the BY-TIPG cells this population rapidly decreased to about 20% (Fig. 6B). In addition, the thick tube-network vacuoles appeared to develop faster in the BY-TIPG cells than in the BY-GV cells, with these populations comprising up to almost 70 and 40% of the cells, respectively, after 12 h culture (Fig. 6C). Furthermore, the large vacuoles appeared earlier in the BY-TIPG cells than in the BY-GV cells (Fig. 6D). These observations suggest that overexpression of NtTIP1;1 accelerated the regeneration of large vacuoles. A time-sequential examination of the endogenous NtTIP1;1 gene expression patterns during vacuolar regeneration in the BY-GV miniprotoplasts showed twin peaks of expression at 8 and 32 h after miniprotoplast preparation (Fig. 6E). These times correspond to the transition points from the thin tube-network to the thick tube-network vacuoles (Fig. 6C), and from the thick tube-network vacuoles to large vacuoles (Fig. 6D), respectively.

**Effects of NtTIP1;1 overexpression on protoplast division**

To understand the additional role of NtTIP1;1 in cell growth, we examined whether overexpression of NtTIP1;1 affected cell division in protoplasts. After 2 d under low auxin concentrations, cell numbers reached about 6.0×10⁴ cells ml⁻¹ for the BY-GV protoplasts and 8.0×10⁴ cells ml⁻¹ for the BY-TIPG protoplasts (Fig. 7A), whereas under high auxin concentrations the divided BY-TIPG and BY-GV cell populations were comparable (data not shown). The higher dividing activity of the BY-TIPG cells was confirmed by monitoring expression of the cyclin B gene, NictaCycB1;2. The relative NictaCycB1;2 expression level in BY-TIPG cells at 1 d of culture under low auxin concentrations was 3-fold higher than that in the BY-GV cells under the same conditions (Fig. 7B), but only about 0.5-fold the expression level of the BY-TIPG cells under high auxin concentrations (data not shown). We also monitored expression of the vacuolar proton pump (H⁺-PPase), NtTVP9 (Lerchl et al., 1995), since a previous report suggested that overexpression of the Arabidopsis H⁺-PPase AVP1 promoted auxin transport and cell division during organ development (Li et al. 2005). The relative expression level of NtTVP9 in the BY-TIPG cells at 1 d of culture under low auxin concentrations was 4-fold higher than that in the BY-GV cells (Fig. 7C). These results indicate that overexpression of NtTIP1;1 enhanced the expression of NtTVP9.
Fig. 6 Vacuolar regeneration in tobacco miniprotoplasts and effects of NtTIP1;1 overexpression. (A) Typical vacuolar structures in BY-GV (upper panels) and BY-TIPG (lower panels) cells during miniprotoplast culture. The series of vacuolar structures observed could be categorized as: thin tube-networks (conversion from remnants of the vacuolar network); thick tube-networks (isotropic swelling and increase in the diameter of the vacuolar network); and large vacuoles (from a subregion of the vacuolar network). Scale bars represent 10 µm. (B–D) Changes in populations of miniprotoplasts with: (B) vacuolar remnants or thin tube-network vacuoles, (C) thick tube-network vacuoles and (D) large vacuoles, in BY-TIPG (filled squares) and BY-GV (open squares) cells. Data represent mean values (n = 3, ±SE). From 100 to 150 cells were observed in each experiment. Significance was determined by Student’s t-test with *P < 0.05. (E) Quantitative reverse transcription–PCR analysis of NtTIP1;1 gene expression during vacuolar regeneration in BY-GV miniprotoplasts. The results were standardized on the basis of expression levels of the constitutive tobacco actin gene, Tob103. Data represent mean values (n = 3, ±SE).

Discussion

NtTIP1;1 possesses aquaporin activity

As vacuoles occupy a large volume of plant cells, vacuolar enlargement would be expected to promote cell enlargement directly. In this study, we investigated vacuolar development and subsequent cell growth by focusing on a particular vacuolar aquaporin, NtTIP1;1. We have demonstrated that NtTIP1;1 is localized primarily on the VM (Fig. 2A) and that it has water permeation activities on the VM, as shown by the rapid...
swelling of vacuoles isolated from BY-2 cells stably expressing NtTIP1;1–GFP (BY-TIPG) (Fig. 3). The calculated osmotic water permeability coefficient ($P_f$) of the VM in BY-TIPG cells was about 2.5-fold that of BY-GV cells, and this corresponds to differences in NtTIP1;1 gene expression levels between the cells (Fig. 2B). These results demonstrate that NtTIP1;1–GFP possesses aquaporin activity.

**Acceleration of cell growth and vacuolar regeneration by NtTIP1;1 overexpression**

We have shown in this study that NtTIP1;1 overexpression promoted cell volume increases during protoplast and miniprotoplast culture (Figs. 4, 5), in agreement with previous reports of γTIP overexpression (Reisen et al. 2003, Lin et al. 2007). In addition, however, we found that NtTIP1;1 overexpression enhanced vacuolar regeneration in miniprotoplasts (Fig. 6A–D), and that the peak expression levels of endogenous NtTIP1;1 corresponded to the transition points from the thin tube-network to thick tube-network vacuoles (Fig. 6E). These results suggest that vacuolar aquaporin activity could enhance vacuolar and cellular enlargement in protoplasts and miniprotoplasts.

To increase vacuolar and cellular volumes, water uptake through the plasma membrane (PM) as well as through the VM is required. Most previous studies have discussed the high water permeability of the VM compared with that of the PM (Url 1971, Kiyosawa and Tazawa 1977, Maurel et al. 1997, Niemetz and Tyerman 1997), and indeed the water permeability of the VM was found to be about 100-fold that of the PM (Maurel et al. 1997, Gerbeau et al. 1999). However, Murai-Hatano and Kuwagata (2007) measured the water permeability of the PM and VM using radish protoplasts and their isolated vacuoles, and demonstrated comparable and high levels of osmotic water permeability in the PM and VM, suggesting that vacuolar aquaporins may be a limiting factor of water uptake by plant cells.

The factors generally limiting plant cell expansion are thought to be cell wall pressure and the osmotic potential that are generated by osmotic substances present in the vacuoles. In our experimental system using protoplasts and miniprotoplasts, there was an absence of cell wall pressure at least at the start of the culture. In normal cultured cells with cell walls, NtTIP1;1 overexpression had no effect on cell size (data not shown). Therefore, cell wall pressure may be considered as the primary factor limiting cell enlargement in BY-2 cells, whereas water permeability through the VM may be only a secondary factor.

In addition, it is possible that overexpression of NtTIP1;1 indirectly affects the accumulation of osmotic substances in vacuoles and also vesicular transport to vacuoles, as suggested by previous studies (Reisen et al. 2003, Ma et al. 2004, Lin et al. 2007). In Arabidopsis, repression of AtTIP1;1 gene
expression by RNA interference affected global transcript abundance, including of genes involved in carbon metabolism and cellulose synthesis (Ma et al. 2004). Indeed, as NtTVP9 expression was up-regulated by NtTIP1;1 overexpression in this study (Fig. 7C), there is a similar possibility of global transcript changes in the BY-TIPG cells. Future transcriptome and metabolome approaches will clarify this possibility.

**Acceleration of cell division by NtTIP1;1 overexpression**

In the BY-TIPG protoplast cultures, cell division activity was found to be enhanced under low auxin concentrations (Figs. 4B, 7). In Panax ginseng, the PgTIP1 gene, encoding a γTIP, was found to be constitutively expressed in habituated cells that could grow in phytohormone-free medium, but not in non-habituated cells (Lin et al., 2007). These results are indicative of the potential involvement of vacuolar aquaporins in cell division. One possible explanation for the acceleration of cell division by vacuolar aquaporins might be that the increased cell volumes would drive cell division. However, the volumes of dividing cells were not significantly larger than those of elongating cells (data not shown). Another possibility is that up-regulation of NtTVP9 promoted auxin transport in the BY-TIPG cells and resulted in an acceleration of cell division, as previously reported for overexpression of Arabidopsis H+-PPase AVP1 (Li et al., 2005). Further investigations are needed to clarify which factor(s) enhance cell division in the BY-TIPG cells.

**Materials and Methods**

**Plant materials and culture conditions**

A tobacco BY-2 (Nicotiana tabacum L. cv. Bright Yellow 2) suspension culture was diluted 95-fold with modified Linsmaier and Skoog medium at weekly intervals as described (Nagata et al. 1992). The cell suspension was agitated on a rotary shaker at 130 r.p.m. at 27°C in the dark. Transgenic BY-2 cell lines stably expressing GFP-AtVAM3 (BY-GV cells, Kutsuna and Hasezawa 2002) or NtTIP1;1-GFP (BY-TIPG cells) were maintained similarly to the non-transformed BY-2 cell line.

**FM4-64 staining**

To observe the VM in BY-TIPG cells, N-(3-triethylammoniumpropyl)-4-[6-(4-(diethylamino)phenyl)hexatrienyl]-pyridinium dibromide (FM4-64; Molecular Probes, Invitrogen, Carlsbad, CA, USA) was added to the cell suspensions to a final concentration of 32 μM. The cells were incubated for 2 min, washed with fresh culture medium, and then observed as described previously (Kutsuna and Hasezawa 2002).

**Tobacco transformation and gene expression analysis**

The tobacco γTIP cDNA, NtTIP1;1, was PCR amplified from a tobacco BY-2 cDNA library using primers designed on the basis of the N. glauca NgMIP3 sequence (accession No. AF290619). The open reading frame region of NtTIP1;1 was first subcloned into pENTR/D-TOPO vector (Invitrogen) and then into the pGWBS5 binary vector (Nakagawa et al. 2007) using the Gateway system (Invitrogen). The tobacco BY-TIPG cell line was established by transformation with the binary vector construct using the Agrobacterium tumefaciens strain, LBA4404, according to the method of Mayo et al. (2006).

Real-time quantitative PCR was conducted in a Smart Cycler 2 system (Cepheid, Sunnyvale, CA, USA) using SYBR Premix Taq (TAKARA BIO INC, Shiga, Japan) according to the manufacturer’s specifications. Values of relative transcripts were calculated with actin Tob103 fragments (Moniz de Sa and Drouin 1996) amplified with primers 5′-ATTACCAATTGCTGCCAGAG-3′ and 5′-ATCCCTCAGATCACACTG-3′. A 292 bp fragment of the NtTIP1;1 cDNA was amplified with the PCR primers 5′-TTCCCAAGCTTCC GGTTTCG-3′ and 5′-CACCAAGGTAACGCGGGGATT-3′. A 285 bp fragment of the Nt-vag1 cDNA (Rouquié et al. 1998) was PCR amplified with primers 5′-TGCAAAGATCTCGACACCTGAAA-3′ and 5′-CACGGGTGTGACACGTTTCG-3′. A 202 bp fragment of the NictaCycB1;2 cDNA was amplified with the PCR primers 5′-TATGTTGATCTGTACCATG-3′ and 5′-CACCAAGGTAACGCGGGGATT-3′. A 188 bp fragment of the NtTVP9 cDNA (Lerchl et al. 1995) was PCR amplified with primers 5′-GGGTGCTTGTGC CGGTGTGCTTGG-3′ and 5′-CAGAGGGTCGCAATGATGTCCA-3′.

**Preparation of protoplasts, miniprotoplasts and isolated vacuoles**

To prepare BY-2 protoplasts, 3-day-old cell cultures were incubated with an enzyme solution [1% cellulase Y-C (Kyowa Chemical Products Co., Ltd., Osaka, Japan), 0.1% pectolyase Y-23 (Kyowa Chemical Products Co.), 0.35 M mannitol, pH 5.5] at 30°C for 60 min. Miniprotoplasts were prepared from BY-2 protoplasts by density gradient centrifugation with Percoll solution [30% Percoll (GE Healthcare Ltd., Amersham, UK), 0.7 M mannitol, 20 mM MgCl2] at 15,000xg for 30 min. Miniprotoplasts were collected from the pellet, washed twice with 0.7 M mannitol and the vacuoles then collected from the supernatant. Protoplasts and miniprotoplasts, in a state of elongation, were cultured at 27°C in a modified FMS medium [1% sucrose, 4.6 mg l-1 Murashige and Skoog Plant Salt Mixture (Wako Pure Chemical Industries Ltd., Osaka, Japan), 0.001% thiamine-HCl, 0.1% myo-inositol, 5.4 × 10⁻⁷ mM naphthalene acetic acid, 4.4 × 10⁻⁶ mM
benzyladenine (Hasezawa and Syōno 1983)] with 0.35 M mannitol for protoplasts and 0.5 M for miniprotoplasts. Protoplasts, in a state of division, were cultured at 27°C in a modified FMS medium [1% sucrose, 4.6 mg l−1 Murashige and Skoog Plant Salt Mixture (Wako Pure Chemical Industries Ltd.), 0.001% thiamine-HCl, 0.1% myo-inositol, 5.4 × 10−6 mM naphthalene acetic acid, 4.4 × 10−6 mM benzyladenine (Hasezawa and Syōno, 1983)] with 0.35 M mannitol for protoplasts and 0.5 M for miniprotoplasts.

The osmotic water permeability (\(P_f\)) was calculated according to Ohshima et al. (2001) from the following equation:

\[
P_f = \frac{(V_f - V_i)}{(S \cdot t \cdot V_w \cdot \Delta \pi)}
\]

where \(V_i\) and \(V_f\) are the initial and final vacuolar volumes (cm³), respectively, \(S\) is the initial vacuolar surface area (cm²), \(t\) is the period taken to reach the maximum vacuolar volume(s), \(V_w\) is the molar volume of water (18×10⁻³ l mol⁻¹) and \(\Delta \pi\) is the osmotic gradient (mol l⁻¹).

Morphometric analysis of cells

To quantify the shape of cells, regions of the cell images were manually segmented using Adobe Photoshop. Based on these images, the cell shape was approximated as an ellipse and the size and major and minor axis lengths of the cell calculated using the plugin; KBI MorphoMeasure (http://hasezawa.ib.k.u-tokyo.ac.jp/zp/Kbi/ImageJkbiPlugins) of ImageJ (Abramoff et al., 2004).

The sequence of NtTIP1;1 has been deposited in GenBank under the accession number, AB371711.

Supplementary data

Supplementary data are available at PCP online.

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