Phosphorylation of Plasma Membrane Aquaporin Regulates Temperature-Dependent Opening of Tulip Petals

Abul Kalam Azad 1,2, Yoshihiro Sawa 1, Takahiro Ishikawa 1 and Hitoshi Shibata 1,3

1 Faculty of Life and Environmental Sciences, Shimane University, Matsue, Shimane, 690-8504 Japan
2 The United Graduate School of Agricultural Sciences, Tottori University, Koyama-Minami, Tottori, 680-0954 Japan

The opening and closing of tulip petals was reproduced in the dark by changing the temperature from 5°C to 20°C for opening and 20°C to 5°C for closing. The opening process was accompanied by 3H2O transport through the stem from the incubation medium to the petals. A Ca2+-channel blocker and a Ca2+-chelator inhibited petal opening and 3H2O transport. Several proteins in the isolated plasma membrane fraction were phosphorylated in the presence of 25 µM Ca2+ at 20°C. The 31-kDa protein that was phosphorylated, was suggested immunologically as the putative plasma membrane aquaporin (PM-AQP). This phosphorylated PM-AQP clearly reacted with the anti-phospho-Ser. In-gel assay revealed the presence of a 45-kDa Ca2+-dependent protein kinase in the isolated plasma membrane. Phosphorylation of the putative PM-AQP was thought to activate the water channel composed of PM-AQP. Dephosphorylation of the phosphorylated PM-AQP was also observed during petal closing at 5°C, suggesting the inactivation of the water channel.

Keywords: Ca2+ — CDPK — Dephosphorylation — Petal oscillation — Phosphorylation — Water transport.

Abbreviations: AQP, aquaporins; BAPTA, O,O′-bis(2-aminopheny)-labeled glycol-N,N,N′,N′-tetraacetic acid; [Ca2+]cyt, cytosolic free Ca2+ concentration; CDPK, Ca2+-dependent protein kinase; K2, potassium phosphate; MBP, myelin basic protein; 2-ME, 2-mercaptoethanol; PM, plasma membrane; PM-AQP, plasma membrane aquaporin; RR, ruthenium red; TCA, trichloroacetic acid.

Introduction

Plant movement or oscillation occurs in many plants. Leaf shrinking in Mimosa pudica, leaf folding and unfolding in Samanea saman, and stomatal movements in almost all of plants are known to occur. Leaves move in time frames ranging from milliseconds to several hours as a result of changes in the shape and/or the volume of motor cells; such movement takes place in plants in response to external environmental changes or stimuli (Morillon et al. 2001). Many external stimuli such as cold or heat shock, touch, and red as well as blue light lead to transient elevations in the [Ca2+]cyt and free Ca2+ is known as a ubiquitous second messenger in many plant signaling pathways (Sze et al. 2000, Sanders et al. 1999, Haley et al. 1995, Barnes et al. 1997, Long and Jenkins 1998, Baum et al. 1999). The closing movement of stomata requires the elevation of [Ca2+]cyt (McAinsh and Hetherington 1998, Allen et al. 2000). Blue and red light causes the movement of leaves and leaflets by changing the osmotic volume and turgor in motor cells (Moshelion et al. 2002b, Suh et al. 2000, Satter and Galston 1981, Satter et al. 1988). Some specialized plant cells (e.g., stomata and motor cells of the pulvini of M. pudica, or of the stamens of Mahonia spp.) change their volume very rapidly by exchanging water with surrounding cells (Moshelion et al. 2002a, Fleurat-Lessard et al. 1997).

It has been established that water transport across biological membranes occurs not only through the lipid bilayer, but also through specific water channel proteins, namely, aquaporins (AQP) (Suga et al. 2002, Johansson et al. 2000, Preston et al. 1992). AQP found throughout nature from bacteria to mammals are also commonly referred to as belonging to the major intrinsic protein superfamily (Karlsson et al. 2003, Tyerman et al. 2002), and 35 different AQP genes have been identified both in Arabidopsis and maize (Karlsson et al. 2003). Plant AQP are present in both the plasma membrane (PM) (plasma membrane intrinsic proteins, PIPs) and tonoplasts (tonoplast intrinsic proteins, TIPs) (Maurel 1997, Schaffner 1998, Johansson et al. 2000), which facilitate transcellular and intracellular water transport, thus changing the osmotic pressure across the PM and tonoplasts, respectively (Ohshima et al. 2001). Large and rapid turgor variations in response to external stimuli in Mimosa pudica are regulated by tonoplast AQP and H+-ATPase (Fleurat-Lessard et al. 1997). Efficient transmembrane water transport is also essential for guard and pulvinus cells to exert their functions by swelling and shrinking followed by massive water flux across PM-AQP (Johansson et al. 2000).

Protein phosphorylation and dephosphorylation are considered important regulatory mechanisms by which the activity of key enzymes and receptor molecules are altered within cells in response to a wide variety of external stimuli (Komina et al. 2002, Kerk et al. 2002). The elevation of [Ca2+]cyt acts to elicit downstream events in signaling pathways (Sze et al. 2000), and as one of the pathways, the activation of protein kinase by Ca2+ and the subsequent promotion of phosphorylation in plants has been well established (Komina et al. 2002, Johansson et al.)

608

5Corresponding author; E-mail shibata@life.shimane-u.ac.jp
Aquaporin regulates movement of tulip petals

Results

Temperature-dependent petal opening and closing

Temperature changes were observed as the crucial physiological stimuli in tulip petal opening and closing. All incubations were performed in a temperature-regulated growth chamber in the dark. Tulip petals with 2-cm stems were immersed in distilled H_2O at the cut end and were pre-incubated at least for 2 h at 5°C in order to ensure almost complete closure. After transferring the flowers to 20°C, the petals began to open (expressed as petal aperture) by increasing the petal aperture without a lag time with an increase in incubation time (Fig. 1). Opening proceeded almost linearly, and reached a maximum aperture (completely opened) after 100 min. The petals remained in an open state during further incubation at 20°C. The petal opening process was accompanied by ³H₂O transport from the incubation medium to the petals, and the amount of ³H₂O detected in the petals was roughly proportional to the petal aperture. However, the ³H₂O in the petals began to increase slightly for a period of up to 45 min, which may reflect the time required for the ³H₂O to reach from the medium to the petals via the stem because the cut end of the stems of the closed petals had been placed in a test tube containing ³H₂O, and then incubation was initiated immediately at a temperature of 20°C. When the petals were fully open, the ³H₂O content remained almost constant. After transferring the open petals to 5°C, the petals began to close without any lag period. Closing was concomitant with a rapid decrease in the ³H₂O content of the petals, which then reached an almost closed state. In contrast, closed petals under conditions of continual incubation at 10°C scarcely opened; negligible ³H₂O transport to the petals was observed in these petals, in comparison with the case at 20°C. These results indicated that the temperature change is critical for the opening of tulip petals, which is accompanied by water transport to the petals; a temperature change is also crucial for petal closing, accompanied by water loss from the petals.

Water transport to different parts of the flowers was determined by incubation with ³H₂O at 20°C for 2 h and by further incubation for 2 h at 5°C (Table 1). The ³H₂O content in all parts of the flowers incubated at 20°C was almost three-fold that of flowers incubated at 5°C. The distinct distribution of ³H₂O in both groups of flowers indicated that ³H₂O transport from the medium to the upper portion of the petals most likely occurred via the stem, peduncle, and lower and middle parts of petal, as expected. The male and female parts of the flower were not the main sites of ³H₂O transport. Table 1 also shows that more than 60% of the ³H₂O was lost within 2 h when the flowers were transferred from 20°C to 5°C. In order to determine the direction of flow of the final destination of the lost water, the flowers were pre-incubated with ³H₂O at 20°C for 2 h, and then were incubated in distilled H₂O at 5°C. A very negligible amount of ³H₂O was recovered in the distilled H₂O after 2 h, suggesting that the ³H₂O was not transported back-
Aquaporin regulates movement of tulip petals. This finding indicates the involvement of transpiration in \(^{3}H_{2}O\) loss, since the stomata regulate transpiration, i.e., the loss of water from plants (Jones 1998). Fig. 2 demonstrates that following the start of incubation of the tulips at 20\(^{\circ}\)C to achieve petal opening, the transpiration rate gradually increased and reached a constant level (1.75 \(\mu \text{l} \text{ min}^{-1} \text{ flower}^{-1}\)) after 100 min. However, the transpiration rate decreased dramatically by subsequent transfer of the flowers to 5\(^{\circ}\)C, and then reached an almost constant level after 90 min. These results, shown in Fig. 1 and 2, revealed that continuous water transport, as well as simultaneous and constant transpiration, are involved in the opening of petals and in the maintenance of that open state.

The effects of inhibitors on tulip petal opening and closing are shown in Table 2. Ruthenium red (RR), a Ca\(^{2+}\) channel blocker (Tsuji et al. 2000, Xu et al. 1999) and \(O,O'\)-bis(2-aminophenyl)ethylene-glycol-\(N,N',N'\),\(N'\)-tetraacetic acid (BAPTA), a Ca\(^{2+}\) chelator (Gressel et al. 2002), both exerted adverse inhibitory effects on petal opening. In addition, these chemicals inhibited the transport of \(^{3}H_{2}O\). These findings indicate that a transient elevation in [Ca\(^{2+}\)]\(_{cyt}\) may be a crucial factor for temperature-dependent tulip petal opening. However, these inhibitors had no effect on closing, independent of [Ca\(^{2+}\)]\(_{cyt}\).

Aquaporin phosphorylation under petal opening condition

Microsomal membrane prepared from petals was incubated with \(\gamma\)-\(^{32}\)P]ATP for in vitro protein phosphorylation. Total \(^{32}\)P incorporation into the protein, expressed as total dpm mg\(^{-1}\) protein, proceeded in a temperature- and time-dependent manner. The phosphorylation of proteins became saturated after 30 min of incubation. An aliquot containing an equal amount of protein was separated by SDS-PAGE for autoradiography (Fig. 3A). Several polypeptides of 90, 75, 52, and 31 kDa were phosphorylated prominently, depending on incubation temperature (Fig. 3A, B), incubation time up to 30 min (Fig. 3C), and the Ca\(^{2+}\) concentration (Fig. 3D). The maximum \(^{32}\)P-labeling of these polypeptides was observed at 20\(^{\circ}\)C and at a Ca\(^{2+}\) concentration of 25 \(\mu \text{M}\) after incubation for 30 min, when the radioactivity after autoradiography was compared with the intensity, as expressed by photo-stimulated-luminescence (PSL) (Fig. 3B–D). In the absence of added Ca\(^{2+}\), a slight phosphorylation proceeded at 20\(^{\circ}\)C.

**Table 1** \(^{3}\)H\(_{2}\)O content in different tulip parts after incubation for 2 h at 20\(^{\circ}\)C, and further incubation for 2 h at 5\(^{\circ}\)C

<table>
<thead>
<tr>
<th>Different parts of flower</th>
<th>At 20(^{\circ})C for 2 h ((\mu)l)</th>
<th>At 5(^{\circ})C for 2 h ((\mu)l)</th>
<th>Lost (^{3})H(_{2})O within 2 h at 5(^{\circ})C (%)</th>
<th>Recovered (^{3})H(<em>{2})O in dH(</em>{2})O at 5(^{\circ})C ((\mu)l/flower)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petal</td>
<td>4.299</td>
<td>1.397</td>
<td>67.5</td>
<td>0.0033</td>
</tr>
<tr>
<td>Upper part</td>
<td>0.147</td>
<td>0.067</td>
<td>54.4</td>
<td></td>
</tr>
<tr>
<td>Middle part</td>
<td>0.222</td>
<td>0.097</td>
<td>56.6</td>
<td></td>
</tr>
<tr>
<td>Bottom part</td>
<td>3.930</td>
<td>1.233</td>
<td>68.6</td>
<td></td>
</tr>
<tr>
<td>Male part</td>
<td>0.545</td>
<td>0.217</td>
<td>60.2</td>
<td></td>
</tr>
<tr>
<td>Female part</td>
<td>0.405</td>
<td>0.143</td>
<td>64.7</td>
<td></td>
</tr>
<tr>
<td>Peduncle</td>
<td>4.070</td>
<td>1.550</td>
<td>61.9</td>
<td></td>
</tr>
<tr>
<td>Stem (1 cm just below peduncle)</td>
<td>12.890</td>
<td>4.010</td>
<td>68.9</td>
<td></td>
</tr>
</tbody>
</table>

\(\text{a}\) Completely closed flowers kept at 5\(^{\circ}\)C were incubated at 20\(^{\circ}\)C for 2 h with \(^{3}\)H\(_{2}\)O in K-P buffer (described in Materials and Methods) and then were transferred to 5\(^{\circ}\)C for another 2 h.

\(\text{b}\) Different parts were collected to estimate the \(^{3}\)H\(_{2}\)O content.

\(\text{c}\) Lost water was calculated by subtracting the \(^{3}\)H\(_{2}\)O content at 5\(^{\circ}\)C from that of at 20\(^{\circ}\)C.

\(\text{d}\) To check the destination of lost water at 5\(^{\circ}\)C, the flowers were incubated with \(^{3}\)H\(_{2}\)O at 20\(^{\circ}\)C for 2 h and the stems were rinsed extensively with dH\(_{2}\)O, and then the flowers were incubated at 5\(^{\circ}\)C with only dH\(_{2}\)O for another 2 h.

**Fig. 2** Water transpiration rate during petal opening at 20\(^{\circ}\)C and closing at 5\(^{\circ}\)C. Following pre-incubation at 5\(^{\circ}\)C, completely closed flowers with 5 cm stems were incubated at 20\(^{\circ}\)C in test tubes containing H\(_{2}\)O for 2 h, and then the flowers were transferred to 5\(^{\circ}\)C for another 2 h. The total weight of each flower, H\(_{2}\)O, and test tube was measured in 15 min intervals. The transpiration rate at 20\(^{\circ}\)C (filled diamonds) and 5\(^{\circ}\)C (filled triangles) was calculated from the loss of weight, assuming that 1 g of H\(_{2}\)O is equivalent to 1 ml.
Table 2  Effects of inhibitors on petal opening and closing

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Opening (5°C → 20°C)</th>
<th>Closing (20°C → 5°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Petal aperture b (cm)</td>
<td>3H2O per petal c (dpm)</td>
</tr>
<tr>
<td>None (control)</td>
<td>5.3 (100%)</td>
<td>9.24 x 10^4 (100%)</td>
</tr>
<tr>
<td>Ruthenium red (50 µM)</td>
<td>1.6 (30%)</td>
<td>0.96 x 10^4 (10.4%)</td>
</tr>
<tr>
<td>BAPTA (2 mM)</td>
<td>1.8 (34%)</td>
<td>1.21 x 10^4 (13.1%)</td>
</tr>
</tbody>
</table>

*Completely closed flowers with 2-cm stems kept at 5°C, assuming the opposite petal aperture to be 0 cm, were immersed at the cut end in 3H2O containing different inhibitors, and then incubated at 20°C.

*b Opposite petal apertures after 2 h of incubation at 20°C.

*c 3H2O content after 2 h of incubation at 20°C.

*d Completely opened flowers with 2-cm stems were incubated at 5°C with dH₂O for 2 h, and then the petal apertures were measured.

Fig. 3  In vitro phosphorylation of microsomal membrane proteins. (A) Representative autoradiogram of phosphorylated microsomal membrane depending on temperature. Lanes 2–5, phosphorylation was carried out at 10, 15, 20, or 25°C, respectively in the presence of 25 µM Ca²⁺. The molecular masses of the standards are shown in lane 1. 32P-labeled microsomal membrane was separated by SDS-PAGE, and phosphorylated peptides were visualized and analyzed after autoradiography. Radioactivity was expressed as photo-stimulated luminescence (PSL), and the results were plotted in panels B (temperature-dependency), C (time course at 20°C), and D (Ca²⁺-dependency at 20°C for 30 min). In panels B–D, filled diamonds indicate the dpm for 32P incorporated into microsomal membrane; filled squares, filled triangles, ×, and filled circles indicate the PSL of 90-, 75-, 52-, and 31-kDa proteins, respectively.
Aquaporin regulates movement of tulip petals

The \(^{32}\)P-phosphorylated microsomal membrane was separated into PM and tonoplast fractions by the two-phase partition method (Ohshima et al. 2001). As shown in Fig. 4A, almost all of the phosphorylated peptides were distributed in the PM (lane 3), and a slight amount of the protein was found to be phosphorylated in the tonoplast-enriched fraction (lane 4) under the conditions for protein phosphorylation in vitro. Hereafter, we focused on the 31-kDa protein which was phosphorylated at \(20^\circ\)C, because the AQP range in size from 26 to 34 kDa (Karlsson et al. 2003, Tyerman et al. 2002). Using commercially available anti-phospho-Ser, anti-phospho-Thr, and anti-phospho-Tyr, the phosphorylated amino acid residues of the proteins were examined. Immunoblot analysis using anti-phospho-Ser (Fig. 4B) showed seven distinct signals, including 52 and 31 kDa polypeptides in the PM. The 31 kDa phosphorylated PM protein was characterized by immunoblot analysis using an anti-PM-AQP antibody developed against an identical sequence, ‘PPAPLFEPGELSSWS’, present in many plant PM-AQP such as PIP\(_{a2}\) (Craterostigma plantagineum, T09791), AQP1 (Nicotina tabacum, AF024511), PAQ1 (Radish, AB012044), Mip1 (Solanum tuberosum, STU18311), PIP1b2 (Brassica oleracea, AF299050), and PIP1B (Arabidopsis thaliana, Q06611). The conserved amino acid sequence synthesized to raise antisera was common to that used by Ohshima et al. (2001) and they showed reactivity of the prepared antisera to PIPs of radish and a CAM plant. A distinct single spot was detected unequivocally in the PM from petals and stems (Fig. 4C), but there was no signal in the tonoplast-enriched fraction from either of the tissues (Fig. 4D). We detected reactivity in the PM from bulbs, roots, and leaves (Fig. 4E), indicating the ubiquitous expression of PM-AQP in tulip plants. Anti-phospho-Thr and anti-phospho-Tyr conferred no distinct band of 31-kDa protein; this finding may reflect a very weak signal, or may be due to the lack of a site for phosphorylated Thr and Tyr. To date, reports about the putative phosphorylation sites for AQP have primarily specified Ser residues (Karlsson et al. 2003, Johansson et al. 2000, Maurel et al. 1995), supporting the results shown in Fig. 4B.

Characterization of membrane-bound CDPK

The properties of membrane-bound protein kinase were investigated by an in-gel assay method to determine the molec-
Aquaporin regulates movement of tulip petals

Aquaporin regulates movement of tulip petals. Myelin basic protein (MBP) from bovine brain was embedded in the separating gel as the substrate for the kinase. After electrophoresis and protein renaturation, the gel was incubated with \( [\gamma-\text{32P}]\text{ATP} \) in the presence or absence of Ca\(^{2+}\). Only one distinct kinase activity corresponding to a 45-kDa protein was detected following incubation with Ca\(^{2+}\) (Fig. 5A, lane 2). Without Ca\(^{2+}\), a very faint band appeared at the corresponding position (Fig. 5B). Many protein kinases have been reported as being autophosphorylated (Ritchie and Gilroy 1998), but no autophosphorylation signal was observed when SDS-PAGE was performed without the added kinase substrate (Fig. 5C). Therefore, it is likely that a Ca\(^{2+}\)-mediated pathway operates the signal transduction for the putative PM-AQP phosphorylation through a 45-kDa membrane-bound CDPK, concomitant to previous reports on the membrane-bound protein kinase acting on the water channel proteins in plant cells (Johansson et al. 2000, Harvengt et al. 2000).

Aquaporin dephosphorylation during petal closing

As reversible protein phosphorylation is an essential regulatory mechanism in many cellular processes (Kerk et al. 2002, Janssens and Goris 2001), the dephosphorylation of PM-AQP was observed subsequent to its phosphorylation. Flowers with 5-cm stems were incubated at 20°C and then transferred to 5°C. Then the amounts of phosphorylated PM-AQP were compared immunologically in petals of both temperatures. Immunoblot analysis using anti-phospho-Ser clearly showed that more than 50% of the phospho-Ser of PM-AQP in the petals was dephosphorylated during further incubation at 5°C (Fig. 6A). However, the 52-kDa protein prominently phosphorylated at Ser site at 20°C remained unchanged following incubation at 5°C. Another set of experiments using anti-PM-AQP demonstrated that the amount of PM-AQP was almost constant in both the petals and the stems (Fig. 6B) after incubation at both temperatures, whereas the same samples showed that the intensity of the phospho-Ser in the PM-AQP decreased at 5°C in

**Fig. 5** Characterization of membrane-bound CDPK by in-gel kinase assay. PM proteins were separated on 12.5% SDS-PAGE containing MBP in the separating gel. Following the removal of SDS from the gel, and after the renaturing of the proteins, the gel was incubated with the kinase assay buffer containing \([\gamma-\text{32P}]\text{ATP}\) in the presence (A, lane 2) or absence (B) of Ca\(^{2+}\) and was used for autoradiography. Molecular masses (kDa) of the standards were shown (A, lane 1) and the arrow indicates the corresponding position of protein kinase. (C) Autophosphorylation of CDPK without embedding MBP in the separating gel.

**Fig. 6** In vivo phosphorylation and dephosphorylation of PM-AQP. (A, C and D) Immunodetection using anti-phospho-Ser. Completely closed flowers with 5-cm stems were incubated with 5 mM H\(_3\)PO\(_4\) at 20°C and then the flowers were transferred to 5°C. PM was prepared from petals and stems incubated under both conditions. PM from petals was used in panel A, and that from petals and stems was applied in panel C. The same PM used in panel C was applied in panel B for the immunodetection of PM-AQP by anti-PM-AQP. For panel D, completely closed flowers with 5-cm stems were incubated in the same medium at 20°C in the presence of 50 \(\mu\text{M}\) RR, and the PM prepared from petals and stems was used for the immunodetection using anti-phospho-Ser.
Aquaporin regulates movement of tulip petals

both tissues (Fig. 6C). But when the flowers were incubated at 20°C in the presence of RR, no signal of phosphorylation was observed in the range corresponding to PM-AQP in either of the tissues (Fig. 6D), suggesting that RR inhibited petal opening as well as water transport (Table 2). As far as we know, these results may provide the first direct evidence of phosphorylation and reversible phosphorylation of PM-AQP as regards its regulation of water transport in the process of temperature-dependent tulip petal oscillation.

Discussion

Although many studies and reviews regarding flowering time are available focusing on short-day and long-day light regimes (Koornneef et al. 1998), to our knowledge, this is the first report to focus on the relationship between tulip petal oscillation and temperature-dependent petal transport regulated at the molecular level. Closed petals that were maintained in this state at 5°C for 2 h began to open after transferring the flowers to 20°C. The open state returned to a closed state when opened flowers were transferred from 20°C to 5°C. Our data (Fig. 1, Table 2) showed that temperature-dependent petal opening was accompanied by water transport from other parts of the plant to the petals. Water moved through the stems to the petals via peduncles, and thereby would increase the turgor pressure in guard cells. Due to the elastic properties of cell walls, petals can reversibly increase their volume, which leads to petal opening. The volume of guard cells increases by 40–100%, depending on the species (Taiz and Zeiger 1998). In this study, the open state was maintained until the temperature was changed to 5°C. Two physiological events, water transport and simultaneous transpiration, have been found to play a significant role in maintaining the petal-open state, and water availability to plants and stomatal conductance are reported to be critical for transpiration (Jones 1998). Our study indicated negligible water transport at 5°C. However, when the flowers were transferred from 20°C to a 5°C, the ¹H₂O content sharply decreased (Fig. 1) while the transpiration rate was maintained (Fig. 2). The most likely reason for these results might be that 60% of the inner surface stomata, as well as 40% of the outer surface stomata lead to the accelerated transpiration of water due to their open state, which was maintained for up to 15 min after the transfer of the flowers from 20°C to 5°C (data not shown). After 60 min of exposure to 5°C, the decreases in the ¹H₂O content and in the rate of transpiration were insignificant, leading the flower to close completely after 120 min. Moreover, water transport at 5°C was negligible. Our data (Fig. 1, 2) therefore suggested that continuous water transport took place at an invariable rate from source to petal, and that simultaneous transpiration was necessary to maintain tulip petal opening.

The release of Ca²⁺ from intracellular and extracellular Ca²⁺ stores is controlled in response to specific stimuli through Ca²⁺-permeable ion channels (McAinsh and Hetherington 1998, Thuleau et al. 1994, Allen et al. 1995). RR blocks the Ca²⁺ channel inhibiting an increase in [Ca²⁺]cyt, and BAPTA chelates intracellular Ca²⁺ when the tissues or cells are immersed in the solution containing these compounds (Tsujii et al. 2000, Borutaite et al. 2000), and BAPTA can penetrate the plant cuticle to chelate internal Ca²⁺ (Gressel et al. 2002). Incubation with RR and with BAPTA severely inhibited the petal opening and the water transport (Table 2), thus suggesting the temperature change as a stimulus leading to an increase in [Ca²⁺]cyt.

A tulip tonoplast water channel protein, γ-TIP, has been cloned (X95650), and this channel protein has a predicted protein sequence of 259 amino acids with 26.6 kDa (Balk and Boer 1999). Most members of the AQP superfamily have similar molecular masses ranging from 26 to 34 kDa (Karlsson et al. 2003, Tyerman et al. 2002), and TIPs (23–26 kDa) have been shown to be smaller than PIPs (Maeshima 2001). The phosphorylated 31-kDa PM protein was identified in almost all tissues of tulip as the putative AQP immunologically using prepared polyclonal antibody against the conserved amino acid sequence in many plant PM-AQP (Fig. 4C, E).

The changes in the amount of phospho-Ser in PM-AQP in vivo (Fig. 6A, C) unequivocally revealed the involvement of the phosphorylation of PM-AQP during petal opening at 20°C and dephosphorylation during petal closing at 5°C. Below 10°C, water transport was found to be negligible, compared to that at 20°C, and PM-AQP was not phosphorylated at 10°C (Fig. 3A, B). Our results suggested that at 20°C, the phosphorylation of PM-AQP would gate the water channel, thereby facilitating rapid water transport from the stems to the petals; at 5°C, dephosphorylation provoked the PM-AQP gate to be closed, thus decreasing water transport.

The Ca²⁺-binding properties are capable of translating Ca²⁺ signals into qualitative messages (Chaudhury et al. 1999), and modulating regulatory enzymes such as CDPK (Ueoka-Nakanishi et al. 1999) to cause phosphorylation of PM-AQP (Karlsson et al. 2003, Johansson et al. 2000). It may be possible based on these results that temperature-dependent tulip petal oscillation possibly post-translationally regulated via the temperature-dependent phosphorylation and dephosphorylation of the putative PM-AQP by a PM-associated CDPK and a protein phosphatase. We therefore hypothesized that low temperatures lead to the closure of PM-AQP channels via dephosphorylation of PM-AQP, thus decreasing water transport to close petals. However, like other stimuli such as touch, heat shock, drought, and red light (Sze et al. 2000, Sanders et al. 1999, Ueoka-Nakanishi et al. 1999), temperature changes to 20°C may lead to transient elevation in [Ca²⁺]cyt, thus activating CDPK, causing the phosphorylation of PM-AQP, and the gating of the water channel. Rapid water transport would then result in tulip petal opening. As the recent studies showed that AQP are specific for the water channel proteins and the co-transporter of glycerol and/or small solutes with water (Karlsson et al. 2003) as well as of NH₄⁺ and some important
Aquaporin regulates movement of tulip petals 615

non-electrolytes (Tyerman et al. 2002), the transport of such compounds may also occur during petal opening. The volume and turgor regulation of the cell with water channel activity can rectify overall hydraulic resistance of the cell-to-cell pathway to change the hydraulic pathways within whole tissues affecting the water potential gradient required for water flow, and therefore during petal closing, a different water potential gradient between cytosol and apoplasts may lead to intracellular water flow to apoplasts (Tyerman et al. 2002) and finally water is transpirated through stomata. At present, we have no evidence of a 20°C-sensing mechanism in tulip petals. We have currently purified and characterized a soluble protein phosphatase 2A as holoenzyme as well as free catalytic subunit. Although the both preparations dephosphorylated the phosphorylated putative PM-AQP at 20°C, only the holoenzyme preparation could act at 5°C on the phosphorylated PM-AQP with higher substrate specificity (unpublished data). However, further study is required to clone the putative PM-AQP to show the involvement of the specific PM-AQP from the superfamily in tulip petals for temperature-dependent opening and closing.

Materials and Methods

Sources of tulip and chemicals

Tulips (Tulipa gesnerina L. cv. Ile de France) were grown at a farm associated with Shimane University, and 2-day-old flowers were harvested, and stored at −80°C. All chemicals were from Wako Pure Chemical Industries, Osaka, Japan unless noted otherwise. [γ-32P]ATP and H2O were purchased from ICN Biomedicals Inc., and NEN (Boston, MA, U.S.A.), respectively. Biotin-conjugated primary antibodies against phospho-Ser, phospho-Thr, and phospho-Tyr, avidin-peroxidase conjugate (secondary antibody), and protein phosphatase inhibitor (cocktail 1) were obtained from Sigma.

Analyses of petal oscillation and water transport

Flowers with 2-cm stems were incubated either at 20°C or 5°C in 10 ml of 10 mM KP, pH 7.0, containing 2.5×104 dpm H2O supplemented with or without inhibitors. Opposite petal distances (petal aperture) were measured as a reflection of the degree of opening and closing. For estimating the amount of H2O transported into the petals, each petal or a selected portion of the petal was cut into several segments and incubated in 5 ml MeOH for 72 h in the dark at room temperature. The radioactivity was then measured in each aliquot using a liquid scintillation counter (Backman, LS6000SE).

Preparation of microsomal and plasma membranes

Microsomal membrane was prepared at 4°C by the method described by Briskin et al. (1987). Briefly, frozen tulip petals were crushed into small pieces and homogenized using a chilled mortar and pestle. Two milliliters of homogenization medium containing 0.2 M sucrose, 25 mM Tris-MES, pH 7.8, 1 mM DTT, 3 mM EDTA, 15 mM 2-ME, 0.1% (w/v) BSA, and 0.5% (w/v) insoluble PVP was used per gram of tissue. The homogenate was then filtered through four layers of cheesecloth and the pH of the filtrate was adjusted to 7.8 with concentrated Tris buffer. After successive centrifugation of the filtrate at 3,000×g for 5 min and at 13,000×g for 15 min, the resulting supernatant was centrifuged at 144,000×g for 1 h. The pellet was resuspended in the same buffer and stored at −80°C as a source of microsomal membrane. PM was purified using an aqueous polymer two-phase partition method (Ohshima et al. 2001). A microsomal membrane sample corresponding to 50 mg proteins suspended in 10 ml of 5 mM KP, pH 7.8, containing 0.33 M sucrose and 3 mM KCl was added to 28 ml of a polymer-phase mixture, giving a final concentration of 6.2% (w/w) Dextran T-500, 6.2% (w/w) polyethylene glycol 3400, 0.33 M sucrose, 3 mM KCl, and 5 mM KP, pH 7.8. The membrane phase system was inverted and returned to the upright position 20 times for thorough mixing, and was kept on ice for 20 min. The mixture was then centrifuged at 1,500×g for 3 min at 0°C. The upper phase was carefully removed with a Pasteur pipette without disturbing the interface. The upper and lower phases were repartitioned three times for further purification. The pooled upper phase (PM) and the lower phase (tonoplasts) were diluted with 5 mM KP, pH 7.8, containing 0.33 M sucrose, and precipitated by centrifugation at 144,000×g for 1 h. The pellets were washed with the same diluting buffer and suspended in 25 mM Tris-HCl, pH 7.8, containing 0.25 M sucrose, and then stored at −80°C until further use. PM obtained by this procedure was shown to be highly purified (close to 100%) (Ohshima et al. 2001, Kjellborn and Larsson 1984).

Protein concentrations were determined according to the method of Bradford (1976) using protein assay reagent (Bio-Rad) with BSA as the standard protein.

In vitro phosphorylation

For in vitro phosphorylation, 60 µg of microsomal membrane proteins were added to a reaction mixture containing 50 mM Tris-MES, pH 6.5, 10 mM MgCl2, 5 mM DTT, 10 µM ATP (including 185 kBq of [γ-32P]ATP), 0.2 mM EGTA, 100 µM CaCl2, and 5 mM NaF in a total volume of 40 µl and the mixture was incubated at 25°C for 30 min unless otherwise noted. The reaction was initiated by the addition of ATP containing [γ-32P]ATP, and was stopped by the addition of 40 µl of 2-fold concentrated SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-ME, and 0.001% bromophenol blue). In the Ca2+-dependent phosphorylation assays, the Ca2+ concentration was expressed as described by Askerlund (1996). After phosphorylation, an aliquot was absorbed on Whatman cellulose filter paper (3MM) to measure the total amount of 32P incorporated into the proteins. The filter paper was washed four times with 5% (v/v) TCA and then with 95% ethanol, and the radioactivity was measured by a liquid scintillation counter. Another aliquot of the sample was separated by SDS-PAGE, and the phosphorylated polypeptides were visualized and analyzed after autoradiography by MacBAS-1500 (Fujiﬁlm, Japan). For the in vivo phosphorylation, each tulip with a 5-cm stem was immersed in 25 mM Tris-HCl, pH 7.5, containing 5 mM H2PO4, 0.25 mM KCl, and 0.5 mM MgSO4 (Komina et al. 2002). Tulips were then incubated at 20°C. After incubation, the PM was prepared as described above using the same homogenization medium containing 2 µl of cocktail 1 (a mixture of protein phosphatase inhibitors) per each milliliter of medium.

Antibody preparation and immunodetection of PM aquaporin

The polypeptide consisted of 15 amino acids, PPAPLFEPGEL-SSWS, which is a highly conserved sequence found in the N-terminal portion of many plant PM-AQP (Ohshima et al. 2001) was conjugated with keyhole limpet hemocyanin, and then injected into rabbits. The IgG fraction was used as antibody against plant PM-AQP. Immunoblotting was performed using the standard method described below under “Phospho-amino acid analysis”.

In vitro phosphorylation
**Phospho-amino acid analysis**

PM was phosphorylated according to the same method mentioned above without [γ-32P]ATP in the presence of 200 μM ATP and 10 mM NaF in the total 40 μl reaction mixture for 4 h at 20°C. Following SDS-PAGE, proteins on 12.5% polyacrylamide gel were transferred to a PVDF membrane by electroblotting using a semi-dry apparatus (Taitec, Japan) at 2 mA cm−2 for 1 h. The membrane was blocked with 0.5% BSA in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 2 mM KH2PO4) for 1 h with gentle agitation and then was incubated with monoclonal primary antibody for 12 h. The dilutions used for anti-phospho-Ser, anti-phospho-Thr, and anti-phospho-Tyr were 1: 2,000, 1: 1,000, and 1: 1,000 respectively. The membrane was then washed three times, 5 min each, with PBS containing 0.1% Tween-20, and then the membrane was incubated for 3 h under conditions of gentle agitation with 1: 2,000 diluted secondary antibody, an avidin-peroxidase conjugate containing 0.2% BSA. Following the washing procedure described above, the color was developed using 2 mM 4-methoxy-1-naphthol (Aldrich Chemical Com., U.S.A.) and 3 M H2O2 as substrates.

**In-gel protein kinase assay**

The in-gel protein kinase activity was detected by slight modifications of the procedure described by Osawa and Matsumoto (2001). In brief, 20 μg of PM proteins per each lane were separated on 12.5% SDS-PAGE containing 0.36 mg ml−1 of MBP embedded in the separating gel. After electrophoresis, SDS was removed by washing the gel with 50 mM Tris-HCl, pH 8.0 containing 20% (v/v) 2-propanol for 1 h at room temperature with continuous gentle agitation. The solution was changed twice, and the gel was incubated with 250 ml of buffer A (50 mM Tris-HCl, pH 8.0, 5 mM 2-ME) for another 1 h under the same conditions, and the buffer was again changed twice. The proteins in the gel were denatured with buffer A containing 6.0 M guanidine hydrochloride under continuous agitation at room temperature for 1 h and then the gel was incubated in buffer A containing 0.05% (v/v) Tween-20 at 4°C for 16 h under continuous agitation, and the buffer was changed four times. The gel was pre-incubated in the kinase assay buffer (40 mM Tris-HCl, pH 8.0, 100 μM CaCl2, 50 mM NaCl, 20 mM KCl, 10 mM MgCl2, 0.1 mM EGTA, and 2 mM DTT) at room temperature for 1 h under continuous agitation and the buffer was changed at least twice. Finally, the gel was incubated in 25 ml of the kinase assay buffer containing 50 μM ATP plus 3.7 MBq [γ-32P]ATP for 75 min at room temperature under continuous agitation. The gel was washed for 2 h with 5% (v/v) TCA containing 1% (w/v) sodium pyrophosphate at room temperature, and the solution was changed four times to remove the unincorporated [γ-32P]ATP. The dried gel was exposed to sensitized X-ray film for 24 h at room temperature for autoradiography. To investigate the autophosphorylation of the membrane-bound kinase, we used the same method, without using MBP in the separating gel.

**References**


Aquaporin regulates movement of tulip petals


(Received December 12, 2003; Accepted March 2, 2004)