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Water Transport Activity of the Plasma Membrane Aquaporin PM28A Is Regulated by Phosphorylation

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PM28A is a major intrinsic protein of the spinach leaf plasma membrane and the major phosphoprotein. Phosphorylation of PM28A is dependent in vivo on the apoplastic water potential and in vitro on submicromolar concentrations of Ca^{2+}. Here, we demonstrate that PM28A is an aquaporin and that its water channel activity is regulated by phosphorylation. Wild-type and mutant forms of PM28A, in which putative phosphorylation sites had been knocked out, were expressed in Xenopus oocytes, and the resulting increase in osmotic water permeability was measured in the presence or absence of an inhibitor of protein kinases (K252a) or of an inhibitor of protein phosphatases (okadaic acid). The results indicate that the water channel activity of PM28A is regulated by phosphorylation of two serine residues, Ser-115 in the first cytoplasmic loop and Ser-274 in the C-terminal region. Labeling of spinach leaves with ^{32}P-orthophosphate and subsequent sequencing of PM28A-derived peptides demonstrated that Ser-274 is phosphorylated in vivo, whereas phosphorylation of Ser-115, a residue conserved among all plant plasma membrane aquaporins, could not be demonstrated. This identifies Ser-274 of PM28A as the amino acid residue being phosphorylated in vivo in response to increasing apoplastic water potential and dephosphorylated in response to decreasing water potential. Taken together, our results suggest an active role for PM28A in maintaining cellular water balance.

INTRODUCTION

Transmembrane water flow accompanies many physiological processes in plants, including the transcellular movement of water in the transpiration stream, phloem loading, osmotic adjustments between the vacuole and the cytosol, stomatal movement, and cell expansion. The recent discoveries that both the plasma membrane and the tonoplast contain aquaporins (water channel proteins) have changed our view of how plant cells may be able to regulate transmembrane water movement (reviewed in Chrispeels and Maurel, 1994; Maurel, 1997). Such regulation may be especially important during periods of water deficit, whether locally in a tissue or generally in the soil. Aquaporins are integral membrane proteins and belong to the major intrinsic protein (MIP) family of channel-forming proteins. Most MIP homologs are aquaporins, that is, they function as water-specific channels, although a few MIP homologs have been shown to transport solutes other than water, for example, glycerol, as in the case of the yeast MIP homolog Fps1 (reviewed in Park and Saier, 1996).

Since the first aquaporin was identified in human erythrocytes (AQP1; Preston et al., 1992), aquaporins have been found in many organisms, including bacteria, plants, and animals. In plants, aquaporins are encoded by multigene families. In Arabidopsis, six aquaporins belonging to the plasma membrane intrinsic protein (PIP) family have been characterized (Daniels et al., 1994; Kammerloher et al., 1994). At least five additional expressed PIP-like genes are present in Arabidopsis, but the corresponding proteins have not been characterized (Weig et al., 1997). Proteins with high sequence similarity to the Arabidopsis plasma membrane aquaporins have been identified in several other plant species, but either they have not been assayed for water channel activity or their subcellular location has not been established.

Transcriptional induction of aquaporin genes in response to water deficiency has been demonstrated. The MIP homolog clone 7a of pea and RD28, an aquaporin of the plasma membrane of Arabidopsis, were identified by differential hybridization using desiccated plant tissues (Guerrero et al., 1990; Yamaguchi-Shinozaki et al., 1992). The mRNA corresponding to TRAMP, a MIP homolog in tomato, was induced when leaves were wilted (Fray et al., 1994).

A number of studies indicate that the activity of aquaporins may be regulated by phosphorylation. α-TIP, an aquaporin in the tonoplast of bean seeds, is phosphorylated in vivo (Johnson and Chrispeels, 1992), and the mammalian

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aquaporin AQP2 can be phosphorylated in vitro (Lande et al., 1996). By expressing these aquaporins in Xenopus oocytes, it was found that phosphorylation led to an increase in water channel activity (Kuwahara et al., 1995; Maurel et al., 1995). Kuwahara et al. (1995) suggested direct regulation of water transport by the phosphorylation of AQP2 in addition to regulation by the vasopressin-induced fusion of AQP2-containing vesicles with the plasma membrane. Contradictory results were presented by Lande et al. (1996), who could not increase the water permeability of AQP2-enriched endosomes by phosphorylation of AQP2. NOD26, a MIP homolog in soybean root nodules, is also phosphorylated (Weaver et al., 1991). Recent experiments using expression in oocytes and isolated symbiosome membrane vesicles show that NOD26 has a water channel conductance 30-fold lower than that of AQP1 and that it permits the passage of glycerol and formamide (Rivers et al., 1997).

We recently showed (Johansson et al., 1996) that one of the most abundant proteins of the spinach leaf plasma membrane, a 28-kD polypeptide designated PM28A, is a MIP homolog with considerable sequence homology with the plasma membrane aquaporins RD28 (Yamaguchi-Shinozaki et al., 1992; Daniels et al., 1994) and PIP2a, PIP2b, and PIP2c (Kammerloher et al., 1994). We have identified both PM28A as the major phosphoprotein of the plasma membrane after in vitro phosphorylation by using isolated plasma membrane vesicles and Ser-274 as the phosphorylation site. We have also shown that a polypeptide of 28 kD corresponds to the major phosphoprotein upon in vivo phosphorylation and that the phosphorylation of this polypeptide is dependent on the apoplastic water potential. We now report that PM28A is an aquaporin and that its water channel activity when expressed in Xenopus oocytes is regulated by phosphorylation at two different sites: Ser-115, a serine residue conserved in all plant plasma membrane aquaporins, and Ser-274, which is conserved in the PIP2 subfamily. In addition, we identified Ser-274 of PM28A as the amino acid phosphorylated and dephosphorylated in vivo in response to changing apoplastic water potential. Our results suggest an active role for PM28A in cellular water balance.

RESULTS

PM28A Is an Aquaporin

The water channel activity of PM28A was assayed by expression of *pm28a* complementary RNA (cRNA) in Xenopus oocytes. cRNA was synthesized in vitro using the *pm28a* cDNA clone as a template, and the cRNA was injected into oocytes. After 3 days of incubation in isotonic buffer, the oocytes were transferred to a hypotonic buffer, and the relative increase in volume as a consequence of water uptake was measured. As shown in Figure 1, oocytes injected with *pm28a* cRNA have a swelling rate five to 10 times higher than that of water-injected oocytes, demonstrating that PM28A is an aquaporin. The swelling rate of 0.097 units per minute corresponds to an osmotic membrane permeability coefficient, P_f, of 1.1×10^{-2} cm/sec (see Methods for calculations). This value is similar to values for other aquaporins reported in the literature (e.g., Kammerloher et al., 1994; Daniels et al., 1996).

Analysis of Water Transport Activity of PM28A Mutants

Using isolated plasma membrane vesicles, we showed that PM28A is phosphorylated on Ser-274 in vitro (Johansson et al., 1996). An examination of the amino acid sequence shows that there are at least six potential phosphorylation sites at the cytoplasmic side, as shown in Figure 2A. Site-directed mutagenesis was used to convert the codons for serine at these sites to codons for alanine. The resulting mutants are designated S6A, S36A, S115A, S188A, S192A, and S274A. The mutated clones were transcribed into cRNA, and water transport assays were performed as described above. As can be seen in Figure 2B, five of the mutants exhibited water transport activity comparable to that of the wild type. S115A, however, showed only ~50% of the wild-type activity (Figures 2B and 3). This result and the observation that Ser-274 is phosphorylated in vitro prompted us to analyze the double mutant S115A/S274A. This mutant had

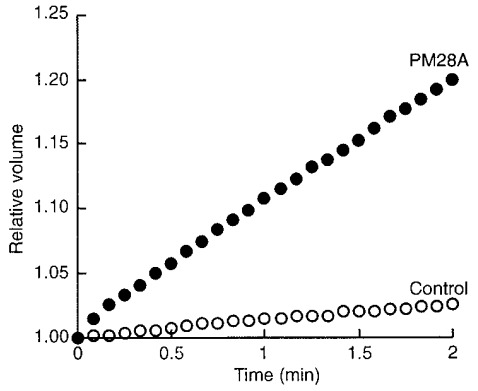

Figure 1. Changes in Cell Volume of Xenopus Oocytes Expressing PM28A.

Oocytes were injected with 50 ng of cRNA encoding PM28A (closed circle) or with water (open circle) (control oocytes). After 3 days of incubation in isotonic buffer, the oocytes were transferred to a fivefold dilution of the same buffer at time 0. Osmotic water permeability (P_f) was calculated from the initial swelling rate. P_f (PM28A) = 1.1×10^{-2} cm/sec and P_f (control) = 0.15×10^{-2} cm/sec.

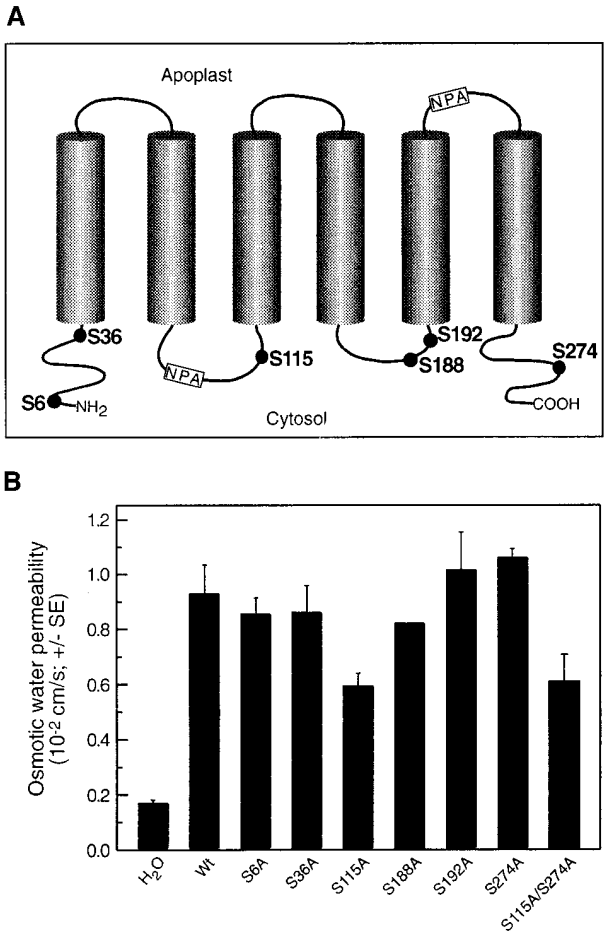

Figure 2. Water Transport Activity of Wild-Type and Mutant Forms of PM28A.

(A) Model of PM28A showing potential phosphorylation sites on the cytoplasmic side of the membrane. The six predicted transmembrane regions are represented by cylinders, and the two highly conserved Asn-Pro-Ala acid (NPA) boxes are indicated. Serine residues at potential phosphorylation sites are labeled with their amino acid numbers.

(B) Osmotic water permeability of wild-type and mutant forms of PM28A. Serine residues at potential phosphorylation sites were converted to alanine residues by using site-directed mutagenesis. Oocytes were injected with 50 nL of H_20 or with 50 nL of cRNA (1 ng/nL) encoding wild-type PM28A (Wt) or mutated PM28A (S6A, S36A, S115A, S188A, S192A, S274A, and S115A/S274A). The oocytes were incubated for 3 days before osmotic permeability was assayed. The values are means of two to four experiments using different batches of oocytes. A minimum of seven oocytes was assayed for each construct in each experiment. s, sec.

water transport activity similar to S115A and ~50% lower than the activity of S274A (Figures 2B and 3).

To test whether these differences were due to different protein expression levels, we performed sulfur-35 labeling of wild-type and mutant PM28A. Newly injected oocytes were incubated in ^{35}S-methionine and ^{35}S-cysteine for 2 days. Total homogenates (six oocytes) were obtained from the wild type and mutants of interest (S115A, S274A, and S115A/S274A), and the proteins were resolved by SDS-PAGE. Autoradiography of the gel showed that the wild-type and mutant forms were expressed to a similar level (data not shown).

Effect of K252a and Okadaic Acid on Water Transport Activity

Oocytes expressing wild-type and mutant PM28A were incubated with either the protein kinase inhibitor K252a or the protein phosphatase inhibitor okadaic acid for 30 min before measuring water transport activity. K252a shows broad-range substrate specificity, inhibiting protein kinase A, protein kinase C, and several other protein kinases (Kase et al., 1987; Tischler et al., 1990). Okadaic acid is an inhibitor of protein phosphatase 2A and, to a lesser extent, protein phosphatase 1 (Cohen, 1989).

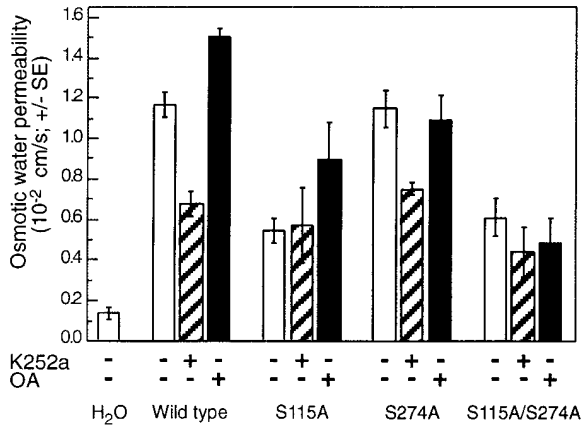

Figure 3. Effect of a Protein Kinase Inhibitor (K252a) and a Phosphatase Inhibitor (Okadaic Acid) on Osmotic Water Permeability of Wild-Type and Mutated PM28A.

Oocytes were injected with 50 ng of cRNA encoding wild-type PM28A or mutated PM28A (S115A, S274A, and S115A/S274A) or with 50 nL of H_2O. The oocytes were preincubated in Barth's solution supplemented with 1 μM K252a or 5 μM okadaic acid (OA) or with 0.1% DMSO (controls) for 30 min before measuring osmotic water permeability. The values are means of two (S115A/S274A) or three (H_2O, wild type, S115A, and S274A) experiments using different batches of oocytes. For each experiment, at least seven oocytes were assayed. Plus and minus signs indicate the presence or absence of K252a and OA. s, sec.

The results presented in Figure 3 show that the water transport activity of wild-type PM28A was inhibited ~50% by K252a, thereby decreasing to the same level as the water transport activity of S115A. Okadaic acid, on the other hand, activated PM28A by ~30%. K252a had no effect on S115A but inhibited S274A to about the same extent as it inhibited the wild type. These data suggest that in the wild-type protein, Ser-115 is phosphorylated and that phosphorylation at this site is prevented by the protein kinase inhibitor K252a. Okadaic acid activated S115A to the same extent as it activated the wild type but had no effect on S274A activity, suggesting that phosphorylation of Ser-274, but not of Ser-115, is enhanced by adding the phosphatase inhibitor. Consistent with this interpretation, neither K252a nor okadaic acid had any effect on the double mutant S115A/S274A. Mutants with amino acid changes at other potential phosphorylation sites (S6A, S36A, S188A, and S192A) responded to the inhibitors in a manner similar to the wild type, that is, inhibition by K252a and stimulation by okadaic acid (data not shown). Taken together, these data suggest that the water channel activity of PM28A is regulated by phosphorylation at two serine residues, Ser-115 and Ser-274.

PM28A Is Phosphorylated on Ser-274 in the Plant

The results of the experiments with PM28A expressed in oocytes indicate the involvement of two phosphorylated serine residues (Ser-115 and Ser-274) in the regulation of water transport activity. To investigate the situation in the plant, a phosphorylation experiment in which pieces of spinach leaves were incubated in ^{32}P-orthophosphate was performed. After incubation for 2 hr, the plasma membrane fraction was isolated, and the polypeptides were separated by SDS-PAGE. Previously, we showed that this treatment leads to a heavily labeled polypeptide band at 28 kD (Johansson et al., 1996). The 28-kD band was cut from the gel and digested with the proteinase LysC. The resulting peptides were separated by reversed-phase HPLC, and the eluted fractions were examined for radioactivity. As shown in Figure 4A, radioactivity was found mainly in one peak, although a second smaller radioactive peak was also present. No additional peaks with radioactivity significantly above background could be detected. The peptides corresponding to the two radioactive peaks were sequenced. Both had the sequence ALGSFRSNPTN, which is identical to the C terminus of PM28A. This sequence contains two serine residues available for phosphorylation—Ser-274 and Ser-277. To identify the phosphorylated amino acid(s), we measured radioactivity for each fraction released during sequencing. As shown in Figure 4B, the main peak corresponds to a peptide phosphorylated at Ser-274 only. The minor peak corresponds to a peptide phosphorylated at both Ser-274 and Ser-277.

In a separate experiment, the synthetic peptide ALGSFRSNPTN was added to the LysC-derived peptides and

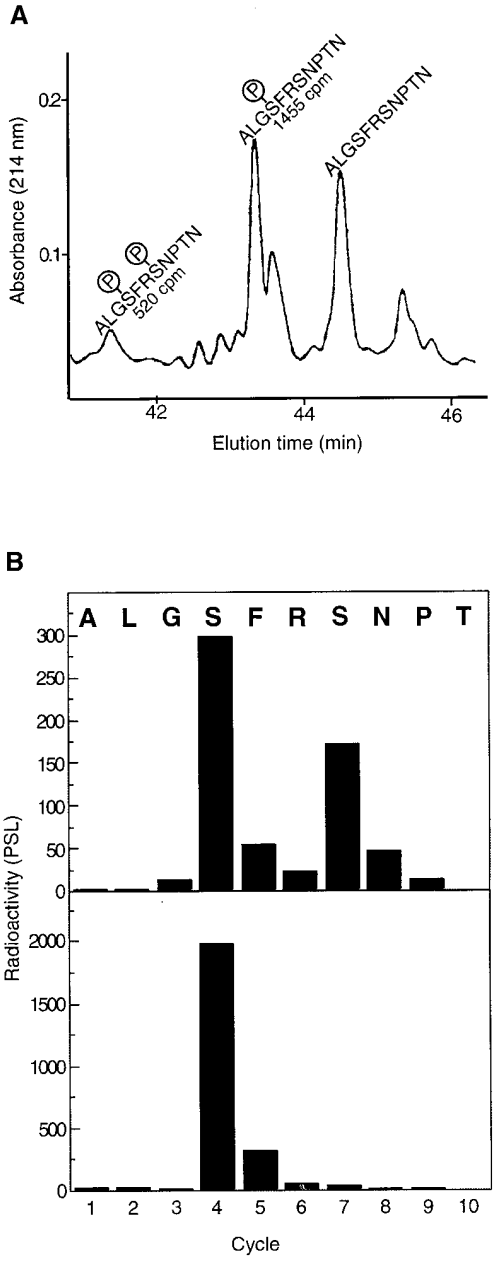

Figure 4. Identification of Phosphorylated Amino Acids of PM28A.

(A) Part of the reversed-phase HPLC chromatogram showing separation of peptides resulting from digestion of in-the-plant phosphorylated PM28A with LysC. Radioactivity was measured for each fraction eluted. The two peaks corresponding to the fractions that contained radioactivity are labeled with their counts per minute values. The peaks corresponding to the double-phosphorylated, single-phosphorylated, and unphosphorylated C-terminal peptide of PM28A are shown. These were all identified by sequencing.

(B) Radioactivity released for each cycle during sequencing of the 520-cpm peak (top) and the 1455-cpm peak (bottom). The identified amino acids are shown in one-letter code above the graphs. The first serine in the peptides corresponds to Ser-274 and the second to Ser-277.

fractionated by reversed-phase HPLC. The purpose of this experiment was to identify the peak corresponding to the unphosphorylated peptide and to determine the degree of phosphorylation of PM28A (Figure 4A). The peak identified by cochromatography was also sequenced to confirm its identity. The relative amounts of unphosphorylated, phosphorylated, and double-phosphorylated PM28A were determined by integration of the peak areas, taking into account minor contaminations in the double-phosphorylated and unphosphorylated peaks evident from second choices in the sequences obtained. Approximately 5% of PM28A was phosphorylated on both Ser-274 and Ser-277, whereas >50% of PM28A was phosphorylated on Ser-274 only under the conditions used (high apoplastic water potential; Johansson et al., 1996).

Because no phosphorus-32 phosphorylation of Ser-115 was obtained in the plant, a number of experiments were designed to trigger phosphorylation at this site. Thus, activators of animal protein kinase A activity in vivo known to function in the oocyte system, such as the cAMP analog 8-Br-cAMP, the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX), and the adenylate cyclase activator forskolin (Kuwahara et al., 1995; Maurel et al., 1995), were included either alone or in combination in the medium used for the infiltration of leaf pieces. However, phosphorylation could still be detected only on Ser-274 (data not shown). Similarly, inclusion of abscisic acid (ABA), a proposed signal compound in response to drought stress (reviewed in Bray, 1997), in the infiltration medium did not elicit phosphorylation of Ser-115, and neither did the presence of okadaic acid (data not shown).

DISCUSSION

Preventing water loss under conditions of osmotic stress or water limitation is crucial to maintaining plant growth because the lowering of turgor that results from water loss quickly leads to the cessation of cell elongation and growth. Work with maize has shown that shoot growth is more sensitive to water deficit than root growth (Sharp et al., 1988). The physiological responses of plants to drought stress suggest that plants have evolved mechanisms that allow them to sense turgor and activate signaling pathways that ultimately regulate proteins and genes that help the cells to withstand water deficit (reviewed in Bray, 1997). One such response is the synthesis of osmotically active compounds, for example, polyols, sugars, amino acids, and amines, that increase the osmotic pressure in the cell, thereby preventing further water loss and maintaining turgor. Another response may be to slow down water loss by decreasing the osmotic water permeability of the plasma membrane. Here, we present evidence that such a mechanism operates in plant cells and is coupled to the reduced phosphorylation of a plasma membrane aquaporin.

Plant plasma membrane aquaporins have been identified in Arabidopsis (Daniels et al., 1994; Kammerloher et al., 1994; Kaldenhoff et al., 1995), and they can be divided into two subfamilies, PIP1 and PIP2, based on sequence similarities. In spinach, we have identified two PIP homologs that are highly expressed in leaf tissue (Johansson et al., 1996). These are PM28A, which is closely related to the PIP2 subfamily, and PM28B, which is closely related to the PIP1 subfamily. The presence of these two subfamilies of plasma membrane aquaporins may be a common characteristic among plants. We showed earlier that decreased water potential in the apoplast causes decreased phosphorylation of a 28-kD protein in the plasma membrane (Johansson et al., 1996). We now identify this protein as PM28A, show that PM28A is an aquaporin (Figure 1), and show that reduced phosphorylation of Ser-115 and Ser-274 results in reduced water transport activity of PM28A when expressed in oocytes (Figures 2 and 3). Results from phosphorylation experiments in the plant show that Ser-274 and, to a lesser extent, Ser-277 at the C terminus of PM28A are phosphorylated in vivo under hypoosmotic conditions (Figure 4). This identifies PM28A as the 28-kD polypeptide phosphorylated and dephosphorylated in vivo in response to changing apoplastic water potential, suggesting an active role for PM28A in regulation of cell turgor.

Phosphorylation at Two Different Sites Regulates the Water Channel Activity of PM28A in Oocytes

When potential phosphorylation sites in PM28A (Figure 2A) were mutated and the different mutant proteins were expressed in oocytes, only the change of Ser-115 to Ala significantly lowered osmotic water permeability (Figure 2B). Moreover, water transport by this mutant was not affected by the protein kinase inhibitor K252a, which lowered the activity of wild-type *pm28a*-injected oocytes (Figure 3). This indicates that the low activity of the S115A mutant is due to its inability to become phosphorylated on this residue rather than to changes in structure or stability of the protein. Unexpectedly, the S274A mutant showed the same activity as did the wild type. However, water transport by this mutant was not further stimulated by okadaic acid, in contrast to water transport activity of the wild type (Figure 3). This suggests that Ser-274 is not phosphorylated in oocytes unless protein phosphatases are inhibited. Unlike the situation in the plant in which close to 60% of Ser-274 was found to be phosphorylated (Figure 4B), the level of phosphorylation in the oocyte system is not known. Therefore, it is possible that the increase in water transport caused by okadaic acid (Figure 3) is due to the phosphorylation of only a minor fraction of Ser-274, which would underestimate the possibility for regulation via the phosphorylation/dephosphorylation of this site. Mutants with amino acid changes at other potential phosphorylation sites responded to the inhibitors in a manner similar to that of the wild type, that is, inhibition by K252a

and stimulation by okadaic acid (data not shown). Taken together, our results suggest the involvement of two phosphorylation sites, Ser-115 and Ser-274, in the regulation of water transport activity of PM28A.

Ser-274 Is Phosphorylated in the Plant

Phosphorylation in the plant resulted in a strong phosphorus-32 labeling of Ser-274 (Figure 4); however, no labeling of Ser-115 could be detected under the conditions used. Ser-274 resides in a consensus phosphorylation site recognized by vertebrate protein kinase C (Ser-X-Arg, where X stands for any amino acid) (Ohno and Suzuki, 1995) in the C-terminal region. This site is conserved only among the closest relatives of PM28A, such as the Arabidopsis proteins RD28 and PIP2a and PIP2b, that is, among the aquaporins that may be grouped with the PIP2 subfamily. In vitro (isolated plasma membrane vesicles), Ser-274 is phosphorylated in a Ca^{2+}-dependent manner by a plasma membrane–associated protein kinase (Johansson et al., 1996).

There are several possible reasons for the lack of phosphorus-32 labeling of Ser-115 in the plant. First, Ser-115 may not be a regulatory site in the plant and thus is not phosphorylated in the plant. Other possibilities are that the proper protein kinase was not activated under the conditions used or that Ser-115 was already phosphorylated but that no turnover took place due to an inactivated protein phosphatase. Alternatively, protein phosphatase activity was high, and all label was lost during purification, despite the presence of the phosphatase inhibitor NaF in the grinding medium. In an attempt to modify the conditions to favor phosphorylation of Ser-115, activators of animal protein kinase A activity were included during incubation of the leaf pieces. The cAMP analog 8-Br-cAMP, the phosphodiesterase inhibitor IBMX, and the adenylate cyclase activator forskolin were used, either alone or in combination. However, phosphorylation could still be detected only on Ser-274 and to a lesser extent on Ser-277 (data not shown). These activators have been used to increase phosphorylation of aquaporins expressed in oocytes (Kuwahara et al., 1995; Maurel et al., 1995), but it is not clear whether they activate plant protein kinases.

ABA synthesis is induced during drought and is thought to play a major role in mediating the cellular responses to osmotic stress (reviewed in Bray, 1997). Therefore, we investigated whether ABA might modulate the phosphorylation status of PM28A. However, inclusion of ABA in the incubation buffer did not affect the phosphorylation of PM28A; neither did the presence of the protein phosphatase inhibitor okadaic acid (data not shown).

Ser-115 Is Highly Conserved

Ser-115 is located in the first cytoplasmic loop and resides in a typical protein kinase A recognition sequence (Arg-Lys-X-Ser) (Pinna and Ruzzene, 1996) as well as in a typical consensus phosphorylation site (Leu-X-Arg-X-X-Ser) for CDPK (calmodulin-like domain protein kinase; Harmon et al., 1987; Harper et al., 1991; Bachmann et al., 1996) and protein kinase C (Arg-Lys-X-Ser-X-X-Arg) (Kennelly and Krebs, 1991). A search of the databases (Altschul et al., 1990) resulted in 40 plant protein sequences with high homology to PM28A and thus to established plant plasma membrane aquaporins. Sequence comparisons between these 40 homologs showed that the serine residue corresponding to Ser-115 of PM28A is highly conserved. Indeed, it is present in a similar position and amino acid environment in all 40 homologs, and a corresponding serine residue is even found in some of the tonoplast aquaporins, for example, α-TIP (Maurel et al., 1995). The consensus sequence obtained for the 40 plasma membrane aquaporin homologs is Leu-Ala-Arg-Lys-Leu/Val-Ser-Leu-X-Arg, which means that phosphorylation by any of the above-listed protein kinases is at least theoretically possible.

Concluding Remarks

In Arabidopsis, 23 different MIP genes have been identified (Weig et al., 1997). Tissue- and cell-specific expression together with different mechanisms of post-translational regulation of the aquaporins in this family are likely to provide cells and organs with the possibility to fine-tune water flow. Hitherto, only a few aquaporins have been shown to be regulated by phosphorylation. In α-TIP, phosphorylation of three serine residues, two in the N terminus (Ser-7 and Ser-23) and one in the first cytoplasmic loop (Ser-99), increases water transport activity when the protein is expressed in oocytes (Maurel et al., 1995). NOD26, which functions as a weak water channel (Rivers et al., 1997), is phosphorylated by a CDPK on Ser-262 in the C-terminal region (Weaver and Roberts, 1992). Whether this stimulates water transport activity has not yet been investigated. AQP2 is also phosphorylated at the C terminus (Kuwahara et al., 1995), but whether this increases water channel activity directly or only indirectly by promoting fusion of AQP2-containing vesicles with the plasma membrane is still being debated (Lande et al., 1996).

In this study, we demonstrate that phosphorylation of Ser-115 and Ser-274 activates the spinach leaf aquaporin PM28A when expressed in oocytes, and the phosphorylation of one of these residues (Ser-274) is demonstrated in the plant. Figure 5 presents a model for the role of PM28A in controlling cellular water balance. The model suggests that when the water potential in the apoplast is high, the aquaporin is phosphorylated, the channel is open, and water flow across the plasma membrane is essentially unrestricted. The protein kinase phosphorylating PM28A on Ser-274 is membrane associated and activated by submicromolar concentrations of Ca^{2+} (Johansson et al., 1996). The osmosensor in the plasma membrane may therefore simply be a stretch-activated Ca^{2+} channel, known to be present in plant

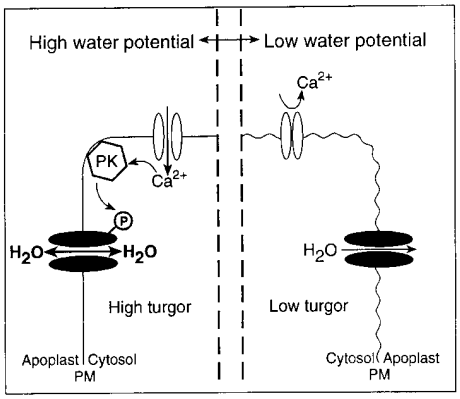

Figure 5. Hypothetical Model for the Regulation of Water Flow through the Plasma Membrane Water Channel PM28A.

PK is the Ca^{2+}-dependent protein kinase associated with the plasma membrane (PM) and responsible for the phosphorylation of Ser-274. The Ca^{2+} channel might be of the mechanosensitive type (stretch activated) and directly respond to changes in the apoplastic water potential.

plasma membranes (reviewed in Garrill et al., 1996). Alternatively, osmosensing and subsequent signal transduction may be more complex, and Ca^{2+} could also be released from the vacuole. Activation of water transport based on a phosphorylation-triggered fusion of periplasmic vesicles with the plasma membrane, as has been suggested for AQP2 (Fushimi et al., 1997), is not likely for PM28A because the presence of PM28A in the plasma membrane is constitutively high and not affected by subjecting plants to drought stress (Johansson et al., 1996). Furthermore, in vitro phosphorylation of PM28A shows that the protein is phosphorylated in situ in the plasma membrane (Johansson et al., 1996).

The model shown in Figure 5 further suggests that when the leaf experiences water deficiency and cell turgor begins to fall, the Ca^{2+} channel is closed, the protein kinase is thereby inactivated, the aquaporin is dephosphorylated, and water flow through the aquaporin becomes restricted. This would give PM28A an active role in cellular water balance, hindering water loss under desiccating conditions and buying time for the plant to take other measures to cope with the situation, such as biosynthesis of osmotically active compounds (reviewed in Bray, 1997).

METHODS

In Vivo Phosphorylation

Spinach (*Spinacia oleracea*) leaves (20 g) were cut in small pieces and incubated in 10 mM Mes–1,3-*bis*(*tris*[hydroxymethyl]methylamino)propane, pH 6.0, 0.1 mM EDTA, 0.1 mM EGTA, and 5 mCi ^{32}P-labeled Pi. Vacuum was applied until the leaf pieces darkened and sank. The reaction mixture was then incubated for 120 min at 20°C with occasional swirling. After removal of the incubation buffer, the leaf pieces were rinsed, and the plasma membranes were isolated essentially as described by Larsson et al. (1994). The tissue was homogenized at 4°C in 50 mM 3-(*N*-morpholino)propanesulfonic acid–KOH, pH 7.5, 5 mM EDTA, 5 mM DTT, 5 mM ascorbate, 0.33 M sucrose, 0.2% casein (boiled enzymatic hydrolysate, C-0626; Sigma), 0.2% BSA (protease free, A-3294; Sigma), 0.5 mM phenylmethylsulfonyl fluoride (added after filtration of the homogenate), and 50 mM NaF. Plasma membranes were isolated from the microsomes (10,000 to 50,000*g* pellet) by partitioning in an aqueous polymer two-phase system. The final plasma membrane pellet was resuspended in 0.33 M sucrose, 5 mM K-phosphate, pH 7.8, and 50 mM KCl. Polypeptides were separated by SDS-PAGE, and phosphorylated polypeptides were detected using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The band corresponding to PM28A was cut from the gel and digested.

Protein Digestion and Amino Acid Sequencing

The gel piece containing PM28A was incubated with LysC from *Achromobacter lyticus* (Wako, Osaka, Japan), essentially as described by Rosenfeld et al. (1992). The eluted peptides were separated using a SMART chromatography station equipped with a μRPC SC C2/C18 2.1/10 column (Pharmacia, Uppsala, Sweden). The gradient was from 0.1% trifluoroacetic acid (TFA) in H_2O to 50% acetonitrile/0.085% TFA for 75 min with a flow of 100 μL/min. Peaks were monitored at 214 and 280 nm, and automatic peak collection was used.

Amino acid sequencing was performed on an ABI 476A sequencer, according to the manufacturer's instructions (Applied Biosystems/Perkin-Elmer, Foster City, CA). Determination of the phosphorylated amino acid was done by covalent sequencing of the radioactive peptide on arylactivated membranes (Millipore, Bedford, MA). After several washes with neat TFA in an ABI 477A sequencer, the AZT amino acid was extracted with neat TFA, collected, and concentrated by evaporation. Each fraction, corresponding to one released amino acid, was spotted on thin layer chromatography plates, and radioactive spots were visualized and quantified using a PhosphorImager (Fuji, Tokyo, Japan).

Protein Determination and SDS-PAGE

Protein concentration was determined essentially as in Bearden (1978), using BSA as the standard. SDS-PAGE was performed according to Laemmli (1970), with minor modifications. Gradient gels (8 to 15%) were used. Samples were solubilized at 20°C for 15 min in standard sample buffer (Laemmli, 1970). Gels were run overnight at 9 mA and 14°C and stained with Coomassie Brilliant Blue R 250.

Vector Construction, Site-Directed Mutagenesis, and In Vitro Transcription

The coding sequence of *pm28a* was subcloned into the BglII site of pXβG-ev1, a pSP64T-derived pBluescript KS− vector containing 5′ and 3′ untranslated sequences of a *Xenopus laevis* β-globin gene (Preston et al., 1992). The mutants S6A, S36A, S115A, S188A, S192A, S274A, and S115A/S274A were synthesized using a recombinant polymerase chain reaction (PCR) technique (Higuchi, 1990). Two separate PCR reactions were performed, using the sense or

antisense sequence of the mutated region and the T3 or T7 promoter vector sequences as primers. The sequences of the primers are as follows: S6A, 5′-CTAAGGAAGTAGCTGAAGAAGCACA-3′ (sense) and 5′-TGTGCTTCTTCAGCTACTTCCTTAG-3′ (antisense); S36A, 5′-TCAAATTGTGGGCTTTCTGGAGAGC-3′ (sense) and 5′-GCTCTCCAGAAAGCCCACAATTTGA-3′ (antisense); S115A, 5′-GCAAGGAAGGTGGCCTTACTAAGGGC-3′ (sense) and 5′-GCCCTTAGTAAGGCCACCTTCCTTGC-3′ (antisense); S188A, 5′-ACCCTAAGAGGGCCGCACGTGACTC-3′ (sense) and 5′-GAGTCACGTGCGGCCCTCTTAGGGT-3′ (antisense); S192A, 5′-GCGCACGTGACGCTCACGTGCCTAT-3′ (sense) and 5′-ATAGGCACGTGAGCGTCACGTGCGC-3′ (antisense); S274A, 5′-AAGGCGTTGGGAGCCTTCAGAAGCAA-3′ (sense) and 5′-TTGCTTCTGAAGGCTCCCAACGCCTT-3′ (antisense).

The two PCR products were gel purified and used as templates in a second PCR reaction with the following *pm28a* start and end sequences as primers: 5′-GAAGATCTATGTCTAAGGAAGTAAGT-3′ (start) and 5′-GAAGATCTTTAATTGGTAGGGTTGCT-3′ (end). These primers contained BglII sites at the 5′ end to facilitate cloning. The final PCR product was ligated into the BglII site of pXβG-ev1. All PCR reactions were performed using Vent DNA polymerase (New England Biolabs, Beverly, MA), and the mutated clones were sequenced to confirm the altered sequence. After linearizing the vector, capped complementary RNA (cRNA) encoding PM28A or the mutants was synthesized in vitro using T3 RNA polymerase. cRNA was purified and diluted to 1.0 μg/μL in RNase-free water.

Oocyte Preparation and Injection

Stage V and VI oocytes were isolated from Xenopus and injected with 50 nL of cRNA (1.0 μg/μL) or 50 nL of RNase-free water. The oocytes were incubated for 3 days at 18°C in Barth's solution (10 mM Hepes-NaOH, pH 7.4, 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, and 0.82 mM $MgSO_4$) before water permeability measurements.

Osmotic Water Permeability Analysis

Individual oocytes were transferred from Barth's solution (Osm_{in} is 200 mosmol) to a cuvette perfused with Barth's solution diluted to 40 mosmol (Osm_{out}) at 20°C. Oocyte swelling was followed by video microscopy. Pictures were stored at 5-sec intervals, and cell volume was calculated from the cell section area. Osmotic water permeability (P_f) was determined from the initial slope of the time course of relative cell volume by using the formula $P_f = V_0[d(V/V_0)/dt]/[S \times V_w \times (Osm_{in} - Osm_{out})]$, where initial oocyte volume, V_0, is 9×10^{-4} cm^3; initial oocyte surface area, S, is 0.045 cm^2; and molar volume of water, V_w, is 18 cm^3/mol (Zhang and Verkman, 1991).

For inhibition studies, oocytes were incubated for 30 min in Barth's solution containing 1 μM K252a (Calbiochem, San Diego, CA), 5 μM okadaic acid (Calbiochem), or 0.1% DMSO (control oocytes) at 20°C before water permeability measurements.

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