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Multiple Phosphorylations in the C-terminal Tail of Plant Plasma Membrane Aquaporins

ROLE IN SUBCELLULAR TRAFFICKING OF AtPIP2;1 IN RESPONSE TO SALT STRESS

Sodana Prak‡, Sonia Hem§¶, Julie Boudet‡, Gaëlle Viennois‡, Nicolas Sommerer§, Michel Rossignol§, Christophe Maurel‡, and Véronique Santoni‡

Aquaporins form a family of water and solute channel proteins and are present in most living organisms. In plants, aquaporins play an important role in the regulation of root water transport in response to abiotic stresses. In this work, we investigated the role of phosphorylation of plasma membrane intrinsic protein (PIP) aquaporins in the Arabidopsis thaliana root by a combination of quantitative mass spectrometry and cellular biology approaches. A novel phosphoproteomics procedure that involves plasma membrane purification, phosphopeptide enrichment with TiO_2 columns, and systematic mass spectrometry sequencing revealed multiple and adjacent phosphorylation sites in the C-terminal tail of several AtPIPs. Six of these sites had not been described previously. The phosphorylation of AtPIP2;1 at two C-terminal sites (Ser^{280} and Ser^{283}) was monitored by an absolute quantification method and shown to be altered in response to treatments of plants by salt (NaCl) and hydrogen peroxide. The two treatments are known to strongly decrease the water permeability of Arabidopsis roots. To investigate a putative role of Ser^{280} and Ser^{283} phosphorylation in aquaporin subcellular trafficking, AtPIP2;1 forms mutated at either one of the two sites were fused to the green fluorescent protein and expressed in transgenic plants. Confocal microscopy analysis of these plants revealed that, in resting conditions, phosphorylation of Ser^{283} is necessary to target AtPIP2;1 to the plasma membrane. In addition, an NaCl treatment induced an intracellular accumulation of AtPIP2;1 by exerting specific actions onto AtPIP2;1 forms differing in their phosphorylation at Ser^{283} to induce their accumulation in distinct intracellular structures. Thus, the present study documents stress-induced quantitative changes in aquaporin phosphorylation and establishes for the first time a link between aquaporin subcellular localization.


Aquaporins form a family of channel proteins that mediate the transport across membranes of water, small neutral solutes, and occasionally ions (1–3). Aquaporins are present in all living kingdoms and in plants. Aquaporins exhibit a characteristic high multiplicity of forms with for instance 35 members in Arabidopsis (4, 5). Based upon their amino acid sequence homology, plant aquaporins can be classified into four subfamilies (4–6). One of these corresponds to the plasma membrane intrinsic proteins (PIPs). The PIPs with 13 members in Arabidopsis represent the most abundant aquaporins in the plasma membrane (PM) and can be further divided into two sequence homology groups (AtPIP1 and AtPIP2). Aquaporins are 25–35-kDa proteins that share a typical organization with six transmembrane α-helices interrupted by five connecting loops (loops A–E) (7, 8). In PM aquaporins, the N and C termini as well as loops B and D are exposed in the cytosol, whereas loops A, C, and E face the cell wall.

Plants need to continuously adjust their water status in response to changing environmental conditions, and aquaporins play an important role in these processes (3, 9, 10). In particular, physiological and genetics studies have provided compelling evidence for a role of aquaporins in the regulation, in response to abiotic stresses, of root water transport, i.e. root hydraulic conductivity (Lpr) (10, 11). For instance, exposure of Arabidopsis plants to salt (100 mM NaCl) induced a rapid (half-time, 45 min) and significant decrease (~70%) in Lpr, that was maintained for at least 24 h (12). Whereas the long term effect of this NaCl stress can be accounted for by an overall transcriptional down-regulation of aquaporins, the molecular mechanisms involved in the early inhibition of Lpr by NaCl are not fully understood yet. These mechanisms involve a slight decrease in overall abundance of AtPIP1 proteins as soon as 30 min after exposure to NaCl and a trafficking of AtPIP1 and AtPIP2 isoforms between the PM and intracellular compartments that may contribute to reducing the abundance of AtPIPs at the PM and therefore the hydraulic conductivity of salt-stressed root cells (12). Chilling is another stress that leads to inhibition of Lpr, and a relationship

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1 The abbreviations used are: PIP, plasma membrane intrinsic protein; GFP, green fluorescent protein; Lpr, root hydraulic conductivity; PM, plasma membrane; TiO_2, titanium dioxide; WT, wild type; ER, endoplasmic reticulum.
between aquaporin regulation and reactive oxygen species was established in this context (13). In cucumber for instance, hydrogen peroxide (H2O2) accumulated in response to chilling, and treatment of roots with exogenous H2O2 inhibited Lp, to the same extent as chilling. In Arabidopsis a rapid decrease in Lp, can also be observed in response to 2 mM H2O2. Because of its amplitude (>70%) and rapidity (half-time, ≈8 min) this decrease is undoubtedly due to a down-regulation of root aquaporins.

Post-translational modifications are central for regulating protein structure and function and thereby for modulating and controlling protein catalytic activity, subcellular localization, stability, and interaction with other partners. Qualitative and quantitative information about post-translational modifications and in particular measurements of their dynamic changes are now critically needed to understand the complexity of cell regulations. Protein phosphorylation is one of the most important and best characterized post-translational modifications. Virtually all cellular processes are regulated in one or multiple ways by reversible phosphorylation, and the identification of the protein kinases and phosphatases, their substrates, and the specific sites of phosphorylation involved is crucial for the understanding of cell signaling. Besides classical methods relying on in vivo and in vitro labeling or immunodetection of phosphorylated proteins, MS is now widely used for studies on protein phosphorylation (14, 15). Different instrumentation such as ESI- and MALDI-MS systems are now amenable to phosphoprotein analysis (16), and sample preparation procedures have been optimized to enhance phosphopeptide recovery and detection by MS (17). In particular, immobilized metal affinity chromatography (18) or titanium dioxide (TiO2) microcolumns (19) have proved powerful for the selective enrichment of phosphorylated peptides.

Phosphorylated serine residues have been identified in the N-terminal and C-terminal tails of various plant aquaporins (20–25). In particular, two phosphorylation sites were identified in the C terminus of Arabidopsis AtPIP2;1 (26) and At-PIP2;6 (25) and spinach SoPIP2;1 (22) (Table I). AtPIP2;7 also shows double phosphorylation, but only one phosphosite was clearly identified (26) (see Table I). Also all plant AtPIPs shows a conserved putative phosphorylation site in loop B (22, 23). Based on functional analyses in Xenopus oocytes, it was proposed that phosphorylation of SoPIP2;1 at this site and at Ser262 (in the C terminus) was able to regulate its water transport activity (22). A molecular mechanism for phosphorylation-dependent gating of PIPs has recently been proposed from the atomic structures of SoPIP2;1 in its open and closed conformations (8). Mammalian aquaporins also carry multiple phosphorylation sites, and by contrast to plant aquaporins, phosphorylation of mammalian aquaporin-2 is not involved in gating but rather regulates the shuttling of the protein between the PM and intracellular compartments (27, 28).

The purpose of this work was to study the role of plant PM aquaporin phosphorylation in regulating the root water permeability in response to NaCl and H2O2 treatments. For this, a systematic inventory of phosphorylation sites in the C terminus of AtPIP aquaporins was performed, and novel phosphoresidues were discovered. Because of the emerging role of stimulus-dependent trafficking of plant aquaporins between the PM and intracellular compartments (12, 29, 30), the role of aquaporin phosphorylation in this process was investigated. The results point to a specific phosphorylated site in the C terminus of AtPIP2;1 that regulates the trafficking of this aquaporin in control conditions and in response to an NaCl treatment.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Endoproteinase Lys-C was purchased from Calbiochem. Synthetic PIP2 peptides (277SLGSFRSAANV297), either unmodified or singly phosphorylated at Ser280, were isotopically labeled on Arg261 with 13C and 15N to induce a 10-Da mass increment (Sigma). The same PIP2 peptide but diphosphorylated was isotopically labeled on Ala284 and Ala285 with 13C to induce a 6-Da mass increment (NeoMPS, Strasbourg, France). TiO2 beads were obtained by disassembling TiO2 guard columns purchased from GL Sciences Inc. (Tokyo, Japan). The 3M Empore™ C8 disks were from 3M Bioanalytical Technologies (St. Paul, MN). GEloader tips were from Eppendorf (Hamburg, Germany). 2,5-Dihydroxybenzoic acid and α-cyano-4-hydroxycinnamic acid were from Sigma-Aldrich. All other chemicals and reagents were of the highest commercially available grade.

**Plant Materials and Treatments—**Arabidopsis thaliana ecotype Columbia (Col-0), plants were cultivated in hydroponic conditions as described previously (31). Briefly plants were cultivated in a growth chamber at 20 °C with an 8-h light (150 microeinstein m−2 s−1)/16-h dark cycle at 70% relative humidity. Plants were mounted on 35 × 35 × 0.6-cm polystyrene rafts floating in a basin filled with 8 liters of nutrient medium (1.25 mM KNO3, 0.75 mM MgSO4, 1.5 mM Ca(NO3)2, 0.5 mM KH2PO4, 0.1 mM Na2SiO3, 50 μM FeEDTA, 50 μM H3BO3, 12 μM MnSO4, 1 μM ZnSO4, 0.7 μM CuSO4, 0.24 μM MoO3Na3) and cultivated for up to 7 weeks. The effects of NaCl and H2O2 were then studied by complementing the nutrient solution with 100 mM NaCl for 2 and 4 h or 2 mM H2O2 for 15 min prior to root excision. Transgenic seedlings were cultivated for 8 days on half-strength Murashige and Skoog medium (32) without any antibiotic selection. The plantlets were then transferred for 2 or 4 h into a nutrient solution as described above complemented or not with 100 mM NaCl.

**Purification of PIPs—**A microsomal fraction was obtained from roots (31). Plasma membrane vesicles were purified by aqueous two-phase partitioning of the microsomal fraction in a mixture of polyethylene glycol 3350/dextran T-500, 6.4% (w/w) each in the presence of 5 mM KCl, as described previously (31). Protein concentration was measured using a modified Bradford procedure (31). The mean yield of PM extraction was 20 μg of protein/g of fresh weight. Extrinsic membrane proteins were stripped with a urea and NaOH treatment according to a previously described procedure (31). The abundance of AtPIP2 isoforms in PM samples was evaluated by an ELISA using an antibody raised against the last 17 amino acids of the AtPIP2;1 sequence as described previously (33). The mean yield of AtPIP2 isoform was 5.3 pmol of PIP2/μg of PM proteins. Proteins were separated by SDS-PAGE on 12% acrylamide gels (31).

**Protein Digestion and Phosphopeptide Purification—**The migrating band at 28 kDa was excised from SDS-PAGE and prepared for proteolytic digestion as described previously (31). Gel pieces containing 350 pmol of AtPIP2 aquaporins were reswollen in the presence of

Lys-C at an enzyme: aquaporin ratio of 1:25 at 37 °C for 16 h. The supernatant of the digest was collected, and the remaining peptides were extracted in 0.1% TFA, 60% acetonitrile by sonication for 15 min. Supernatants were pooled, and the final volume was reduced to 10 µl using a centrifuge evaporator. To build up a TiO₂ microcolumn, a small piece was stamped out of an Empore C₈ disk by using a 200-µl pipette tip and placed at the constricted end of the GELoader tip, and TiO₂ beads in suspension in acetonitrile were packed (34). The protein digest was then diluted in a loading buffer containing 80% acetonitrile and 0.1% TFA and loaded on the column, and the column was washed with 30 µl of loading buffer. Phosphopeptides were eluted with 3 µl of NH₄OH at pH 12. 0.8 µl of eluted peptides was mixed with 0.8 µl of 20 mg/ml 2.5-hydroxybenzoic acid dissolved in acetonitrile, water, and phosphoric acid (50:44:6, v/v/v) and spotted onto the MALDI target for crystallization. The quantification of AtPIP2;1 C-terminal phosphorylation was performed by adding the synthetic labeled peptides corresponding to the C terminus of AtPIP2;1 (unmodified:singly phosphorylated:diphosphorylated, 1:1:3) to the protein digest prior to loading onto the TiO₂ column. The flow-through was desalted using ZipTip columns (Millipore, Bedford, MA). The desalted sample (0.8 µl) was mixed with 0.8 µl of matrix solution (α-cyano-4-hydroxycinnamic acid at half-saturation in 1:1 (v/v) H₂O/acetonitrile, 0.1% TFA) and spotted onto the MALDI target.

**Mass Spectrometric Analysis—MALDI-TOF MS and MS/MS analyses were performed, in positive reflector mode, using an UltraFlex II mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a smartbeam™ laser. MS/MS spectra were obtained by PSD-LIFT™ without adding a collision gas. MS data were analyzed using the FlexAnalysis software (Bruker Daltonics). All MS and MS/MS spectra shown were externally calibrated and are raw data spectra, i.e. without recalibrating, smoothing, or base-line subtracting.**

MS and MS/MS spectra annotation was performed manually. *De novo* sequencing was performed and was facilitated by the knowledge of all aquaporin sequences. All peptides proposed as phosphorylated were first checked for the presence of the major fragment ion [MH - H₂PO₄]⁻ = MH – 98 Da corresponding to the loss of the phosphate moiety. In addition, all MS/MS spectra were carefully checked manually for assignment of phosphorylation sites.

**Gene Constructs and Expression in Transgenic Plants**—Mutagenesis of AtPIP2;1 C-terminal phosphorylation sites was carried out by PCR on a cDNA of AtPIP2;1 fused by its N terminus to the green fluorescent protein (GFP) (GFP-PIP2;1). For this, we used a sense primer containing an XhoI restriction site: 5’-TTTTCTCGAGATGGTGAGCAGACAGGGCGAGG-3’. The antisense primer allows the introduction of an XbaI restriction site as well as the desired mutation. The mutagenic primers used to generate the following mutations (bold characters) were: S280A, 5’-TTT TAG ATT AGA CTT TGA AAC-3’; S283A, 5’-TTT TAG ATT AGA CTG TGG CAG CAG CTC TG-3’; and S283D, 5’-TTT CTA GAT TAG ACG TG-3’. The fragments amplified by PCR were digested by XhoI and XbaI and cloned in a pBluescript vector. The presence of the mutations was checked by DNA sequencing (Geno-Plu, Lille, France). The GFP-PIP2;1 sequences were placed under the control of a cauliflower mosaic virus 35S and RbcS terminator by cloning into the EcoRI and ClaI sites of a pGREEN vector (35). The constructs were then transferred into Agrobacterium tumefaciens strain GV3101 by electroporation with a selection for tetracycline, hygromycin as described previously (12). Two, three, two, and two independent lines were obtained for the GFP-PIP2;1, GFP-PIP2;1-S280A, GFP-PIP2;1-S283A, and GFP-PIP2;1-S283D genotypes, respectively.

**Fig. 1. MALDI-TOF MS spectrum of phosphopeptides from plant PM aquaporins.** The 28-kDa band from root PM enriched in hydrophobic proteins was digested by Lys-C. Phosphopeptides were enriched using a TiO₂ column. All C-terminal phosphopeptides expected from root AtPIP aquaporins cannot be simultaneously recorded onto the same MS spectrum. The present spectrum illustrates the presence of the singly (m/z 1188.54) and diphosphorylated (m/z 1268.51) peptides of AtPIP2;1 and/or AtPIP2;2 (see text) and of the singly (m/z 1217.53) and diphosphorylated (m/z 1297.56) peptides of AtPIP2;7. *, metastable decomposition of peptide with m/z 1268.51, a.i., absolute intensity.

**RESULTS**

A Phosphoproteomics Analysis Reveals Novel Phosphorylation Sites in the C-terminal Tail of AtPIP Aquaporins—A PM fraction was purified from Arabidopsis roots by aqueous two-phase partitioning and enriched in hydrophobic proteins with a urea and NaOH treatment (31). This extract was used to make a systematic inventory of the C-terminal phosphorylations of AtPIP2 isoforms. For this, the extract was first treated with the endoproteinase Lys-C, which is predicted to release the C-terminal tail of all AtPIP2 aquaporins. Phosphorylated C-terminal peptides were then enriched using TiO₂ microcolumns (19). A typical MALDI MS spectrum is shown in Fig. 1. The candidate phosphopeptides were initially assigned by MALDI-TOF MS from 79.96-Da mass increments per phosphate moiety relative to the unmodified peptides. During MALDI-TOF MS, phosphopeptides also lose phosphoric acid...
Phosphorylation-dependent Trafficking of Plant Aquaporin

**TABLE I**

**Phosphorylation sites in the C-terminal tail of PIP aquaporins**

The first and second columns describe the name of the aquaporin and the peptide sequence, respectively. The third column (n_ph) refers to the number of phosphorylation sites present in the peptide. pS, phosphorylated serine; pT, phosphorylated threonine.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sequence</th>
<th>n_ph</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtPIP2;2/1/2/2/2/3</td>
<td>277SLGpSFR^282</td>
<td>1</td>
<td>25, 31^a</td>
</tr>
<tr>
<td>AtPIP2;2/1/2/2/2/3</td>
<td>277SLGpSFRSAANV^287</td>
<td>1</td>
<td>Present work</td>
</tr>
<tr>
<td>AtPIP2;2/1/2/2/2/3</td>
<td>277SLGpSFRpSAANV^287</td>
<td>2</td>
<td>25, 26, and present work</td>
</tr>
<tr>
<td>AtPIP2;4</td>
<td>277ALGpSFGSFRSAF^291</td>
<td>1</td>
<td>Present work</td>
</tr>
<tr>
<td>AtPIP2;4</td>
<td>277ALGpSFGSFRSAF^291</td>
<td>2</td>
<td>Present work</td>
</tr>
<tr>
<td>AtPIP2;4</td>
<td>277ALGpSFGSFRSAF^291</td>
<td>3</td>
<td>Present work</td>
</tr>
<tr>
<td>AtPIP2;4</td>
<td>277ALGpSFGSFRpSAF^291</td>
<td>3</td>
<td>Present work</td>
</tr>
<tr>
<td>AtPIP2;6</td>
<td>285pSQLHELHA^269</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td>AtPIP2;7</td>
<td>270ALGpSFRSNATN^280</td>
<td>1</td>
<td>Present work</td>
</tr>
<tr>
<td>AtPIP2;7</td>
<td>270ALGpSFRSNATN^280</td>
<td>2</td>
<td>26, 34,^b and present work</td>
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<td>3</td>
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<td>22</td>
</tr>
<tr>
<td>SoPIP2;1</td>
<td>270ALGpSFRpSNPTN^291</td>
<td>2</td>
<td>22</td>
</tr>
</tbody>
</table>

^a One phosphorylation site was detected but not identified.

^b Two phosphorylation sites were detected, but only Ser^277 was identified as phosphorylated.

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**FIG. 2.** Phosphopeptide sequencing by MALDI-TOF/TOF of the C-terminal tail of AtPIP2;1. A, MS/MS spectrum of singly phosphorylated 277SLGSFRSAANV^287 (m/z 1188.53). y7 and y9 ions allowed identification of Ser^280 as the phosphorylated residue. B, MS/MS spectrum of the corresponding diphosphorylated peptide (m/z 1268.54). y4, y5, y7, and y9 ions allowed identification of the two phosphorylated residues as Ser^280 and Ser^283. *, fragment ions arising from loss of phosphoric acid (–98 Da), pS, phosphorylated serine; [MH]^+, precursor ion; [MH – P]^-, precursor ion with a loss of one metaphosphoric acid (–200 Da); [MH – P – 18]^–, precursor ion with a loss of one phosphoric acid (–98 Da); [MH – 2P – 18]^–, precursor ion with a loss of one metaphosphoric acid (–200 Da) and one phosphoric acid (–98 Da); [MH – 2P – 2 x 18]^–, precursor ion with a loss of two phosphoric acids (–196 Da).

As H₃PO₄ (98 Da) with the concomitant production of metastable ions with an apparent mass loss of 83 Da. Their presence was utilized as reliable indicators for phosphopeptides. A computational analysis of the mass spectra and comparison with the known aquaporin sequences allowed prediction of the presence of putative singly and diphosphorylated peptides of AtPIP2;1, AtPIP2;2, AtPIP2;3, AtPIP2;4, and AtPIP2;7 (Table I). In addition, triphosphorylated forms could be assigned to AtPIP2;4 and AtPIP2;7 isoforms (Table I). The putative phosphopeptides assigned to AtPIP isoforms were then sequenced by MALDI-TOF/TOF for confirmation and for identification of the phosphorylated residues. The positioning of the phosphorylated residue(s) was more specifically based on the identification of dehydroalanine residue-containing ions in the MS/MS spectrum.

We note that AtPIP2;1 and AtPIP2;2 are among the most abundant aquaporins in roots (12, 31, 37). The phosphopeptides derived from these isoforms were systematically detected in MS spectra. By contrast, phosphopeptides attributed to the less abundant AtPIP2 isoforms such as AtPIP2;4 and AtPIP2;7 were only occasionally detected as shown in Fig. 1.

Because they share identical C-terminal sequences, the AtPIP2;1, AtPIP2;2, and AtPIP2;3 that were predicted to be singly and diphosphorylated could not be distinguished in our study. By contrast to AtPIP2;1 and AtPIP2;2, AtPIP2;3 is barely expressed in roots (31), and for the sake of simplifica-
tion, these peptides were attributed to AtPIP2;1 here. Using this approach, the sequencing of the peptides at m/z 1188.54 and m/z 1268.51 revealed single and diphosphorylation of AtPIP2;1 on Ser280 and Ser283, respectively (Fig. 2). The fragmentation of the C-terminal peptides of AtPIP2;4 revealed a single phosphorylation on Ser286 (peptide at m/z 1617.55) and a diphosphorylation on Ser276 and Ser283 (peptide at m/z 1697.68). The MS/MS analysis of a putative low abundance triphosphorylated peptide of AtPIP2;4 (peptide at m/z 1777.63) revealed that it actually corresponded to a mixture of two isobaric forms of the AtPIP2;4 C-terminal tail (Fig. 3C). Sequencing showed a conserved phosphorylation at residues Ser280 and Ser283 and an additional phosphorylation of either Ser286 or Ser289. The C-terminal tail of AtPIP2;7 was found to be singly phosphorylated on Ser273 (peptide at m/z 1217.53), diphosphorylated on Ser273 and Ser276 (peptide at m/z 1297.49), or triphosphorylated on Thr279 in addition to the two Ser residues (peptide at m/z 1377.51) (Fig. 4). Table I summarizes all phosphorylation sites identified in this work. Table I shows that six new phosphorylation sites were identified in the C-terminal tail of aquaporins of Arabidopsis in addition to four previously known phosphorylation sites. This work also allowed the discovery that not only Ser residues but also a Thr residue can be phosphorylated in plant aquaporins.

In theory, by considering all peptide forms, two, three, and four phosphorylation sites should result in peptides with four, eight, and 16 phosphorylation states, respectively. However, a lower number of peptides was observed in AtPIP2;1, AtPIP2;4, and AtPIP2;7, suggesting that phosphorylation events in these proteins might be interdependent. It appeared that phosphorylation of the most distal residues in a C-terminal sequence was only observed in association to phosphorylation of upstream neighboring Ser residue(s). In AtPIP2;1 for instance, Ser283 was never found to be singly phosphorylated, and its phosphorylation was always associated to that of Ser280. Similarly in AtPIP2;7 phosphorylation of Thr279 was linked to that of Ser276, which was itself linked to that of Ser273. In AtPIP2;4, the phosphorylation of the distal residues Ser286 and Ser289 appeared to be linked to phosphorylation of both Ser280 and Ser283.

C-terminal Phosphorylation of AtPIP2;1 Is Quantitatively Modified following Treatments of Plants with NaCl or H2O2—AtPIP2;1 is one of most abundant aquaporins in Arabidopsis root and therefore must significantly contribute to LP, and to

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**Fig. 3.** Phosphopeptide sequencing by MALDI-TOF/TOF of the C-terminal tail of AtPIP2;4. A, MS/MS spectrum of singly phosphorylated 277ALGSFGSFGSRFSFA291 (m/z 1617.55). b2 and y1 ions allowed identification of Ser280 as the phosphorylated residue. B, MS/MS spectrum of the corresponding diphosphorylated peptide (m/z 1697.68). y4, y5, y6, and y7 ions allowed identification of phosphorylated Ser276 and Ser283. C, MS/MS spectrum of the corresponding triphosphorylated peptide (m/z 1777.63) that is a mixture of two forms: b7 ion allowed determination of the phosphorylation of the two residues Ser280 and Ser283; y4, y5, and y6 ions indicated that Ser286 can be phosphorylated; y7bis, y8bis, and y9 ions showed that alternatively Ser289 can carry the third phosphorylation. *, pS; [MH]+, [MH−P]−, [MH−2P−18]+, [MH−2P−2×18]+, and [MH−3P−3×18]+ are as explained in the legend of Fig. 2.

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**Table I** shows that six new phosphorylation sites were identified in the C-terminal tail of aquaporins of Arabidopsis in addition to four previously known phosphorylation sites. This work also allowed the discovery that not only Ser residues but also a Thr residue can be phosphorylated in plant aquaporins.
its regulation. In addition, AtPIP2;1 displays a less complex phosphorylation pattern than other AtPIP2 isoforms. For these reasons, AtPIP2;1 was chosen as a model root aquaporin, and qualitative and/or quantitative changes in its C-terminal phosphorylation status in response to NaCl and H2O2 treatments were investigated. MS/MS sequencing of the singly and diphosphorylated forms (m/z 1188.53 and m/z 1268.54) of AtPIP2;1 in plants exposed to a 2- or a 4-h treatment with 100 mM NaCl revealed that, as in control conditions, the singly phosphorylated residue was Ser273 (supplemental Fig. 1) and that diphosphorylation had occurred on the phosphorylated residues. *, pS, [MH]+, [MH – P]+, [MH – P – 18]+, [MH – 2P – 18]+, and [MH – 2P – 2 × 18]+ are as explained in the legend of Fig. 2. pT, phosphorylated threonine.

The unmodified C-terminal AtPIP2;1 peptide and its singly and diphosphorylated forms were quantified using a strategy adapted from the absolute quantification method (38). For this, we used three reference synthetic peptides that, with respect to endogenous peptides, had incorporated stable isotopes. This labeling induced a mass increment of 10, 10, and 6 Da with respect to the unmodified, singly phosphorylated, and diphosphorylated endogenous peptides, respectively (Fig. 5). The reference peptides were introduced into the peptide digest prior to the purification of phosphopeptides with TiO2 microcolumns. The phosphorylated and unmodified peptides were quantified in the MALDI-labeled MS spectra arising from the elution of the microcolumns and from their flow-through, respectively. More specifically, native peptides were quantified from the ratio of the monoisotopic peak to the isotopically labeled (M+H) peptide and its singly phosphorylated (M+H-P) peptide (Fig. 5) was also observed in these experiments (Fig. 6). By contrast, an H2O2 treatment induced a statistically significant 2-fold decrease in abundance of the unmodified form (Mann and Whitney, p < 0.05) (Fig. 6A). This decrease was accompanied by a slight relative (20%) increase in the abundance of the diphosphorylated form (Fig. 6B).
The Phosphorylation of Ser^{283} Is Involved in the Targeting of AtPIP2;1 to the PM and in Its Intracellular Accumulation upon a NaCl Treatment—The role of specific phosphorylated residues in gating plant aquaporins has been well described (8, 21, 22). By contrast, the role of phosphorylation in the regulation of plant aquaporin trafficking has not yet been investigated. We previously showed that a fusion of AtPIP2;1 with GFP labels the PM of root cells and that an NaCl treatment induces the additional labeling of intracellular structures, suggesting a relocalization mechanism in response to NaCl (12). The finding that an NaCl treatment decreased the phosphorylation of Ser^{283} of AtPIP2;1 prompted us to investigate the role of this modification in the subcellular trafficking of the protein. For this, GFP was fused to the N-terminal tail of AtPIP2;1, either wild type (WT) or carrying Ser to Ala mutations at positions 280 (S280A) or 283 (S283A) or a Ser to Asp mutation at position 283 (S283D). The fusion proteins were expressed in transgenic Arabidopsis, and their expression in epidermal cells at 1 cm from the apex was observed by laser-scanning confocal microscopy. In normal growth conditions, root cells of plants expressing the fusions of GFP with WT-PIP2;1 (GFP-PIP2;1) or the PIP2;1-S280A mutant (GFP-PIP2;1-S280A) showed a labeling pattern consistent with predominant localization of the proteins in the PM (Fig. 7A). By contrast, plants expressing GFP-PIP2;1-S283A showed an intracellular reticulation pattern in 40% of root cells (Fig. 7, A and B). Because of a pronounced localization around the nucleus and its fuzzy aspect throughout the cell, this intracellular staining was partly assigned to endoplasmic reticulum (ER) structures (39). Interestingly plants expressing GFP-PIP2;1-S283D, whereby the introduced mutation is supposed to mimic a constitutive phosphorylation at position 283, displayed a consistent PM staining in root cells.
Altogether these observations indicated that residue Ser^{283}, and very likely its phosphorylation, is necessary for a proper targeting of AtPIP2;1 to the PM.

To investigate the role of C-terminal phosphorylation of AtPIP2;1 in its salt-induced subcellular relocalization, we used the same set of transgenic plants as above. Microscopic observations of root epidermal cells were performed specifically at 1 cm from the apex with an intracellular staining. Data were obtained from transgenic plants that express the following constructs: GFP-PIP2;1 (n = 9 plants from two independent transgenic lines), GFP-PIP2;1-S280A (n = 7 plants from two independent transgenic lines), and GFP-PIP2;1-S283D (n = 6 plants from two independent transgenic lines). The numbers on the graph correspond to the total number of observed cells. The error bars represent S.D.

(Fig. 7, A and B). Altogether these observations indicated that residue Ser^{283}, and very likely its phosphorylation, is necessary for a proper targeting of AtPIP2;1 to the PM.

(Fig. 8, A and B). The figure shows typical laser-scanning confocal micrographs of the fluorescence emitted by root epidermal cells at 1 cm from the apex with an intracellular staining. Data were obtained from transgenic plants that express the following constructs: GFP-PIP2;1 (n = 13 plants from two independent transgenic lines), GFP-PIP2;1-S280A (n = 9 plants from three independent transgenic lines), GFP-PIP2;1-S283A (n = 7 plants from two independent transgenic lines), and GFP-PIP2;1-S283D (n = 6 plants from two independent transgenic lines). The numbers on the graph represent the total number of observed cells. The error bars represent S.D.

(Fig. 8). The figure shows typical laser-scanning confocal micrographs of the fluorescence emitted by root epidermal cells at 1 cm from the apex with an intracellular staining. Data were obtained from transgenic plants that express the following constructs: GFP-PIP2;1 (n = 13 plants from two independent transgenic lines), GFP-PIP2;1-S280A (n = 9 plants from three independent transgenic lines), GFP-PIP2;1-S283A (n = 7 plants from two independent transgenic lines), and GFP-PIP2;1-S283D (n = 6 plants from two independent transgenic lines). The numbers on the graph correspond to the total number of observed cells. The error bars represent S.D.

(Fig. 7). Fig. 7. Role of C-terminal phosphorylation in the subcellular localization of AtPIP2;1. A, the figure shows typical laser-scanning confocal micrographs of the fluorescence emitted by root epidermal cells at 1 cm from the apex with an intracellular staining. Data were obtained from transgenic plants that express the following constructs: GFP-PIP2;1 (n = 13 plants from two independent transgenic lines), GFP-PIP2;1-S280A (n = 9 plants from three independent transgenic lines), GFP-PIP2;1-S283A (n = 7 plants from two independent transgenic lines), and GFP-PIP2;1-S283D (n = 6 plants from two independent transgenic lines). The numbers on the graph correspond to the total number of observed cells. The error bars represent S.D.

(Fig. 8). Fig. 8. Role of C-terminal phosphorylation in the subcellular localization of AtPIP2;1 in response to salinity. The figure shows typical laser-scanning confocal micrographs of the fluorescence emitted by root epidermal cells at 1 cm from the apex with an intracellular staining. Data were obtained from transgenic plants that express the following constructs: GFP-PIP2;1 (n = 13 plants from two independent transgenic lines), GFP-PIP2;1-S280A (n = 9 plants from three independent transgenic lines), GFP-PIP2;1-S283A (n = 7 plants from two independent transgenic lines), and GFP-PIP2;1-S283D (n = 6 plants from two independent transgenic lines). The numbers on the graph correspond to the total number of observed cells. The error bars represent S.D.
cells of plants expressing GFP-PIP2;1 (Figs. 8, A and B, and 9B). Although these bodies might be related to the endosome/prevacuolar compartment (40), their precise nature remains uncertain. Salt-treated plants expressing GFP-PIP2;1-S280A and GFP-PIP2;1-S283D showed the same proportion of cells with these spherical structures (Figs. 8, C, D, G, and H, and 9B). By contrast, this type of labeling was almost not visible in root cells expressing GFP-PIP2;1-S283A (Figs. 8, E and F and 9B). These data suggested that residue Ser283, and very likely its phosphorylated form, is required during the salt-induced relocalization of AtPIP2;1 in intracellular spherical bodies.

**DISCUSSION**

The present work reports an original proteomics strategy to investigate the phosphorylation of AtPIP aquaporins in the Arabidopsis root. Three major steps were involved: (i) an enrichment in aquaporins from purified root PM, (ii) a subsequent enrichment in corresponding phosphopeptides by affinity purification on TiO2 columns, and (iii) the identification of phospho-residues by MALDI/TOF-TOF. The present work was focused on the C-terminal tail of AtPIPs, and overall nine sites were identified in AtPIP2;1 (and/or AtPIP2;2 and AtPIP2;3), AtPIP2;4, and AtPIP2;7. In support for the enhanced resolution of our successive enrichment procedure, we note that six of these sites had not been described previously.

AtPIP2;6 recently has been reported to be phosphorylated (25) but is not expressed in roots (12, 31). The present work also shows that bioinformatics predictions of phosphorylation sites cannot be substituted by an experimental identification of sites by MS. For instance, three Ser residues (Ser273, Ser277, and Ser280) were predicted using NetPhos software to be phosphorylated in the C-terminal tail of AtPIP2;1, whereas the experimental data established the phosphorylation of Ser280 and that of an unpredicted site (Ser283). Similar discrepancies were observed for AtPIP2;4 and AtPIP2;7 (not shown). Our analysis also identified the phosphorylation of a Thr residue in PI2;7, a modification that had never been formally described in aquaporins. A remarkable feature of AtPIPs is the presence of multiple, up to three, adjacent phosphorylations on their C-terminal tail. The amino acid sequence alignment of SoPIP2;1, AtPIP2;1, AtPIP2;4, AtPIP2;6, and AtPIP2;7 revealed a correspondence between the first two adjacent
phosphorylated Ser residues of these proteins (Fig. 10). Whereas independent phosphorylation at n sites yields in theory 2^n peptide forms, we observed a reduced number of phosphorylated forms in AtPIP2;1, AtPIP2;4, and AtPIP2;7. In all three isoforms, phosphorylation of a site was apparently linked to phosphorylation of the closest site, upstream in the peptide sequence, or exceptionally to the second closest site in the case of Ser289 of AtPIP2;4. Interdependent phosphorylation events could result from either a processive or a distributive functioning of the protein kinase along the C-terminal tail (41–43). In the case of PIP2;1, we observed that an S280A mutation did not alter the cellular expression of a GFP-PIP2;1 fusion, whereas an S283A mutation did (Fig. 7). This suggests that phosphorylation of Ser283 can occur in the GFP-PIP2;1-S280A form in the absence of phosphorylation at position 280. Therefore, we favor a distributivity mechanism whereby a similar protein kinase would act on the two sites with a greater efficiency on Ser280. Similar mechanisms may also be present in animal aquaporins because up to four phosphorylated serine residues have been identified in the C-terminal tail of mammalian aquaporin-2, and a putative interdependency was observed between phosphorylation of two of these sites (Ser256 and Ser261) (44).

Although the present study represents a comprehensive analysis of C-terminal phosphorylation of AtPIP2 aquaporins expressed in roots, other aquaporin phosphorylation sites surely remain to be uncovered. AtPIP1 isoforms represent in AtPIP2 abundant proteins in the 28-kDa SDS-PAGE band (31, 33). Although the C-terminal tail of AtPIP1 is not predicted to be phosphorylated, we attempted to detect phosphopeptides derived from this domain. However, the digestion of AtPIP1 isoforms with several different proteolytic enzymes that should release C-terminal AtPIP1 did not allow detection of these domains (data not shown). A phosphorylation site conserved in loop B of all plant PIP isoforms has been postulated based on immunodetection with an anti-phosphopeptide antibody (45) or functional characterization of site-directed mutants in Xenopus oocytes (22). Here again, the corresponding native or phosphorylated peptide could not be detected by MS possibly because of low efficiency of the enzyme digestion, the confinement of these peptides into acrylamide after digestion, or also a low ionization efficiency of these peptides. With recent developments in Fourier transform mass spectrometry and the introduction of dissociation modes other than CID, top-down proteomics may be useful to uncover additional phosphorylation sites (46, 47).

An absolute quantification procedure was developed to quantify the relative abundance of the unmodified, singly, and diphosphorylated forms of AtPIP2;1. Plants growing in normal conditions showed a 1:1:2 relative abundance ratio, indicating that AtPIP2;1 is mainly diphosphorylated in the root PM. We also investigated the effects on AtPIP2;1 phosphorylation of NaCl and H$_2$O$_2$ treatments, two stimuli known to typically induce a rapid inhibition of Lp$_p$ in Arabidopsis (12). In these experiments it was of importance to systematically sequence all singly and diphosphorylated forms of AtPIP2;1. Because no phosphorylation of Ser277 was observed in any of the treatments, our quantitative data can truly be interpreted as reversible changes of phosphorylation at Ser280 and Ser283. NaCl induced a 30% decrease in the level of Ser283 phosphorylation together with a tendency for an increased relative abundance of the singly and unphosphorylated forms (Fig. 6A). By contrast, an H$_2$O$_2$ treatment increased by 20% the relative abundance of the diphosphorylated form and decreased the abundance of the unmodified form (Fig. 6B). Thus, the AtPIP2;1 phosphorylation status appears to be highly sensitive to environmental stimuli acting on root water transport. However, the changes in AtPIP2;1 phosphorylation were not unequivocally associated to changes in Lp$_p$. Because they were of modest amplitude, neither one of these changes may be sufficient to account for the strong decrease in Lp$_p$ induced by the two stimuli. Thus, additional mechanisms including altered phosphorylation of other root aquaporins (AtPIP2;4, AtPIP2;7, or others) or other as yet unidentified regulatory mechanisms may contribute to the decrease in Lp$_p$. Phosphorylation at Ser274 of SoPIP2;1, the spinach homologue of AtPIP2;7, was shown to be decreased in leaves under reduced water potential (hyperosmotic treatment) (22). By contrast, phosphorylation of the corresponding residue in AtPIP2;1 (Ser280) was insensitive to NaCl treatment, whereas phosphorylation of Ser283 was decreased. In nitrogen-fixing nodules of soybean roots, phosphorylation of the aquaporin Nodulin-26 on a C-terminal serine residue (Ser262) was enhanced upon a water stress (21). These different observations may be explained by differences in the aquaporin isoform and the tissue considered. Recent results on mammalian aquaporin-2 have also revealed reciprocal changes in phosphorylation of two C-terminal serine residues (Ser256 and Ser261) in response to vasopressin exposure, suggesting that these residues may serve distinct roles in aquaporin-2 regulation (44, 48). Overall these different studies point to a critical role for aquaporin phosphorylation in response to various physiological contexts and emphasize the need for a global view of aquaporin.
phosphorylation dynamics. In these respects, the present study justifies the development of novel, more comprehensive MS-based strategies based on multiple reaction monitoring and/or stable isotope labeling (14).

Functional and structural analyses in spinach SoPIP2;1 have indicated a role for Ser\(^{274}\) in gating the aquaporin (8, 22). By contrast, a possible role for this or the equivalent site in AtPIP2;1 (Ser\(^{280}\)) in controlling aquaporin trafficking has remained unexplored. In addition, the functional significance of the adjacent phosphorylation site (Ser\(^{283}\) in AtPIP2;1) has remained totally unknown. Here we found that this phosphosite was specifically involved in the response of Arabidopsis roots to NaCl. Therefore, we focused on the role of the two sites (Ser\(^{280}\) and Ser\(^{283}\)) in AtPIP2;1 trafficking under normal or NaCl stress conditions. For this, we expressed in transgenic Arabidopsis GFP-PIP2;1 fusions carrying Ser to Ala mutations to abolish phosphorylation or Ser to Asp mutations to possibly mimic a constitutive phosphorylation. A S280A mutation did not affect the localization profile of the fusion protein when compared with that of wild type GFP-PIP2;1 (Fig. 7). By contrast, an S283A but not an S283D mutation prevented a proper transfer of the protein at the PM (Fig. 7). These results allow us to exclude a detrimental effect of Ser\(^{283}\) removal and rather indicate that phosphorylation of this residue, but not of Ser\(^{280}\), is necessary for the subcellular trafficking of AtPIP2;1. The perinuclear staining displayed by GFP-PIP2;1-S283A suggests an accumulation in the ER. Therefore, phosphorylation of AtPIP2;1 at Ser\(^{283}\) seems to favor export of the protein from the ER. A similar model was proposed for mammalian aquaporin-2 whereby phosphorylation of Ser\(^{256}\) by two distinct protein kinases, with different subcellular localizations, mediates the exit of the aquaporin from the Golgi complex and subsequently its translocation from vesicular compartments to the PM (28).

One of the marked effects of an NaCl treatment was to exacerbate the staining by PIP2;1-GFP of diffuse intracellular structures. These intracellular structures were similar to those stained by GFP-PIP2;1-S283A in resting conditions. Mutant analysis showed in addition that the NaCl effects were less pronounced specifically in GFP-PIP2;1-S283D and therefore may be counteracted by phosphorylation of Ser\(^{283}\) (Fig. 9A). These results suggest that NaCl acts on AtPIP2;1 with unphosphorylated Ser\(^{283}\) to favor its intracellular accumulation. However, we cannot distinguish at present between (i) an intracellular retention or a misrouting of neosynthesized proteins on their route to the PM and (ii) a relocalization of proteins from the PM into intracellular compartments. NaCl also induced the labeling of intracellular spherical bodies in transgenic plants expressing GFP-PIP2;1. The precise nature of these structures remains to be elucidated, but due to their size (1–5 \(\mu\)m) and spherical form, they may be related to the prevacuolar/multivesicular body compartment\(^2\) and devoted to protein degradation. GFP-PIP2;1-S280A and GFP-PIP2;1-S283D, but not GFP-PIP2;1-S283A, labeled the spherical bodies in NaCl-treated roots (Fig. 9B) suggesting that phosphorylation at position 283 was required for relocalization of AtPIP2;1 in this compartment. In summary, an NaCl treatment induced an intracellular accumulation of AtPIP2;1 by exerting specific actions onto AtPIP2;1 forms differing in their phosphorylation at Ser\(^{283}\) to induce their accumulation in distinct intracellular structures. It is noteworthy that NaCl also induced dephosphorylation of Ser\(^{283}\) as was observed on a purified PM fraction. This may reflect a compensatory mechanism to prevent the relocalization of the phosphorylated AtPIP2;1 in spherical bodies and thus to slow down its degradation. Therefore, a fine and reversible tuning of aquaporin density at the cell surface may be achieved.

In conclusion, aquaporin phosphorylation appears to be a significant target in plants under stress. The present study documents salt-induced quantitative changes in aquaporin phosphorylation and establishes, for the first time, a link with aquaporin subcellular localization. Similar links will have to be investigated in contexts such as stress or nutrient responses where information on aquaporin phosphorylation or trafficking has recently emerged (25).\(^2\)

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