Salinity and Hyperosmotic Stress Induce Rapid Increases in Phosphatidylinositol 4,5-Bisphosphate, Diacylglycerol Pyrophosphate, and Phosphatidylcholine in Arabidopsis thaliana Cells*

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In animal cells, phosphoinositides are key components of the inositol 1,4,5-trisphosphate/diacylglycerol-based signaling pathway, but also have many other cellular functions. These lipids are also believed to fulfill similar functions in plant cells, although many details concerning the components of a plant phosphoinositide system, and their regulation are still missing. Only recently have the different phosphoinositide isomers been unambiguously identified in plant cells. Another problem that hinders the study of the function of phosphoinositides and their derivatives, as well as the regulation of their metabolism, in plant cells is the need for a homogenous, easily obtainable material, from which the extraction and purification of phospholipids is relatively easy and quantitatively reproducible. We present here a thorough characterization of the phospholipids purified from [32P]orthophosphate- and myo-[2-3H]inositol-radiolabeled Arabidopsis thaliana suspension-cultured cells. We then show that NaCl treatment induces dramatic increases in the levels of phosphatidylinositol 4,5-bisphosphate and diacylglycerol pyrophosphate and also affects the turnover of phosphatidylcholine. The increase in phosphatidylinositol 4,5-bisphosphate was also observed with a non-ionic hyperosmotic shock. In contrast, the increase in diacylglycerol pyrophosphate and the turnover of phosphatidylcholine were relatively specific to salt treatments as only minor changes in the metabolism of these two phospholipids were detected when the cells were treated with sorbitol instead of NaCl.

Phosphoinositides are quantitatively minor phospholipids that play an important role in the transduction of physiological signals, such as hormones, growth factors, and neurotransmitters in animal cells (1). One of the key early events triggered by these physiological stimuli is the activation of phosphoinositide-specific phospholipase C (PI-PLC), resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) to the two second messengers, inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol, which induce Ca2+ release from internal stores and stimulate protein kinase C, respectively (1, 2). During the last decade, it has become evident that in addition to serving as precursors to Ins(1,4,5)P3 and diacylglycerol, phosphoinositides actively participate in other cellular functions: they have been shown to regulate the dynamics of the actin cytoskeleton through the interaction with actin-binding proteins (3, 4), and to potentiate the activation of protein kinase C (5) and PI-PLC (6, 7). In addition, phosphoinositides phosphorylated at the D3-hydroxy group of the inositol headgroup are required for specific vesicle trafficking steps (8, 9) and are able to activate the recently identified novel protein kinases Akt/PKB and phosphoinositide-dependent kinases (10). Recently, a new 3-phosphorylated phosphoinositide, phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P2), was identified and shown to accumulate in yeast cells subjected to hyperosmotic or NaCl stress (11).

Contrasting with the very detailed picture available for the components of the phosphoinositide system, and their function and regulation in animal cells, it is still not clear for plant cells whether any physiological factors act by stimulating PI-PLC-catalyzed PtdIns(4,5)P2 hydrolysis. However, it has been demonstrated that micro-injected “caged” Ins(1,4,5)P3 can release Ca2+ from internal stores (12) and is also able to trigger stomatal closure (13, 14). There is also evidence that phosphoinositides may participate in the regulation of cytoskeletal structures in plant cells (15, 16). A number of reports also suggest that a wide range of signals, such as light, hormones, and stress, may mediate their effect through phosphoinositide metabolism (17), although the effects reported were often very limited and the identity of the lipid or inositol phosphate species affected not always clearly demonstrated. Interestingly,

1 The abbreviations used are: PI-PLC, phosphoinositide-specific phospholipase C; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)P3, inositol 1,4,5-trisphosphate; PtdIns(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PtdIns(5)P, phosphatidylinositol 5-phosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PtdIns, phosphatidylinositol; PtdInsP, phosphatidylinositol monophosphate; DGPP, diacylglycerol pyrophosphate; PtdCho, phosphatidylcholine; [32P]orthophosphate; [2-3H]inositol; glycerophosphoinositol 4-phosphate; PLC, phospholipase C; GroPInsP, glycerophosphoinositol 4-phosphate; PLC, phospholipase C; GroPInsP, glycerophosphoinositol 4-phosphate; TSL, thin-layer chromatography; GroP, glycerophosphate; PtdInsP, phosphatidylinositol bisphosphate; GroPInsP, glycerophosphoinositol 3-phosphate; GroPIns(4)P, glycerophosphoinositol 4-phosphate; GroPInsP, glycerophosphoinositol 4-phosphate; PtdIns(3,4)P2, glycerophosphoinositol 5,4-bisphosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdOH, phosphatidic acid; PtdGro, phosphatidylglycerol; PtdInsP, phosphatidylinositol 3,4-bisphosphate.
hyperosmotic stress was recently shown to induce an increase in PtdIns(3,5)P₂ synthesis in Chlamydomonas moewusii and in some higher plant cells (18).

With the exception of phosphatidylinositol 5-phosphate (PtdIns(5)P) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃), all the phosphoinositide isomers found in animal and yeast cells have also been identified in plant cells (11, 18–21). cDNA clones representing functional enzymes necessary for the synthesis of these phospholipids, phosphatidylinositol (PtdIns) 4-kinase (22), phosphatidylinositol monophosphate (PtdInsP) kinase (23), PtdIns 3-kinase (24), have also been characterized in plants. Several PI-PLC isoforms have been cloned from plant species (25–28).

Salinity is a common and detrimental environmental stress imposed on land plants (29). Salt tolerance is mediated by multiple determinants (30), a notion reflected in the observation that the expression of numerous genes is altered in plants subjected to high salt conditions (31). Knowledge of the mechanisms by which plants perceive and transduce salinity stress should provide crucial information to successfully improve salt tolerance in plants through genetic engineering. In Arabidopsis thaliana, the expression of one PtdInsP kinase gene (23) and one PI-PLC gene (25) is increased by salt stress, suggesting that phosphoinositides may participate in the response to high salinity. There is also evidence that cytotoxic Ca²⁺ is a likely component of the transduction cascade (32–34). Here we present results clearly showing that increased synthesis of PtdIns(4,5)P₂ and diacylglycerol pyrophosphate (DGPP) and also increased turnover of phosphatidylicholine (PtdCho), may be involved in the response to high salinity in plant cells.

**EXPERIMENTAL PROCEDURES**

**Plant Material and Growth Conditions—** A. thaliana (ecotype Fi-3) cells were grown in Murashige and Skoog (MS) medium supplemented with myo-[2-³²P]inositol for 5 days, and subcultured for another 2 days in the same growth medium. Cells were then collected and resuspended to an A₆0₀₀ = 1.0 and processed further as indicated above for ³²P labeling.

**Chemicals—** ³²P-Oxophosphate (³²P-P), myo-[2-³²P]inositol, and [methyl-³²P]Choline chloride were purchased from Amersham Pharmacia Biotech. All cell culture chemicals were of cell culture grade and purchased from Sigma. Standard lipids were from Sigma. Monomethylamine (40% aqueous solution) was from Fluka. Standard [³¹H]glycerophosphate (³¹H[Gro]Ph₃), and [³¹H]inositol-radiolabeled yeast cells as described in Ref. 11.

**³²P Labeling and Cell Treatment—** Cells were collected 2 days after sub-culture, filtered through a metal mesh and washed with phosphate-free MS medium. They were resuspended to an A₆0₀₀ = 1.0 and incubated at 26 °C and 130 rpm for at least 15 min before addition of ³²P-P (0.1–0.2 μCi/ml). The cells were further incubated in the same conditions for 90 min, or the specified time when indicated otherwise, and then treated with NaCl or sorbitol for 15 min or as otherwise indicated. The treatment was terminated by addition of two volumes of ice-cold methanol, vortexing, and freezing in liquid nitrogen.

**Myo-[2-³²P]inositol Labeling—** Cells were grown in inositol-free MS medium supplemented with myo-[2-³²H]inositol for 5 days, and subcultured for another 2 days in the same growth medium. Cells were then collected and resuspended to an A₆0₀₀ = 1.0 and processed further as indicated above for ³²P labeling.

**Enzymes—**

**Sucrose Phosphate Synthase (SuPSase) and Phosphoglucomutase—** Enzymes were purified from S. cerevisiae (35). The enzymes were dialyzed against a buffer containing 100 mM Mops, pH 7.5, 0.1% NaN₃, and 1 mM dithiothreitol.

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**RESULTS**

**Separation of ³²P-Radiolabeled Phospholipids—** The pattern of ³²P-radiolabeled phospholipids extracted from suspension-cultured A. thaliana cells and separated by one-dimensional thin-layer chromatography (TLC) or HPLC is shown in Fig. 1.
TLC is presented in Fig. 1. In order to identify some of the radiolabeled phospholipids, non-radiolabeled commercial phospholipids were mixed with the purified radiolabeled samples, prior to TLC separation. As shown in Fig. 1, the addition of excess non-radiolabeled lipids affected the migration of the corresponding radiolabeled lipids. In this way, and by comparison to the TLC profiles obtained with other plant species (37), the different radiolabeled phospholipids were tentatively identified as indicated in Fig. 1. The TLC alkaline solvent system used here resolved two phosphorylalanine isomers, PtdIns(3,5)P2 and PtdIns(4,5)P2, obtained from 32P-radiolabeled yeast cells (data not shown), and has previously been shown to separate phosphorylalanine-3,4-bisphosphate (PtdIns(3,4,5)P2) from PtdIns(4,5)P2 (20), as well as PtdIns(4,5)P2 from PtdIns(3,5)P2 (18) from plants. However, it does not permit a clear separation of the different PtdInsP isomers (see, e.g., Ref. 38).

Identification of [32P]- and [3H]inositol-radiolabeled Phospholipids—In order to confirm the identity of the different 32P-radiolabeled phospholipids we analyzed their deacylation products by chromatography on a strong anion-exchange HPLC column. Fig. 2A shows that two glycerophosphoinositol mono-phosphate isomers, [3H]glycerophosphoinositol-3-phosphate ([3H]GroPIns(3)P) and [3H]glycerophosphoinositol-4-phosphate ([3H]GroPIns(4)P), and two glycerophosphoinositol bisphosphate isomers, [3H]glycerophosphoinositol-3,5-bisphosphate ([3H]GroPIns(3,5)P2) and [3H]glycerophosphoinositol-4,5-bisphosphate ([3H]GroPIns(4,5)P2), purified from myo-[2-3H]inositol-radiolabeled yeast cells, as described (11), were well resolved from each other on the Partisphere SAX column.

The profile obtained with the water-soluble fraction after deacylation of phospholipids extracted from 32P-radiolabeled A. thaliana cells comprised peaks eluting from the column exactly as authentic [3H]GroPIns(3)P, [3H]GroPIns(4)P, and [3H]GroPIns(4,5)P2, respectively (Fig. 2A). [32P]GroPIns(4,5)P2 represented approximately 90% of the total [32P]GroPInsP, which closely agrees with the levels reported for Spirodela polyrhiza (39, 40), Conmelina communis (19), and Chlamydomonas eugametos (20). The identity of the A. thaliana derived, 32P-radiolabeled peaks that did not co-chromatograph with any of the standard [3H]GroPInsP was deduced from similar profiles published for other plant systems (39, 41, 42) and from the myo-[2-3H]inositol labeling profile (Fig. 2C), which showed that two peaks detected with 32P-labelling did not incorporate myo-[2-3H]inositol. The 32P-radiolabeled bands tentatively identified as PtdIns(4,5)P2, PtdInsP, and DGPP in Fig. 1 were individually scraped from a TLC plate, deacylated, and the resulting water-soluble products chromatographed individually on the Partisphere SAX column (Fig. 2B). Approximately 95% of the radioactivity present in each of the PtdIns(4,5)P2 and PtdInsP bands was recovered in the water-soluble phase after deacylation, demonstrating that these lipids did not contain significant amounts of phosphoglycerolipids, some of which have been shown to co-chromatograph with PtdInsP as TLC (43, 44). The water-soluble product obtained from the deacylation of the putative [32P]PtdIns(4,5)P2 band eluted from the column as a single peak at the same position as yeast [3H]GroPIns(4,5)P2 (Fig. 2B). The deacylation product obtained from the PtdInsP band contained two radiolabeled compounds, which behaved like yeast [3H]GroPIns(3)P and [3H]GroPIns(4)P, respectively (Fig. 2B). The [32P]phosphatidylinositol 4-phosphate ([32P]PtdIns(4)P)/[32P]phosphatidylinositol 3-phosphate ([32P]PtdIns(3)P) ratio calculated from the deacylation product of the PtdInsP TLC band was similar to that deduced from the HPLC profile obtained from total lipid deacylates (Fig. 2A). Finally, the deacylation product of the putative [32P]DGPP band eluted as a single peak at 76 ml (Fig. 2B), matching exactly the elution of one of the products present in the total 32P-labeled lipid deacylate (see Fig. 2A). Mild acid hydrolysis of fraction 76 produced two radioactive compounds, which, on polyethyleneimine-cellulose TLC plates, chromatographed exactly as authentic GroP and P (data not shown). This confirmed that the original lipid putatively identified as DGPP indeed was DGPP.

We also examined the incorporation of myo-[2-3H]inositol into lipids of A. thaliana cells (Fig. 2C). This demonstrated that the three 32P-radiolabeled GroPInsP isomers identified in Fig. 2A as representing [32P]GroPIns(3)P, [32P]GroPIns(4)P, and [32P]GroPIns(4,5)P2 also incorporated myo-[2-3H]inositol, and that the other peaks observed in Fig. 2A, except for [32P]GroPIns, were absent in the total lipid deacylate from myo-[2-3H]inositol-radiolabeled cells. The [3H]GroPIns(3)P/[3H]GroPIns(3)P ratio was similar to the one determined for 32P-radiolabeled samples.

PtdIns(4,5)P2 was the only PtdInsP2 isomer we could detect in both 32P and myo-[2-3H]inositol-radiolabeled A. thaliana cells, under the conditions tested. In addition, we were unable to detect any PtdIns(3,4,5)P3, an isomer that has so far never been detected in plant cells, although the amount of radioactive incorporated into polyphosphoinositides would probably not have allowed us to detect this compound as in animal cells levels of PtdIns(3,4,5)P never exceed 0.1% of the radioactivity in PtdIns(4)P and PtdIns(4,5)P2 even under stimulated conditions.

Time Course of 32P Incorporation into A. thaliana Phospholipids—The incorporation of 32P into most phospholipids was detectable after a few minutes of labeling (Fig. 3). However, the radiolabeling of the quantitatively minor lipids (i.e., the polyphosphoinositides, DGPP, and phosphatidic acid (PtdOH)) reached a maximum much earlier than for the other phospholipids. For PtdInsP, PtdIns(4,5)P2, PtdOH, and DGPP, a maximum was reached between 15 and 30 min, while the levels of [3P]phosphatidyglycerol ([3P]PtdGro), [3P]phosphatidylethanolamine, [3P]PtdIns, and some additional unidentified lipids continued to increase well beyond the time scale of the experiment. This obviously reflects the fact that the quantitatively minor lipids all possess a monoester phosphate group, which approaches isotopic equilibrium with the added 32P, much more rapidly than the diester phosphate group present in the other phospholipids because of the much higher rate of turnover of phosphomonoesters. Since under these conditions (i.e., short term radiolabeling) the monoester phosphates will be at isotopic equilibrium with the 32P, and the diester phosphates of these compounds will scarcely be radiolabeled, any change in radioactivity can be interpreted as a change in mass for the polyphosphoinositides and DGPP, as long as the monoester phosphates of these compounds will have reached isotopic equilibrium. This is most commonly demonstrated by extracting a fixed mass of cells at different times during a short term radiolabeling regime and extracting the lipids and quantifying the amount of 32P associated with each lipid, as in Fig. 3. When the level of 32P in a given compound reaches a plateau the monoester phosphate of that compound has attained isotopic equilibrium. Fig. 3 shows that the 32P incorporation into the quantitatively minor phospholipids reached equilibrium after 30 min of labeling with 32P. However, depending on the experiment, it was sometimes necessary to label the cells for 90 min in order to reach a stable and reproducible background 32P incorporation into the phospholipids, as shown, for example in Fig. 1. Therefore, the cells were routinely radiolabeled with 32P, for 90 min before lipids were extracted or before application of any treatments. As previously observed with plant cells, the
FIG. 2. HPLC analysis of the water-soluble products after deacylation of lipids from $^{32}$P-radiolabeled A. thaliana cells. A. thaliana cells were $^{32}$P-radiolabeled as indicated in Fig. 1 (A and B), and the extracted lipids were deacylated. A., the water-soluble products were mixed with $[^3H]$GroPInsP, of known structure (purified from $[^3H]$inositol-radiolabeled yeast), and separated on a Partisphere SAX column. ---, $^3H$ incorporation (dpm); ●, $^{32}$P incorporation (dpm). B., the bands corresponding to lipids tentatively identified as PtdIns(4,5)P$_2$, PtdInsP, and DGPP were scraped individually from a TLC plate, de-acylated and the corresponding water-soluble products analyzed by HPLC as in (A); ●, PtdIns(4,5)P$_2$; ■, PtdInsP; □, DGPP. C, A. thaliana cells were cultured in full MS medium in the presence of myo-[2-$^3H$]inositol and their lipids extracted and analyzed by HPLC as in A. All peaks are identified as the lipids they are derived from.
steady-state level of PtdIns(4,5)P₂ was much lower than that of PtdInsP (see, e.g., Fig. 1) (17, 38).

Effect of Salt and Hyperosmotic Stresses on Phospholipids—NaCl (0.4 M) induced large increases in the steady-state levels of several ³²P-radiolabeled phospholipids, namely PtdIns(4,5)P₂ and DGPP. The levels of radioactivity also increased in an additional phospholipid, which, on TLC, chromatographed exactly like authentic soybean PtdCho (Fig. 4). To confirm that this lipid indeed was PtdCho, we briefly radiolabeled cells with [methyl-¹⁴C]choline. As shown in Fig. 4D, this lipid incorporated choline, and no other lipid appeared to be radiolabeled under these conditions. The increased incorporation of ³²P-radiolabel into PtdCho is suggestive of an increase in turnover of this lipid, but since the diester phosphate of PtdCho is nowhere near isotopic equilibrium, the increase in radioactivity does not allow any conclusions about changes in the steady-state levels of this lipid to be drawn from data gathered under this experimental regime. The accumulation of [³²P]PtdIns(4,5)P₂ was consistently detectable within minutes of NaCl addition, and the levels rose 8–25-fold after 10–20 min (Figs. 4B and 5). The effect of NaCl on the incorporation of radiolabel into PtdCho was very similar whether cells were radiolabeled briefly with ³²P or [methyl-¹⁴C]choline (Fig. 4, A and D). The steady-state levels of PtdIns(4,5)P₂ remained high for at least 1 h, and returned to levels close to those of control cells after 2–6 h (Figs. 4B and 7). In contrast, no decrease in [³²P]PtdCho was observed 6 h following salt stress (Fig. 4B). After a lag phase of a few minutes, during which [³²P]DGPP was not affected by the salt treatment, it reached concentrations 10–40 times higher than in control cells after 15 min, and continued to rise slowly for at least 1 h (Fig. 4B, 6, and 7). Thereafter, DGPP remained elevated between 1 and 2 h of treatment and started to decrease significantly after 6 h (Fig. 4, A and B). Of the other phospholipids, only PtdInsPs were affected by the salinity treatment, whereas PtdIns and PtdOH showed no alterations (Fig. 4, A and C). However, we consistently detected a sharp drop in PtdOH levels, by approximately 50%, during the first few minutes following the addition of NaCl (Fig. 4, A and C). By 15 min, PtdOH concentration had returned to its value before addition of salt. HPLC analyses of [³H]inositol-radiolabeled lipids revealed that of the two PtdInsP isomers, PtdIns(4)P was the one that responded to the salinity stress (Fig. 6). Simultaneously to the large increase in PtdIns(4,5)P₂, PtdIns(4)P decreased by almost 50%, while PtdIns(3)P was unaffected. This decrease in the levels of PtdInsP is exactly what would be predicted to occur if activation of PtdIns(4)P 5-kinase(s) preceded activation of the plant PtdIns 4-kinase(s). To determine whether the accumulation of PtdIns(4,5)P₂, DGPP, and PtdOH induced by the salt treatment was solely due to the hyperosmotic shock imposed, or whether it also reflected a NaCl-specific response, we analyzed the effect of sorbitol at a concentration equivalent to the osmolarity produced by the NaCl concentration used. Figs. 6 and 7 show that sorbitol (0.8 M) had similar effects on PtdIns(4,5)P₂ and DGPP to those described above for NaCl; however, these effects were not as pronounced as with NaCl.

![Figure 3](image-url)
especially for DGPP. Interestingly, sorbitol had almost no effect on the incorporation of $^{32}$Pi into PtdCho (Fig. 7).

**DISCUSSION**

The strongest evidence that a phosphoinositide/Ca$^{2+}$ signal transduction system operates in plant cells has been provided by the identification of homologues of the components of the classical animal phosphoinositide system, such as phosphoinositide-metabolizing enzymes (22, 23, 25, 26, 28) and inositol lipids themselves (19, 21, 38, 45, 46). In the present study, we have identified phosphoinositides as well as other phospholipids in suspension-cultured *A. thaliana* cells, and provide evidence that some of these lipids may play an important function in the response to salt stress, and hyperosmotic stress.

PtdIns(4)P was the major polyphosphoinositide in *A. thaliana* cells, representing at least 90% of the total polyphosphoinositides, while PtdIns(4,5)P$_2$ was present in low amounts, in unperturbed cells. However, PtdIns was by far the most abundant inositol lipid in these cells. Such a distribution has been observed in several other plant systems (see, e.g., Refs. 19 and 38). We were not able to detect PtdIns(5)P, PtdIns(3,4)P$_2$, PtdIns(3,5)P$_2$ or PtdIns(3,4,5)P$_3$. PtdIns(3,4)P$_2$ (19–21) and PtdIns(3,5)P$_2$ (11, 18) have been detected in plant cells. In *C. communis*, the main PtdInsP$_2$ isomer in guard cells has been identified as PtdIns(3,4)P$_2$ (19), whereas in mesophyll protoplasts, PtdIns(4,5)P$_2$ was readily detectable but the presence of PtdIns(3,4)P$_2$ could not be confirmed (41). In *A. thaliana* cells, PtdIns(3,5)P$_2$ and PtdIns(3,4)P$_3$, if present, represent very minor phosphoinositides. This suggests that in plants, the distribution of the different phosphoinositide isomers varies greatly between different species and/or cell types. This could reflect cell-specific functions of some of the phosphoinositides. It is

**FIG. 4.** Effects of NaCl on phospholipids extracted from $^{32}$P$_{i}$-radiolabeled or [methyl-$^{14}$C]choline-radiolabeled *A. thaliana* cells. Two-day-old *A. thaliana* cells were incubated with phosphate-free MS medium in the presence of $^{32}$P$_{i}$ (0.1–0.2 mCi/ml) (A–D), or [methyl-$^{14}$C]choline chloride (3.33 mCi/ml) (D) for 90 min. MS or NaCl (final concentration 0.4 M) was added to the cells. 0.3–1-ml aliquots were removed at the indicated times, and the lipids were extracted and analyzed by TLC with the alkaline solvent system, as described under “Experimental Procedures.” The radiolabeled phospholipids were visualized with a PhosphorImage. The incorporation into the different phospholipids was quantified from the phosphorimage. A, phosphorimage (upper panel). An excess of authentic PtdCho was added to one of the samples and visualized with iodine vapors (lower panel). B and C, graphic representation. ●, PtdIns(4,5)P$_2$; □, PtdInsP; ▲, PtdOH; ○, PtdIns; △, DGPP; Δ, PtdCho. D, after labeling with $^{32}$P$_{i}$ or [methyl-$^{14}$C]choline, cells were treated with MS or NaCl (0.4 M) for 15 min before their lipids were extracted and analyzed by TLC. The data presented are representative of five independent experiments.
also possible that the level of some of these lipids is very low in unperturbed cells but is specifically increased under certain conditions, as shown here for PtdIns(4,5)P2. For example, PtdIns(3,5)P2, present in very low amounts in non-stimulated yeast and plant cells, was recently shown to be specifically induced by challenging these cells with hyperosmotic solutions (11, 18).

Unstimulated A. thaliana cells also contained low levels of DGPP. High levels of a non-identified lipid were detected in 32P-radiolabeled protoplasts from C. communis (41). This lipid probably represented DGPP, since it eluted from a Partisphere SAX column at a position very similar to the lipid we identified as DGPP in A. thaliana cells. The high levels detected in C. communis protoplasts were probably due to the high osmolarity (0.45 M mannitol, 20 mM KCl) conditions, and possibly also the electroporation, used to label the protoplasts.

Osmotic stress can affect phosphoinositide metabolism in plant cells, but so far most reports have not shown dramatic alterations in the turnover of any phosphoinositide isomer. For example, Cho et al. (47) did not see any effect of hyperosmotic shock on PtdInsP2 levels in carrot cells grown in suspension culture, but observed a 25% decrease in PtdInsP levels. The unicellular green algae Dunaliella salina responded to hyperosmotic shock by increasing its PtdInsP2 content by approximately 30% after 10 min of treatment (48). As mentioned earlier, hyperosmotic stress was very recently shown to stimulate the turnover of PtdIns(3,5)P2 in C. moewusii and higher plant cells (18). This response was maximal at 150–200 mM NaCl. At higher concentrations, no effect on PtdIns(3,5)P2 levels were observed (18). The turnover of PtdIns(4,5)P2 in C. moewusii, tomato, pea, and alfalfa cells was not affected by 150 mM NaCl (18). This is in contrast to the more dramatic effects on PtdIns(4,5)P2 we observed for A. thaliana cells. PtdIns(4,5)P2 levels increased by 8–25 times in A. thaliana cells subjected to hyperosmotic stress. The increase in PtdIns(4,5)P2 did not depend on the chemical nature of the osmotic agent used; sorbitol and NaCl had the same effect, indicating that this response was due to hyperosmolarity. Such rapid and large effects on PtdIns(4,5)P2 have not been identified in plants previously, and are indicative of a possible in-
involved the classical (4,5)P₂-inositol pathway in response to hyperosmotic stress in plants. In preliminary experiments, in which the effect of increasing NaCl concentrations was tested, an increase in PtdIns(4,5)P₂ level was detected already at 50 mM NaCl (data not shown); the increase was, however, more pronounced at 400 mM. Clearly, the effect of hyperosmotic stress on phosphoinositide turnover varies significantly between plant species.

The expression of a PtdInsP kinase gene can be induced by salt stress (23). However, this induction was observed only 1 h after salt stress. The 8–25-fold increase in PtdIns(4,5)P₂ occurs within minutes and is therefore almost certainly due to the activation of endogenous PtdIns 4-kinase and PtdIns(4)P 5-kinase activities rather than to newly synthesized enzymes. On the other hand, the PI-PLC isoform found to be overexpressed under salt stress (25) may well contribute to the re-adjustment of PtdIns(4,5)P₂ levels seen after 20 min of salt treatment. This hydrolysis of excess PtdIns(4,5)P₂ could also represent an initial step in a cascade leading ultimately to growth adjustment.

It is possible that during the early period of increasing accumulation of PtdIns(4,5)P₂, some of this lipid is hydrolyzed to Ins(1,4,5)P₃ and diacylglycerol, which could be involved in the response to hyperosmolarity/salinity stress. For example, it was recently shown that cytosolic free calcium concentration in whole A. thaliana seedlings increased following a hyperosmotic/salt shock (34). This increase in cytosolic calcium was inhibited by U73122, an inhibitor of PI-PLC-dependent processes in animal cells, thus suggesting that PI-PLC may be involved in the early response to hyperosmotic/salt stresses. Alternatively, PtdIns(4,5)P₂ may participate in salinity signaling in plant cells through association with actin-binding proteins and re-organization of the cytoskeleton. PtdIns(4,5)P₂ may also trigger its effects by regulating various enzymes or cellular functions such as vesicle trafficking (8), or ion channels (49), an area that is virtually unexplored in plant systems.

NaCl-specific alterations in the levels of some lipids were also observed. DGPP also accumulated upon non-saline and NaCl stresses, but salinity induced significantly stronger responses. An increase in DGPP can also be triggered by low concentrations of mastoparan (37). However, the function of this lipid is at present unknown. Since PtdOH is the direct precursor of DGPP, and we did not detect any decrease in PtdOH, PtdOH production is probably stimulated during salt stress, for example from the PI-PLC-catalyzed hydrolysis of PtdIns(4,5)P₂ or through direct DAG kinase stimulation.

In response to salinity, osmotic stress, and other stresses, some plants, e.g. spinach, barley, and sugar beet, synthesize and accumulate non-toxic solutes, such as glycine betaine, proline, and various sugars, thus maintaining turgor and the driving force for water uptake (50). Glycine betaine is synthesized from choline in a two-step reaction (51). The free choline used for the synthesis of glycine betaine is believed to be derived from PtdCho or phosphocholine, although it has been shown that free choline is almost exclusively used for PtdCho synthesis (52). It is possible that salinity stimulates the synthesis of a specific pool of PtdCho that is used more efficiently for the synthesis of glycine betaine.

In conclusion, our results show that, in A. thaliana suspension-cultured cells, salinity induces quick and dramatic increases in several phospholipids, PtdIns(4,5)P₂, DGPP, and, to a lesser extent, in the turnover of PtdCho, and that the alterations in DGPP and PtdCho metabolism are, at least in part, specific to salinity, whereas the PtdIns(4,5)P₂ increase is truly a response to hypertonicity and not merely to increases in sodium or chloride. The elucidation of the downstream targets of these lipids should help understand the molecular mechanisms of salinity and hopefully direct us toward possible strategies to alleviate its detrimental effects on plant growth. With the recent findings that depending on plant species, PtdIns(3,5)P₂ (18) or PtdIns(4,5)P₂ (this work) synthesis is affected by hyperosmotic stress, and that distinct phosphoinositide distributions exist between cell types (19, 41), it seems now obvious that the phosphoinositide system in plants is as complex as in animal and yeast cells. These differences may reflect cell-specific functions of phosphoinositides, in addition to species differences.

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REFERENCES
1. Berridge, M. J. (1993) Nature 361, 315–325
2. Berridge, M. J., and Irvine, R. F. (1989) Nature 341, 197–205
3. Lassing, I., and Lindberg, U. (1985) Nature 314, 472–474
4. Janney, P. A., and Stossel, T. P. (1987) Nature 325, 362–364
5. Oh, E. S., Woods, A., Lim, S. T., Theibert, A. W., and Couchman, J. R. (1998) J. Biol. Chem. 273, 10624–10629
6. Falasca, M., Logon, S. K., Lehto, V. P., Baccante, G., Lemmon, M. A., and Schlesinger, J. (1998) EMBO J. 17, 414–422
7. Bae, Y. S., Cantley, L. G., Chen, C. S., Kim, S. R., Kwon, S. R., and Suh, S. G. (1998) J. Biol. Chem. 273, 4465–4469
8. De Camilli, P., Rmr, S. D., McPherson, P. S., and Novick, P. (1996) Science 271,
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9. Wurmsner, A. E., Gary, J. D., and Emr, S. D. (1999) J. Biol. Chem. 274, 9129–9132
10. Wyman, M. P., and Pirolo, L. (1998) Biochim. Biophys. Acta 1436, 127–150
11. Deve, S. K., Cooke, F. T., Douglas, M., Sayers, L., Parker, P. J., and Michell, R. H. (1997) Nature 380, 187–192
12. Alexandre, J., Lassalles, J. F., and Kado, R. D. (1990) Nature 342, 57–59
13. Blatt, M. F., Thiel, G., and Trentham, D. R. (1990) Nature 346, 766–769
14. Gilroy, S., Read, N. D., and Trewavas, A. J. (1990) Nature 346, 769–771
15. Yang, W., Burkhardt, W., Cavallius, J., Merrick, W. C., and Boss, W. F. (1993) J. Biol. Chem. 268, 392–398
16. Drøbak, B. K., Watkins, P. A. C., Valenta, R., Dove, S. K., Lloyd, C. W., and Staiger, C. J. (1994) Plant J. 6, 389–400
17. Mikami, J., Katagiri, T., Iuchi, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11398–11402
18. Meijer, H. J. G., Divecha, N., van den Ende, H., Musgrave, A., and Munnik, T. (1994) J. Biol. Chem. 269, 1059–1060
19. Parmar, P. N., and Brearley, C. A. (1997) Plant J. 11, 255–263
20. Munnik, T., Irvine, R. F., and Musgrave, A. (1994) Biochem. J. 298, 269–273
21. Brearley, C. A., and Hanke, D. E. (1995) Biochem. J. 311, 1001–1007
22. Xue, H.-W., Pical, C., Brearley, C., Elge, S., and Muller-Rober B. (1999) J. Biol. Chem. 274, 5738–5745
23. Mikami, J., Katagiri, T., Iuchi, S., Yamaguchi-Shinozaki, K., and Shinozaki, K. (1996) Plant J. 15, 563–568
24. Weulers, P., Takegawa, K., Emr, S. D., and Chrisspeels, M. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11398–11402
25. Hirayama, T., Ohto, C., Mizoguchi, T., and Shinozaki, K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3963–3967
26. Shi, J., Gonzales, R. A., and Bhattacharryya, M. K. (1995) Plant J. 8, 381–390
27. Pical, C., Kopka, J., Muller-Rober, J., Hetherington, A. M., and Gray, J. E. (1997) Physiol. Plant. 114, 747
28. Kopka, J., Pical, C., Gray, J. E., and Muller-Rober, B. (1998) Plant Physiol. 116, 239–250
29. Epstein, E., Norlyn, J. D., Rash, D. W., Kingsbury, R. W., Kelley D. B., Cunningham, G. A., and Wrena, A. P. (1988) Science 201, 399–404
30. Niu, X., Bressan, R. A., Hasegawa, P. M., and Pardo, J. M. (1995) Plant Physiol. 106, 735–742
31. Zhu, J.-K., Hasegawa, P. M., and Bressan, R. A. (1997) Crit. Rev. Plant Sci. 16, 253–277
32. Lynch, J., Polito, V. S., and Lauschli, A. (1989) Plant Physiol. 90, 1271–1274
33. Liu, J., and Zhu, J.-K. (1998) Science 280, 1943–1945
34. Knight, H., Trewavas, A. J., and Knight, M. R. (1997) Plant J. 12, 1067–1078
35. Liu, B., Joshi, H. C., Wilson, T. J., Silflow, C. D., Palevitz, B. A., and Snustad, D. P. (1994) Plant Cell 6, 303–314
36. Xu, D., and Michell, R. H. (1999) in Signal Transduction: A Practical Approach (Milligan, G., ed) pp. 255–281, Oxford University Press, Oxford
37. Munnik, T., de Vrije, T., and Musgrave, A. (1996) J. Biol. Chem. 271, 15709–15715
38. Munnik, T., Musgrave, A., and de Vrije, T. (1994) Planta 189, 89–98
39. Brearley, C. A., and Hanke, D. E. (1992) Biochem. J. 283, 255–260
40. Brearley, C. A., and Hanke, D. E. (1993) Biochem. J. 290, 145–150
41. Brearley, C. A., Parmar, P. N., and Hanke, D. E. (1997) Biochem. J. 324, 123–131
42. Brearley, C. A., and Hanke, D. E. (1997) in Signalling by Inositides (Shears, S., ed) pp. 1–31, IRL Press, Oxford
43. Lester, R. L., Becker, G. W., and Kauf, K. (1978) in Inositol Metabolism in Plants (Wells, W., and Eisenberg, F., Jr., eds) pp. 83–102, Academic Press, New York
44. Drøbak, B. K., Ferguson, J. B., Dawson, A. P., and Irvine, R. F. (1988) Plant Physiol. 87, 217–222
45. Irvine, R. F., Letcher, A. J., Landor, D. J., Drøbak, B. K., Dawson, A. P., and Musgrave, A. (1989) Plant Physiol. 90, 888–892
46. Côté, G. G., De Plass, A. L., Quarmby, L. M., Tate, B. F., Morse, M. J., Satter, R. L., and Crain, R. C. (1989) Plant Physiol. 90, 1422–1428
47. Cho, M. H., Shears, S. B., and Boss, W. F. (1993) Plant Physiol. 103, 637–647
48. Einsehr, K. F., Peeler, T. C., and Thompson, G. A., Jr. (1988) J. Biol. Chem. 263, 5775–5779
49. Ashcroft, F. M. (1998) Science 282, 1059–1060
50. Rhodes, D., and Samaras, Y. (1994) in Cellular and Molecular Physiology of Cell Volume Regulation (Strange, K., ed) pp. 347–361, CRC Press, Boca Raton, FL
51. Nuccio, M. L., Russell, B. L., Nolte, K. D., Rathinasabapathi, B., Gage, D. A., and Hanson, A. (1998) Plant J. 16, 487–496
52. Hanson, A. D., and Rhodes, D. (1983) Plant Physiol. 71, 692–700