RESEARCH COMMUNICATION

Rapid turnover of phosphatidylinositol 3-phosphate in the green alga *Chlamydomonas eugametos*: signs of a phosphatidylinositide 3-kinase signalling pathway in lower plants?

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When *Chlamydomonas eugametos* gametes were incubated in carrier-free [^{32}P]P_i, the label was rapidly incorporated into PtdIns*P* and PtdInsP_2 and, after reaching a maximum within minutes, was chased out by recirculating unlabelled P_i in the cell. This pulse–chase labelling pattern reflects their rapid turnover. In contrast, ^{32}P incorporation into the structural lipids was slow and continued for hours. Of the radioactivity in the PtdIns*P* spot, 15% was in PtdIns3*P* and the rest in PtdIns4*P*, and of that in the PtdInsP_2 spot, 1% was in PtdIns(3,4)P_2 and the rest in PtdIns(4,5)P_2, confirming the findings by Irvine, Letcher, Stephens and Musgrave [(1992) Biochem. J. **281**, 269–266]. When cells were labelled with carrier-free [^{32}P]P_i, both PtdIns*P* isomers incorporated label in a pulse–chase-type pattern, demonstrating for the first time in a plant or animal system that D-3 polyphosphoinositides turn over rapidly in non-stimulated cells, with kinetics similar to those shown by the D-4 isomers. In animal systems such lipids are already established as signalling molecules, and the data suggest that a similar role must be sought for them in lower plants such as *Chlamydomonas*.

INTRODUCTION

In animals, and more recently in plants, a lot of attention has been focused on polyphosphoinositides and their metabolism, because they are the precursors of second messengers. Thus PtdIns(4,5)P_2 is synthesized by sequential phosphorylation of PtdIns and PtdIns4*P* and is then hydrolysed by phospholipase C (PLC) to produce the messengers Ins(1,4,5)P_3 (hereafter called simply InsP_3) and diacylglycerol (DAG). InsP_3 triggers the release of Ca^{2+} from internal stores, while DAG together with Ca^{2+} activates protein kinase C. Since external factors such as hormones can activate PLC, this InsP_3/Ca^{2+}/DAG signalling mechanism transduces extracellular information into a cell response (Berridge, 1993).

Interest in phosphoinositides has been heightened by the recent discovery in animals that those phosphorylated at the D-3 position of the inositol ring [PtdIns3*P*, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3] represent a novel signal-transduction pathway that is distinct from the classical 'PI' (phosphoinositide) system described above. Since their formation is quickly coupled to cell responses, yet they cannot be hydrolysed by the known phospholipases, the lipids themselves are seen as the second messengers. They have been suggested to regulate the cell cycle, trigger differentiation and activate terminally differentiated cells (Downes and Carter, 1991; Irvine, 1992; Panayotou and Waterfield, 1992; Fry and Waterfield, 1993; Stephens et al., 1993b). There is still some controversy as to how D-3 phosphoinositides are synthesized and which of them is biologically active, but that may depend on the stimulus and the cell in question (Cunningham et al., 1990; Stephens et al., 1991, 1993a,b; Hawkins et al., 1992, 1993; Jackson et al., 1992; Kunz et al., 1993; Schu et al., 1993). Because they are all formed by the same family of enzymes, the transduction pathway is referred to as 'PI 3-kinase signalling' (Panayotou and Waterfield, 1992; Stephens et al., 1993b).

In plants, InsP_3/Ca^{2+}/DAG signalling is slowly being established (Trewavas and Gilroy, 1991; Drøbak, 1992, 1993; Musgrave et al., 1992, 1993; Coté and Crain, 1993), but as yet there are only a few reports of D-3 phosphoinositides being present (Irvine et al., 1992; Brearley and Hanke, 1992, 1993; Parmar and Brearley, 1993; Munnik et al., 1994) and none indicating that they have a function in signalling. However, a feature that can be expected of all signalling lipids compared with structural lipids is that they rapidly turn over, either because as precursors they are constantly being synthesized and broken down to produce a signal, or because, as signals themselves, they must be quickly broken down to prevent non-regulated stimulation of the cell. This property of D-3 phosphoinositides has not yet been established for any cell, plant or animal, but is demonstrated here for PtdIns3*P* in non-stimulated *Chlamydomonas eugametos* gametes. It is shown to turn over as fast as PtdIns4*P* and much faster than the structural lipids. While rapid turnover in itself does not prove that PtdIns3*P* is a signal, in the light of 'PI signalling' in animals, it does suggest it.

EXPERIMENTAL

Cell cultures

Chlamydomonas eugametos strain UTEX 10 (*mt*$^-$) derived from the Culture Collection of Algae (University of Texas, Austin, TX, U.S.A.), and strain 17.17.2 (*mt*$^+$) (Schuring et al., 1987) were grown in Petri dishes on agar-containing M1 medium (Wiese, 1965). Cultures were grown at 20 °C in a light/dark regime (12 h/12 h) with an average photon flux of 30 $\mu E \cdot s^{-1} \cdot m^{-2}$ (Philips TL 65W/33 fluorescent tubes).

Abbreviations used: DAG, diacylglycerol; Gro*P*Ins, glycerophosphoinositol; *mt*$^+$/*mt*$^-$, mating-type plus or minus; PtdEtn, phosphatidylethanolamine; PEI, polyethyleneimine; PLC, phospholipase C; PI, phosphoinositide.

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Swimming-gamete suspensions were obtained by flooding 3–4-week-old plate cultures with 20 ml of 10 mM Hepes/1 mM $MgCl_2$/1 mM $CaCl_2$/1 mM KCl, pH 7.4 (HMCK). After 2–16 h, cells were harvested and washed twice with HMCK by centrifugation for 10 min at 500 *g*. Cells were resuspended in HMCK at a final concentration of (1–2) × 10^7 cells/ml.

[^{32}P]Phospholipid labelling

Cells [(1–2) × 10^7 cells/ml] were incubated in 100 μCi of carrier-free $^{32}PO_4^{3-}$ (Amersham International, s'-Hertogenbosch, The Netherlands)/ml of HMCK, pH 7.4. At subsequent times, labelling was stopped by withdrawing 100 μl samples and adding them to 10 μl of 50% (w/v) $HClO_4$, after which the lipids were extracted.

Lipid analysis

Lipids were extracted in 900 μl of chloroform/methanol/HCl (100:100:1, by vol.) in a 2 ml Eppendorf tube while being vigorously mixed for 1 min. After 450 μl of chloroform and 337.5 μl of 0.9% NaCl had been added, the tubes were rigorously vortexed and centrifuged for 10 s at 10000 *g* in a microcentrifuge. The aqueous upper phase was discarded and the lower organic phase was washed twice with 400 μl of methanol/water/HCl (50:50:1, by vol.). Lipids were dried under a stream of N_2, dissolved in chloroform, and separated by t.l.c.

Lipids were chromatographed on impregnated [1% oxalate/2 mM EDTA in methanol/water (2:3, v/v)] silica-gel 60 t.l.c. plates (Merck, Darmstadt, Germany) with an alkaline [chloroform/methanol/25% NH_3/water (45:35:2:8, by vol.)] or an acidic [propan-1-ol/2 M acetic acid (13:7, v/v)] solvent system (Munnik et al., 1994; Auger et al., 1989). Routinely, impregnated t.l.c. plates were heat-activated for at least 30 min at 115 °C, and lipids were applied immediately after cooling. Plates were equilibrated for 1 h above 100 ml of the particular solvent to enhance resolution of the phospholipids, and were subsequently developed until the solvent front had travelled 20 cm. Separated radiolabelled phospholipids were detected by autoradiography. Individual spots were excised from the t.l.c. plate to measure the radioactivity by liquid-scintillation counting, or to deacylate the lipids for further analysis, as described below.

Deacylation

Lipids were deacylated by a method modified from that of Clarke and Dawson (1981). T.l.c.-separated ^{32}P-labelled polyphosphoinositides were scraped off the plates and directly deacylated by incubation in 750 μl of monomethylamine reagent (Clarke and Dawson, 1981) for 30 min at 53 °C. Samples were cooled to room temperature and centrifuged at 10000 *g* for 2 min to collect the supernatant. The silica pellet was washed with an additional 250 μl of methylamine. After re-centrifugation (2 min, 10000 *g*), the supernatants were combined and dried overnight by rotary evaporation. To remove the fatty-acyl groups, samples were dissolved in water and extracted twice with butan-1-ol/light petroleum (b.p. 40–60 °C)/ethyl formate (20:40:1, by vol.). The aqueous lower phase containing glycerophosphoinositides was dried by rotary evaporation, dissolved in water and analysed by h.p.l.c. or polyethyleneimine (PEI)-cellulose t.l.c. as described below.

Resolution of glycerophosphoinositides

Deacylated t.l.c.-purified ^{32}P-labelled polyphosphoinositides were separated by anion-exchange h.p.l.c. using a 5 μm Partisil SAX column (Whatman, Maidstone, Kent, U.K.) and a shallow discontinuous gradient of $(NH_4)_2H_2PO_4$, pH 3.8, at a flow rate of 1 ml/min, as described by Stephens et al., (1991, 1993c). Routinely, ^{32}P-labelled Gro*P*Ins*P*s and Gro*P*InsP_2s were co-injected with [^{3}H]inositol-labelled Gro*P*Ins4*P* and Gro*P*Ins-(4,5)P_2 respectively, together with AMP, ADP and ATP to monitor detection at 260 nm and evaluate the reproducibility of the h.p.l.c. runs. Fractions were collected at 15–30 s intervals and measured for radioactivity by liquid-scintillation counting.

Alternatively, Gro*P*InsP_2 isomers were separated by PEI-cellulose t.l.c. as described by Stephens et al. (1993c). In brief, t.l.c.-purified ^{32}P-labelled PtdInsP_2 spots were deacylated, the products dissolved in 20 mM HCl/10 mM KH_2PO_4 (2 μl) and spotted on to PEI-cellulose plates (CamLab, Cambridge, U.K.) in 1 μl aliquots. Vials and pipette tips were rinsed twice with 1 μl volumes of 20 mM HCl/10 mM KH_2PO_4 and also applied. The plates were developed in 0.48 M HCl and the radioactive spots detected by autoradiography.

RESULTS AND DISCUSSION

Phospholipid turnover

In *Chlamydomonas eugametos* gametes, PtdIns*P* and PtdInsP_2 have been shown to be typical signal precursors in that their chemical abundance is low (accounting for 0.45 and 0.33% of the phospholipids respectively), and their metabolic turnover is extremely rapid compared with that of the structural phospholipids (Brederoo et al., 1991). This was established by ^{32}P incorporation and pulse–chase experiments, in which the label was chased out by non-radioactive PO_4^{3-}. However, this rapid turnover can be demonstrated by simply incubating gametes in carrier-free [^{32}P]P_i and following the amount of ^{32}P incorporated into each phospholipid. A typical pulse–chase labelling pattern is obtained because the small amount of labelled phosphate is rapidly taken up, incorporated into the minor lipids PtdIns*P* and PtdInsP_2 and, after reaching a maximum within minutes, is chased out by recirculating non-radioactive phosphate already present in the cell (Figure 1a). Polyphosphate was recently demonstrated in *Chlamydomonas* (Hentrich et al., 1993) and

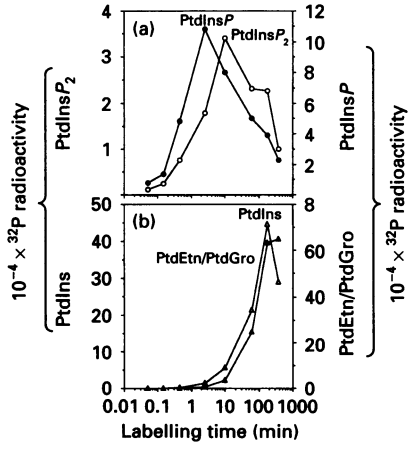


Figure 1 ^{32}P-labelling pattern of phospholipids using carrier-free [^{32}P]P_i

Gametes (*mt*$^+$) were incubated with carrier-free [^{32}P]P_i and their lipids extracted at the times indicated. Phospholipids were separated by alkaline t.l.c., revealed by autoradiography and quantified by liquid-scintillation counting. (**a**) Minor lipids PtdIns*P* (●) and PtdInsP_2 (○); (**b**) structural lipids PtdIns (△) and PtdEtn/PtdGro (▲).

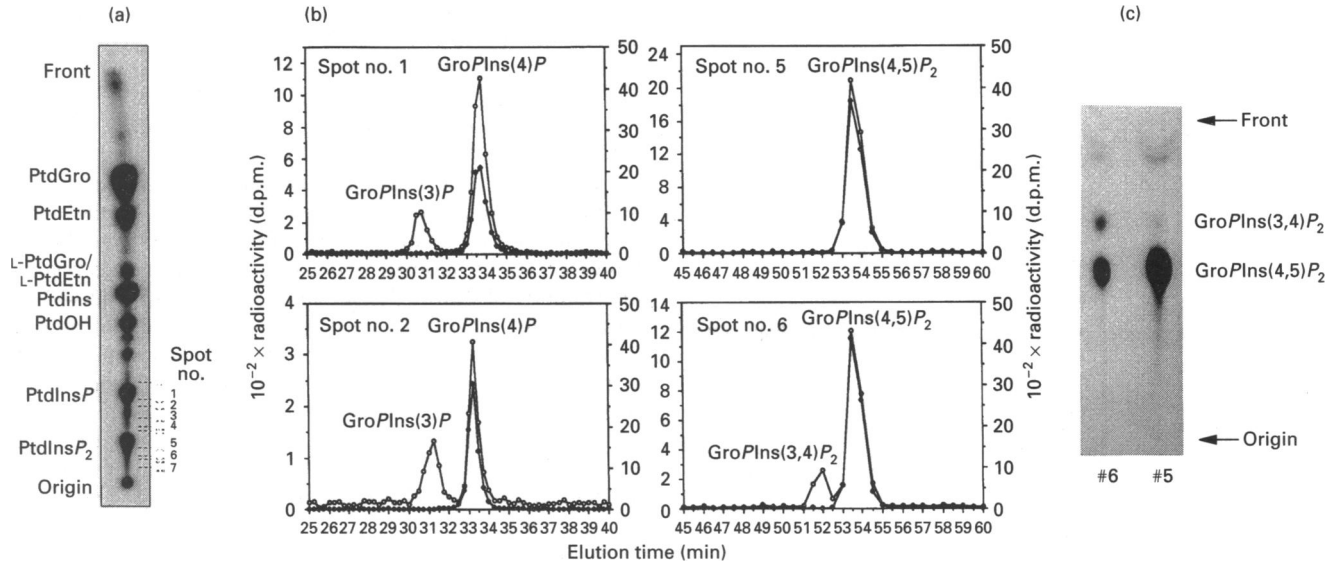


Figure 2 Enrichment of D-3- and D-4-phosphorylated polyphosphoinositides

Gametes (*mt*⁻) were labelled for 60 min with carrier-free [^{32}P]P_i, their lipids extracted and separated by alkaline t.l.c. Lipid spots that migrated in the region of PtdIns$P$ and PtdInsP_2 (spot nos. 1–7) were isolated, deacylated, and analysed by h.p.l.c. together with ^{3}H-labelled GroPIns4P or GroPIns(4,5)P_2. (**a**) Alkaline t.l.c.; (**b**) h.p.l.c. profiles of the corresponding regions representing D-3/D-4-phosphorylated PtdIns$P$ (nos. 1–2) and PtdInsP_2 (no. 5–6) isomers. Open symbols, ^{32}P; closed symbols, ^{3}H. (**c**) PEI-cellulose t.l.c. of deacylated PtdInsP_2 isomers (nos. 5–6).

could be the source of this non-radioactive phosphate. In contrast, the incorporation into the structural lipids PtdIns, PtdEtn and PtdGro is relatively slow and continues for many hours (Figure 1b).

While the time taken to reach maximum ^{32}P incorporation into the polyphosphoinositides varied between experiments (3–60 min), the relative labelling pattern was always reproduced. The radioactivity in PtdInsP_2 peaked later than in PtdIns$P$, probably because it can incorporate two radioactive phosphates consecutively rather than because it turns over more slowly.

PtdIns3P and PtdIns(3,4)P_2 in *C. eugametos*

While PtdIns4P and PtdIns(4,5)P_2 are the major polyphosphoinositides in most plants (Irvine et al., 1989; Drøbak, 1992), Irvine et al. (1992) recently demonstrated that PtdIns3P and PtdIns(3,4)P_2 are present in *C. eugametos*, even though the latter, illustrated as an Ins(1,3,4)P_3 peak after deacylation and deglyceration, hardly came above the background. Quarmby et al. (1992) also reported finding PtdIns3P in *C. reinhardtii*, but made no mention of PtdIns(3,4)P_2. Our first attempts to detect PtdIns(3,4)P_2 were also negative, but, since D-3 phosphoinositides have been reported to migrate slightly more slowly than D-4 isomers in the alkaline t.l.c. systems that we use (Whitman et al., 1988; Stephens et al., 1991), a more thorough analysis was made of the PtdIns$P$ and PtdInsP_2 spots and neighbouring regions. As illustrated in Figure 2(a), seven ^{32}P-labelled-lipid sections of a chromatogram were isolated, the fatty acids removed by deacylation with methylamine and the resulting headgroups separated by h.p.l.c. (Stephens et al., 1993c). ^{3}H-labelled GroPIns4P or GroPIns(4,5)P_2 were co-chromatographed as internal standards. The h.p.l.c. profiles from the four sections containing essentially all the PtdIns$P$ and PtdInsP_2 are illustrated in Figure 2(b). The D-3 isomers were indeed concentrated in the trailing-edge fractions of the two phosphoinositide spots. The enrichment was such that GroPIns(3,4)P_2 formed a clear peak accounting for 17% of the total radioactivity in fraction 6, although it was absent from fraction 5, where most of the PtdIns(4,5)P_2 migrated. Similarly, 34% of the radioactivity in fraction 2 was in GroPIns3P. However, on average about 15% of the radioactivity in the PtdIns$P$ pool was in the 3-isomer, while about 1% of that in the PtdInsP_2 pool was in the 3,4-isomer. From the relative lipid composition of *C. eugametos* (Brederoo et al., 1991), we therefore estimate that 0.07% and 0.003% of the total phospholipid is present as PtdIns3P and PtdIns(3,4)P_2 respectively. To illustrate further the presence of PtdIns(3,4)P_2, deacylated samples were chromatographed on PEI-cellulose as described by Stephens et al. (1993c). As predicted, a GroPIns(3,4)P_2 spot migrated in front of a more dominant GroPIns(4,5)P_2 spot in fraction 6, but not in fraction 5 (Figure 2c).

These results not only provide strong evidence for the presence of PtdIns(3,4)P_2 in *C. eugametos*, but illustrate how one can concentrate these chemically low abundance D-3 isomers for detection in plant extracts. They also support the recent claim (Gaudette et al., 1993) that the alkaline t.l.c. system can be used for the separation and analysis of PtdIns(3,4)P_2 and PtdIns(4,5)P_2. Other t.l.c. systems that separate D-3/D-4 isomers have been reported (Stephens et al., 1991; Pignataro and Ascoli, 1990; Walsh et al., 1991; Munnik et al., 1994). Having once established identity of the spots, t.l.c. provides a cheaper and much faster alternative to h.p.l.c.

Turnover of D-3- and D-4-phosphorylated polyphosphoinositides

Since D-3- and D-4-phosphorylated isomers of PtdIns$P$ and PtdInsP_2 are present in *C. eugametos*, we tried to assess the labelling characteristics of all four lipids. Accordingly, gametes were labelled with carrier-free [^{32}P]P_i and, at subsequent times, their lipids extracted and separated by t.l.c. Lipid spots were isolated, deacylated and the glycerophosphoinositides separated by h.p.l.c. Unfortunately, we were unable to attribute radioactivity to GroPIns(3,4)P_2 consistently because of the minimal amounts present. The incorporation of ^{32}P in both GroPIns$P$ isomers with time is presented in Figure 3. The pulse–chase-type

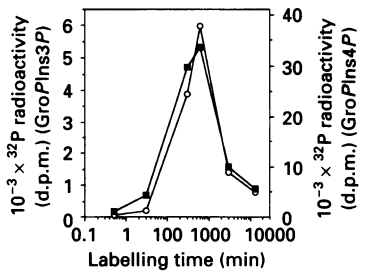
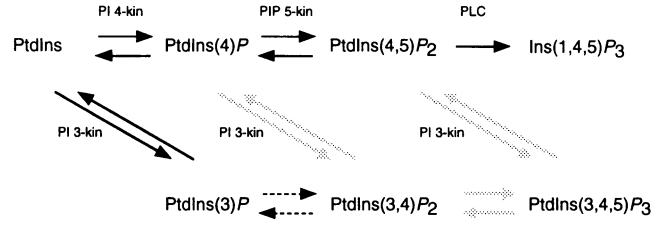


Figure 3 Time-dependent ^{32}P-incorporation into PtdIns4*P* and PtdIns3*P*

Cells were labelled for different periods of time with carrier-free [^{32}P]P_i, their lipids extracted, separated by acidic t.l.c. (propanol/acetic acid) and revealed by autoradiography. PtdIns*P* spots were scraped off the silica plates, deacylated and the resulting Gro*P*Ins*P*s were analysed by h.p.l.c. together with a ^{3}H-labelled Gro*P*Ins4*P* standard. Radioactivity levels in the respective peaks were measured by liquid-scintillation counting. ○, [^{32}P]Gro*P*Ins3*P*; ■, Gro*P*Ins4*P*.



Scheme 1 Biosynthesis of D-3- and D-4-phosphorylated inositides in plants

All arrows represent the possible metabolic reactions between polyphosphoinositides that have been identified in animal cells. The reactions that are thought to occur in yeast are indicated by ⟶, while those in plants are represented by ⟶ and ⇢. Abbreviations: PI 3-kin, phosphatidylinositol 3-kinase etc.; PLC, phospholipase C.

pattern was again found, with the two PtdIns*P* isomer peaks being practically superimposable. The labelling pattern of Gro*P*Ins(4,5)P_2 was similar, while the structural lipids continued incorporating radioactivity throughout the course of the experiment, as already illustrated in Figure 1. Clearly the labelling pattern of PtdIns3*P* is that of a compound rapidly turning over, as expected of a lipid with a signal function itself or, like PtdIns4*P*, as a signal precursor.

Apart from in the lower plant *Chlamydomonas*, PtdIns3*P* has also been identified in the higher plants *Dianthus caryophylus* (carnation), *Spirodela polyrhiza* and *Commelina communis*, while PtdIns(3,4)P_2 has only been reported for the last two (Irvine et al., 1992; Munnik et al., 1994; Brearley and Hanke, 1992, 1993; Parmar and Brearley, 1993). One can ask whether other D-3 phosphoinositides are present and which of them is biologically active. In yeast for example, PtdIns3*P* is seen as the active lipid because no other D-3 inositides are present and because 50% of the PtdIns*P* pool is present as the D-3 isomer (Auger et al., 1989; Hawkins et al., 1993). It may be involved in membrane trafficking within the cell (Herman et al., 1992; Schu et al., 1993; Stack et al., 1993). In animal cells, PtdIns(3,4,5)P_3 is thought to be the active lipid with the others being formed as breakdown products as follows: $-(3,4,5)P_3 \rightarrow -(3,4)P_2 \rightarrow -3P$ (see Scheme 1; Stephens et al., 1991; Hawkins et al., 1992). However, in unstimulated cells, PtdIns3*P* is relatively abundant, whereas PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are hardly detectable. On stimulation, the levels of PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 rapidly increase to the level of PtdIns3*P*, whose concentration remains surprisingly constant (Stephens et al., 1991, 1993b; Hawkins et al., 1992; Jackson et al., 1992). This indicates that, in some animal cells, the major part of the PtdIns3*P* pool is metabolically independent of PtdIns(3,4,5)P_3, suggesting that it has a function in its own right, consistent with our view of it in yeast and the rapid turnover now reported for *Chlamydomonas*.

We have not yet found a PtdInsP_3 in *Chlamydomonas*, but even if present, its breakdown is unlikely to produce the relatively large amount of PtdIns3*P*, especially since the PtdIns(3,4)P_2 pool is too small (1:20) to be its immediate precursor. In addition, Brearley and Hanke (1993) recently found in *Spirodela* that, after short-term ^{32}P labelling, the phosphate with the highest specific radioactivity in PtdIns(3,4)P_2, was at the 4-position, implying that it was synthesized from PtdIns3*P*. Therefore, we suggest that PtdIns3*P* is an active plant lipid and that it is directly synthesized from, and broken down to, PtdIns (Scheme 1).

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