Pathway of synthesis of 3,4- and 4,5-phosphorylated phosphatidylinositols in the duckweed *Spirodela polyrhiza* L.

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[³H]Inositol and [³²P]_i labelling of the aquatic plant *Spirodela polyrhiza* L. revealed the presence of PtdIns(3,4)₂P₂, in addition to PtdIns3P, PtdIns4P and PtdIns(4,5)₂P₂ previously identified [Brearley and Hanke (1992) *Biochem. J.* **283**, 255–260]. PtdIns(3,4,5)₃P₃ was not detected. Throughout a 40 min [³²P]_i labelling period the specific radioactivity of the γ -phosphate of ATP and of the ATP pool as a whole increased. Chemical and enzymic dissection of phosphoinositides obtained from plants labelled for 35 min with [³²P]_i showed that over 99.7% of the label in PtdIns3P and PtdIns4P was accounted for by the monoester phosphates. The 3- and 4-monoester phosphates of PtdIns(3,4)₂P₂ accounted for 23.1% and 76.6% respectively of

the label, whereas the 4- and 5-monoester phosphates of PtdIns(4,5)₂P₂ accounted for 21.1% and 78.6% respectively. These results are consistent with the synthesis of PtdIns(4,5)₂P₂ via PtdIns4P. The labelling of the individual phosphates of PtdIns(3,4)₂P₂ is, however, inconsistent with synthesis from PtdIns(4,5)₂P₂ via PtdIns(3,4,5)₃P₃, but instead suggests that PtdIns(3,4)₂P₂ is synthesized by 4-phosphorylation of PtdIns3P. These results afford the first evidence that in plants *in vivo*, synthesis of PtdIns(4,5)₂P₂ follows the pathway described in animal cells and also that plants possess PtdIns3P 4-kinase activity similar to that reported from animal cells.

INTRODUCTION

It is widely assumed that in plants the synthesis of PtdIns(4,5)₂P₂ follows the pathway described for animal cells. This assumption is based on the following evidence. Experiments *in vitro* have demonstrated the presence in isolated membrane fractions of kinase activities capable of using [³²P]ATP to phosphorylate PtdIns in the 4-position and PtdIns4P in the 5-position (Sommarin and Sandelius, 1988) and lyso-PtdIns to a lyso-PtdInsP (Wheeler et al., 1991). Labelling studies *in vivo* have shown that tissues labelled briefly with [³²P]_i incorporate a large proportion of the total label in phospholipids into the 4-phosphomonoester of PtdIns4P (Drøbak et al., 1988; Hanke et al., 1990a) and only a very small proportion of the label into PtdIns(4,5)₂P₂ even after prolonged labelling. Thus the evidence that PtdIns(4,5)₂P₂ synthesis *in vivo* proceeds via PtdIns4P is largely circumstantial.

Recently we have rigorously identified PtdIns4P, PtdIns(4,5)₂P₂ and PtdIns3P in *Spirodela polyrhiza* (Brearley and Hanke, 1992). In the present paper we have set out to determine the metabolic route by which PtdIns(4,5)₂P₂ is synthesized, to look for other 3-phosphorylated phosphatidylinositols and to examine the metabolic relationship between 3- and 4-phosphorylated lipids.

MATERIALS AND METHODS

Reagents

myo-[2-³H]Inositol and [³²P]_i (carrier-free) were obtained from Amersham International (Amersham, Bucks., U.K.). D-[1-³H]-Inositol 1,4,5-trisphosphate and D[1-³H]inositol 1,3,4,5-tetrakisphosphate were obtained from New England Nuclear–DuPont (Stevenage, Herts., U.K.). Alkaline phosphatase (bovine intestinal; type 5521) was obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

Tissue

The aquatic monocotyledonous plant *Spirodela polyrhiza* L. was maintained in axenic culture as described by Smart and Trewavas (1983).

Tissue extraction

After labelling, the washed plants were cooled in liquid N₂, ground in a liquid-N₂-cooled mortar and pestle and extracted with 0.7 ml of 3.5% (w/v) HClO₄. The cell debris was pelleted by centrifugation at 13000 *g*_{max} for 5 min in a refrigerated microfuge, and the lipids were extracted by a method modified from those of Boss (1989) and Stephens et al. (1991). To the cell debris was added 1.5 ml of chloroform/methanol (1:2, v/v) and the sample was mixed vigorously. Further additions to the sample, each with vigorous mixing, were in turn 2.4 M HCl (0.5 ml), 1 mM EDTA (0.5 ml), and chloroform (0.5 ml). The sample was centrifuged for 5 min at 3000 *g*_{max} and the lower organic phase was removed. The upper aqueous phase was extracted with 2 × 0.5 ml of chloroform, and the lower organic phases obtained were pooled with the original organic phase. The pooled organic phase was washed with 2 × 2 ml of 1 M HCl/methanol (1:1, v/v) containing 1 mM inositol, 1 mM H₃PO₄ and 1 mM CDTA (*trans*-1,2-diaminocyclohexane-*NNN'*-tetra-acetic acid).

Lipid deacylation

Lipids were deacylated by the method of Hawkins et al. (1986).

Deglyceration of glycerophosphoinositol phosphates

Glycerol was cleaved from desalted GroPInsP₂ species by mild treatment with sodium periodate as described by Whitman et al. (1988).

H.p.l.c. of inositol phosphates

Water-soluble products of deacylation were resolved on a 25 cm Partisphere SAX h.p.l.c. column (Whatman) by using the gradient of Stephens et al. (1989). The column was eluted with a gradient derived from buffers A (water) and B [$1.5 \text{ M } (\text{NH}_4)_2\text{HPO}_4$ adjusted to pH 3.8 with H_3PO_4] at a flow rate of 1 ml/min: 0 min, 0% B; 5 min, 0% B; 45 min, 12% B; 52 min, 20% B; 64 min, 100% B; 70 min, 100% B.

The InsP_3 products of GroPInsP_2 deglyceration were resolved on a 25 cm Partisphere SAX h.p.l.c. column (Whatman) as described by Wreggett and Irvine (1989). The column was eluted isocratically with 550 mM NaH_2PO_4 .

Desalting the h.p.l.c. fractions

Fractions containing phosphate were diluted 5–10-fold with water adjusted to pH 6–7 with triethylamine and desalted as described by Stephens et al. (1988).

Ghost preparation

Erythrocyte ghosts were prepared by the method of Hawkins et al. (1984), divided into batches, cooled in liquid N_2 and stored at -70°C until used.

Preparation of [^3H]Ins(1,3,4) P_3

D-[1- ^3H]Inositol(1,3,4,5) P_4 was incubated with erythrocyte ghosts under conditions designed to remove the 5-phosphate (Stephens et al., 1991) and described below. The products were resolved by h.p.l.c. on a Partisphere 5 SAX column with a gradient derived from buffers A (water) and B (2.5 M NaH_2PO_4) at a flow rate of 1 ml/min: 0 min, 0% B; 60 min, 100% B. The InsP_3 product of ghost treatment was desalted and re-chromatographed on the isocratic system described above. This procedure yielded a single peak of radioactivity which was shown, by chromatographing a mixture of the InsP_3 product and [^3H]Ins(1,4,5) P_3 , to be eluted before that compound, confirming that the product was Ins(1,3,4) P_3 .

Dephosphorylation of GroPInsP species

Desalted preparations of ^{32}P -labelled GroPInsP species were mixed with their ^3H -labelled counterparts and dephosphorylated with alkaline phosphatase (method modified from Stephens et al., 1989).

Dephosphorylation of GroPInsP_2 species

Desalted preparations of ^{32}P -labelled GroPInsP_2 were mixed with their respective ^3H -labelled standard compounds and selectively dephosphorylated with erythrocyte ghosts under conditions designed (Stephens et al., 1991) to remove either the 5-phosphate or the 3-phosphate from inositol phosphates.

$\text{GroPIns}(4,5)\text{P}_2$ was incubated with erythrocyte ghosts at a protein concentration of 2 mg/ml in 12.5 mM Hepes (pH 7.0)/1 mM EGTA/10 mM MgCl_2 for 60 min at 37°C .

Putative $\text{GroPIns}(3,4)\text{P}_2$ was incubated with erythrocyte ghosts at the same protein concentration, but in a buffer comprising 12.5 mM Hepes, pH 7.0, 1 mM EGTA and 5 mM EDTA for

120 min at 37°C . Ghost preparations were washed three times by centrifugation in the relevant buffer before addition to the GroPInsP_2 substrates. Reactions were terminated by addition of 20 μl of 70% (w/v) HClO_4 . The samples were put on ice for 15 min and the ghosts were pelleted in a microfuge. The supernatants were neutralized with 2 M NaOH/50 mM Tris and applied to a Partisphere SAX column eluted with $(\text{NH}_4)_2\text{HPO}_4$ as described above.

Incubation of $\text{GroPIns}(4,5)\text{P}_2$ with ghost membranes in the presence of MgCl_2 and in the absence of EDTA yielded a single GroPInsP product, which when mixed with [^{32}P]GroPIns3P was eluted after that compound and at the retention time expected of GroPIns4P .

Labelling of the ATP pool

Plants (approx. 200 mg fresh wt.) were inoculated into 2 ml of phosphate-free media containing 0.4 mCi of [^{32}P]P_i and incubated for 40 min. At intervals, plants were removed, frozen in liquid N_2 , extracted with HClO_4 and processed by h.p.l.c. on a Partisphere SAX column eluted with a gradient derived from buffers A (water) and B (2.5 M NaH_2PO_4) at a flow rate of 1 ml/min: 0 min, 0% B; 60 min, 100% B; 70 min, 100% B. The ^{32}P content of the ATP peak was determined by Čerenkov counting of collected fractions, and the ATP content was determined by A_{254} measurement.

Estimation of the labelling of the γ -phosphate

ATP-containing fractions (1–2 nmol) from h.p.l.c. were desalted and mixed with an excess of unlabelled ATP. Partial conversion into ADP and glucose 6-phosphate was effected by incubation of the ATP samples with hexokinase with D-glucose as substrate.

Hexokinase incubation

Desalted ATP fractions were taken up in 0.5 ml of 20 mM Hepes, pH 7.5, containing 1 mM ATP, 6 mM MgCl_2 and 2 mM D-glucose and incubated with 2.8 units of yeast hexokinase (Boehringer) for 2 min at 25°C . Reactions were terminated by boiling for 2 min. Products of the incubation were analysed by SAX h.p.l.c. on the isocratic system described above.

RESULTS

Pulse-chase experiment

Plants (approx. 180 mg fresh wt.) were inoculated into 5 ml of phosphate-free medium containing 1.0 mCi of [^{32}P]P_i and incubated for 12 min. The plants were washed briefly with half-strength Hunter's medium (see Smart and Trewavas, 1983), which contains 1.15 mM phosphate, and incubated in the same medium. At intervals up to 60 min later, plants (approximately equal masses) were removed, and the lipids were extracted, deacylated and processed for h.p.l.c. (all steps as described in the Materials and methods section).

The results are presented in Table 1 as a percentage of the total radioactivity recovered in glycerophosphoinositols and glycerol phosphate for each interval of labelling. Glycerol phosphate, the deacylation product of phosphatidic acid, is included, as phosphatidic acid is the immediate precursor of PtdIns. Deacylation products of PtdIns, PtdIns3P, PtdIns4P and PtdIns(4,5) P_2 were identified. GroPIns4P accounted for between 57 and 74% of the label recovered. The extensive labelling of GroPIns4P and the decline in labelling of glycerol phosphate, even after only 12 min, suggesting rapid turnover of phosphatidic

Table 1 Pulse-chase analysis of [32 P] P_i labelling of phosphoinositides and phosphatidic acid (PtdOH)

Spirodela plants were pulse-labelled for 12 min in phosphate-free half-strength Hutner's medium containing [32 P] P_i , washed and incubated (chased) for a further 60 min in half-strength Hutner's medium containing phosphate. Glycerophosphate and GroPIns P species obtained by deacylation of 32 P-labelled lipids were resolved by h.p.l.c. on Partisphere SAX and their radioactivity was determined by Čerenkov counting of collected fractions (all steps as described in the text). Abbreviation: n.d., not detected.

Phospholipid	Distribution of ^{32}P label among phosphatidic acid and phosphoinositides (%)						
	Chase period (min)...	0	5	10	15	30	60
PtdOH		21.4	24.1	14.3	14.6	10.0	11.7
PtdIns		15.2	5.8	13.0	4.5	10.0	9.6
PtdIns3 <i>P</i>		1.6	2.3	6.5	1.7	1.9	7.6
PtdIns4 <i>P</i>		57.6	64.9	63.4	74.7	73.8	67.2
PtdIns(3,4) P_2		0.6	n.d.	n.d.	0.7	0.3	0.3
PtdIns(4,5) P_2		3.6	2.9	2.8	3.9	4.0	3.6

acid, are both consistent with previous studies of short-term labelling of these phospholipids in other plant tissues (Drøbak et al., 1988; Hanke et al., 1990a,b). Low levels of labelling were detected in GroPIns(4,5) P_2 , and only traces of labelling in a compound which was eluted approx. 2 min before GroPIns(4,5) P_2 and which was tentatively identified as GroPIns(3,4) P_2 . Thus the results of this experiment could not be used to identify product-precursor relationships among the phosphoinositides.

We therefore increased the amount of tissue and radioisotope in an attempt to characterize further the putative [32 P]-GroPIns(3,4) P_2 , and in addition labelled tissue from [3 H]inositol to see whether the unidentified compound could be labelled from *myo*-inositol.

Identification of GroPIns(3,4) P_2 in deacylated preparations

Plants were inoculated into either 2.4 ml of half-strength Hutner's medium containing 600 μ Ci of [3 H]inositol or 2 ml of phosphate-free half-strength Hutner's medium containing 2 mCi of [32 P] P_i . Plants were incubated for 45 h or 35 min respectively. At the end of the incubation, the plants were washed with water, weighed ([3 H]inositol-labelled tissue, 203 mg fresh wt.; [32 P] P_i -labelled tissue, 280 mg fresh wt.), extracted and processed for h.p.l.c. (all steps as described in the Materials and methods section).

Deacylation of phospholipid extracts from [32 P] P_i - and [3 H]-inositol-labelled *Spirodela* gave the profiles detailed in Figure 1 on h.p.l.c. The profiles are essentially as described previously (Brearley and Hanke, 1992), but show in addition a minor peak which was eluted approx. 2 min before GroPIns(4,5) P_2 , and in the 32 P-labelling experiment another peak approx. 6 min after GroPIns(4,5) P_2 . The latter peak was shown by mixing with [3 H]Ins(1,3,4) P_3 and [3 H]Ins(1,4,5) P_3 and subsequent chromatography to be [32 P]Ins(1,4,5) P_3 , generated presumably by a side reaction during deacylation.

The former peak, assumed to be GroPIns(3,4) P_2 , was characterized further. The peak was desalted and subjected to mild periodate treatment to remove the glycerol moiety. The products were mixed with [3 H]Ins(1,4,5) P_3 and [3 H]Ins(1,3,4) P_3 , prepared by erythrocyte-ghost treatment of Ins(1,3,4,5) P_4 in the presence of MgCl $_2$, and subjected to h.p.l.c. on the isocratic system of Wreggett and Irvine (1989). The 32 P-labelled product of deglyceration co-chromatographed precisely with the [3 H]Ins(1,3,4) P_3

Figure 1 Anion-exchange separation of deacylation products from (a) [3 H]inositol- and (b) [32 P] P_i -labelled *Spirodela polyrrhiza* L.

Lipid extracts were deacylated and the water-soluble products were applied to a Partisphere SAX h.p.l.c. column. Fractions (0.5 or 1.0 min) were collected and radioactivity was measured either by scintillation counting of 10 μ l portions of fractions or by Čerenkov counting of the whole fraction. All steps are described in the Materials and methods section.

standard and was eluted before [3 H]Ins(1,4,5) P_3 (results not shown).

Labelling of the ATP pool

The kinetics of labelling of the ATP pool in *Spirodela* were investigated in a separate experiment to those used to prepare lipid deacylates. The results of this experiment are shown in Figure 2. The specific radioactivity of the ATP pool increased progressively over the 40 min labelling period.

Labelling of the γ -phosphate of ATP

The successful resolution of glucose 6-phosphate, ADP and ATP by h.p.l.c. (results not shown) allowed the quantification of the

Figure 2 Incorporation of [^{32}P]P_i into the γ -phosphate of ATP

Plants were incubated in a modified Hutner's medium containing 0.2 mCi/ml [^{32}P]P_i for various times before extraction with HClO₄ and analysis by Partisphere SAX h.p.l.c. The specific radioactivity of ATP was determined by A_{254} measurement and Čerenkov counting of h.p.l.c. fractions. The specific radioactivity of the γ -phosphate of ATP was determined after hexokinase-mediated transfer of the γ -phosphate to glucose and measurement of radioactivity in the products of the reaction after separation by h.p.l.c. The extent of the reaction was determined from A_{254} measurement of ADP and ATP (all steps as described in the text). Symbols: ○, ATP; ●, γ -phosphate of ATP.

^{32}P content of all three compounds. Measurement of the ADP and ATP content by A_{254} afforded determination of the extent of metabolism of ATP, and hence the ^{32}P content of the γ -phosphate of ATP, transferred stoichiometrically to glucose, was determined (Figure 2).

The specific radioactivity of the γ -phosphate of ATP increased over the 40 min labelling period (Figure 2). This allowed us to use the strategy defined by Stephens and Downes (1990) to determine, from the relative labelling of individual phosphate groups, the sequence of addition of these phosphates to the lipid. That is, over a period during which the specific radioactivity of the ATP pool is increasing, any phosphate added at a particular point in a metabolic sequence will be more strongly labelled than its predecessors and less so than its successors. Thus, as long as a phosphate in a given position is not removed and replaced subsequent to the addition of another phosphate in a different position, then the rank order of specific radioactivity of the individual phosphates reveals the order in which the individual phosphates were esterified to the inositol moiety. Such an approach makes the assumption that the γ -phosphate of ATP is the phosphate donor and that there is effectively only one metabolically active pool of ATP contributing to the labelling.

Enzymic dissection of GroPInsPs

Having demonstrated that the specific activity of the γ -phosphate of ATP increased throughout a 40 min labelling period, we were able to dissect enzymically the GroPInsP species obtained in the previous experiment to identify the relative labelling of individual phosphate groups within each individual GroPInsP. Samples of desalted ^{32}P -labelled GroPIns3P and GroPIns4P were mixed with their ^3H -labelled counterparts and treated with alkaline

Table 2 Distribution of ^{32}P label among individual phosphate groups of phosphoinositides

GroPInsP and GroPInsP₂ species obtained by deacylation of ^{32}P -labelled lipids were purified on Partisphere SAX h.p.l.c., desalted, mixed with their individual ^3H -labelled counterparts and enzymically cleaved, with either alkaline phosphatase (GroPInsP species) or erythrocyte ghosts (GroPInsP₂ species). The products of enzymic cleavage were separated by h.p.l.c. on Partisphere SAX and their radioactivity was determined by dual-label scintillation counting (all steps as described in the text).

Phosphoinositide	Identity of phosphate...	³² P label distribution in phosphoinositides (%)			
		1	3	4	5
PtdIns3P		0.2	99.8		
PtdIns4P		0.3		99.7	
PtdIns(3,4)P ₂		0.3*	23.1	76.6	
PtdIns(4,5)P ₂		0.3*		21.1	78.6

* Assumes diester phosphate accounts for 0.3% of label (see the text).

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Figure 3 Dephosphorylation of [^{32}P]GroPIns(3,4)P₂ by human erythrocyte ghosts

GroPIns(3,4)P₂ samples extracted from [^3H]inositol- or [^{32}P]P_i-labelled *Spirodela* and purified by h.p.l.c. on Partisphere SAX were desalted separately. Samples were mixed and incubated with human erythrocyte ghosts in the presence of 5 mM EDTA and in the absence of MgCl₂ for 120 min. The reaction was quenched with HClO₄, the protein was removed by centrifugation, and the supernatant was neutralized and applied to a Partisphere SAX h.p.l.c. column. Fractions were collected and their radioactivity was measured by dual-label scintillation counting. Symbols: ○, ^3H ; ●, ^{32}P .

phosphatase (Brearley and Hanke, 1992) to liberate the phosphate in the 3- and 4-positions respectively as [^{32}P]P_i. Unchanged substrate GroPInsP and the GroPIns and P_i products were resolved by h.p.l.c. on Partisphere SAX (results not shown). Determination of the $^3\text{H}/^{32}\text{P}$ ratio of GroPIns and GroPInsP by dual-label scintillation counting allowed calculation of the relative labelling of the monoester and diester phosphates (Table 2). In both GroPIns3P and GroPIns4P the monoester phosphate accounted for over 99.7% of the ^{32}P label. This reflects the rapid turnover of the monoester phosphate compared with the diester

Figure 4 Dephosphorylation of [^{32}P]GroPIns(4,5) P_2 by human erythrocyte ghosts

GroPIns(4,5) P_2 samples extracted from [^3H]inositol- or [^{32}P] P_i -labelled *Spirodela* and purified by h.p.l.c. on Partisphere SAX were desalted separately. Samples were mixed and incubated with human erythrocyte ghosts in the presence of 10 mM MgCl_2 and in the absence of EDTA for 0 or 60 min. The 0 min sample showed a single peak of $^3\text{H}/^{32}\text{P}$ radioactivity which was eluted in the position of GroPIns(4,5) P_2 (results not shown). The Figure shows the results from the 60 min treatment. The reaction was quenched with HClO_4 , the protein was removed by centrifugation, and the supernatant was neutralized and applied to a Partisphere SAX h.p.l.c. column. Fractions were collected and their radioactivity was measured by dual-label scintillation counting. Symbols: $\circ$, ^3H ; $\bullet$, ^{32}P .

phosphate. The high level of labelling of the monoester phosphate contrasts with our previous observations (Brearley and Hanke, 1992), when after prolonged labelling (3 days), and presumably extensive turnover of the diester phosphate, the diester phosphate was itself appreciably labelled.

Similar analyses were made of the distribution of ^{32}P label between the monoester and diester phosphates of [^{32}P]GroPIns(3,4) P_2 and [^{32}P]GroPIns(4,5) P_2 . The use of erythrocyte ghost 3- and 5-phosphohydrolase activities afforded a means to remove selectively the 3- and 5-phosphates. The results of these experiments are shown in Figures 3 and 4. Thus incubation of GroPIns(4,5) P_2 with ghost membranes in the presence of MgCl_2 and absence of EDTA yielded a single GroPIns P product (Figure 3), which, when mixed with [^{32}P]GroPIns3 P and rechromatographed, was eluted after that compound and in precisely the position expected of GroPIns4 P (results not shown).

Incubation of GroPIns(3,4) P_2 with ghost membranes in the presence of EDTA and absence of MgCl_2 also yielded a single GroPIns P product (Figure 4), which, when mixed with [^{32}P]GroPIns3 P and rechromatographed, was eluted after that compound and in precisely the position expected of GroPIns4 P (results not shown).

Measurement of the $^3\text{H}/^{32}\text{P}$ ratio of the substrate and GroPIns P products and of the ^{32}P content of the P_i peak allows determination of the extent of reaction and of the ^{32}P content of the individual phosphomonoester released by ghost treatment. These results (summarized in Table 2) show that the 3-monoester phosphate accounted for 23.1 % of the ^{32}P label in GroPIns(3,4) P_2 and the 5-monoester phosphate for 76.6 % of the label in

GroPIns(4,5) P_2 . Assuming that the diester phosphate accounts for only 0.3 % of the label in these compounds, as in GroPIns3 P and GroPIns4 P , 76.6 and 21.1 % of the label reside respectively in the 4-monoester phosphate of GroPIns(3,4) P_2 and GroPInsP(4,5) P_2 . In two other independent experiments with labelling periods of 30–40 min the 5-monoester phosphate accounted for 68.6 % and 68.9 % of the label in GroPIns(4,5) P_2 .

The weak labelling of the diester phosphate, assumed above, was confirmed, for GroPIns(4,5) P_2 at least, in an independent labelling experiment whereby GroPIns(4,5) P_2 was dephosphorylated to yield a mixture of products including GroPIns (results not shown). It seems not unreasonable to assume that the diester phosphates of the higher phosphoinositides are labelled to a similar extent as their GroPIns P counterparts.

DISCUSSION

There are to date few rigorous identifications of phosphoinositides in the plant kingdom (Irvine et al., 1989; Coté et al., 1989; Brearley and Hanke, 1992), and there is even less evidence that phosphoinositide-derived second messengers mediate physiological responses to environmental stimuli. Nevertheless, the case for such a role has recently been highlighted by work (Blatt et al., 1990; Gilroy et al., 1990) implicating Ins(1,4,5) P_3 involvement in abscisic acid-induced stomatal closure. This case would be considerably strengthened by the identification of phosphoinositides in stomatal guard cells, although it has also yet to be unequivocally demonstrated that the Ins P_3 identified in other plant cells is the 1D-*myo*-inositol 1,4,5-trisphosphate isomer, or that the Ins P_3 is derived from PtdIns(4,5) P_2 .

Although it is widely assumed that PtdIns(4,5) P_2 synthesis in plants follows the pathway described for animal cells, there is no evidence that the pathway operates *in vivo*. In fact, direct evidence that the pathway operates in an intact hormone-sensitive animal cell has only recently been provided (Stephens et al., 1991).

By analysis of the distribution of [^{32}P] P_i incorporated into individual phosphate esters of phosphoinositides during a short labelling period, we have shown that the phosphate in the 4-position of PtdIns4 P is added after the diester phosphate and that the phosphate in the 5-position of PtdIns(4,5) P_2 is added after that in the 4-position. In doing so, we have demonstrated for the first time that the pathway PtdIns $\rightarrow$ PtdIns4 P $\rightarrow$ PtdIns(4,5) P_2 is functional *in vivo* in the plant kingdom.

Extensive labelling of *Spirodela* with [^3H]inositol also facilitated identification of GroPIns(3,4) P_2 . At no stage during either [^3H]inositol or [^{32}P] P_i labelling did we observe a compound with the chromatographic characteristics of a putative GroPIns(3,4,5) P_3 , which should be eluted between Ins(1,4,5) P_3 and Ins(1,3,4,5) P_4 , which are resolved on this gradient.

The identification of GroPIns(3,4) P_2 reported here shows that the plant kingdom has the full complement of phosphoinositides commonly identified in animal cells except PtdIns(3,4,5) P_3 , identified in certain stimulated animal cells (Stephens et al., 1991). Nevertheless, the possibility arises that PtdIns(3,4,5) P_3 may yet be found in stimulated plant cells.

Together with our prior identification of PtdIns3 P (Brearley and Hanke, 1992), the identification of PtdIns(3,4) P_2 raises the further question of how the 3-phosphorylated lipids are synthesized. The almost exclusive labelling of the 3-phosphomonoester in preference to the phosphodiester of PtdIns3 P indicates that the 3-phosphate is added after the diester phosphate. This does not, however, prove that PtdIns3 P is made by 3-phosphorylation of PtdIns. The distribution of label between the monoester phosphates of PtdIns(3,4) P_2 precludes the possibility that PtdIns(3,4) P_2 is synthesized by 3-phosphorylation of PtdIns4 P .

Rather, the 3-phosphate is added before the 4-phosphate, which also discounts 3-phosphorylation of $\text{PtdIns}(4,5)\text{P}_2$ and subsequent 5-dephosphorylation as a route of synthesis of $\text{PtdIns}(3,4)\text{P}_2$. $\text{PtdIns}3\text{P}$ may thus be made either by 3-phosphorylation of PtdIns or by 4-dephosphorylation of $\text{PtdIns}(3,4)\text{P}_2$. In summary, the metabolic evidence presented here suggests that, of the three possible metabolic schemes variously proposed for the synthesis of 3-, 4- and 5-phosphorylated phosphoinositides in animal cells by Stephens et al. (1991), by Whitman and Cantley (1988) and by Majerus et al. (1990), the scheme proposed for platelets by Majerus et al. (1990) fits best the evidence presented here, i.e. that successive 3- and 4-phosphorylation of PtdIns generates $\text{PtdIns}3\text{P}$ and $\text{PtdIns}(3,4)\text{P}_2$. By extension, we might expect to find $\text{PtdIns}(3,4,5)\text{P}_3$ synthesized by 5-phosphorylation of $\text{PtdIns}(3,4)\text{P}_2$.

By corollary, we can now assume that plants are likely to possess not only the PtdIns 4-kinase and $\text{PtdIns}4\text{P}$ 5-kinase activities described by Sommarin and Sandelius (1988) and the lysophosphatidylinositol kinase activity described by Wheeler et al. (1991), but also kinase activities, previously unsuspected, capable of phosphorylating PtdIns in the 3-position and/or $\text{PtdIns}3\text{P}$ in the 4-position. In this context it is noteworthy that a $\text{PtdIns}3\text{P}$ 4-kinase activity, distinct from PtdIns 4-kinase, has recently been characterized in human erythrocytes (Graziani et al., 1992). A re-examination of the enzymology of plant phosphoinositide metabolism would therefore appear timely.

It is also likely that the early report of phosphoinositide phosphomonoesterase activity in the unicellular green alga *Dunaliella salina* (Einspahr et al., 1989) will be substantiated in higher plants.

It is clear from the above that our perception of inositol involvement in cell signalling in plants will need to be expanded to accommodate the 3-phosphorylated lipids identified in *Spirodela polyrrhiza* L. The possibility arises that, as in animal cells, inositol phosphate second messengers other than $\text{Ins}(1,4,5)\text{P}_3$ may yet be identified.

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REFERENCES

- Blatt, M. R., Thiel, G. and Trentham, D. R. (1990) *Nature* (London) **346**, 766–769
- Boss, W. F. (1989) in *Second Messengers in Plant Growth and Development* (Boss, W. F. and Morré, D. J., eds.), pp. 29–56, Wiley-Liss, New York
- Brearley, C. A. and Hanke, D. E. (1992) *Biochem. J.* **283**, 255–260
- Coté, G. G., De Pass, A. L., Quarby, L. M., Tate, B. F., Morse, M. J., Satter, R. L. and Crain, R. C. (1989) *Plant Physiol.* **90**, 1422–1428
- Drøbak, B. K., Ferguson, I. B., Dawson, A. P. and Irvine, R. F. (1988) *Plant Physiol.* **87**, 217–222
- Einspahr, K., Peeler, T. C. and Thompson, G. A., Jr. (1989) *Plant Physiol.* **90**, 1115–1120
- Gilroy, S., Read, N. D. and Trewavas, A. J. (1990) *Nature* (London) **346**, 769–771
- Graziani, A., Ling, L. E., Endemann, E., Carpenter, C. and Cantley, L. C. (1992) *Biochem. J.* **284**, 39–45
- Hanke, D. E., Biffen, M. and Davies, H. (1990a) *Symp. Soc. Exp. Biol.* **44**, 193–205
- Hanke, D. E., Davies, H., Biffen, M., Connett, R. J. A. and Freathy, T. C. (1990b) in *Plant Growth Substances 1988* (Pharis, R. P. and Rood, S. B., eds.), pp. 161–172, Springer-Verlag, Berlin
- Hawkins, P. T., Kirk, C. J. and Michell, R. H. (1984) *Biochem. J.* **218**, 785–793
- Hawkins, P. T., Stephens, L. R. and Downes, C. P. (1986) *Biochem. J.* **238**, 507–516
- Irvine, R. F., Letcher, A. J., Lander, D. J., Drøbak, B. K., Dawson, A. P. and Musgrave, A. (1989) *Plant Physiol.* **89**, 888–892
- Majerus, P. W., Ross, T., Cunningham, T., Caldwell, K., Bennett, A. B., Jefferson, A. and Bansal, V. (1990) *Cell* **63**, 459–465
- Smart, C. C. and Trewavas, A. J. (1983) *Plant Cell Environ.* **6**, 507–514
- Sommarin, M. and Sandelius, A. S. (1988) *Biochim. Biophys. Acta* **958**, 268–278
- Stephens, L. R. and Downes, C. P. (1990) *Biochem. J.* **265**, 435–452
- Stephens, L. R., Hawkins, P. T., Barker, C. J. and Downes, C. P. (1988) *Biochem. J.* **253**, 721–733
- Stephens, L. R., Hawkins, P. T. and Downes, C. P. (1989) *Biochem. J.* **259**, 267–276
- Stephens, L. R., Hughes, K. T. and Irvine, R. (1991) *Nature* (London) **351**, 33–39
- Wheeler, J. J., Gross, W., Assefa, A. and Boss, W. F. (1991) *Biochim. Biophys. Acta* **1086**, 310–316
- Whitman, M. and Cantley, L. (1988) *Biochim. Biophys. Acta* **948**, 327–344
- Whitman, M., Downes, C. P., Keeler, M., Keller, T. and Cantley, L. (1988) *Nature* (London) **322**, 644–646
- Wreggett, K. A. and Irvine, R. F. (1989) *Biochem. J.* **262**, 997–1000