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Three Distinct Lipid Kinase Activities Are Present in Spinach Chloroplast Envelope Membranes: Phosphatidylinositol Phosphorylation Is Sensitive to Wortmannin and Not Dependent on Chloroplast ATP

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Chloroplast envelope membranes display properties that are important in lipid synthesis, regulation of metabolites, and protein transport, as well as in signal transduction. The recent discovery showing that phosphorylation of lipids occurs in envelope membranes provides a new approach for understanding the role of chloroplast lipids in these processes. The present investigation shows that three major lipid kinase activities are at least present in envelope membranes. These activities greatly depend on external conditions, such as pH, ATP concentrations, temperature, and chloroplast ATP and wortmannin sensitivity. Two types of phosphorylated lipid couples displayed similar intrinsic responses toward these biochemical parameters, namely phosphatidic acid (PA) and its lysoderivative (LPA) and monogalactosyl-phosphate-diacylglycerol (MGpDG) and its lysoderivative (LMGpDG), but not phosphatidylinositol-monophosphate (PIP) and its lysoderivative (LPIP). Phosphorylation of phosphatidylinositol was not dependent on chloroplast ATP, but was sensitive toward wortmannin in intact chloroplasts and outer envelope membrane vesicles.

Key Words: **chloroplast envelope; lipid phosphorylation; lipid kinase; monogalactosyldiacylglycerol; phospholipid; *Spinacia oleracea*.**

Abbreviations used: DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; DGPP, diacylglycerol pyrophosphate; DGK, diacylglycerol kinase; HPTLC, high-performance TLC; LPA, L-α-lysophosphatidic acid; LPIP, L-α-lysophosphatidyl-inositol 4-monophosphate; MGDG, monogalactosyldiacylglycerol; MGpDG, monogalactosyl-phosphate-diacylglycerol; LMGpDG, lysomonogalactosyl-phosphate-diacylglycerol; PA, L-α-phosphatidic acid; PI, L-α-phosphatidyl-inositol; PIP, L-α-phosphatidyl-inositol 4-monophosphate; PIP_2, L-α-phosphatidyl-inositol 4,5-diphosphate; $PI(3,5)P_2$, L-α-phosphatidyl-inositol 3,5-diphosphate; $PI(4,5)P_2$, L-α-phosphatidyl-inositol 4,5-diphosphate; IP_3, inositol 1,4,5-triphosphate; $IP(1,4,5)P_3$, inositol 1,4,5-triphosphate; PS, L-α-phosphatidyl-L-serine; TLC, thin-layer chromatography.

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Lipid phosphorylation is one of the main pathways involved in signal transduction, enzyme activation, and stress responses in eukaryotes. As in animal cells, several lipids, namely DAG, PA, PI, and PIP, are known to undergo phosphorylation in plants, thereby generating new lipid classes (1). So far, most of the knowledge in this field essentially originates from studies in plasma membranes, but very little is known about the presence of lipid kinase activities in membranes of plant organelles. Indeed, chloroplasts, mitochondria, the endoplasmic reticulum, glyoxysomes and other vesicular membranes are all putative sites for lipid phosphorylation. Concerning the chloroplast, lipid phosphorylation occurs in envelope membranes, but not in thylakoid membranes, and galactolipids are substrates for CTP-dependent kinase(s) (2, 3).

Diacylglycerol, which can be produced by the enzymatic activity of phospholipase C within membranes, can itself be a second messenger molecule and a substrate for DAG kinase (DGK) (4). This enzyme has been mainly characterized in plasma membranes (5, 6) and cloned from *Arabidopsis thaliana* cDNA (AtDGK1; D63787) (7). Interestingly, AtDGK1 exhibits sequence similarities with mammalian DGK, thereby suggesting similar activities to the animal membrane enzyme. DAG, which is a transient compound found in the cell, exists in plants as a wide range of molecular species (8), thereby rendering difficult the study of DAG kinase(s) substrate specificity and its cell location. The presence of a DGK activity associated with chloroplast envelope membranes was first described in (2). In addition, DAG was reported to be the main hydrolysis product appearing during the preparation of envelope membranes (9). This observation certainly increases the difficulty for future assessments of the role of the DGK activity within chloroplast envelope membranes.

Phosphatidic acid (PA), that is generated by the lipid biosynthesis pathway or is the product of DGK activity, is itself the substrate for another lipid kinase present

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in plant plasma membranes, namely phosphatidic acid kinase. Its phosphorylated product (DGPP) was first identified in microsomal membranes of *Catharanthus roseus* cell cultures (10, 11), but also in Chlamydomonas (12), yeast and bacteria (4 and references therein). However, the role of DGPP in plant membranes is still controversial and its presence in other plant membranes and cell compartments has not been reported yet.

The phosphorylation mechanism using PI, as the substrate molecule, is one of the best-known lipid biochemical reactions occurring in plants (13). Indeed, it seems now clear that two types of PI kinase (PI-3- and PI-4-kinases) are involved in the vesicle trafficking and membrane biogenesis. Both are inhibited at various concentrations by wortmannin. Concerning PI-4-kinase, two different genes have been cloned from *A. thaliana*, i.e., PI-4-kinase-α and PI-4-kinase-β. PI-4-kinase activity has been found in different subcellular compartments suggesting that PIP displays different physiological functions depending on its specific location. In addition, the generated phosphorylated phosphatidylinositol can be substrate for PI(3)P(5)-kinase or PI(4)P(5)-kinase and produce $PI(3,5)P_2$ or $PI(4,5)P_2$. The latter is postulated to be involved in cell elongation, due to its high turnover rate linked to the production of $IP(1,4,5)P_3$. Indeed, IP_3 is suspected to mediate Ca^{2+} oscillations involved in plant growth controlling pathways. Up to now, the existence of PI kinase activities within chloroplast envelope membranes was only supported by *in vitro* labeling experiments using [γ-^{32}P]ATP (2).

More and more, chloroplast envelope membranes appear to be a cellular subcompartment that carries vital functions for all plant cells, not only regarding plastid biogenesis but also interactions with other organelles in controlling and regulating exchanges and export of carbon assimilates (14, 15). Lipid phosphorylation obviously offers one possible mechanism for achieving this purpose. The present investigation was aimed at characterizing the different lipid kinase activities present in chloroplast envelope membranes by varying several parameters such as ATP concentrations, endogenous chloroplast ATP, pH, temperature, and wortmannin sensitivity. Our results show that (i) at least three distinct lipid kinase activities occur in chloroplast envelope membranes and (ii) the PI phosphorylation process is confined to the chloroplast outer envelope membrane.

MATERIALS AND METHODS

Materials. Spinach (*Spinacia oleracea L.*) leaves were purchased from the local market. [γ-^{32}P]ATP (110 TBq $\times$ mmol^{-1}) was obtained from Amersham-Rhan (Zürich, Switzerland) or Hartmann Analytic (Braunschweig, Germany); PA, LPA, PS, and phosphoinositides were purchased from Sigma. Thin-layer chromatography plates (TLC- or HPTLC-plates, Silica Gel 60 precoated) were provided by Merck (Darmstadt, D). Autoradiography BIOMAX-MS films were purchased from Kodak (Eastman Kodak Co., New York, NY).

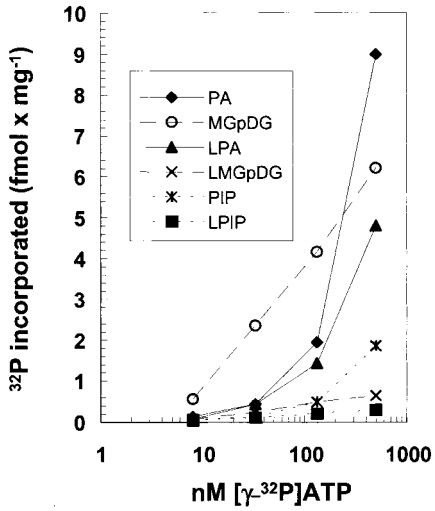

FIG. 1. Lipid phosphorylation in chloroplast envelope membranes in the presence of increasing concentrations of [γ-^{32}P]ATP. Envelope membranes were incubated with 8, 33, 130, and 500 nM of [γ-^{32}P]ATP (110 TBq $\times$ mmol^{-1}), respectively. Lipids were then extracted, separated by TLC, revealed by autoradiography, and estimated by Cerenkov counting, as indicated under Materials and Methods. The graph represents the ^{32}P incorporated (fmol $\times$ mg^{-1}) in each envelope lipid after a 5-min incubation.

Preparation of intact chloroplasts and envelope membranes. Intact chloroplasts were purified as described in (16). Envelope membranes were basically isolated from intact chloroplasts according to the method of Douce and Joyard (17). Outer envelope membranes were prepared according to the method of Keegstra and Youssif (18), adapted by Siegenthaler and Dumont (19). The protein concentration of chloroplast envelope membranes was determined as described by Bradford (20) and Lowry (21).

Lipid kinase activity. Aliquots of chloroplast envelope membranes (200 μg) were incubated in the presence of 0.01 mM [γ-^{32}P]ATP (0.375 TBq $\times$ mmol^{-1}), 50 mM Mops–NaOH, pH 7.6, and 5 mM $MgCl_2$, at 25°C in a total volume of 200 μl (unless otherwise indicated in the legends of the figures). The reaction was started by the addition of envelope membranes and stopped after 5 min by laying the samples in a ice cold glass vial containing 1.5 ml of cold chloroform/methanol (1:2).

Two-phase extraction of membrane lipids. After laying the samples on 1.5 ml of cold chloroform/methanol (1:2, v/v), 100 nmol of unlabeled PA and LPA, 600 nmol of a mixture of unlabeled phosphoinositides (PIP, PIP_2, PI, and PS) used as carriers, 100 μmol MgATP, 0.8 ml HCl/EDTA–Na_2 (1.25 N:0.5 mM) and 0.5 ml cold chloroform were successively added. After vortexing thoroughly, the formation of the two phases was terminated by centrifugation at 3000*g* for 2 min. The lower phase was washed with 1 ml of cold 1 N methanol/HCl (1:1, v/v) and then with 1 ml of cold methanol/H_2O/25% NH_3 (10:8:2, v/v/v). The lower phase containing the lipids was dried under nitrogen and dissolved in 100 μl chloroform/methanol (3:1, v/v) (2).

Separation of phospholipids. The samples were spotted on silica gel plates which were preactivated with a solution of potassium oxalate and heated at 110°C for 20 min just before use (22). The plates were developed with chloroform/acetone/methanol/acetic acid/water (40:15:13:12:8, v/v/v/v/v) at room temperature for 45 min. The lipids were visualized under UV light after spraying a solution of

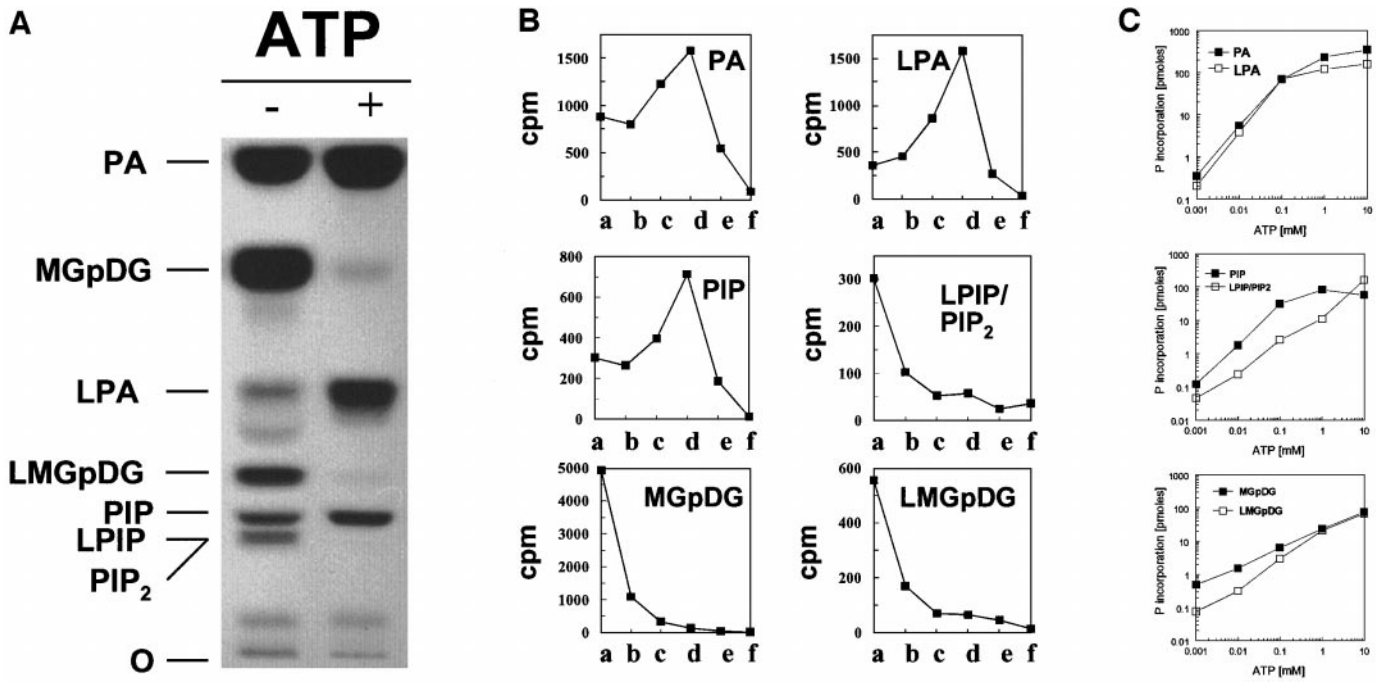

FIG. 2. Effect of various concentrations of cold ATP on the [^{32}P] labeling of envelope membrane lipids. The phosphorylation of envelope vesicles, lipid extraction and TLC analysis were performed as indicated under Materials and Methods. In A, minus (−) ATP corresponded to 10 μCi [γ-^{32}P]ATP (110 TBq × mmol^{-1}) and plus (+) ATP to 10 μCi [γ-^{32}P] ATP (110 TBq × mmol^{-1}) supplemented with 0.01 mM unlabeled ATP. In B, the graphs represent the effect of increasing concentrations of cold ATP (a to f, see below) on the [^{32}P] incorporation in each lipid. All media contained 0.37 MBq of [γ-^{32}P]ATP (110 TBq × mmol^{-1}). The specific activities are, respectively: a (0 mM ATP), 110 TBq × mmol^{-1}; b (0.001 mM ATP), 3.75 TBq × mmol^{-1}; c (0.01 mM ATP), 0.375 TBq × mmol^{-1}; d (0.1 mM ATP), 37.5 GBq × mmol^{-1}; e (1 mM ATP), 3.75 GBq × mmol^{-1}; f (10 mM ATP), 0.375 GBq × mmol^{-1}. C shows the phosphate incorporation in each lipids (shown in B) versus the ATP concentrations. Calculations were done on the basis of the phosphate incorporation in the presence of carrier free [γ-^{32}P]ATP (33 nM), assuming that the phosphate is equally incorporated at low and high ATP concentrations.

acetone/water (60:40, v/v) containing 0.01% primuline (w/v). The ^{32}P-labeled lipids were detected using Kodak X-Omat AR films, scrapped from the plate, and quantified by Cerenkov counting.

Separation of PIP isomers. Envelope labeled PIP was scrapped from the silica gel plate and extracted with a solvent made of 2 ml chloroform/methanol (53:37, v/v) and 1 ml 500 mM KCl. The mixture was then thoroughly shaken (Vortex) for 1 min, and thereafter spin down at 2100*g* for 1 min. The bottom phase was evaporated under a stream of N_2 and finally dissolved in 20 μl of a solvent containing chloroform/methanol (3:1, v/v). The lipids were separated on borated HPTLC–NH_2 plates as described by Hegewald (23). Finally, the lipids were visualized as explained previously.

RESULTS

When spinach chloroplast envelope membranes are incubated in the presence of [γ-^{32}P]ATP, four main labeled lipids (PA, LPA, PIP, and LPIP) can be separated on TLC plates (2), as well as two newly identified lipids, namely MGpDG and L-MGpDG (3). Figure 1 shows that increasing concentrations of carrier free [γ-^{32}P]ATP enhanced, though to different extents, the labeling of all phosphorylated lipids. Although the labeling of PA, LPA and MGpDG was much greater than that of LMGpDG, PIP, and LPIP, the labeling curves displayed similarities within the phosphorylated lipid couples PA/LPA, MGpDG/LMGpDG.

Figure 2A illustrates the incorporation of ^{32}P in envelope membrane lipids in the absence (−) and presence (+) of 0.01 mM nonradioactive ATP. It can be seen that the incorporation of ^{32}P in MGpDG, LMGpDG and LPIP was nearly totally abolished by ATP. In contrast, the incorporation of ^{32}P in PA, LPA, and PIP was enhanced by an addition of ATP in the medium. To further investigate this phenomenon, envelope membranes were incubated in the presence of a constant concentration of [γ-^{32}P]ATP (0.37 MBq, 33 nM) and supplemented with increasing concentrations of nonradioactive ATP. Figure 2B shows that under these conditions, the incorporation of ^{32}P in lipids displayed two quite different types of response. The first corresponded to an increase of ^{32}P incorporation in PA, LPA and PIP, up to an ATP concentration of 0.1 mM (a = 0 mM ATP, b = 0.001 mM, c = 0.01 mM, d = 0.1 mM), followed by a strong decrease of ^{32}P incorporation in these three lipids with ATP concentrations greater than 0.1 mM (e = 1 mM ATP, f = 10 mM). The second type of response, which concerns MGpDG, LMGpDG and LPIP, corresponded to a strong decrease of ^{32}P incorporation when ATP was added to the medium (b to f). These results may explain why MGpDG and LMGpDG were first not detected in chloroplast enve-

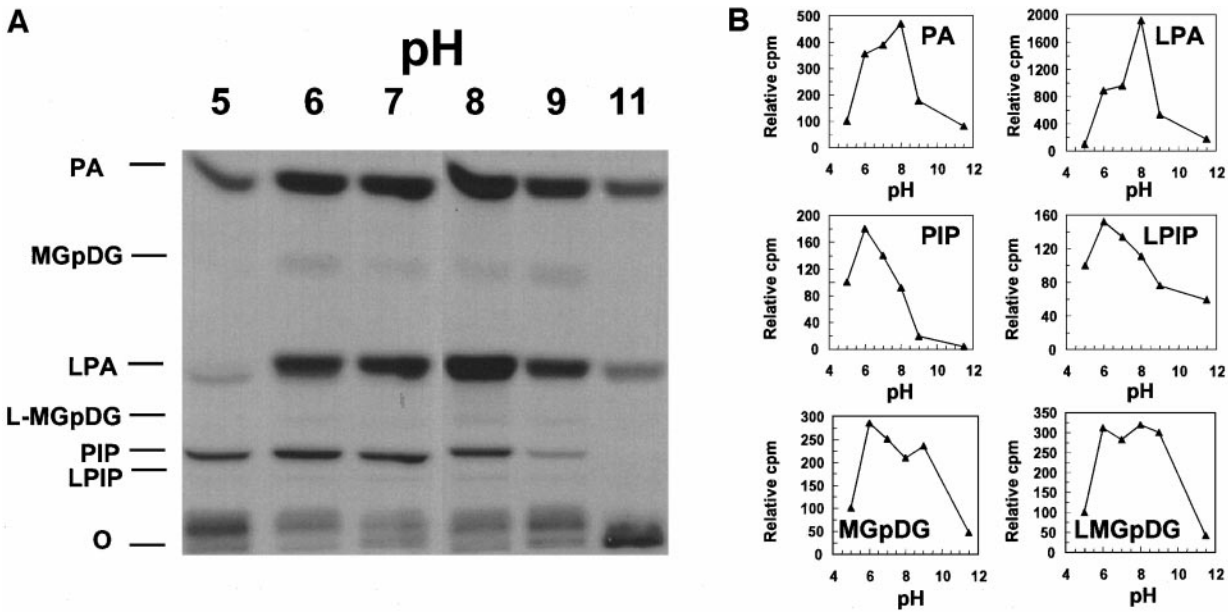

FIG. 3. Effect of pH on the labeling of PA, LPA, PIP, LPIP, MGpDG, and LMGpDG in envelope vesicles incubated with 0.01 mM [γ-^{32}P]ATP (0.375 TBq $\times$ mmol^{-1}). The buffers used were Mes (pH 5 to 7), Mops (pH 7 to 9) and Hepes (pH 9 to 11). The overlapping pH values were closed to each other (<10%). A illustrates the autoradiography of the phosphorylated lipids and B the [^{32}P]incorporation in each lipid after Cerenkov counting of the scrapped ^{32}P-labeled lipids. For comparison, the 100% values represented in the graphs (relative cpm) corresponded to 1970, 510, 1440, 120, 160, and 50 cpm for PA, LPA, PIP, LPIP, MGpDG, and LMGpDG, respectively.

lope preparations (2). Indeed, in this early report, the incubation medium contained 0.01 mM nonradioactive ATP, thus preventing the detection of these phosphorylated lipids. If one considers that the radioactive phosphate is incorporated into lipids with the same efficiency in the absence or presence of nonradioactive ATP (Fig. 2C), the MGpDG and LMGpDG couple and the PA and LPA couple have therefore their own intrinsic phosphorylation processes. This observation is however not valid for PIP and LPIP (Figs. 2B and 2C), as also illustrated in Fig. 1.

Figure 3 illustrates the effect of pH on the incorporation of ^{32}P in the six major phosphorylated lipids of envelope membranes. The pH-dependency offers a good criterion for distinguishing between three kinds of lipid-kinase activities (Fig. 3B). The first resulted in the formation of PA and LPA and had a pH optimum of 8. The second displayed an optimum pH at 6 and was responsible for the incorporation of ^{32}P into PIP and LPIP. The last catalyzed the incorporation of ^{32}P into MGpDG and LMGpDG, and had a broader pH optimum extending from 6 to 9. At pH 5, a new phosphorylated lipid was detectable just under the Rf position of LPA (Fig. 3A). This new phosphorylated lipid could be ^{32}P-labeled PA (diacylglycerol pyrophosphate, DGPP), since it comigrated with it on TLC plate (unlabeled DGPP was a kind gift of Dr. J. B. Wissing, data not shown). Interestingly, greater pH inhibited the incorporation of ^{32}P in this lipid (DGPP), thereby being undetectable at neutral pH.

The time dependency of ^{32}P incorporation in envelope membrane lipids was investigated by using intact chloroplasts (Fig. 4). The rate profiles of ^{32}P incorporation in the two pairs of lipids (i.e., MGpDG/LMGpDG and PA/LPA, respectively) were quite different. The maximal level of the two phosphorylated galactolipids was

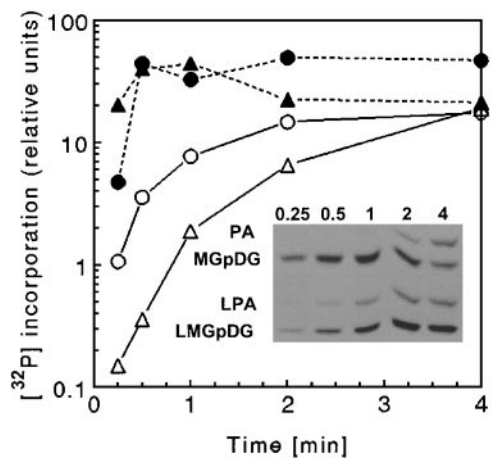

FIG. 4. Time-dependent lipid phosphorylation in intact chloroplasts. Intact chloroplasts (0.12 mg Chl) were incubated in the presence of [^{32}P]Pi (1.85 MBq), 10 mM $MgCl_2$, 330 mM sorbitol, 50 mM Mops–NaOH, pH 7.6, 1 mM $NaHCO_3$ and light (12,000 Lux) at 20°C (in a final volume of 200 μl), before lipid extraction. Lipids were then separated by TLC and autoradiographed (inset). The time-dependent ^{32}P incorporation in PA (●), LPA (○), MGpDG (▲), and LMGpDG (△) was determined by Cerenkov after scrapping labeled lipids.

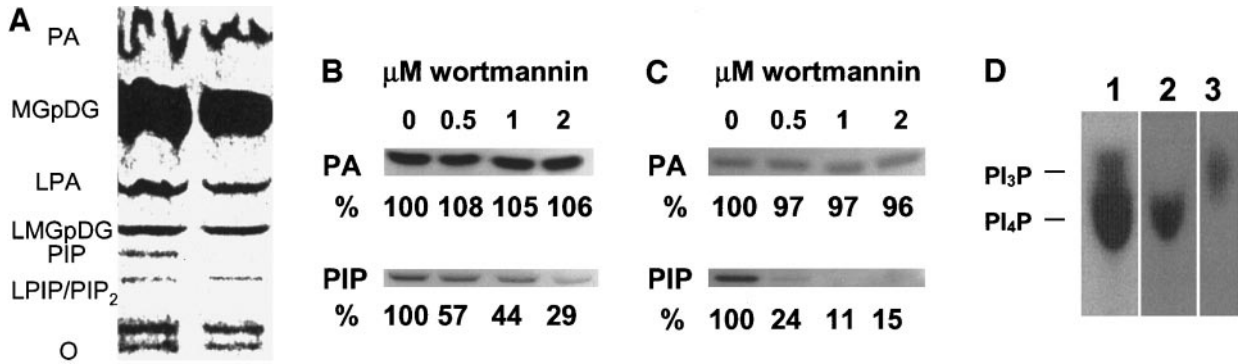

FIG. 5. Intact chloroplasts were treated with (+) or without (−) 10 μM wortmannin at 4°C for 5 min before phosphorylation (as described in the legend to Fig. 4, except that [^{32}P]Pi was replaced by [γ-^{32}P]ATP (0.37 MBq)). Lipids were then extracted, separated by TLC and autoradiographed (A). Total (B) or outer (C) envelope membranes were incubated in the presence of increasing concentrations of wortmannin for 5 min before adding [γ-^{32}P]ATP. The figure shows the autoradiography of the lipids PA and PIP and their relative [^{32}P] incorporation. In D, lane 1 shows the separation of scrapped and eluted [^{32}P]PIP (equivalent to that depicted in Fig. 2A, in the absence of wortmannin) using HPTLC–NH_2–borate-impregnated plates. The separation of PI-4-P and PI-3-P, scrapped from lane 1 and extracted, was achieved on the same impregnated plates (lanes 2 and 3).

reached after 30 s. By contrast, the rate of PA and LPA phosphorylation was slower and 4 min was required to reach the beginning of the plateau. On the other hand, phosphorylated PI derivatives were not detected in this experiment, indicating that chloroplastic ATP was not a substrate source for PI or LPI kinases.

Another approach for investigating different types of lipid kinases in chloroplast envelope membranes was to use the selective properties of wortmannin toward PI kinase activities. This drug specifically inhibits PI-3-kinase activity, but also some isoforms of PI-4-kinase activity (13, 24). To this end, intact chloroplasts were preincubated with wortmannin (+) before lipid phosphorylation in the presence of [γ-^{32}P]ATP. The data show that PI, only, is not phosphorylated in the presence of the drug, thereby suggesting that wortmannin specifically inhibited (93% inhibition) envelope PI kinase activities (Fig. 5A). To confirm this point, total envelope membranes (Fig. 5B) and outer envelope membranes (Fig. 5C) were incubated with various concentrations of wortmannin. As can be seen, the PI-kinase activity of both envelope preparations, but not the DGK activity (serving as control in this experiment), was inhibited by wortmannin. It is noteworthy that in outer envelope membranes, the activity was much more sensitive to the drug (76% inhibition, compared to 43% inhibition in total envelope membranes, at a concentration of 0.5 μM wortmannin). This observation is not surprising since PI and PI phosphorylated derivatives are known mainly confined to the outer envelope membranes (3, 25). The third set of experiments consisted of separating the labeled PIP in total envelope membranes using a TLC system allowing the identification of PI_3P from PI_4P (23). Figure 5D clearly shows that PIP appearing in one band under standard TLC systems (lane 1), segregates into two isoforms, likely to be PI_3P and PI_4P, on borate-impregnated HPTLC–NH_2 plates (lanes 2 and 3).

DISCUSSION

The results presented here show that at least three different envelope lipid kinase activities, leading to the formation of three phosphorylated lipid couples, i.e., PA/LPA, MGpDG/LMGpDG and PIP/LPIP, take place within the chloroplast envelope membranes. Each of the two first activities generating the lipid pairs PA/LPA and MGpDG/LMGpDG were equally regulated by ATP concentrations (Figs. 1 and 2), pH (Fig. 3), temperature (data not shown) and endogenous chloroplast ATP (Fig. 4). Concerning the formation of PIP and LPIP by a unique lipid kinase, the data were not clear-cut, although the kinase sensitivity leading to their formation was similar regarding pH and chloroplast ATP. However, the LPIP and PIP formation profiles were different with respect to ATP concentrations (Figs. 1 and 2) and temperature variations (data not shown). This difference is probably due to the fact that the phosphorylated lipids co-migrating with LPIP and PIP_2 markers were not only LPIP (2) when performing phosphorylation experiments in the presence of 0.01 mM ATP, but also PIP_2 as the result of a PIP kinase activity preferentially working in the presence of carrier free [γ-^{32}P]ATP (see Fig. 2). Indeed, PIP_2 is only a minor component of the total cellular lipids (4), thereby rendering difficult its identification in chloroplast envelope membranes.

Interestingly, the data presented in Fig. 4 show that ATP formed in intact chloroplast in the presence of [^{32}P]Pi and light is not a substrate for inositol lipids within the envelope membranes. This observation suggests that their phosphorylation occurs at the outer

side of the inner envelope membrane, i.e., in the vicinity of the envelope intermembrane space or at the cytosolic face of the outer envelope membranes. A marked inhibition (93%) of the PIP labeling was found in intact chloroplasts pretreated with wortmannin (Fig. 5A), thereby attesting the accessibility of PI-kinase(s) by wortmannin in intact chloroplasts. The data presented in Figs. 5B and 5C show that the sensitivity of PI-kinase(s) toward wortmannin was also found in total and outer envelope membrane vesicles. The inhibition is higher in outer envelope membrane preparations than in the whole envelope fractions, thus strongly suggesting the confinement of PI substrate (25) and phosphorylated products [see above and in (2)] in the outer envelope membrane. Figure 5D clearly shows that PI-3-P, as well as PI-4-P are produced in the envelope membranes, thus suggesting the presence of PI_3- and PI_4-kinases in the outer envelope membranes. This could also explain why the formation of PIP and LPIP in the presence of increasing ATP concentrations (Figs. 1 and 2) and at different temperatures (data not shown) did not display the same pattern, and therefore, could result from the activity of the two distinct lipid kinases. PI-3-P kinase was also found to be associated with the cytoskeleton of plant cell from *Daucus carota L.* (26). Like in the plastid envelope membranes, PI_3-kinase of the cytoskeleton was inhibited with high levels of wortmannin, in opposition to the mammal and yeast cell enzyme (24). However, in chloroplast envelope membranes, the major phosphorylated pool of phosphatidylinositol corresponded to PI-4-P (Fig. 5D), thereby indicating that the PI_4-kinase(s) of envelope membranes was also sensitive to wortmannin, but rather at elevated concentrations (500 nM), as already reported for some other PI_4-kinases (24, 27). The optimum pH for PI_4-kinase(s) was around 6 in envelope membranes (Fig. 3) and not similar to that of other plant material compartments, i.e., pH 8 (28, 29) or 6.5–7 (30).

According to the evolutionary theory, plastids in plant cells may originate, owing to an endosymbiotic process, from photosynthesizing prokaryotes such as cyanobacteria (31, 32). Indeed. the presence of PI, PIP, LPIP (PIP_2?) in the outer envelope membrane of chloroplasts could result from engulfing plasma membranes of the phagotrophic organism, since phosphorylated PI has been reported to be mainly localized in plasma membrane of plant and animal cells (13).

A particular feature, that is still unsolved, concerns the origin of phosphorylated lysoderivatives in chloroplast envelopes. According to our data, this compounds likely derive from postphosphorylation phospholipase activities. Indeed, (i) large unlabeled LDAG and LMGpDG pools were never found in any chloroplast fractions and (ii) LMGpDG has been reported to be induced after CTP-chase experiments *in vitro* (3) and *in organello* after 2 min incubation with [^{32}P]Pi (Fig. 4).

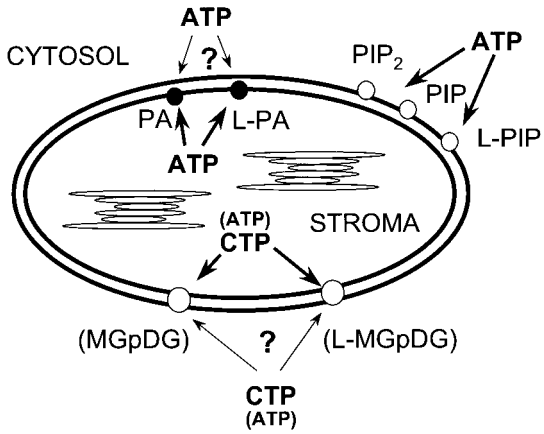

FIG. 6. Schematic representation of the different lipid phosphorylation sites in chloroplast envelope membranes. The PI phosphorylated derivatives are located in outer envelope membranes and use cytosolic ATP as substrate. PA and LPA are preferentially located in the inner envelope membranes using chloroplast, as well as external sources of ATP. Concerning MGpDG and LMGpDG, their CTP-dependent phosphorylation has been clearly demonstrated (3) and likely occurs at the inner face of the inner envelope membrane.

The experiments involving increasing amounts of ATP (Figs. 1 and 2) show that envelope lipid phosphorylation is under the close control of ATP concentration levels. This may indicate that the energetic charge of the cell, in particular within the chloroplast, may be one of the prominent factors regulating envelope lipid phosphorylation. In this respect, one can postulate that MGpDG is constitutively present in the envelope membranes, since the labeling of MGDG is the strongest at low ATP concentrations (nanomolar range, Figs. 1 and 2). Interestingly, such an observation was also reported for the chloroplast phosphoglucomutase (a 67-kDa phosphoprotein) (16, 33), but not for the pool of phosphorylated SSu and LHCII trapped in the envelope membranes (34). Therefore the activity of the 67-kDa phosphoglucomutase [associated with envelope vesicles preparations (33)] might be linked to MGDG phosphorylation [possibly by CTP (3)], when considering the hexose-phosphate turn-table. Indeed MGpDG is itself a potential source of disposal energy, particularly if the phosphorylated galactolipid (galactose 6-P) is removed by hydrolysis from the lipid bone and further transformed into glucose 6-P.

One of the roles of DGK activity in envelope membranes could be the regulation of the biosynthetic pathways of lipids, conjointly with the PA phosphatase of the envelope membrane (35). Other roles for DGK activity products in envelope membranes could be related to the activation of protein kinases (36, 37), PI-4-P kinases (38) and PKC-like enzyme (39). Furthermore, PA and LPA could be involved in signal transduction systems (40–42).

In conclusion, the results of this investigation, as well as those reported earlier (2, 3), strongly suggest

that at least three distinct lipid kinase activities are present in chloroplast envelope membranes. In Fig. 6, the topology of the phosphorylated lipids and their nucleoside triphosphate substrates is indicated.

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