Evidence for lipid kinase activities in spinach chloroplast envelope membranes

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Abstract Three spinach chloroplast envelope membrane preparations (i.e. whole, outer and inner membranes) were incubated in the presence of [γ-^{32}P]ATP. After lipid extraction and separation by TLC, four main phosphorylated lipids were detected by autoradiography in whole envelope preparations. These phospholipids were identified by comparing their Rf with that of lipid markers and by a deacylation procedure. They were found to be phosphatidic acid (PA) and lyso-PA, L-α-phosphatidyl-inositol 4-monophosphate (PIP) and lyso-PIP. These lipids were not equally distributed in the outer and inner envelope membranes. Chloroplast envelope membranes were verified not to be contaminated by plasma membranes. It is concluded that lipid kinase activities are associated with spinach chloroplast envelope membranes.

Key words: Acyl lipid; Chloroplast envelope membrane; Lipid kinase; (Lyso) phosphatidic acid; (Lyso) L-α-phosphatidyl-inositol 4-monophosphate; *Spinacia oleracea L.*

1. Introduction

It has been shown that lipids such as diacylglycerol, phosphoinositides, phosphatidate and lysophosphatidate display important roles in lipid biosynthesis as well as in signal transduction processes and regulation of protein kinase activity in animal and plant cells [1–7]. The chloroplast which is actively involved in lipid biosynthesis [8] and needs to communicate with the rest of the cell by exchanging several metabolites, ions and messengers may use similar mechanisms to sense its environment. Although lipid kinase activities in plant cell membranes, especially in plasma membranes [7,9,10], have been extensively studied over this last decade, lipid phosphorylation in chloroplast envelope membranes has not yet been reported. The aim of this investigation is to determine whether spinach chloroplast envelope membranes display lipid kinase activities. This has been achieved by incubating whole, outer and inner chloroplast envelope membranes with [γ-^{32}P]ATP followed by the identification and localization of the lipid kinase products.

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Abbreviations: DAG, diacylglycerol; DGDG, digalactosyldiacylglycerol; GP, L-glycerol-3-phosphate; GPC, L-α-glycerophosphorylcholine; GPG, L-α-glycerophosphorylglycerol; GPIP, L-α-glycerophospho-D-myo-inositol-4-monophosphate; $GPIP_2$, L-α-glycerophospho-D-myo-inositol-4,5-bis-phosphate; HPTLC, high performance TLC; LMW, low molecular weight; LPA, L-α-lysophosphatidic acid; LPIP, L-α-lysophosphatidyl-inositol 4-monophosphate; MGDG, monogalactosyldiacylglycerol; Mops, 3-(*N*-morpholino)propane sulfonic acid; PA, L-α-phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, L-α-phosphatidyl-inositol; PIP, L-α-phosphatidyl-inositol 4-monophosphate; PIP_2, L-α-phosphatidyl-inositol 4,5-diphosphate; PS, L-α-phosphatidyl-L-serine; SQDG, sulfoquinovosyldiacylglycerol; TLC, thin layer chromatography; Tris, Tris(hydroxymethyl)aminomethane

2. Materials and methods

2.1. Materials

Spinach (*Spinacia oleracea L.*) leaves were purchased from the local market. Spinach plasma membranes were a kind gift of Dr. C. Larsson, Department of Plant Biochemistry, Lund University, Sweden. [γ-^{32}P]ATP 110 TBq mmol^{-1} was obtained from Amersham-Rhan (Zürich, Switzerland); PA, LPA, phosphoinositides, GP, GPIP and $GPIP_2$ were purchased from Sigma. Thin layer chromatography plates (TLC or HPTLC plates, silicagel 60 pre-coated) were provided by Merck (Darmstadt, Germany).

2.2. Preparation of envelope membranes

The intact chloroplasts were purified according to [11]. Envelope membranes were isolated from intact chloroplasts according to the method of Douce and Joyard [12]. Inner and outer envelope membranes were prepared according to the method of Keegstra and Youssif [13] adapted by Siegenthaler and Dumont [14]. The protein concentration of plasma and envelope membranes were determined as described in [15] or [16].

2.3. Lipid kinase assay

Aliquots of whole, inner and outer envelope or plasma membranes (200 μg) were incubated in the presence of [γ-^{32}P]ATP (10 μCi), 0.01 mM ATP, 50 mM Mops-NaOH pH 7.6 and 5 mM $MgCl_2$, at 25°C in a total volume of 200 μl. The reaction was started by the addition of ATP and stopped after 5 min by laying the samples on 1.5 ml of cold chloroform/methanol (1:2) in glass vials.

2.4. Protein kinase assay

Aliquots of whole envelope or plasma membranes (20 μg) were incubated in the presence of [γ-^{32}P]ATP (1 μCi), 0.01 mM ATP, 50 mM Mops-NaOH pH 7.6 and 5 mM $MgCl_2$ at 25°C in a total volume of 20 μl. The reaction was started by the addition of ATP and stopped after 5 min by adding 20 μl of sample cocktail (0.125 M Tris-HCl pH 6.8, 0.01% (w,v) bromophenol blue, 4% (w,v) SDS, 12.5% glycerol and 10% mercaptoethanol). The mixture was heated at 95°C for 5 min.

2.5. Two-phase extraction of membranes lipids

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

2.6. 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 [17]. The plates were developed with chloroform/

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aceton/methanol/acetic acid/water (40:15:13:12:8) at room temperature for 45 min. The lipids were visualized under UV light after spraying a solution of aceton/water (60:40) containing 0.01% primuline (w,v). The ^{32}P-labelled lipids were detected using Kodak X-Omat AR films.

2.7. Determination of the nature of phosphorylated lipids

In order to determine the identity of the labelled lipids, they were scrapped from the TLC plates and submitted to a mild deacylation procedure as described in [18]. The resulting labelled polar head groups (deacylation products) were spotted on a HPTLC silicagel 60 plate and developed conjointly with polar head markers in a solvent containing methanol/H_2O (40:10), 26 mM formic acid and 1 mM EDTA-Na_2. The plate was sprayed with a solution containing 1,5% $(NH4)_2MoO_4$ (w/v) in 70% $HClO_4$+0,7% HCl and the polar head markers were visualized as blue spots after 1 h UV light (254 nm) exposure. Finally, the phosphorylated head groups were detected by autoradiography.

2.8. SDS-PAGE

Membrane proteins were loaded on SDS polyacrylamide gels and separated by electrophoresis according to [19].

2.9. 1,3-β-glucan synthase assay

1,3-β-glucan synthase assay was performed according to [20].

3. Results and discussion

After phosphorylation of isolated spinach chloroplast envelope membranes with [γ-^{32}P]ATP, four main labelled lipids (A, B, C, D) were separated on TLC plates, as shown in lane 2 of Fig. 1. The labelling of the lipids A and B was stronger than that of the lipids C and D. A faint additional band (★), also appeared (but not in all experiments) between the Rf of lipids A and B. Based on the Rf of lipid markers, lipids A, B, C and D might be assigned to PA, LPA, PIP and PIP_2, respectively. Lipids usually found in envelope membranes [12] are also shown in Fig. 1 (lane 1). It can be seen that, with the exception of lipid B, none of the phosphorylated lipids comigrated with the envelope membrane lipids that can be visualized by charring.

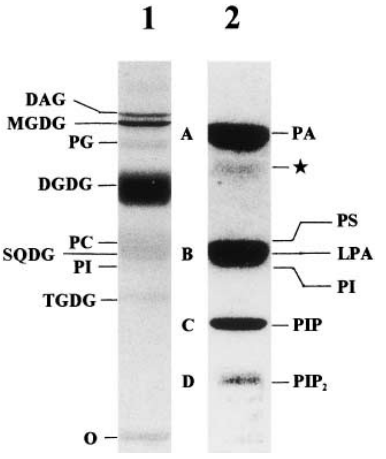

Fig. 1. Separation of lipids and phosphorylated lipids from spinach chloroplast envelope membranes. Lane 1: After extraction and TLC development, lipids were revealed by charring [26]. The names of the lipids are indicated at the left side of lane 1. Lane 2: Autoradiography of the phosphorylated lipids. The envelope membrane lipids were phosphorylated as described in Section 2. The lipids were extracted in the presence of unlabelled lipid carriers (PA, LPA, PS, phosphoinositides), then separated on TLC and revealed by autoradiography. A, B, C and D correspond to the four major phosphorylated lipids. The migration position of unlabelled lipid markers is indicated on the right side of lane 2. The star (★) corresponds to a non-identified phosphorylated lipid.

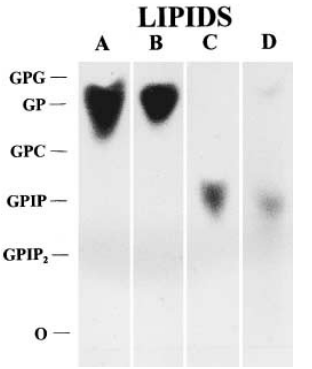

Fig. 2. Autoradiography of the polar heads of phosphorylated envelope lipids A, B, C and D shown in Fig. 1. Phosphorylated envelope membrane lipids A, B, C and D were scrapped and deacylated as described in Section 2. The resulting polar heads were separated on HPTLC plates in the presence of reference markers (GPG, GP, GPC, GPIP and $GPIP_2$). The polar heads were visualized under UV light as described in Section 2 and then detected by autoradiography.

In order to confirm the identity of the lipids A, B, C and D, they were submitted to a mild deacylation and the resulting polar head group of each one was separated on HPTLC and its Rf compared to that of the expected marker. Fig. 2 shows that the polar head of the two lipids A and B was the same and, therefore, corresponded to GP. These results reveal that lipids A and B differ only by the number of their acyl chains and correspond to PA and LPA, respectively, as was expected from their Rf value (see Fig. 1). The polar head group of lipids C and D was the same and comigrated with GPIP. This result confirms the identity of the lipid C as being PIP. In contrast, the deacylation of the lipid D (which was presumed to be PIP_2, see Fig. 1) should result in the formation of $GPIP_2$, which was obviously not the case. This observation strongly suggests that the lipid D corresponds in fact to LPIP. Indeed, when PIP was treated in vitro with the phospholipase A_2 from porcine pancreas, the resulting lyso-derivative (LPIP) comigrated, under our experimental conditions, with PIP_2 (data not shown).

Fig. 3 shows that the lipid pattern and the intensity of the labelled bands were different in whole, outer and inner envelope membrane preparations. In general, the relative intensity

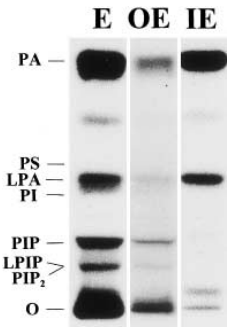

Fig. 3. Comparison of the ^{32}P-labelled lipid pattern in the whole envelope (E), outer (OE) and inner (IE) envelope membranes. The positions of the unlabelled lipid markers are indicated on the left side of the figure. O, origin of the migration. Note that the lipid marker PIP_2 displays the same Rf as LPIP, as explained in the text.

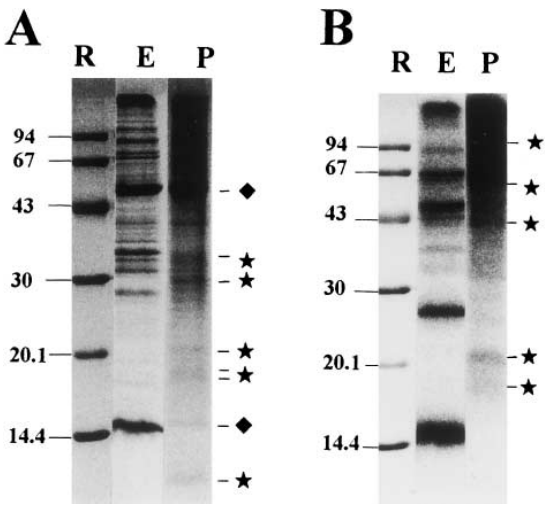

Fig. 4. Comparison of proteins and phosphorylated proteins in spinach chloroplast envelope and plasma membranes, as revealed by Coomassie blue staining (A) and autoradiography (B). R, LMW protein markers; E, envelope membranes; P, plasma membranes; ◆, large and small subunits of the RubisCo which are essentially associated with envelope membranes; ★, position of the main protein bands of plasma membrane which are not seen in envelope membranes.

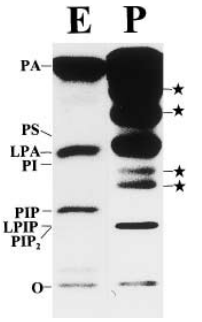

Fig. 5. Comparison of the autoradiography patterns of phosphorylated lipids in envelope (E) and plasma (P) membranes. The position of the unlabelled lipid markers are indicated on the left side of the figure. O, origin of the migration; ★, labelled lipid bands which are not found in envelope membranes.

of the labelled lipids was greater in whole envelope vesicles than in the outer and inner ones. Labelled PIP and LPIP were exclusively localized in the outer envelope membrane. In contrast, labelled PA and LPA were mainly found in the inner one. The presence of small amounts of PA and LPA (ca. 15%) in the outer membrane could be due to a small contamination by the inner membrane and/or to some DAG kinase activity.

To the best of our knowledge, it is the first time that the presence of phosphorylated lipids is reported in the chloroplast envelope membranes. It was therefore of utmost importance to demonstrate that chloroplast envelopes are not contaminated by other membrane systems. Among them, the plasma membrane is the best candidate since this membrane fraction was reported to contain the highest DAG and PI kinase activities of all plant cell membrane systems [7,21]. The DAG kinase was therefore proposed to be a specific marker for plasma membranes [23]. In this respect, Table 1 shows the phosphate incorporation into PA, LPA, PIP and LPIP from chloroplast envelope membranes. When these values were compared with plant plasma membrane DAG and PI kinase activities, both envelope enzymatic activities were found to be about 2000 times [10] and 3000 times [10,22] weaker, respectively. However, such comparative analyses should be considered with caution because the lipid kinase assays (e.g. the ATP concentration) differ markedly.

Three tests were designed to show that our chloroplast envelope preparations were devoid of plasma membrane contaminants. Firstly, as can be seen in Fig. 4A, the protein patterns of envelope (E) and plasma membranes (P) were quite different. In particular, the bands marked with a star corresponded to the proteins which were found exclusively in the plasma membrane. In addition, the corresponding autoradiographic patterns showing the phosphorylated proteins reveals the presence of specific bands in each membrane (see stars in Fig. 4B). Secondly, the comparison of the autoradiography of phosphorylated lipids in envelope and plasma membranes (Fig. 5) shows that plasma membranes contained several phosphorylated lipids which were not detected in envelope membranes. This figure also shows that the lipid kinase activity was greater in plasma membranes than in envelope membranes. Thirdly, Fig. 6 shows that the 1,3-β-glucane synthase activity, which is specifically associated with plant plasma membrane [24], was detected only in this membrane but not in chloroplast envelope membranes. Thus, these three tests, i.e. the comparison of the phosphorylated proteins (Fig. 4) and phosphorylated lipids (Fig. 5), as well as of the 1,3-β-glucane synthase activity (Fig. 6) in the two types of membranes exclude a possible contamination of envelope membranes by plasma membranes. These results are in accordance with previous reports [12,25].

In conclusion, this investigation shows for the first time that lipid kinase activity products are present in chloroplast envelope membranes upon phosphorylation in vitro with [γ-^{32}P]ATP (Fig. 1). Compared to the lipid kinase activity in plasma membranes, the envelope one is much weaker (see Fig. 5), probably explaining why this activity escaped to observation. The presence of two phosphorylated lipid families (PA/LPA and PIP/LPIP) suggests the involvement of at least two lipid kinase activities in the chloroplast envelope. In addition, the simultaneous presence of PA and PIP, and of their respective lyso-derivatives is likely to be due to the activity of

Table 1
Incorporation of [^{32}P] phosphate and total phosphate into the four main labelled envelope membrane lipids

Lipid	Cpm	[^{32}P] incorporated (fmol min^{-1} mg^{-1})	Total P incorporated (fmol min^{-1} mg^{-1})
PA	3465 ± 133	1.05 ± 0.04	630 ± 24
LPA	781 ± 110	0.23 ± 0.03	138 ± 18
PIP	527 ± 54	0.15 ± 0.016	90 ± 9
LPIP	57 ± 13	0.017 ± 0.003	10.2 ± 1.8

Results are expressed in cpm or fmol min^{-1} mg^{-1} protein as mean values ± S.D. (n=4). Total P incorporated (fmol min^{-1} mg^{-1})=[^{32}P] incorporated (fmol min^{-1} mg^{-1}) × concentration of total ATP in the reaction mixture (fmol)/concentration of the total [^{32}P]ATP in the reaction mixture (fmol).

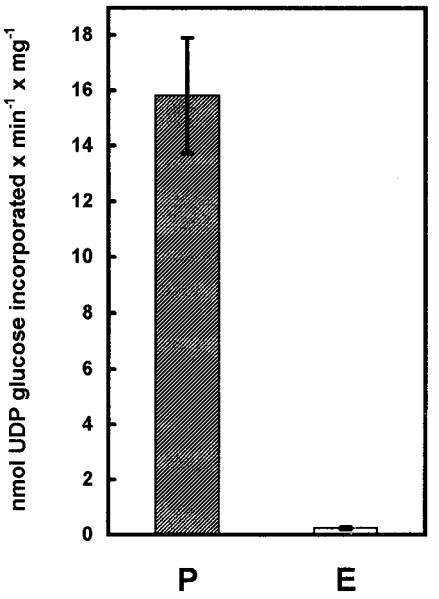

Fig. 6. Comparison of the 1,3-β-glucane synthase activity in the plasma (P) and envelope (E) membranes.

an endogenous phospholipase. Although the physiological role of the envelope lipid kinase(s) is unknown, one can predict that phosphorylated lipids may play important functions in the chloroplast envelope, e.g. in signal transduction and metabolic regulation as shown in other membrane systems [1–5,7].

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References

[1] Kanoh, H., Sakane, F., Imai, S.-I. and Wada, I. (1993) Cell. Signal. 5, 495–503.
[2] Moolenaar, W.R. (1995) Curr. Opin. Cell Biol. 7, 203–210.
[3] Berridge, M.J. (1987) Ann. Rev. Biochem. 56, 159–193.
[4] Trewavas, A. and Gilroy, S. (1991) Reviews 7, 356–357.
[5] Coté, G.G. and Crain, R.C. (1994) BioEssays 16, 39–46.
[6] Munnik, T., Vrije, T., Irvine, R.F. and Musgrave, A. (1996) J. Biol. Chem. 271, 15708–15715.
[7] Boss, W.F. (1989) in: Second Messengers in Plant Growth and Development (Boss, W.F. and Morré, D.J., Eds), vol. 6, pp. 29–56, Alan R. Liss, Inc., New York.
[8] Ohlrogge, J. and Browse, J. (1995) The Plant Cell 7, 957–970.
[9] Heim, S., Bauleke, A., Wylegalla, C. and Wagner, K.G. (1987) Plant Sci. 49, 159–165.
[10] Hanenberg, A., Wissing, J.B. and Wagner, K.G. (1995) Plant Sci. 112, 53–63.
[11] Mourioux, G. and Douce, R. (1981) Plant Physiol. 67, 470–473.
[12] Douce, R. and Joyard, J. (1982) In: Methods in Chloroplast Molecular Biology (Edelman, M., Hallick, R.B. and Chua, N.H., Eds.), pp. 239–259, Elsevier Biomedical Press, Amsterdam.
[13] Keegstra, K. and Youssif, A.E. (1986) Methods Enzymol. 118, 316–325.
[14] Siegenthaler, P.A. and Dumont, N. (1990) Plant Cell Physiol. 31, 1101–1108.
[15] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randal, R.J. (1951) J. Biol. Chem. 193, 265–275.
[16] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[17] Heim, S. and Wagner, K.G. (1986) Biochem. Biophys. Res. Commun. 134, 1175–1181.
[18] Clarke, N.G. and Dawson, R.M.C. (1981) Biochem. J. 195, 301–306.
[19] Laemmli, U.K. (1970) Nature 227, 680–685.
[20] Ray, P.M. (1977) Plant Physiol. 59, 594–599.
[21] Wissing, J.B., Grabowski, L., Drewitz, E., Hanenberg, A., Wylegalla, C. and Wagner, K.G. (1992) Plant Sci. 87, 29–37.
[22] Sommarin, M. and Sandelius, A.S. (1988) Biochim. Biophys. Acta 958, 268–278.
[23] Wissing, J.B. and Wagner, K.G. (1992) Plant Physiol. 98, 1148–1153.
[24] Van Der Woude, W.J., Lembi, C.A. and Morré, D.J. (1974) Plant Physiol. 54, 333–340.
[25] Blée, E. and Joyard, J. (1996) Plant Physiol. 110, 445–454.
[26] Siegenthaler, P.A., Rawyler, A. and Smutny, J. (1989) Biochim. Biophys. Acta 975, 104–111.