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LINOLENIC ACID BIOSYNTHESIS VIA GLYCEROLIPID MOLECULAR SPECIES IN PEA AND SPINACH LEAVES

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IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—*Pisum sativum*; *Spinacia oleracea*; linolenic acid; phosphatidylcholine; monogalactosyldiacylglycerol; molecular species.

Abstract—Only molecular species of the eukaryotic type (18:3/18:3; 18:2/18:3; 18:2/18:2, 16:0/18:3, etc . . .) are present in the phosphatidylcholine (PC) of pea or spinach leaves. In pea leaves, total monogalactosyldiacylglycerol (MGDG) is mostly represented by two eukaryotic molecular species: 18:3/18:3 (79 mol%) and 18:2/18:3 (9%). In spinach leaves, the prokaryotic molecular species 18:3/16:3 forms 39 mol% of total MGDG; however the eukaryotic species 18:3/18:3 (60 mol%) is dominant. At the end of a 30 min pulse with ^{14}C-oleate, four molecular species of PC were intensively labelled in pea leaves: 18:3/^{14}C-18:1; 18:2/^{14}C-18:1; 16:0/^{14}C-18:1 and 18:1/^{14}C-18:1. During the following 48 hr chase period, clear precursor–product relationships could be observed between ^{14}C-18:1 PC and ^{14}C-18:2 PC molecular species on one side and between ^{14}C-18:2 PC and ^{14}C-18:3 PC molecular species on the other side. In spinach leaves the same ^{14}C-18:1 PC molecular species were labelled more slowly and desaturation was not observed beyond ^{14}C-18:2 PC molecular species. In MGDG from pea leaves, labelled linolenic acid accumulated steadily in 18:3/18:3 MGDG, after a lag time of 1 hr. In MGDG from spinach leaves labelled 18:3/18:2 MGDG was a precursor for labelled 18:3/18:3 MGDG. It is concluded that linolenic acid is synthesized via PC molecular species in the eukaryotic pathway of pea leaves and via MGDG molecular species in the eukaryotic pathway of spinach leaves.

INTRODUCTION

The lipids which contain either saturated or unsaturated C_{16} fatty acids at the *sn*-2 position of their glycerol moieties are derived from phosphatidic acid synthesized within the chloroplast. The biosynthesis of lipids containing such a configuration is referred to as the prokaryotic pathway. The desaturation of palmitic acid to hexadecatrienoic acid is only observed in the prokaryotic pathway of the so-called $C_{16:3}$ plants [1–5]. On the other hand, the lipids which contain either saturated or unsaturated C_{18} fatty acids at the *sn*-2 position of glycerol are derived from diacylglycerol moieties synthesized in endoplasmic reticulum membranes. The synthesis of these lipids is referred to as the eukaryotic pathway and this metabolic route is predominant in plants containing no hexadecatrienoic acid, the so-called $C_{18:3}$ plants [1–5]. In both types of plants, many uncertainties remain concerning the biosynthesis of linolenic acid which is mostly found in the galactolipid molecules of chloroplasts [6]. For instance, in the prokaryotic pathway, it has been postulated that MGDG [7, 8] or PG [9] molecules containing dienoic acids could be the substrates of the chloroplastic desaturases producing trienoic fatty acids. On the other hand, in the eukaryotic pathway, it has been proposed that PC molecules containing linoleic acid could be desaturated into PC molecules containing linolenic acid: this desaturation would occur in endoplasmic reticulum [10, 11]. Alternatively, MGDG molecules containing linoleic acid in position 2 and derived from cytoplasmic PC could be desaturated within the chloroplast into MGDG molecules containing linolenic acid in position 2 [8–10, 12, 13].

The aim of the present paper is to take advantage of the high-resolving power of HPLC [14] to research eventual precursor-product relationships between different molecular species of PC and MGDG in plant leaves; it was expected that such relationships could help to choose between the preceding hypotheses. As a labelled precursor of linolenic acid, we have used ^{14}C-oleate, given externally to entire leaves, to follow the labelling of the metabolic intermediates of the sole eukaryotic pathway. To perform the labelling experiments, we have used two types of plants: on one hand pea, a typical $C_{18:3}$ plant where the eukaryotic pathway is the most active [3]; on the other hand spinach, a $C_{16:3}$ plant, with a prokaryotic pathway as active as the eukaryotic one [3]. Finally, in some experiments, after the labelling of the lipids of entire leaves, we have separated chloroplasts and microsomes to follow separately the labelling of PC and MGDG molecular species in these purified subcellular fractions.

* Abbreviations: DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol.

RESULTS

PC and MGDG molecular species in pea and spinach leaves

Combining HPLC and GLC, we were able to separate and identify eight different molecular species from the total PC of pea leaves and nine different molecular species from the total PC of spinach leaves (Table 1). Only molecular species of the eukaryotic type are present in the PC from both plants. Linolenic acid is mostly present in those molecular species containing two C_{18} fatty acids both in pea and spinach.

MGDG molecular species distributions are different in pea and spinach. In the $C_{18:3}$ plant (pea) nearly 90% of total MGDG is represented by two eukaryotic molecular species: 18:3/18:3 (79 mol%) and 18:2/18:3 (9 mol%). In the $C_{16:3}$ plant (spinach), the presence of 39 mol% 18:3/16:3 (a prokaryotic molecule) is noticeable; however in this plant too, the eukaryotic molecular species 18:3/18:3 is largely dominant (60%) and more than 97% of total MGDG is also represented by two molecular species.

Finally, results in Table 1 point to the importance of the eukaryotic pathway for MGDG biosynthesis in both plants: the main molecular species, 18:3/18:3, represents nearly 80 mol% of total MGDG in pea and *ca* 60% in spinach; this peculiar molecular species can only be synthesized by the eukaryotic pathway.

Labelling of PC molecular species from ^{14}C-oleate in entire leaves

Exogenous ^{14}C-oleic acid is rapidly incorporated and acylated by young pea or spinach leaves, which renders this precursor very suitable for studying lipid classes and molecular species involved in the two successive desaturations leading to ^{14}C-linolenic acid formation.

Labelling of polar lipid classes. Following a 30 min pulse with ^{14}C-oleate, Fig. 1A shows the distribution of ^{14}C-radioactivity between the various polar lipids of pea leaves, during a 48 hr chase period. The same is shown for spinach leaves in Fig. 1B. In pea, at the end of the pulse period (time 0, in abscissa of Fig. 1A); all the ^{14}C-oleate incorporated is found acylated to PC; there is still a small increase in PC labelling during the first hour of chase, up to a maximum. Thereafter, PC radioactivity is constantly decreasing while galactolipid labelling is regularly increasing, first in MGDG, then in DGDG. At the end of the 48 hr chase period, more radioactivity is found in MGDG (50%) and DGDG (26%) than in PC (18%). Some labelling (18% at maximum) is also observed in PE. These results suggest strongly that ^{14}C-oleoyl residues are first acylated to PC molecules, eventually desaturated, and then transferred to galactolipids. Similar results were already obtained by Murphy and Stumpf [15] with greening cucumber cotyledons or Ohnishi and Yamada [16] with greening etiolated oat leaves pulse-labelled with ^{14}C-oleate.

Table 1. Molecular species distributions (in mol%) in PC and MGDG from pea or spinach leaves

Molecular species	Pea		Spinach	
	PC	MGDG	PC	MGDG
18:3/16:3	—	—	—	38.6
18:3/16:2	—	—	—	0.3
18:3/18:3	11.1	79.3	28.3	59.3
18:2/18:3	24.4	8.9	13.8	0.6
18:2/18:2	18.9	6.8	11.6	0.2
16:0/18:3	26.7	5.0	15.5	0.4
18:1/18:3	—	—	9.0	0.6
16:0/18:2	7.0	—	4.7	—
18:1/18:2	2.5	—	6.5	—
16:0/18:1	—	—	3.6	—
18:1/18:1	—	—	6.9	—
18:0/18:3	6.5	—	—	—
18:0/18:2	2.9	—	-	-

The kinetics of labelling of polar lipids from spinach leaves in the same conditions (Fig. 1 B) differ from those observed in pea leaves. Again, PC contain all the radioactivity at the end of the 30 min pulse period, but the decrease in radioactivity of this phospholipid is slower than the decrease observed in pea leaves: after a 48 hr chase period 54% of total radioactivity is still observed in spinach leaf PC while only 18% remained in pea leaf PC. Consequently the progressive increase of galactolipid labelling is also slower than the increase observed in the galactolipid from pea leaves. MGDG remains far less labelled than PC along the whole chase period. Similar results were obtained by Stobart *et al.* [17] with barley leaves and Hawke and Stumpf [18] with maize leaves pulse labelled with ^{14}C-oleate.

^{14}C-Oleate desaturation in total phosphatidylcholine. As it has been clearly demonstrated *in vitro* that oleoyl residues are desaturated into linoleoyl residues while esterified to various PC molecular species [19, 20], it was interesting to look for eventual desaturations of ^{14}C-oleate *in vivo*, in the total PC from pea or spinach leaves.

In pea, results are consistent with a desaturation process following the pathway: 18-1 PC → 18:2 PC → 18:3 PC. Clear precursor-product relationships could be recognized between these three types of molecules (data not shown). Similar relationships were observed previously by Murphy and Stumpf in greening cucumber cotyledons [15].

In spinach, ^{14}C-oleoyl residues were far less desaturated, as compared with pea. At the end of the 48 hr chase period 61% of total PC radioactivity remained in ^{14}C-18:1-PC while no ^{14}C-18:3-PC had appeared. Comparative results were obtained by Stobart *et al.* [17] with barley leaves of by Onishi and Yamada in greening oat leaves [16]. However very small quantities of ^{14}C-18:3 PC were observed by these authors.

Labelling of PC molecular species. PC molecular species, labelled *in vivo*, were separated and analysed by radio-HPLC [20]. After a 30 min pulse with ^{14}C-oleate, pea leaves contained four heavily labelled PC molecular species: 18:3/^{14}C-18:1, 18:2/^{14}C-18:1, 18:1/^{14}C-18:1 and 16:0/^{14}C-18:1 (Fig. 2). It is interesting to notice that these four PC molecular species are the same as those which were labelled *in vitro* from ^{14}C-oleoyl CoA with pea microsomes [21]. In all these molecular species the labelled oleoyl residues have been found mostly in position 2 of *sn*-glycerol. Figure 2A–D shows clearly the progressive desaturation, during the 48 hr chase period,

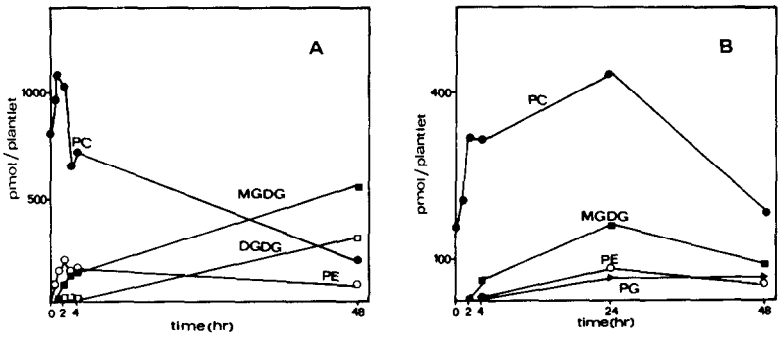

Fig. 1. Kinetics of labelling of the various polar lipids from pea (A) or spinach (B) leaves, during a 48 hr chase period following a 30 min pulse with ^{14}C-oleate. Time 0, on the abscissa, corresponds to the end of the pulse period.

of each one of these PC molecular species, with clear precursor-product relationships allowing to write the four following reactions:

18:3/^{14}C-18:1 PC → 18:3/^{14}C-18:2 PC → 18:3/^{14}C-18:3 PC

18:2/^{14}C-18:1 PC → 18:2/^{14}C-18:2 PC → 18:2/^{14}C-18:3 PC

16:0/^{14}C-18:1 PC → 16:0/^{14}C-18:2 PC → 16:0/^{14}C-18:3 PC

18:1/^{14}C-18:1 PC → 18:1/^{14}C-18:2 PC → 18:1/^{14}C-18:3 PC

To the best of our knowledge, it is the first time that clear precursor–product relationships are evidenced in four families of PC labelled molecular species, showing the progressive desaturation of oleate to linoleate and to linolenate *via* phosphatidylcholine molecules.

The relative labelling of PC molecular species from spinach leaves pulse-labelled with ^{14}C-oleate are shown in Fig. 2E–H. Two PC molecular species were heavily labelled after the 30 min pulse: 16:0/^{14}C-18:1 PC and 18:3/^{14}C-18:1 PC. Two hours later, 18:2/^{14}C-18-1 PC appeared and four hours later: 18:1/^{14}C-18:1 PC. Again the four PC molecular species labelled *in vivo* are similar to those found labelled *in vitro* with microsomes and ^{14}C-oleoyl CoA [21]. As already observed with total PC in spinach leaves, the desaturation of the oleoyl residues esterified in the four labelled PC molecular species did not go further than linoleoyl residues. This is in marked contrast with what had been observed in pea leaves.

Labelling of MGDG molecular species from ^{14}C-oleate in entire leaves

As Fig. 1A suggested a transfer of ^{14}C-labelled acyl residues from PC to galactolipids, it was interesting to follow the labelling of unsaturated C_{18} fatty acids in total MGDG from pea and spinach leaves.

Labelling of unsaturated C_{18} fatty acids in total MGDG

Labelled C_{18} unsaturated fatty acids did not appear in MGDG from pea leaves before 1 hr of chase (data not shown). Only two fatty acids were labelled: 18:3 containing most (90%) of the total radioactivity and 18:2 (10%). No labelled 18:1 was incorporated into MGDG, in good agreement with the general formulation of the eukaryotic pathway in $C_{18:3}$ plants. Almost similar results had been found previously by Stobart *et al.* [17] with barley leaves, particularly no precursor–product relationship had been evidenced by these authors between MGDG-18:2 and MGDG-18:3. The latter acid appeared immediately more intensively labelled in MGDG than the former. However a small incorporation of ^{14}C-18:1 was observed in MGDG by Stobart *et al.* [17] as well as by Ohnishi and Yamada in oat leaves [16] or Murphy and Stumpf [15] with greening cotyledons of cucumber.

In MGDG from spinach leaves, labelled C_{18} unsaturated fatty acid did not appear before 2 hr of chase (data not shown). Three acids were labelled: 18:3 (containing 60% of total MGDG radioactivity after 48 hr of chase), 18:2 (25%) and 18:1 (15%). No clear precursor-product relationship could be evidenced between these labelled fatty acids. The presence of ^{14}C-18:1 in MGDG is not in agreement with the general formulation of the eukaryotic pathway but Thompson *et al.* [22] have recently offered some evidence that *de novo* glycerolipid synthesis in chloroplasts can utilize free fatty acids imported from other cellular compartments. As indicated previously, other authors [15–17] have also found some incorporation of exogenous ^{14}C-18:1 into MGDG.

Labelling of MGDG molecular species

The kinetics of labelling during the chase period of the two MGDG molecular species from pea leaves which appeared labelled after a lag time of 1 hr is shown in Fig. 3A. Clearly labelled linolenic acid accumulated steadily in 18:3/18:3 MGDG whereas the transient labelling of 18:3/18:2 MGDG did not suggest any precursor-product relationship between those two molecular species.

In MGDG from spinach leaves (Fig. 3B), four molecular species appeared labelled after 2 hr lag time. Labelling kinetics show that 18:3/18:2 MGDG can be used as a precursor for 18:3/18:3 MGDG. The two other labelled molecular species (18:3/18:1 and 18: 2/18:2) contained only 10% each, of total MGDG radioactivity, at the moment of their maximum labelling.

DISCUSSION

It is well known that leaf tissues from higher plants are able to incorporate and desaturate ^{14}C-18:1 [15–18, 23].

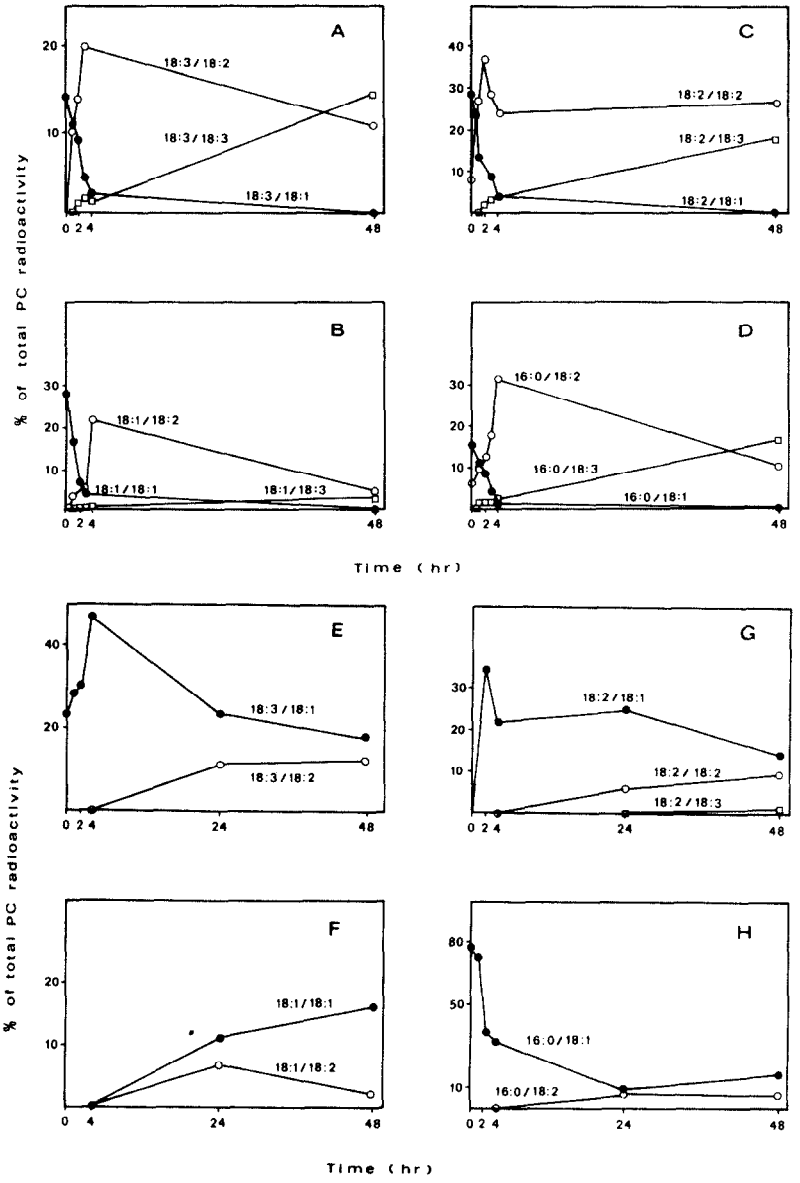

Fig. 2. Evolution of the percentages of total PC radioactivity found in the different PC molecular species from pea (A–D) or spinach (E–H) leaves pulse-labelled with ^{14}C-oleate. Time 0, on the abscissa, corresponds to the end of the pulse period.

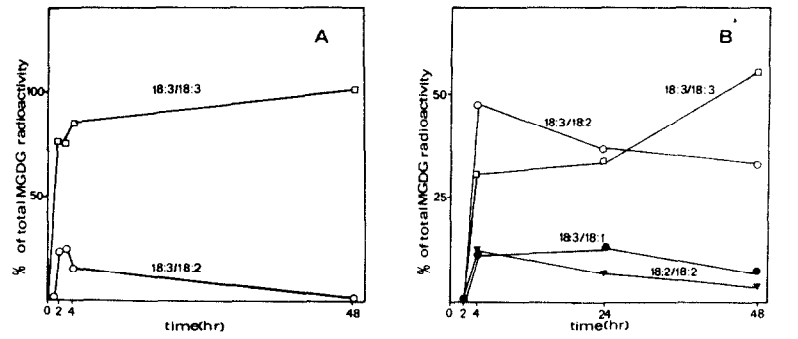

Fig. 3. Evolution of the percentages of total MGDG radioactivity found in the different MGDG molecular species from pea (A) or spinach (B) leaves pulse-labelled with ^{14}C-oleate. Time 0, on the abscissa, corresponds to the end of the pulse period.

In entire tissues, this desaturation proceeds up to ^{14}C-18:3 and most of the ^{14}C-linolenic acid produced accumulates finally in eukaryotic MGDG molecules. One of the main question remaining unsolved by the preceding work was the following: which molecular species (whether from PC or from MGDG) were utilized as substrates for ^{14}C-$C_{18:2}$ desaturation? In this paper, we show that the answer to this question differs according to which plants are chosen for experiments.

In pea, a typical $C_{18:3}$ plant, ^{14}C-18:2 is converted to ^{14}C-18:3 while ^{14}C-acyl residues are esterified to glycero-phosphorylcholine: we show that four families of PC molecular species are utilized for the final formation of ^{14}C-18:3 PC. These progressive desaturations of PC molecular species could be observed also in the microsomal fractions of pea leaves (data not shown) but could not be observed clearly in chloroplast PC. In pea chloroplasts, only the more unsaturated MGDG molecular species (18:3/18:3 and 18:3/18:2) were labelled during the pulse-chase experiment (data not shown); this fact is in good agreement with the proposal that the diacyl glycerol moieties of MGDG molecules originate from cytoplasmic PC [24] as supposed by the eukaryotic pathway.

In spinach, a $C_{16:3}$ plant where the prokaryotic pathway is very active, we have shown that the conversion of ^{14}C-18:1-PC was limited to ^{14}C-18:2-PC and that this occurred in the microsomes (data not shown). A precursor–product relationship could be observed in entire tissue as well as in the chloroplasts of this plant between 18:3/18:2-MGDG and 18:3/18:3-MGDG. Thus in the eukaryotic pathway of spinach the final linoleate desaturation occurs in the chloroplast, while acyl residues are esterified to galactosylglycerol backbones.

Norman and St John [8] studied recently the metabolism of unsaturated MGDG molecular species, separated by HPLC, both in the wild type and in a mutant of *Arabidopsis thaliana* containing reduced levels of trienoic acids. Comparing the kinetics of labelling of the various MGDG molecular species found in the leaves of these normal and mutant $C_{16:3}$ plants, the authors reached the conclusion that in the same $C_{16:3}$ plant where prokaryotic and eukaryotic pathways function simultaneously, there are different sites (cytoplasmic and chloroplastic) and different substrates for linolenic acid synthesis. Procaryotic MGDG (i.e. 18:3/16:3 MGDG) would be formed entirely in the chloroplast; eukaryotic MGDG (i.e. 18:3/18:3 MGDG) would be synthesized from a 18:3/18:3 diacylglycerol residue coming from cytoplasmic PC.

Lem and Williams [11] reached the same conclusion with a $C_{18:3}$ plant. These authors followed by means of argentation TLC, the ^{14}C-labelling of molecular species of MGDG in *Vicia faba* leaves infiltrated with ^{14}C-glucose, and they utilized an inhibitor of linolenic acid biosynthesis (San 9785). As a conclusion of their results, they proposed a model for 18:3/18:3 MGDG biosynthesis in '$C_{18:3}$ plants' incorporating several lipid classes for fatty acid desaturation: phospholipids (the desaturation of which was not inhibited by San 9785) and MGDG (the desaturation of which was inhibited by the herbicide).

Our own conclusions are in good agreement with the main conclusions of the preceding authors. However we add to their respective proposals: (i) the demonstration of linolenate formation in four PC molecular species from pea leaves and we show the absence of linolenate biosynthesis in PC from spinach leaves, (ii) we show that the eukaryotic pathway does not proceed similarly, for the biosynthesis of the eukaryotic 18:3/18:3 MGDG, in a $C_{18:3}$ plant like pea and in a $C_{16:3}$ plant like spinach.

In pea, linolenic acid is synthesized in PC molecular species, outside of the chloroplast; in spinach linolenic acid is finally synthesized in MGDG molecular species, within the chloroplast. The biosynthesis of linolenic acid must be studied at the level of glycerolipid molecular species in other $C_{18:3}$ or $C_{16:3}$ plants before any further generalization may be stated.

EXPERIMENTAL

Plant material. Pea seeds (*Pisum sativum* var. Petit Provençal) were germinated in long day conditions (16 hr of white light) at 24° during 7 days. Experiments were performed with 7-day-old leaves. Spinach seeds were germinated during 30 days at 25° in short day conditions (9 hr of white light). Experiments were performed with 30-day-old leaves.

In vivo *labelling experiments.* Microdroplets of 1-^{14}C oleic acid ammonium salt (57 mCi/mmol) were deposited on the abaxial surface of leaves. The leaves were rinsed after 30 min with water and allowed to stand for different times (0, 1, 2, 4, 24 and 48 hr) before being harvested. Lipids were extracted according to ref. [25] from entire leaves or from microsomes [20] or chloroplasts [24].

Lipid analysis. Separation of PC and MGDG by HPLC was performed with Porasil column (Waters associates) according to the method of ref. [14].

Separation, identification and quantification of molecular species of PC and MGDG were performed by combining HPLC on an O.D.S. ultrasphere column and gaschromatography of the methyl esters prepared from fractions collected at the exit of the column according the method of ref. [14]. The radioactivity of each compound eluted from HPLC was measured by scintillation in a continuous flow apparatus [Flow-one I (Radiomatic instruments)] as described in ref. [20]. Fatty acid methyl esters were also separated by argentation TLC [26] and the radioactivity of separated methyl esters was determined by scintillation counting. Positional analysis of fatty acids in phosphatidylcholine molecular species was determined after hydrolysis with the purified lipase from *Rhizopus arrhizus* [27].

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