Carbon isotope composition of lipidic classes isolated from tobacco leaves

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It is shown that $\delta^{13}C$ values of total fatty acids in each of the main lipidic classes of tobacco leaves reach a uniform value around $-34.3‰$. This finding is in agreement with the common origin for acyl chains demonstrated in plant cells. For all the lipidic classes investigated, the $\delta^{13}C$ values in acyl chains were lower than in the whole lipidic molecules, except for the phosphatidylcholine in which the acyl chains and the polar head had similar values. Metabolic origins of the different part of lipidic molecules are discussed and related to the localization of the isotope fractionation step.

Fatty acid *Lipid* *Phosphatidylcholine* *Carbon fractionation* Nicotiana *Leaf*

1. INTRODUCTION

Carbon isotope fractionation associated with lipid synthesis is a general feature of living matter. The basic design of this fractionation was reported in [1]. A kinetic isotope effect occurs at the rate limiting step of transformation of pyruvate to acetyl CoA catalyzed by the pyruvate dehydrogenase complex. Species-specific differences in the magnitude of ^{13}C depletion could arise from different kinetic effects during the specific pyruvate dehydrogenase reactions and from different flow rates of pyruvate to its other metabolite reactions.

Using a single species, *Nicotiana tabaccum*, we investigated several aspects of lipid metabolism with respect to the carbon isotope composition found in each lipidic class and in their respective fatty acid chains. The study focused on the different lipidic classes relative to their cellular compartmentation, i.e., in chloroplasts for MGDG, DGDG and PG; in the extrachloroplastic compartment for PE and most of PC. If all acyl chains of fatty acids have the same biosynthetic origin in the cell, it could be supposed that the isotopic composition of fatty acids from different lipidic classes would remain essentially constant, whatever the intramolecular localization of the fatty acids in lipidic molecules and the intracellular localization of these lipids. Differences between carbon isotope composition in the acyl chains and the whole lipidic molecule will be expected, depending upon the number of carbon atoms and their isotope composition in the polar head of the molecule. Moreover, the carbon isotope composition of the polar head of the lipidic classes could indicate whether other carbon fractionation steps occur in the building up of the whole molecule.

Abbreviations: C16:0, palmitic acid; C16:1_{Tr}, *trans*-3-hexadecanoic acid; C16:3, hexadecatrienoic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid; MGDG, diacylmonogalactosylglycerol; DGDG, diacyldigalactosylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SL, sulfolipid; FA, fatty acid; PEG, polyethyleneglycol; CoA, coenzyme A; PH, polar head; GLC, gas liquid chromatography; TLC, thin layer chromatography

2. MATERIALS AND METHODS

2.1. *Preparation of total lipidic extract*

Plants (*N. tabaccum* L., var. Samsum) were

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grown under constant environments: 16 h photoperiod, 22°C, for up to 60 days. Batches of 70 g selected mature leaves were extracted as in [2].

2.2. *Separation of lipidic classes*

TLC separation: extracts were spotted on TLC plates (precoated silica gel 60 Merck) and developed in chloroform/acetone/acetic acid/methanol/water, 50:20:10:10:5, v/v, as in [3]. Bands containing lipidic classes were visualized by exposure to iodine vapors and then scrapped; each lipidic class was eluted by chloroform/methanol/water, 1:1:1, v/v.

HPLC separation: the total lipidic extract (about 5 mg per analysis) was injected into a Kontron analytic HPLC apparatus, using a semi-preparative column of silica 10 μ from Waters (μporasil P/N 84175). A linear gradient was performed for 20 min, starting from 100% of solution A (isopropanol/hexane, 8:6, v/v) to 100% of solution B (isopropanol/hexane/water, 8:6:1.5, v/v), at a flow rate of 2 ml per min. Peaks corresponding to MGDG, DGDG, PE, PG and PC were collected and identified by rechromatography on TLC.

2.3. *Fatty acid composition and isolation*

Lipidic extract or lipidic classes were transmethylated according to [4] and fatty acid methyl esters were analyzed by capillary gas column (25 m long, 0.32 mm coated with PEG 20 M) chromatography at 180°C. Concentration was determined by adding an internal standard (heptadecanoate methyl ester). Free fatty acids were prepared by saponification in 0.5 N NaOH for 20 min at 75°C and addition of 12 N HCl. The free fatty acids were extracted by pentane.

2.4. *Carbon isotope analysis*

Samples combustion to CO_2 [7] was performed by standard techniques. The relative abundance of ^{13}C and ^{12}C expressed as $\delta^{13}C$ (‰) with reference to PDB standard (Belemnite from Pee Dee formation, SC, USA) was obtained by double collection. Reproducibility of measurements was 0.4‰ [7]. Analyses were carried out on dry aliquots of lipidic classes and on their free fatty acid preparations. As a control for carbon isotope composition of photosynthates from tobacco leaves, purified starch and cellulose [7] were also analyzed.

3. RESULTS AND DISCUSSION

Lipidic classes obtained by the two different separation procedures exhibited the same characteristic fatty acid composition (table 1), (i) a very large amount of trienoic acid in galactolipids, with hexadecatrienoic acid in MGDG, (ii) *trans-*

Table 1

Fatty acid composition of each lipidic class purified by HPLC or TLC procedures (% total fatty acids per lipidic class)

		16:0	16:1$_{Tr}$	16:3	18:0	18:1	18:2	18:3
MGDG	HPLC	3.3		16.5	1.7	2.7	5.7	69.4
	TLC	6.8		13.9	2.9	1.6	7.9	66.8
DGDG	HPLC	14.0			2.3	15.2	5.3	63.2
	TLC	24.9			4.3	1.7	4.6	64.9
PG	HPLC	28.9	18.8		5.8	7.9	15.5	23.0
	TLC	28.6	21.7		5.1	12.0	14.2	18.2
PC	HPLC	29.8			6.2	10.5	24.1	29.4
	TLC	38.0			7.4	6.0	23.5	25.0
PE	HPLC	35.0			5.3	3.3	21.3	35.0
	TLC	33.2			6.3	3.1	33.5	23.7

hexadecenoic acid in PG [6], (iii) the largest content of linoleic acid in PC and PE [5]. The higher percentage of palmitate in PC isolated by TLC as compared to that isolated by HPLC resulted from a slight contamination by SL which contains a high percentage of this fatty acid. HPLC gave more purified PC. No significant differences between $\delta^{13}C$ values of lipidic classes appeared from two independent TLC separations (table 2, extract A) and from TLC and HPLC separations (table 2, extract B). Only slight differences (less than 1.5‰) occurred for two independent extractions (table 2, compare extract A to extract B). The same observations can be made from the results on free fatty acid preparations (table 2). The average $\delta^{13}C$ values of lipidic classes ranged from −32.80‰ in DGDG, the most enriched in ^{13}C, up to −34.84‰ in PC, the most depleted in ^{13}C. PE, PG and MGDG, with intermediate values near −33.7‰, showed similar enrichment. The $\delta^{13}C$ values for the fatty acid preparations fall in a narrow range: extreme values were −33.84‰ in PG and −34.73‰ in MGDG. The mean value for all preparations was −34.3‰, lower than that of each lipidic class, except for PC. The ^{13}C depletion observed for PC revealed that a specific fractionating step could take place in the synthesis of the polar head of this lipidic molecule.

To clarify this latter point, we calculated $\delta^{13}C$ values of polar heads of MGDG, DGDG and PC (table 3). The fatty acid percentages in the whole lipidic molecule (table 1) were used to obtain the distribution of carbon atoms between fatty acid chains and the polar head (table 3). The $\delta^{13}C$ values calculated from the equation described in the legend of table 3 were similar for MGDG and DGDG, respectively −30.6 and −29.5‰ (mean values from preparations A and B). Note that

Table 2

$\delta^{13}C$ value in each lipidic class and in the respective free fatty acid preparation

	Extract A		Extract B	
	TLC 1	TLC 2	TLC 3	HPLC 4
	Lipidic classes			
MGDG	−33.18	−33.71	−34.00	−34.75
DGDG	−31.98	−32.00	−34.00	−33.25
PG	−33.06	−33.09	−33.98	−34.46
PC	−36.07	−34.91	−33.80	−34.58
PE	−34.03	−34.45	−32.75	−33.21
	Fatty acid preparations			
MGDG	−34.76	−34.35	−34.17	−35.66
DGDG	−33.38	−33.24	−34.92	−34.52
PG	−35.44	–	−32.25	–
PC	−34.88	−35.06	−31.58	−33.74
PE	−34.87	−33.93	–	–

Each value is a mean of two $\delta^{13}C$ determinations on aliquots of dry samples. 1 and 2 were independent TLC procedures applied to the same extract (A), 3 and 4 were respectively TLC and HPLC procedures applied to a second independent extract (B). Note that crude chloroformic extract B and the total fatty acid preparation isolated from it, had $\delta^{13}C$ values of −34.09 and −34.01‰ respectively: extract B contained large amounts of pigments and neutral lipids which had $\delta^{13}C$ values similar to those of fatty acids

Table 3

$\delta^{13}C$ values of polar heads of the lipidic classes for MGDG, DGDG and PC

C atoms		$\delta^{13}C$ values (‰)	
		A	B
MGDG			
TL	44.2	−33.44	−34.37
FA	35.2	−34.55	−34.91
PH	9.0	−29.10	−32.16
DGDG			
TL	49.4	−31.99	−33.62
FA	35.4	−33.31	−34.72
PH	14.0	−28.66	−30.35
PC			
TL	42.8	−35.49	−34.19
FA	34.8	−34.97	−32.66
PH	8.0	−37.75	−41.91

The percentage of carbon atoms coming from fatty acids in the whole lipidic molecule is estimated from data of table 1. We used the following equation to calculate the $\delta^{13}C$ value of the polar head:

$$\delta^{13}C\ (TL)\cdot(C\ \text{per TL}) = \delta^{13}C\ (FA)\cdot(C\ \text{per FA}) + \delta^{13}C\ (PH)\cdot(C\ \text{per PH})$$

with TL, total lipid; FA, fatty acid part; and PH, polar head of the molecule. $\delta^{13}C$ of TL and FA were mean values from 1 and 2, respectively, for A and B

starch and cellulose isolated from these tobacco leaves had $\delta^{13}C$ values of -28.1 and $-28.9‰$, respectively. It can be assumed that the carbon skeleton of the polar head of galactolipids are provided by photosynthates without (or with a weak) additional fractionation step. In contrast, the calculated $\delta^{13}C$ value of the polar head of PC, $-39.8‰$ (table 3, mean value between A and B), was lower than those of the polar heads of galactolipids. It can be assumed that PC originates from a pathway having a particular carbon fractionation step. PC plays a key role as an acyl donor for most of the other lipids [8] and it has a higher turnover rate than all other classes. A rate-limiting step in the complex biosynthesis of PC could possibly be responsible for the fractionation of carbon in its polar head.

To sum up, the similar carbon isotope composition of fatty acids is in good agreement with the usual concept of a single pathway for synthesis of fatty acids in the cells. However, at least in the case of plants, questions arise concerning the relation between the isotope effect occurring at the level of transformation of pyruvate to acetyl CoA catalyzed by the pyruvate dehydrogenase complex and the depletion in ^{13}C of the fatty acids pool.

If it is now proved [9] that fatty acid synthetase is exclusively located in plastid, the origin of acetate remains a matter of speculation: is it produced directly inside the chloroplast [10] or would it come from mitochondria as suggested by [11]? But in all cases, acetate is a precursor, not only for acyl chain, but also for most of cellular components. Therefore, the hypothesis that depletion in ^{13}C of fatty acids only results from pyruvate dehydrogenase action through acetate pool compartmentation remains to be firmly demonstrated. It would be interesting to investigate if another fractionating step more specific for fatty acid synthesis could not be implicated, for example at the level of the decarboxylation of malonyl CoA into acetyl CoA in the chloroplast.

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