Desaturation of oleoyl and linoleoyl residues linked to phospholipids in growing roots of yellow lupin

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When growing lupin roots were fed with [^{14}C]oleate and [^{14}C]linoleate most of the absorbed molecules remained in the free fatty acid pool. Part of the ^{14}C-labelled fatty acid was integrated first into phosphatidylcholine and subsequently into phosphatidylethanolamine. Then, progressive desaturation took place. The kinetics of these processes suggests that desaturation in phosphatidylcholine was followed by transacylation of polyunsaturated fatty acids into phosphatidylethanolamine. To produce good yields of desaturation (about 48%), seeds had to be less than 1-year old. With respect to linoleate desaturation, seedlings had to be 2–7-days old. Increased percentages of oleate desaturation were obtained when the roots were grown on a medium enriched with calcium.

Fatty acid desaturase *Phospholipid* *Acylation* *Seedling* *Age* *Calcium*

1. INTRODUCTION

Higher plants contain large amounts of polyunsaturated fatty acids. The enzymes catalyzing the biosynthesis of linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids have not yet been isolated from plants. However, studies at the subcellular level with labelled precursors have shown that oleate desaturation is localized in the endoplasmic reticulum [1–3] whereas linoleate desaturation has tentatively been localized in chloroplasts [4–7] and/or endoplasmic reticulum [8,9]. The substrate for the desaturation enzymes bound to the endoplasmic membranes is phosphatidylcholine [8,10,11] while galactolipids [12] or phosphatidylglycerol [13] have been proposed as substrates for the chloroplast desaturation system.

Up to now, fatty acid desaturation has been studied in potato tubers [1], green leaves [2] or maturing seeds [14]. This paper shows that oleate and linoleate desaturation can be obtained in a new system: in the roots of yellow lupin seedlings. Acylation of phospholipids and subsequent desaturation of two labelled precursors [^{14}C]oleate and [^{14}C]linoleate, were followed for several hours. We show here that fatty acid desaturation varies according to the different stages of root growth. Moreover, yellow lupin is a calcifuge plant and desaturase activities were increased in yellow lupin roots by calcium ions added to culture medium.

Abbreviations: $C_{16:0}$, palmitic acid; $C_{18:0}$, stearic acid; $C_{18:1}$, oleic acid; $C_{18:2}$, linolenic acid; $C_{18:3}$, linolenic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine

2. MATERIALS AND METHODS

Lupinus luteus L. seeds were obtained from INRA (Dijon), Agricultural Research Station. Seeds were stored at room temperature. For germination, seeds were soaked in calcium hypochloride solution for 1 h, followed by soaking in water for 24 h. Seedlings were grown on a nutrient medium as in [15]; i.e., in the dark, at 28°C; 7 growth stages were defined according to root lengths which ranged from 6–8 mm (2-day old) up to 110 mm (14-day old).

Preparation of labelled precursors: [^{14}C]oleic

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acid (0.2×10^{10} Bq.mmol $^{-1}$) was purchased from CEA (France). [^{14}C]Linoleic acid (0.2×10^{10} Bq.mmol^{-1}) was obtained from Amersham (Bucks). Ammonium salts were prepared by adding 4 ml ammonium ethyl ether to 1 mmol dry, labelled fatty acid. After shaking, the solvent was evaporated under nitrogen and the ammonium salts of fatty acids were dissolved in 1 ml distilled water.

2.1. *Labelling by lipids*

Micro-droplets of the ammonium salts of radioactive fatty acids (7 nmol in 50 μl: 1.40×10^4 Bq) were deposited on the first millimeter of the root. After 30 min, the seedlings were transferred to distilled water, at 22°C, in an illuminated growth chambers (4 W.m^{-2}) with circulating air. After different labelling times, the roots were cut, rinsed with distilled water and homogenized in a Turrax mixer.

2.2. *Lipid analysis*

The extraction of lipids was performed as in [16]. Lipid classes were separated by thin-layer chromatography (TLC) [17,18]. Total fatty acid methyl esters were prepared as in [19] and analyzed by GLC [20]. The fatty acid methyl esters of each lipid class separated by TLC were prepared using 14% borontrifluoride in methanol as in [21]. They were analyzed by GLC [20] or by argentation-TLC. To this end, plates were sprayed with 8% silver nitrate–ethanol solution and developed with an ethyl ether–petroleum ether–acetic acid solvent system (30:70:0.4, by vol.). After autoradiography, the areas corresponding to radioactive methyl esters were scraped off for scintillation counting. The counts were corrected for quenching.

3. RESULTS

3.1. *Lipid composition of yellow lupin roots*

The lipid composition of lupin seedling roots was stable during seedling growth. Phospholipids represented 67.5% of the total lipids while galactolipids represented only 5%, and neutral lipids 27.5%. Phosphatidylcholine (PC, 37.5%) and phosphatidylethanolamine (PE, 24%) were predominant. The major fatty acid in these two

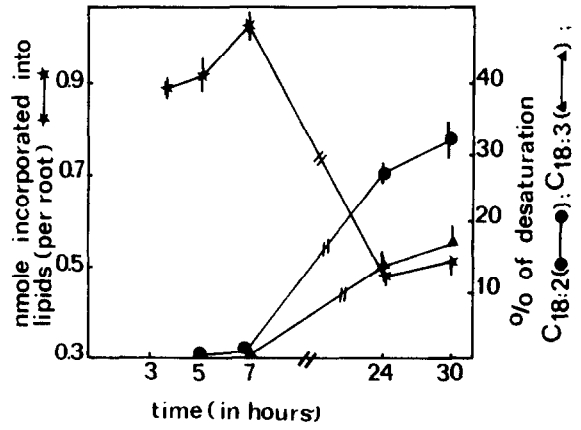

Fig. 1. Incorporation and desaturation of [^{14}C]oleate by 7-days old roots (7.6 nmol of labelled oleate were deposited on the roots). Desaturation yields are expressed as the percentages of total fatty acid radioactivity recovered in polyunsaturated acids. Means of 3 different assays (± SD) are plotted.

lipids was linolenic acid which represented 52% of total fatty acids.

3.2. *Oleate and linoleate desaturase activities*

Fig. 1 shows the incorporation of [^{14}C]oleate into the lipids of 7-day old roots. Maximal incorporation (1.05 nmol/root) was observed 7 h after precursor deposit and was followed by a strong decrease in the amount of labelled molecules (0.47 nmol per mol after 24 h). This decrease was probably due to the oxidation of [^{14}C]oleic acid ($^{14}CO_2$ evolved from respiring roots was trapped by a potassium hydroxide solution). [^{14}C]Oleate was desaturated into [^{14}C]linoleate and [^{14}C]linolenate. The highest desaturation yields were observed 30 h after precursor deposit, when 31.5% and 16% of the total fatty acid radioactivity were recovered in linoleate and linolenate, respectively. However, it was noticed that after 30 h of incubation without nutrients in the culture medium, the seedlings started to fade and the experiments were stopped. Thus, a rather long period of time separates the incorporation of oleate and its subsequent desaturation. [^{14}C]Linoleate was also incorporated into lupin root lipids (fig. 2). As for [^{14}C]oleate, the highest yield of incorporation (2.25 nmol/root) was observed 7 h after precursor deposit, a value twice as high as the maximum of oleate incorporation. The incoporation was followed by a significant decrease in labelling. A

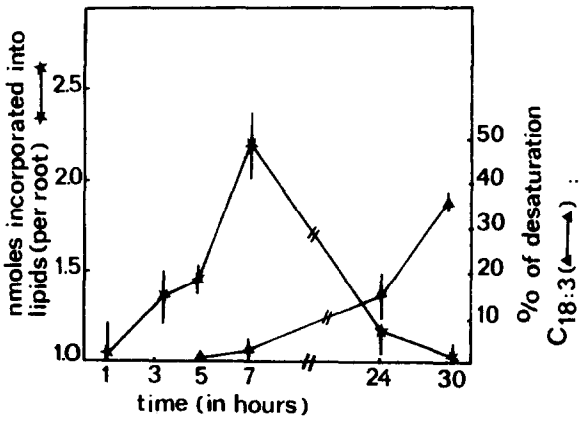

Fig. 2. Incorporation and desaturation of [^{14}C]linoleate by 7-days old roots (7 nmol of labelled linoleate were deposited on the roots). Desaturation yields are expressed as the percentages of total fatty acid radioactivity recovered in linolenic acid. Means of 3 different assays (± SD) are plotted.

lag period, similar to that described previously was observed between the maximum of incorporation and the maximum of desaturation of the precursor. Only 2.5% of [^{14}C]linoleate was desaturated after 7 h of labelling whereas 36% was desaturated after 30 h.

3.3. *Incorporation of the precursors into root phospholipids*

When compared to fig. 1, fig. 3A shows that most of the labelled oleate was recovered as free fatty acid (90% of total labelled lipids after 7 h of incubation). About 10% of [^{14}C]oleate was incorporated into phospholipids, mainly PC and PE. PC contained more heavily labelled carbon atoms than PE at the beginning of incubation but after incubation times longer than 7 h, PE was always more intensively labelled than PC. Later, a gradual decrease in the labelling of both phospholipids was observed. This suggests that some molecules were degraded due to a turnover of membrane phospholipids [22]. Moreover, PC and PE could be acyl donors to other lipid classes such as galactolipids or neutral lipids [5].

When compared with fig. 2, fig. 3B shows that more than 95% of [^{14}C]linoleate was recovered as free fatty acid. However, [^{14}C]linoleate was also esterified into PC and PE. One h after precursor deposit, PC was more radioactive than PE but from 3.5–30 h, PE contained twice as much label

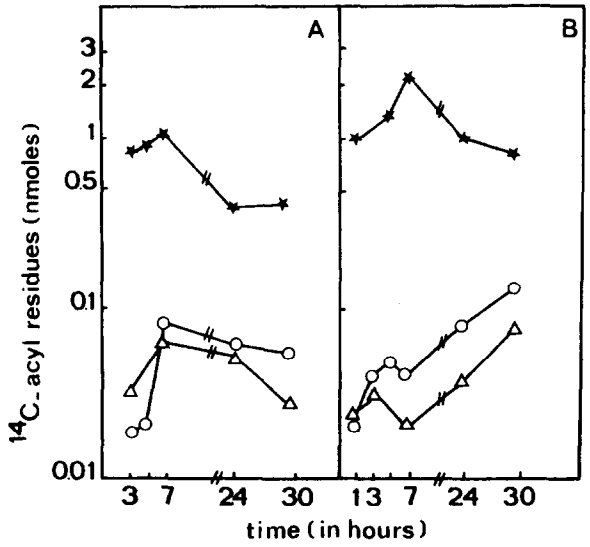

Fig. 3. Acylation of [^{14}C]oleate (3A) or [^{14}C]linoleate (3B) into two phospholipids (PC and PE) and incorporation into neutral lipids. (★—★) ^{14}C-free fatty acids or [^{14}C]acyl residues linked to neutral lipids; (△—△) [^{14}C]acyl residues linked to PC; (○—○) [^{14}C]acyl-residues linked to PE.

as PC. The radioactivity of both phospholipids increased almost continuously with time.

3.4. *Desaturation of the precursors after incorporation into root phospholipids*

When esterified in phospholipid molecules, the labelled acyl-residues were desaturated. Fig. 4A shows the transformation of the oleoyl residues incorporated into phospholipid molecules. During the first 7 h, the desaturation of [^{14}C]oleoyl into [^{14}C]linoleoyl and [^{14}C]linolenoyl residues in PC was low. Later on, the radioactivity of oleoyl residues decreased while that of the polyunsaturated residues increased and finally exceeded that of the oleoyl residues. These results are in good agreement with the hypothesis of a direct desaturation of oleoyl residues esterified in PC. After 30 h, a decrease in the radioactivity of polyunsaturated acids in the PC molecules was observed. The [^{14}C]oleoyl groups in PC remained always more heavily labelled than the [^{14}C]linoleoyl or -linolenoyl groups; however, the labelling of both polyunsaturated residues gradually increased. Fig. 4B shows that in the linoleate desaturase activity affected the PC molecules more strongly than the PE molecules; 30 h after precur-

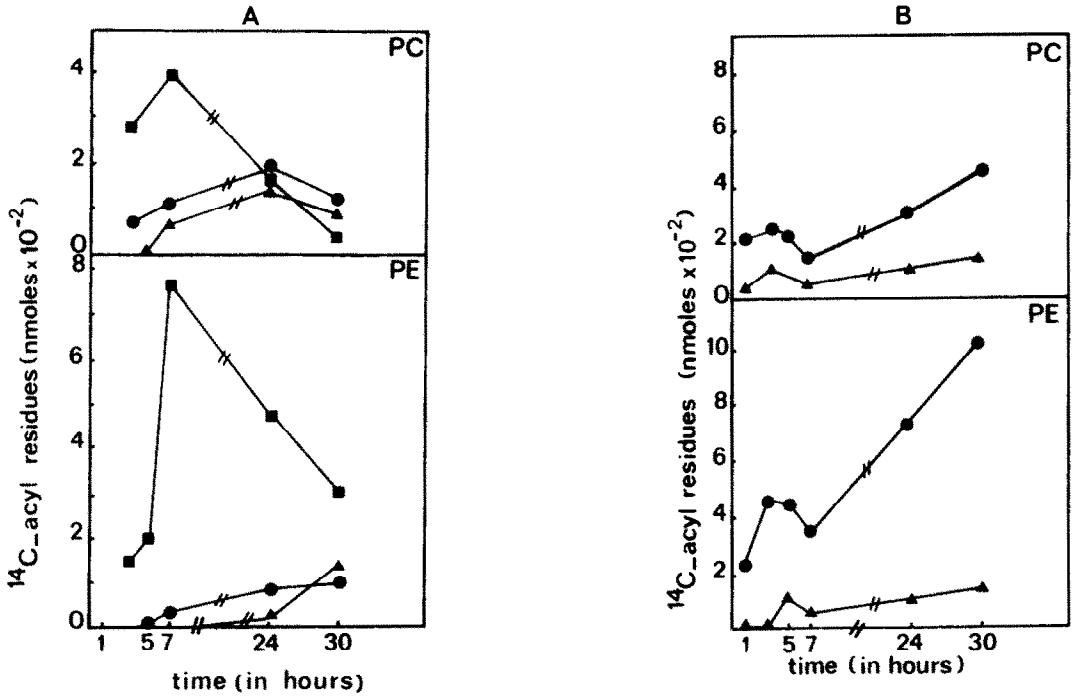

Fig. 4. Desaturation of [^{14}C]oleoyl residues (A) or [^{14}C]linoleoyl residues (B) within PC and PE. (■—■) [^{14}C]oleoyl; (●—●) [^{14}C]linoleoyl; (▲—▲) [^{14}C]linolenoyl residues.

sor deposit, 44% of [^{14}C]linoleoyl–PC and only 14% of [^{14}C]linoleoyl–PE was desaturated into [^{14}C]linolenoyl–PC and PE, respectively.

3.5. *Dependence of desaturation intensity on the growth stages*

Fig. 5A shows that 4–7-day old roots incorporated more radioactive precursor than younger or older roots. Moreover, oleate desaturase activity (measured by the quantity of [^{14}C]linoleate produced) was rather stable during root growth. On the contrary, no linoleate desaturase activity was

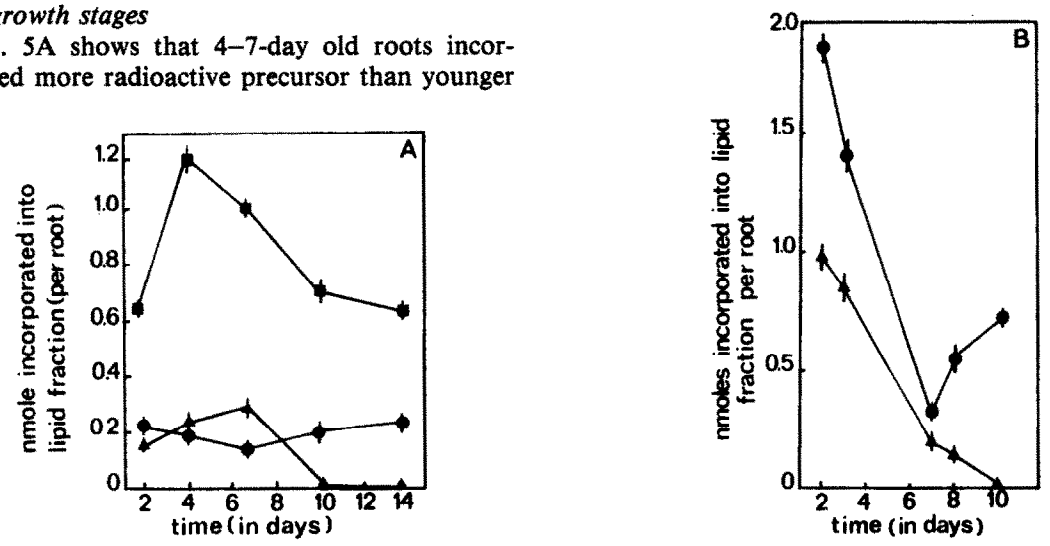

Fig. 5. Variation of oleate desaturase (A) or linoleate desaturase (B) activities with root age. Lipids were extracted 24 h after [^{14}C]oleate deposit or 30 h after [^{14}C]linoleate deposit (same symbols as for fig. 4).

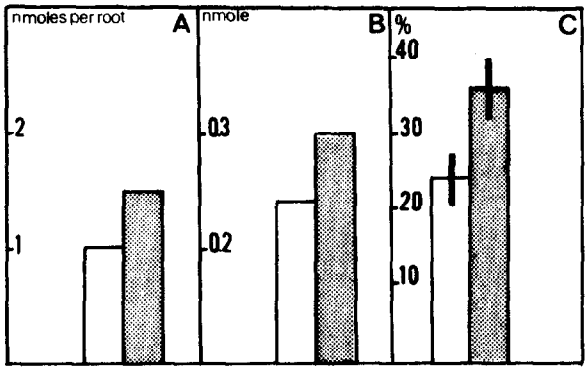

Fig. 6. Incorporation into roots (A), acylation into phospholipids (B) and desaturation (C) of [^{14}C]oleate in yellow lupine roots (10-day old) grown on either a normal (0.1 mM; ▭) or calcium-enriched (50 mM; (shaded) medium.

found in 10–14-day old roots, after having displayed a maximum between the 4th and 7th day of growth.

The maximum yields of [^{14}C]linoleate incorporation into seedlings (fig.5B) and of subsequent [^{14}C]linolenate biosynthesis were observed in 2-day old roots. In older roots, linolenate synthesis decreased gradually until the 10th day.

3.6. *Effect of calcium on oleate desaturation*

As yellow lupin is a calcifuge plant, it was of interest to test the effect of calcium on oleate desaturation. Fig. 6 shows that [^{14}C]oleate incorporation into root lipids, acylation into phospholipids and desaturation into [^{14}C]linoleate increased when 10-day old seedlings were grown on a medium containing 50 mM calcium chloride instead of 0.1 mM, as usual.

4. DISCUSSION

Several enzymes are implied in oleate or linoleate acylation and desaturation, such as acylthiokinases [23], phospholipid-acylases and desaturases [5]. As other plants [8,11], yellow lupin plantlet roots also acylate labelled fatty acids into phospholipids (PC and PE). Any subsequent desaturation of [^{14}C]oleate and [^{14}C]linolenate takes place only under these conditions (at least for the labelling times used (0–30 h).

The kinetics of desaturation shows that PE is a less suitable substrate than PC for the desaturation of both oleate into linoleate and linoleate into linolenate. The lag period observed for polyunsaturated fatty acid synthesis in PE was longer than that in PC. These results suggest that PC could be the real substrate of oleate desaturase and linoleate desaturase, which is in agreement with [8,10,11]. Rapid exchange of acyl residues between PC and PE would occur according to the below scheme;

Such a scheme is in agreement with results obtained on fungi in [24]. Whether or not the exchange of acyl residues between different phospholipid molecules (transacylation reactions) requires the participation of transitory acyl–CoA thioesters, is not yet known but it has been assumed in [28]. Furthermore, in view of our results, one cannot eliminate definitely the hypothesis that desaturation occurs in PE as a real substrate, as suggested in [25].

The activity of the desaturation systems studied developed differently during root growth. Oleate desaturation activity was more or less stable, whereas linoleate desaturation activity was high in very young roots (2-day old), then decreased gradually during root growth. Changes in desaturation activities according to the physiological state of plant tissues have already been suggested [26–29] and are evidenced here once more. Moreover, desaturase activities could only be observed in seedlings less than 1-year old.

Absorption of calcium by the calcifuge plant is more intensive than in a calcicolous plant [30]. Here, it is shown that oleate desaturase activities are stimulated by a high calcium concentration in the culture medium. Since lipid unsaturation increases membrane fluidity and membrane permeability [31], calcifuge plant membranes would be more permeable to calcium in plants grown on a calcium-enriched medium. Such a pro-

$$\begin{array}{l} \qquad\qquad\qquad\qquad\qquad\nearrow \text{oleoyl–PE} \rightarrow \text{linoleoyl–PC} \rightarrow \text{linolenoyl–PC} \\ \text{NH}_4\text{-oleate} \rightarrow \text{oleic acid} \rightarrow \text{oleoyl–CoA} \quad\quad \downarrow \quad\quad \downarrow \quad\quad \downarrow \\ \qquad\qquad\qquad\qquad\qquad\searrow \text{oleoyl–PC} \xrightarrow{?} \text{linoleoyl–PE} \xrightarrow{?} \text{linolenoyl–PE} \end{array}$$

cess could possibly account for the calcifuge status of yellow lupin.

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