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Desaturation of oxygenated fatty acids in *Lesquerella* and other oil seeds

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Abstract. Species of the genus *Lesquerella*, within the Brassicaceae family, have seed oils containing hydroxy fatty acids. In most *Lesquerella* species, either lesquerolic (14-hydroxy-eicosa-11-enoic), auricolic (14-hydroxy-eicosa-11,17-dienoic) or densipolic (12-hydroxy-octadeca-9,15-dienoic) acid dominates in the seed oils. Incubations of developing seed from *Lesquerella* species with 1-^{14}C-fatty acids were conducted in order to study the biosynthetic pathways of these hydroxylated fatty acids. [^{14}C]Oleic (octadeca-9-enoic) acid, but not [^{14}C]linoleic (octadeca-9,12-dienoic) acid, was converted into the hydroxy fatty acid, ricinoleic (12-hydroxy-octadeca-9-enoic) acid, which was rapidly desaturated to densipolic (12-hydroxy-octadeca-9,15-dienoic) acid. In addition, [^{14}C]ricinoleic acid added to *Lesquerella* seeds was efficiently desaturated at the Δ15 carbon. A pathway for the biosynthesis of the various hydroxylated fatty acids in *Lesquerella* seeds is proposed. The demonstration of desaturation at position Δ15 of a fatty acid with a hydroxy group at position Δ12 in *Lesquerella* prompted a comparison of the substrate recognition of the desaturases from *Lesquerella* and linseed. It was demonstrated that developing linseed also was able to desaturate ricinoleate at position Δ15 into densipolic acid. In addition, the linseed Δ15 desaturase was able to desaturate vernolic (12,13-epoxy-octadeca-9-enoic) acid and safflower microsomal Δ12 desaturase was able to desaturate 9-hydroxy-stearate. Thus, hydroxy and epoxy groups may substitute for double bonds in substrate recognition for oil-seed Δ12 and Δ15 desaturases.

Key words: Desaturation – Fatty acid – Hydroxylation – *Lesquerella* – Oil seed

Introduction

A limited number of plant species accumulate seed oils (triacylglycerols) with hydroxylated acyl groups. The best-known example is castor bean (*Ricinus communis*) with seed triacylglycerols in which nearly 90% of the acyl groups are ricinoleate (12-hydroxy-octadeca-9-enoate, 12-OH-18:1). It has been shown that ricinoleate in castor bean is synthesised by direct Δ12 hydroxylation of oleate (octadeca-9-enoate, 18:1) esterified to phosphatidylcholine (Morris 1970; Bafor et al. 1991). The microsomal hydroxylase enzyme is similar in many of its biochemical properties to the microsomal Δ12 desaturase, responsible for the desaturation of oleic acid to linoleic (octadeca-9,12-dienoic) acid, such as substrate and regio-specificity, b_5 electron-transport system and inhibition with cyanide, but not with carbon monoxide (Bafor et al. 1991; Smith et al. 1992). Different species of the genus *Lesquerella*, within the Brassicaceae family, have seed oils containing hydroxy fatty acids. In most *Lesquerella* species, ricinoleic acid is a minor component and either lesquerolic (14-hydroxy-eicosa-11-enoic, 14-OH-20:1), auricolic (14-hydroxy-eicosa-11,17-dienoic, 14-OH-20:2) or densipolic (12-hydroxy-octadeca-9,15-dienoic, 12-OH-18:2) acid dominates (Badami and Patil 1981; Hayes et al. 1995). It can be anticipated that lesquerolic and auricolic acids are synthesized in *Lesquerella* seeds by elongation of ricinoleic and densipolic acid, respectively, with two carbon units. The biosynthesis of densipolic acid is, however, puzzling since it contains a Δ15 double bond. The Δ15 desaturase in *Arabidopsis thaliana* has been shown to have a strong preference for fatty acids with a Δ12 double bond (Browse et al. 1989; Miquel and Browse 1992). If this is a requirement for all Δ15 desaturases, densipolic acid would have to be synthesised via hydration of the Δ12 double bond of alpha-linolenic acid (octadeca-9,12,15-trienoic acid); thus the hydroxylation enzyme in *Lesquerella* would be distinctly different from the castor-bean Δ12 hydroxylase.

In order to resolve the biosynthetic pathways of hydroxylated fatty acids in *Lesquerella* seeds, we have conducted a number of in-vivo experiments with exogenous [^{14}C]fatty acids supplied to developing seeds of two

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Abbreviations: GLC = gas-liquid chromatography; lysoPC = palmitoyl-lysophosphatidylcholine; PC = phosphatidylcholine

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Lesquerella species. On the basis of the results obtained, we have investigated the metabolism of hydroxy- and epoxy-fatty acids in developing linseed and in microsomal fractions of developing safflower seeds. Our results show that a hydroxy group or an epoxy group can substitute for a double bond in substrate recognition for extraplastidial plant desaturases.

Materials and methods

Plant material. Lesquerella auriculata, Lesquerella kathryn (seeds from both species were a generous gift from Dr. A.E. Thompson, USDA, Phoenix, Texs, USA), safflower (*Carthamus tinctorius* L. cv. Gila), and linseed (*Linum usitatissimum* L. cv. Iduna) plants were grown from seeds in a controlled environment in the 16 h photoperiod and 8- h night at 25/20 °C day/night temperatures. Flowers were hand-pollinated for good seed settings. Seeds harvested at mid stage of development (18 d after flowering in the case of safflower and 23–28 d for the other seeds) were used for further manipulations.

Chemicals. Adenosine 5'-triphosphate, CoA, bovine serum albumin (BSA), palmitoyl-lysophosphatidylcholine [lysoPC] and NADH were obtained from Sigma (St. Louis, Mo., USA). [1-^{14}C]Oleic (octadeca-9-enoic, 18:1) acid, specific radioactivity 2.0 GBq·mmol^{-1}, [1-^{14}C]linoleic (octadeca-9,12-enoic) acid, specific radioactivity 2.0 GBq·mmol^{-1}, [1-^{14}C]linolenic (octadeca-9,12,15-trienoic) acid, specific radioactivity 2.0 GBq·mmol^{-1} and [1-^{14}C]acetate (specific radioactivity 2.1 GBq·mmol^{-1}) were obtained from Amersham (Bucks, UK). 9-Hydroxy-stearic [9-(*R*)-hydroxy-octadecanoic, 9-OH-18:0] acid was synthesised by hydrogenation of methyl ester dimorphecolic [9-(*R*)-hydroxy-octadeca-10(*E*),12(*E*)-dienoic] acid by the method devised by Appelqvist (1972) followed by alkaline hydrolysis to yield the free acid. Methyl esters of dimorphecolic acid were isolated from fatty-acid methyl esters of seed oil from *Dimorphoteca pluvialis* (seeds were kindly provided by Dr. R. Kleiman, USDA, Peoria, Ill., USA) by TLC separation on silica gel 60 plates (Merck, Darmstadt, Germany) developed in hexane/diethyl-ether/acetic acid (50:50:1, by vol.). [1-^{14}C]Ricinoleic [12(*R*)-hydroxy-octadeca-9-enoic, 12-OH-18:1] acid (specific radioactivity 0.39 GBq·mmol^{-1}) was synthesised biochemically by the castor-bean microsomal Δ12 hydroxylation of [1-^{14}C]oleate according to Bafor et al. (1991). [1-^{14}C]Vernolic [12(*S*),13(*R*)-epoxy-octadeca-9(*Z*)-enoate] acid, specific radioactivity 63 MBq·mmol^{-1} and [1-^{14}C]12(*S*),13(*R*)-epoxy-octadeca-9(*Z*),15(*Z*)-dienoic acid, specific radioactivity 1.0 GBq·mmol^{-1}, were synthesised biochemically from [1-^{14}C]linoleate and [1-^{14}C]linolenate, respectively, by the microsomal Δ12 epoxygenase from *Euphorbia lagascae* seeds according to Ståhl et al. (1995).

^{14}C-Labelling experiments. Developing embryos were dissected from their seed coats and stored in ice-cold 0.1 M potassium phosphate buffer (pH 7.2) until incubations were initiated. Each incubation contained approximately 20 embryos in 0.2 ml of 0.1 M potassium phosphate buffer (pH 7.2) and either 30 nmol of the ammonium salt of 1-^{14}C-fatty acids, or 16 nmol of [1-^{14}C]acetate. After incubations at 25 °C for times indicated in the tables, the buffer solutions were removed and the embryos were washed with 3 × 1 ml of buffer. The embryos were subsequently either incubated further in fresh buffer at 25 °C for specified times or homogenized with an Ultra-Turrax homogeniser (IKA Werke, Staufen, Germany) in methanol/chloroform (2:1, v/v) followed by extraction of lipids into chloroform according to the method devised by Bligh and Dyer (1959).

Incubations with safflower microsomes. Microsomal fractions were prepared from developing safflower embryos according to Stobart et al. (1986). Microsomal fractions (equivalent to 1.5 mg of protein) were incubated at 25 °C with 100 nmol of 9(*R*)-hydroxy-stearate bound to 10 mg of BSA, 10 μmol of ATP, 2 μmol CoA, 5 μmol of $MgCl_2$, 200 nmol of 16:0-lyso PC in the presence or absence of 2 μmol of NADH, in a total volume of 1 ml 0.1 M potassium phosphate buffer (pH 7.2). The incubations were terminated after 60 min of incubation and the lipids were extracted into chloroform by the method of (Bligh and Dyer 1959).

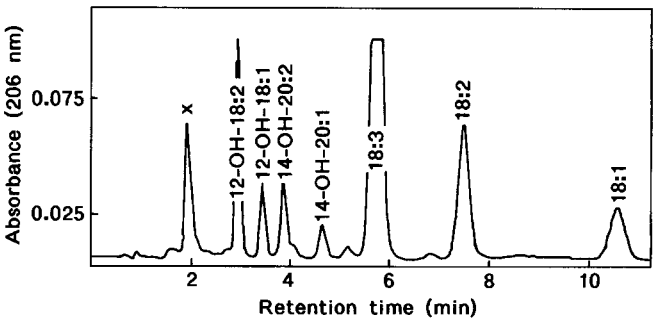

Fig. 1. High-performance liquid chromatographic separation of unsaturated fatty-acid methyl esters prepared from seed extracts of *Lesquerella* species. The sample contained a mixture of methyl esters from *L. fendleri, L. kathryn, L. auriculata* and *L. arctica.* seeds. The peak x is an unretained, UV-absorbant non-fatty-acid compound present in the sample. For separation conditions, see *Materials and methods* section

Analytical procedures. Aliquots of the lipid (chloroform) fractions from the incubations were assayed for radioactivity by liquid scintillation counting or used for preparation of fatty-acid methyl esters by heating in 4% methanolic HCl (w/w). Remaining lipids were separated on silica gel TLC plates (Merck silica 60) in chloroform/methanol/acetic acid/water (170:30:20:7, by vol.). Lipid areas corresponding to phosphatidylcholine (PC), free acid (hydroxylated and non-hydroxylated), neutral complex lipids (all compounds migrating above free fatty acids) and the rest of the lanes were removed for liquid scintillation counting or for methylation by methanolic HCl.

Radioactive distribution between the acyl groups of the methyl esters was determined both by radio gas-liquid chromatography (GLC) and radio-HPLC. The radio-GLC separations were carried out on a 2.5 m × 3 mm i.d. stainless-steel column containing 3% SP-2300 on a Supelcoport 100/120 mesh (Supelco, Bellefonte, P., USA). The HPLC separations were performed on 250 × 4.6 mm i.d. Nucleosil-RP18 (10 μm) column Shandon, Cheshire, UK with methanol/acetonitrile/water (64:28:8, by vol.) as eluent and a flow rate of 2 ml·min^{-1}. Radioactivity of the effluent was monitored in a Ramona 5 LS system (Raylest, Straubenhardt, Germany) by continuous liquid scintillation counting. The HPLC allowed rapid separation of all unsaturated hydroxylated and non-hydroxylated fatty acids present in *Lesquerella* seeds within 11 min (Fig. 1). ^{14}C-Fatty-acid methyl ester peaks with activities as low as 3 Bq could be quantified by the Ramona system, This proved to be essential for measuring the low amount of ^{14}C-hydroxylation products in incubations of *Lesquerella* seeds with added [^{14}C]oleic acid. Acyl composition was determined by GLC analysis of the fatty-acid methyl esters and acyl groups were quantified relative to added heptadecanoic acid (17:0) methyl ester.

Results

Fatty-acid composition of Lesquerella seeds. The total fatty-acid composition of the lipids form seeds of *Lesquerella auriculata* and *Lesquerella kathryn* is shown in Table 1. Both species contain about the same amount of hydroxy fatty acids (44–48%). *Lesquerella kathryn* seeds have only hydroxylated fatty acids with a chain length of C_{18}, with densipolic acid as the dominating acyl group

Table 1. Acyl composition of *Lesquerella kathryn* and *Lesquerella auriculata* seed lipids

Acyl group	Relative abundance of acyl group (%)[a]	
	L. kathryn	*L. auriculata*
16:0	5.5	4.91
16:1	1.7	1.7
18:0	4.3	5.3
$18:1^{\Delta 9}$	30.0	28.6
$18:2^{\Delta 9,12}$	2.9	2.9
$18:3^{\Delta 9,12,15}$	11.3	8.5
12-OH-$18:1^{\Delta 9}$ (ricinoleic acid)	10.3	6.5
12-OH-$18:2^{\Delta 9,15}$ (densipolic acid)	33.9	1.7
14-OH-$20:1^{\Delta 11}$ (lesquerolic acid)	0.0	13.7
14-OH-$20:2^{\Delta 11,17}$ (auricolic acid)	0.0	26.0

[a] Calculated as area % of total fatty-acid methyl-ester peaks from GLC chromatograms

(34%) and with a smaller amount of ricinoleic acid (10%). *Lesquerella auriculata* seeds contain four types of hydroxy fatty acid, the C_{18} fatty acids ricinoleic and densipolic, and the C_{20} acyl groups, lesquerolic and auricolic acids, of which auricolic acid is the most abundant (26%).

Metabolism of [^{14}C]*oleate by L. kathryn seeds.* Detached developing seeds of *L. kathryn* were incubated with ammonium salts of various 1-^{14}C-fatty acids and the incorporation into different lipids and acyl groups was followed during 10- and 40-min incubations (Table 2A, B). In incubations with [^{14}C]oleate, the majority of the label remained in free fatty acids. Only about 7–11% of the label was recovered in PC and nearly all of the remainder of the activity resided in complex neutral lipids (Table 2A). Although radioactivity hydroxylated fatty acid was under the detection limit in an aliquot of the methyl esters of total lipids prepared after 10-min incubation, small amounts of radioactive densipolic and ricinoleic acid were found in PC, with ricinoleic acid being the predominant radioactive hydroxylated acid (Table 2B). No radioactive linoleic or linolenic acid was found. After 40 min of incubation, the ^{14}C-hydroxylated fatty acids amounted to about 2% of the total fatty acids. Densipolic acid was labelled at a considerably higher level than ricinoleic acid in both total lipids and in PC. Also some [^{14}C]linoleate and [^{14}C]linolenate were found, after this period. Incubation times longer than 40 min did not further change the relative proportions between the radioactive acyl groups (data not shown).

Metabolism of [^{14}C]*linoleate by L. kathryn seeds.* Incubations of developing *L. kathryn* embryos with [^{14}C]linoleate showed similar distribution patterns into the different lipids as in the [^{14}C]oleate feeding experiments (Table 2A). However, no radioactive hydroxylated fatty acids were detected (Table 2B). Condiserable amounts of [^{14}C]linolenic acid were formed; the highest percentage was found in PC (16 and 37% at 10 and 40 min, respectively). The relative proportions of the different radioactive acyl groups did not change at incubation times longer than 40 min (data not shown).

Metabolism of [^{14}C]*ricinoleate by L. kathryn seeds.* [1-^{14}C]Ricinoleic acid was taken up and esterified into complex lipids by the *L. kathryn* seeds at a higher rate than both [^{14}C]oleate and [^{14}C]linoleate (Table 2A). A higher proportion of the radioactivity was also incorporated into PC at 10 min (23.5%), but this activity rapidly decreased to only 3% at 40 min of incubation. At 10 min of incubation, about 7% of the radioactivity in total fatty acids resided in densipolic acid, which was about the same as the proportion of labelled densipolic acid in PC at that time (Table 2B). After 40 min of incubation, the proportion of total radioactivity in densipolic acid had increased to 13%. The radioactivity in PC at this time was too low for analysis of the distribution of radioactivity among the acyl groups.

Metabolism of [^{14}C]*ricinoleate by L-auriculata seeds.* When developing seeds of *L. auriculata* were fed [^{14}C]ricinoleic acid, about 21% of the radioactivity was recovered in densipolic acid after 30 min and the proportion of label in this acid had increased to 26% after 120 min (Table 3). A small proportion of the label (2–3%) was also found in lesquerolic acid but no radioactivity was recovered in auricolic acid, which is the major fatty acid in *L. auriculata* seeds. Incubation times longer than 120 min did not change the relative proportions of radioactivity among the acyl groups (data not shown).

Metabolism of [^{14}C]*acetate by L. auriculata seeds.* Since the *L. auriculata* seeds did not metabolize [^{14}C]ricinoleic acid into radioactive auricolic acid, [^{14}C]acetate feeding was performed to investigate if the seeds selected for the experiments were active in auricolic acid synthesis. The results showed that these seeds indeed were very active in this respect. The greatest proportion of the label from [^{14}C]acetate was recovered in auricolic acid at incubation times as short as 30 min. Oleate was the second most highly labelled acid with 40% of the radioactivity (Table 3B). After 120 min of incubation, the radioactivity in oleate had decreased with a corresponding increase in radioactive densipolic acid. At most, only traces of radioactive ricinoleic acid were seen. Neither lesquerolic nor auricolic acids increased in radioactivity between 30 and 120 min of incubation.

Substrates for Δ15 *desaturation in linseed.* The Δ15 desaturation of ricinoleic acid in *L. kathryn* and *L. auriculata* seeds was quite unexpected for us. The next step was to investigate whether the Δ15 desaturase, which is responsible for the synthesis of densipolic acid in *Lesquerella* species, was different in its substrate recognition from the Δ15 desaturase catalyzing the desaturation of linoleic acid to linolenic acid in other oil seeds. A comparison was made between the metabolism of [^{14}C]ricinoleate and that of [^{14}C]linoleate in developing linseed embryos. After 60 min of incubation, the uptake of [^{14}C]linoleate by the linseed embryos was approximately double that of [^{14}C]ricinoleate (Table 4A). About 27% of the label from [^{14}C]linoleate resided in free acids compared to only 1%

Table 2A, B. Distribution of ^{14}C-activity in phosphatidylcholine (PC), free fatty acids (FFA) and complex neutral lipids (**A**) and in acylgroups (**B**) in detached developing *Lesquerella Kathryn* seeds after incubations with ammonium salt of various 1-^{14}C-fatty acids. The seeds were first incubated with ^{14}C-fatty acids for 10 min and then rinsed with buffer and subsequently either extracted or the seeds were incubated a further 30 min before extraction of the lipids

A

Substrate	Incubation time (min)	Uptake of [^{14}C]-fatty acids (nmol)	Distribution of radioactivity (% of total lipid fraction)		
			PC	FFA	Complex neutral lipids
[^{14}C]Oleate	10	6.2	6.7	69.0	15.2
	40	7.1	10.8	56.1	29.5
[^{14}C]Linoleate	10	4.7	7.3	57.0	33.0
	40	3.1	12.8	24.4	58.2
[^{14}C]Ricinoleate	10	10.8	23.5	13.2	64.2
	40	14.4	3.2	13.3	85.0

B

Substrate	Incubation time (min)	Acyl group	Distribution of radioactivity among acyl groups (% radioactivity of each fraction)[a]	
			PC fraction	Total lipid fraction
[^{14}C]Oleate	10	18:1	85.3	100.0
	40		92.9	96.0
	10	18:2	n.d.[c]	n.d.
	40		n.d	1.0
	10	18:3	n.d.	n.d.
	40		n.d.	0.4
	10	12-OH-18:1	0.9	n.d.
	40		0.3	0.4
	10	12-OH-18:2	0.2	n.d.
	40		2.5	1.7
[^{14}C]Linoleate	10	18:2	80.2	93.3
	40		61.8	72.0
	10	18:3	16.3	3.5
	40		37.3	16.6
	10	12-OH-18:1	n.d.	n.d.
	40		n.d.	n.d.
	10	12-OH-18:2	n.d.	n.d.
	40		n.d.	n.d.
[^{14}C]Ricinoleate	10	12-OH-18:1	86.9	92.5
	40		–[b]	85.5
	10	12-OH-18:2	8.3	7.0
	40		–[b]	13.1

[a] Calculated relative to total eluted radioactivity from HPLC column, including unidentified very polar metabolites
[b] Not analyzed because of too low radioactivity in the lipid
[c] n.d. = not detected

of the label from the incubations with [^{14}C]ricinoleate. About 17% of the total activity was recovered as [^{14}C]linolenic acid in the incubation with [^{14}C]linoleate whereas nearly 50% was recovered as [^{14}C]densipolic acid after the feeding with [^{14}C]ricinoleate (Table 4B). The identity of [^{14}C]densipolic acid was established with high certainty by analysing the sample both by reversed-phase HPLC chromatography and radio-GLC. Since the results showed that a hydroxy group at position Δ12 could substitute for a double bond at positions Δ12, 13 for the Δ15 desaturase in linseed, the question arose as to whether a Δ9 unsaturated fatty acid with an epoxy group at Δ12,13 also could be an effective substrate for the linseed desaturase. Therefore, developing linseed was incubated with [^{14}C]vernolic acid (12-epoxy-octadeca-9-enoic acid). After 120 min of incubation about 14% of the

Table 3A, B. Distribution of ^{14}C-activity in phosphatidylcholine (PC), free fatty acids (FFA) and complex neutral lipids (**A**) and among acyl groups (**B**) in detached developing *Lesquerella auriculata* seeds after incubations with ammonium salt of [1-^{14}C]ricinoleic fatty acid or [1-^{14}C]acetate. The seeds were first incubated with ^{14}C-substrates for 30 min and then rinsed with buffer and subsequently either extracted or the seeds were incubated a further 90 min before extraction of the lipids

A

Substrate	Incubation time (min)	Uptake of ^{14}C-label (nmol)	Distribution of radioactivity (% of total lipid fraction)		
			PC	FFA	Complex neutral lipids
[^{14}C]Ricinoleate	30	15.0	14.9	10.0	65.6
	120	13.0	2.6	7.1	89.5
[^{14}C]Acetate	30	5.7	26.5	0.3	68.8
	120	5.0	10.1	1.3	83.9

B

Substrate	Incubation time	Acyl group (min)	Distribution of radioactivity among acyl groups (% radioactivity of total radioactiviity)[a]
[^{14}C]Ricinoleate	30	12-OH-18:1	73.1
	120		68.7
	30	12-OH-18:2	21.4
	120		26.3
	30	14-OH-20:1	2.1
	120		3.4
	30	14-OH-20:2	0.0
	120		0.0
[^{14}C]Acetate	30	18:1	40.0
	120		27.0
	30	18:2	trace
	120		n.d.[b]
	30	18:3	1.7
	120		1.3
	30	12-OH-18:1	trace
	120		n.d.
	30	12-OH-18:2	1.8
	120		12.6
	30	14-OH-20:1	11.2
	120		11.3
	30	14-OH-20:2	44.6
	120		46.3

[a] Calculated relative to total eluted radioactivity from HPLC column, including unidentified very polar metabolites
[b] n.d. = not detected

radioactivity resided in a compound which had the same retention time as 12-epoxy-octadeca-9,15-dienoate, both on reversed-phase HPLC and radio-GLC (Table 4B).

Desaturation of 9-hydroxy-stearate by safflower microsomes. In order to establish if a hydroxy group could substitute for a double bond in the substrate recognition for the Δ12 desaturase, microsomal fractions of developing safflower seeds were utilized. These microsomes have an extremely active NAD(P) H dependent Δ12 desaturase which converts oleate in PC to linoleate (Stobart and Stymne 1985). Incubation of microsomes with 9-hydroxy-stearate, ATP, CoA, 16:0-lysoPC and NADH converted about half of the 9-hydroxy-stearate recovered in the chloroform fraction into an acyl group which eluted just after the added fatty-acid substrate on GLC (Fig. 2B). This product was not seen in incubations in the absence of NADH or in the presence of NADH but in the absence of 9-hydroxy-18:0 (Fig. 2A,C). The reaction product disappeared upon the hydrogenation of the methyl esters with a corresponding percentage increase in methyl esters of 9-hydroxy-stearate (data not shown), thereby demonstrating that safflower microsomes could catalyze an NADH-dependent dehydrogenation of 9-hydroxy-stearate to, tentatively, 9-hydroxy-octadeca-12-enoic acid (iso-ricinoleic acid).

Table 4A, B. Distribution of ^{14}C-activity in phosphatidylcholine (PC), free fatty acids (FFA) and complex neutral lipids (**A**) and among acyl groups (**B**) in detached developing linseed embryos after incubations with the ammonium salt of either [1-^{14}C]linoleic acid (60 min of incubation), [1-^{14}C]ricinoleic acid (60 min of incubation) or [1-^{14}C]vernolic acid (120 min of incubation). The seeds were first incubated with ^{14}C-fatty acids and then rinsed with buffer and subsequently extracted for lipids analysis

A

Substrate	Uptake of [^{14}C]-fatty acids (nmol)	Distribution of radioactivity (% of total lipid fraction)		
		PC	FFA	Complex neutral lipids
[^{14}C]Linoleate	22.9	29.4	27.0	28.4
[^{14}C]Ricinoleate	17.4	17.6	1.3	72.9
[^{14}C]Vernoleate	32.4	17.9	1.8	70.6

B

Substrate	Acyl group	Distribution of radioactivity among acyl groups (% of total radioactivity)[a]
[^{14}C]Linoleate	18:2	38.2
	18:3	16.8
[^{14}C]Ricinoleate	12-OH-18:1	48.6
	12-OH-18:2	49.9
[^{14}C]Vernoleate	12-epoxy-18:1	85.0
	12-epoxy-18:2[b]	13.7

[a] Calculated relative to total eluted radioactivity from HPLC column, including unidentified very polar metabolites

[b] Tentatively identified as 12-epoxy-octadeca-9,15-dienoic acid

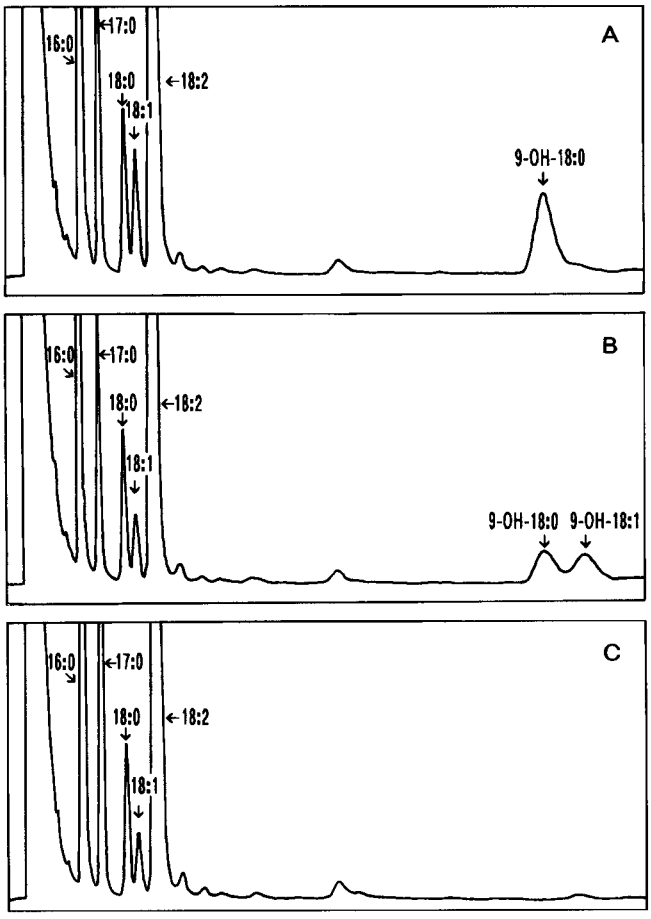

Fig. 2A–C. Gas-liquid chromatography of fatty-acid methyl esters prepared from reaction mixtures of safflower microsomes, 16:0-lysoPC, ATP, and CoA incubated with either 9-OH-stearic acid (**A**), 9-OH-stearic acid and NADH (**B**), or with NADH but without 9-OH stearic acid (**C**). Methyl-heptadecanoate (17:0) was added in the methylation procedure as an internal standard

Discussion

[^{14}C]Oleate but not [^{14}C]linoleate was converted into the hydroxylated fatty acids, ricinoleic and densipolic acid, in developing seeds of *L. kathryn*. Both [^{14}C]linoleate and [^{14}C]ricinoleate were efficiently desaturated by a Δ15 desaturase in *L. kathryn* seeds to yield radioactive linolenic and densipolic acids, respectively, the latter being the dominating fatty acid in these seeds. These results suggest that both ricinoleic and densipolic acids are synthesised in *Lesquerella* via the same pathway as previously has been shown for biosynthesis of ricinoleic acid in castor bean, i.e. via a direct Δ12 hydroxylation of oleate (Morris 1970) but with a subsequent Δ15 desaturation of ricinoleic acid to yield densipolic acid.

Whereas *L. kathryn* seeds only contain C_{18} hydroxylated fatty acids, *L. auriculata* seeds have C_{20} hydroxylated fatty acids as dominating acyl groups. It was shown that *L. auriculata* seeds, like *L. kathryn* seeds, could efficiently convert added [^{14}C]ricinoleic acid into densipolic acid but also, to a small extent, increase its length to produce radioactive lesquerolic acid. However, no radioactive auricolic acid was detected. In sharp contrast to the [^{14}C]ricinoleic acid feeding experiments, the majority of the radioactivity from added [^{14}C]acetate was

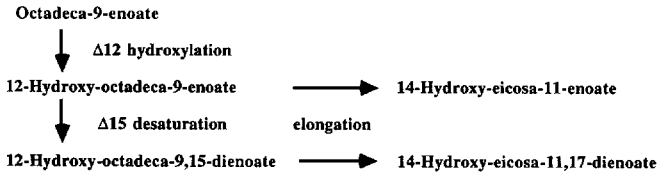

Fig. 3. Proposed biosynthetic pathway for hydroxylated fatty acids in *Lesquerella* seeds

rapidly incorporated into auricolic acid in *L. auriculata* seeds. The demonstration of high incorporation rates of radioactivity from [^{14}C]acetate into C_{20} hydroxylated fatty acids but the extremely low incorporation of radioactivity into these acids from [^{14}C]ricinoleic or [^{14}C]densipolic acids indicates that the added and newly synthesised ^{14}C-labelled hydroxylated C_{18}fatty acids are greatly diluted with an endogenous pool before elongation. The low hydroxylation rates of [^{14}C]oleate compared to the Δ15 desaturation of [^{14}C]linoleate and [^{14}C]ricinoleic acid could also be explained by a great difference in dilution with endogenous substrates for the Δ12 hydroxylation versus Δ15 desaturation. The prime site for Δ12 hydroxylation as well as the Δ15 desaturation in oil seeds is probably PC (Bafor et al. 1991; Stymne et al. 1992). In developing *L. auriculata* and *L. kathryn* seeds, oleate is the dominating acyl group in PC (33 and 44%) and is about 15-fold higher in concentration than the sum of linoleate and ricinoleate (data not shown).

The proposed biosynthetic pathways of the different hydroxylated fatty acids in *Lesquerella* seeds are depicted in Fig. 3. Although we have not been able to demonstrate the elongation of densipolic acid into auricolic acid in *L. auriculata*, we consider this step likely. Before conclusions about the lipid substrates for the different reactions can be made, more detailed time-courses and in-vitro studies of the different enzymatic steps have to be performed. We were unsuccessful in obtaining subcellular fractions from *Lesquerella* seeds active in desaturation and elongation (data not shown). However, based on the available data we would like to suggest the following plausible flow of acyl groups in the synthesis of auricolic acid in *L. auriculata* seeds. Oleate in PC is hydroxylated to ricinoleic acid, which in its turn is rapidly converted to densipolic acid. Densipolic acid is rapidly removed from PC, perhaps by a phospholipase specific for hydroxylated fatty acids (Stähl et al. 1995). It appears, as mentioned above, that [^{14}C]ricinoleic acid and [^{14}C]densipolic acid are diluted with a large pool of endogenous C_{18} hydroxylated fatty acids before elongation to lesquerolic and auricolic acids. Since the only large pool of such acids in the seeds is the neutral lipids (data not shown), it is possible that these lipids can release hydroxylated acyl groups for elongation by yet-uncharacterized mechanisms. In this context it is interesting to note that the polyunsaturated hydroxy fatty acids in *Lesquerella* seed oils are nearly exclusively found in acylglycerol estolides with four or five acyl groups and not in triacylglycerols (Hayes et al. 1995).

Another interesting feature of *Lesquerella* species with C_{20} acids is the very low percentage of C_{20} non-hydroxylated fatty acids (< 3%) in the seed oils (Hayes et al. 1995). The *Lesquerella* genus belongs to the Brassicaceae, whose members frequently have C_{20} and C_{22} monounsaturated fatty acids, derived from elongation of oleic acid, in their seed oils, but always very low amounts of elongated polyunsaturated fatty acids. Thus, it appears that the Δ12 hydroxy group on one hand acts like a double bond in the Δ15 desaturation but on the other hand makes the acyl group favorably compete with other monounsaturates as substrates for elongation. This fact argues for completely different acyl-substrate recognition mechanisms for the desaturases and the elongases.

Both *Lesquerella* and linseed Δ15 desaturases could effectively desaturate ricinoleic acid to densipolic acid. Further, the linseed Δ15 desaturase could also desaturate 12,13-epoxy-octadeca-9-enoic acid (vernolic acid), and the Δ12 desaturase in safflower microsomes could desaturate 9-hydroxy-stearate. It has been shown from studies with *Arabidopsis thaliana* mutated in the plastidic and the extraplastidic Δ12 desaturase genes that the Δ15 desaturase has a strong requirement for the Δ12 double bond since only little, or no, 18: $2^{\Delta 9,15}$ fatty acids were formed in the mutants (Browse et al. 1989; Miquel and Browse 1992). The presence of $18{:}1^{\Delta 12}$ fatty acids has never been reported in plants. It is thus evident that a hydroxy (or an epoxy) group could substitute for a double bond in substrate recognition for both the Δ12 and Δ15 desaturases, which are responsible for the production of the common polyunsaturated fatty acids in plants. Since an epoxy, or a hydroxy, group does not cause the same structural changes as a double bond in a fatty-acid molecule, it is obvious that the substrate-recognition sites of desaturases have considerable flexibility for the three-dimensional structure of the acyl group. Thus, the acyl group may not fit into a preformed 'pocket' of the desaturases but perhaps align with any electrophilic domains, e.g. double bonds, hydroxy groups and epoxy groups, along the acyl chain.

Little systematic evaluation of the substrate-recognition sites of these desaturases has been made since the excellent study by Howling et al. (1972) working with in-vivo feeding experiments with *Chlorella* cells and castor-bean developing endosperm. Since then, it has been shown that there are two Δ12 and two Δ15 desaturases with different subcellular localisations, one in the chloroplast and one in the ER (Browse and Somerville 1991). The in-vivo feeding experiments done by Howling et al. (1972) would have monitored the conversions made by the ER desaturases since exogenously supplied long-chain fatty acids do not readily enter the plastid. Their results showed that monounsaturated acyl groups with 16, 17, 18 or 19 carbons and with a double bond at position Δ9 were all desaturated at the Δ12 carbon, thus demonstrating that the distance between the first double bond and the methyl end was not critical for substrate recognition. In contrast to these results it was later shown that a mutation in the *fad6* gene in *Arabidopsis thaliana* inhibited the chloroplastic desaturation both of $18{:}1^{\Delta 9}$ to $18{:}2^{\Delta 9,12}$ and of $16{:}1^{\Delta 7}$ to $16{:}2^{\Delta 7,10}$ (Browse et al. 1989). Also,

a mutation in the *fad7* gene blocked the chloroplast-located desaturation of both $18:2^{\Delta 9,12}$ to $18:3^{\Delta 9,12,15}$ and $16:2^{\Delta 7,10}$ to $16:3^{\Delta 7,10,13}$ (Browse et al. 1986), thereby suggesting that these desaturases recognized their substrates from the methyl end and should be called omega-6 and omega-3 desaturases. Thus, it appeared that the chloroplastic and ER desaturases responsible for linoleic acid synthesis had totally different substrate-recognition mechanisms. However, when the *fad6* gene was recently expressed in cyanobacteria, the produced desaturase catalysed the Δ12 desaturation of both endogenous $18:1^{\Delta 9}$ and $16:1^{\Delta 9}$ (Hitz et al. 1994). It is therefore obvious that the *fad6* gene neither codes for a omega-6 nor a Δ12 desaturase but inserts the double bond at a methylene-interrupted distance from the first double bond and towards the methyl end. From Howling et al.'s (1972) work with *Chlorella* it appears that the so-called ER Δ12 desaturase will also desaturate $16:1^{\Delta 7}$ at the Δ10 position. Since $18:1^{\Delta 6}$ (petroselinic acid) and $18:2^{\Delta 9,12}$ are present in high amounts in PC of developing carrot seeds but no $18:2^{\Delta 6,9}$ is present (Dutta et al. 1992), it is likely that the ER Δ12 desaturase requires a C_{18} fatty acid with a double bond (or another electrophilic domain) located more than six carbon atoms from the carboxylic end. Howling et al. (1972) reported a number of fatty acids which were not desaturated in in-vivo feeding experiments. However, these negative results do not necessarily mean that the acids actually are ineffective as substrates for the desaturases since it is not known if they were incorporated into phospholipids and thereby accessible for the desaturase enzymes. It can thus be noted that very little desaturation of 9-hydroxy-stearate was observed in incubations with the safflower microsomes in the work presented here unless high amounts of lyso-PC were added to the reaction mixture in order to increase the incorporation of the acyl substrate into phospholipids (data not shown).

It should be stressed that much more systematic work is required to establish the substrate recognition mechanisms of the plant desaturases responsible for the production of polyunsaturated fatty acids. In addition to providing information about the binding sites of the desaturases it will also give information about how seed oils with total novel and useful fatty acids could be created by introducing novel combinations of desaturases and other fatty-acid-modifying enzymes into transgenic oil crops.

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