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Accumulation of Ricinoleic, Lesquerolic, and Densipolic Acids in Seeds of Transgenic Arabidopsis Plants That Express a Fatty Acyl Hydroxylase cDNA from Castor Bean[1]

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A cDNA encoding the oleate 12-hydroxylase from castor bean (*Ricinus communis* L.) has previously been shown to direct the synthesis of small amounts of ricinoleic acid (12-hydroxyoctadec-*cis*-9-enoic acid) in seeds of transgenic tobacco plants. Expression of the cDNA under control of the *Brassica napus* napin promoter in transgenic *Arabidopsis thaliana* plants resulted in the accumulation of up to 17% of seed fatty acids as ricinoleate and two novel fatty acids that have been identified by gas chromatography-mass spectrometry as lesquerolic (14-hydroxyeicos-*cis*-11-enoic acid) and densipolic (12-hydroxyoctadec-*cis*-9,15-dienoic acid) acids. Traces of auricolic acid were also observed. These results suggest that either the castor hydroxylase can utilize oleic acid and eicosenoic acid as substrates for ricinoleic and lesquerolic acid biosynthesis, respectively, or Arabidopsis contains an elongase that accepts ricinoleic acid as a substrate. These observations are also consistent with indirect biochemical evidence that an *n*-3 desaturase is capable of converting ricinoleic acid to densipolic acid. Expression of the castor hydroxylase also caused enhanced accumulation of oleic acid and a corresponding decrease in the levels of polyunsaturated fatty acids. Since the steady-state level of mRNA for the oleate-12 desaturase was not affected, it appears that the presence of the hydroxylase, directly or indirectly, causes posttranscriptional inhibition of desaturation.

Ricinoleic acid (D-12-hydroxyoctadec-*cis*-9-enoic acid) is a hydroxylated fatty acid that accumulates in the seeds of castor bean (*Ricinus communis* L.) plants and other species. In castor 18:1-OH is restricted to the seed TAGs, where it constitutes 85 to 90% of the fatty acids (Atsmon, 1989). Because of the presence of the hydroxyl group, the fatty acid has many industrial uses (Smith, 1985). 18:1-OH also accumulates in the seeds of a variety of distantly related plant species representing at least 10 families, suggesting that its capacity to synthesize the hydroxy fatty acid has arisen many times independently in the course of evolution.

Several species of the genus *Lesquerella* (Brassicaceae) accumulate ricinoleate and other hydroxy fatty acids in seed neutral lipids (Hayes et al., 1995). However, in this genus ricinoleate is generally a minor constituent, and the major hydroxy fatty acids are either densipoleate (12-OH, $18{:}2^{\Delta 9,15}$), lesqueroleate (14-OH, $20{:}1^{\Delta 11}$), or auricoleate (14-OH, $20{:}2^{\Delta 11,17}$) (Hayes et al., 1995). *Lesquerella* has been the object of recent attention (Thompson et al., 1989; Roseberg, 1993) because hydroxylated fatty acids other than ricinoleate might be useful as sources of new monomers for the manufacture of industrial polymers such as nylon. However, these species have not yet been domesticated and will not be amenable to agricultural production in the near future.

Biochemical studies of ricinoleate metabolism in castor have established that the hydroxylase acts on oleate esterified to PC (Bafor et al., 1991). Specific phospholipase activity is thought to remove ricinoleate from membrane lipids immediately after synthesis, and the hydroxy fatty acid is transferred via the Kennedy pathway to TAG, where it accumulates (Bafor et al., 1991; Ståhl et al., 1995). Based on in vitro studies of the substrate specificity of the castor hydroxylase (Howling et al., 1972), the synthesis of both 18:1-OH and 20:1-OH can be explained by the action of a single hydroxylase on oleic acid and eicosenoic acid, respectively. In *Lesquerella* species, ricinoleate can be used as a substrate by one or more modifying enzymes before transfer to the neutral lipid pool. Recently, Engeseth and Stymne (1996) obtained evidence from radioactive labeling experiments with *Lesquerella* embryos that a microsomal *n*-3 desaturase can act on ricinoleate, resulting in the production of densipoleate. Hypothetically, 20:2-OH may be synthesized by the action of an *n*-3 desaturase on 20:1-OH.

We have recently described the isolation of a cDNA clone for the oleate 12-hydroxylase from castor (van de Loo et al., 1995). Constitutive expression of the hydroxylase cDNA in transgenic tobacco resulted in accumulation of low levels of ricinoleate in seed lipids, but not in leaves and roots. It is not known why higher levels of ricinoleate accumulation in transgenic tobacco were not obtained. To explore this

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Abbreviations: DAG, diacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; 35S, cauliflower mosaic virus 35S promoter; TAG, triacylglycerol; TMS, trimethylsilyl; X:Y, a fatty acyl group containing X carbon atoms and Y *cis*-double bonds: 16:0, palmitic acid; 16:3, *cis*-hexadecetrienoic acid (ω3,6,9); 18:0, stearic acid; 18:1, *cis*-oleic acid (ω9); 18:1-OH, ricinoleic acid; 18:2, *cis*-linoleic acid (ω6,9); 18:2-OH, densipolic acid; 18:3, *cis*-linolenic acid (ω3,6,9); 20:1, eicosenoic acid (ω9); 20:1-OH, lesquerolic acid; 20:2, *cis*-eicosadienoic acid (ω6,9); 20:2-OH, auricolic acid; 22:1, erucic acid (ω9).

and related issues in more depth, we have expressed the hydroxylase gene in transgenic *Arabidopsis thaliana* plants using a seed-specific promoter. The results of this experiment, reported here, indicate that the hydroxylase may act on both 18- and 20-carbon fatty acids. In addition, it appears that the Arabidopsis microsomal *n*-3 desaturase accepts ricinoleate and possibly lesqueroleate as substrates. These activities result in the accumulation of ricinoleate, lesqueroleate, densipoleate, and possibly auricoleate in seed lipids of transgenic plants. These results provide an account of the probable mechanisms by which four distinct hydroxylated fatty acids are synthesized, and demonstrate the production of novel hydroxylated fatty acids of industrial interest in transgenic plants.

MATERIALS AND METHODS

All lines of *Arabidopsis thaliana* used here were from the Columbia ecotype. The *A. thaliana fad2* mutant line JB12, which is defective in oleate 12-desaturase activity, has been described (Miquel and Browse, 1992). Seeds of *Lesquerella fendleri* were obtained from Dr. K. Carlson (Oregon State University, Corvallis). Seeds of *Lesquerella kathryn* were generously provided by Dr. Sten Stymne (Swedish University of Agricultural Sciences, Svalöv, Sweden).

DNA Manipulations

Plasmid pA4 is a derivative of the binary Ti plasmid pBI121, which contains the castor (*Ricinus communis* L.) oleate hydroxylase cDNA under transcriptional control of the 35S (van de Loo et al., 1995). The hydroxylase gene has been designated as FAH12.

The napin promoter was amplified by PCR from *Brassica napus* genomic DNA so that it was flanked by synthetic *Hin*dIII and *Bgl*II sites by using the oligonucleotide primers nap1 (GGCGTCGACAAGCTTCTGCGGATCAAGCAGCTTTCA) and nap2 (CATGCCATGGTAGATCTTGTATGTTCTGTAGTGATGAGTTTTGG). The plasmid pNapFah12, which carries the FAH12 coding region under the control of the napin promoter, was constructed in two steps. First, the *Bam*HI-*Xba*I fragment from plasmid pA4 that contains the cDNA was cloned into the corresponding sites of pBI121 to produce pTMP1. Next, a *Hin*dIII-*Bgl*II fragment carrying the napin promoter was substituted for the 35S of pTMP1 by digesting with *Hin*dIII and *Bam*HI, purifying the vector and ligating it to the napin promoter fragment to produce pNapFah12.

To test for the presence of the hydroxylase gene in transgenic plants, the oligonucleotide primers HR1 (CGG TAC CAG AAA ACG CCT TG) and HF2 (GCT CTT TTG TGC GCT CAT TC) were used as PCR primers to amplify a 1-kb fragment that is diagnostic of the presence of a complete hydroxylase gene in transgenic lines. Genomic DNA was extracted, and approximately 100-ng samples were added to a solution containing 25 pmol of each primer, 1.5 units of *Taq* polymerase (Boehringer Mannheim), 200 μM deoxyribonucleotide triphosphate, 50 mM KCl, 10 mM Tris-Cl (pH 9), 0.1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, and 3% (v/v) formamide to a final volume of 50 μL. Amplification conditions were a 4-min denaturation step at 94°C, followed by 30 cycles of 92°C for 1 min, 55°C for 1 min, and 72°C for 2 min, and concluding with a final extension step at 72°C for 5 min.

Northern Analysis of Arabidopsis RNA

RNA from expanding Arabidopsis leaves and siliques was extracted using an RNeasy kit from Qiagen (Chatsworth, CA). The siliques were harvested approximately 10 d after pollination. The seeds constituted 65% of the total weight of the siliques. About 20 μg of total RNA was run in each lane of an agarose gel containing formaldehyde and then transferred to a nylon membrane (Hybond N^+, Amersham) according to the manufacturer's protocol. Northern hybridization was performed using ^{32}P-labeled DNA probes, as described by van de Loo et al. (1995). A plasmid containing a PCR-amplified portion of the Arabidopsis FAD2 gene, graciously supplied by Dr. J. Shanklin (Brookhaven National Laboratory, Uptown, NY), was used as a probe for the FAD2 message. A PCR fragment amplified with primers HR1 and HF2 (described above) was used as a probe for the FAH12 message.

Plant Transformation

Transgenic plants were generated using a modified in planta transformation procedure (Bechtold et al., 1993). Batches of 12 to 15 plants were grown on soil covered with nylon screens for 3 to 4 weeks under continuous light (100 mmol m^{-2} s^{-1} irradiation in the 400- – 700-nm range). Primary bolts were removed 4 d before use to promote growth of multiple secondary bolts. *Agrobacterium tumefaciens* strain GV3101 carrying pA4 or pNapFah12 was grown in liquid cultures to stationary phase in Luria broth medium with 15 mg/L gentamycin and 50 mg/L kanamycin. Cells were harvested and resuspended in infiltration medium (Murashige and Skoog macro- and micronutrient medium containing 10 mg/L 6-benzylaminopurine and 5% Glc). Plants were immersed in the bacterial suspension and then placed under vacuum (600 mm Hg) until tissues appeared uniformly water-soaked. Infiltrated plants were grown at 25°C under continuous light for 4 weeks. Seeds that were bulk harvested from each pot were sterilized in a mixture of bleach, water, and Triton X-100 (30, 70, and 0.1%, respectively), and then germinated on selective medium (1× Murashige and Skoog salts medium enriched with B-5 vitamins, with 50 mg/L kanamycin). Kanamycin-resistant seedlings (the T_1 generation) were transferred to soil for production of T_2 seed.

Fatty Acid Composition of Transgenic Plants

For analysis of leaf fatty acid composition, 0.1 to 0.5 g of fully expanded rosette leaves was harvested from plants grown at 25°C under continuous light. For analysis of roots, seedlings were grown from sterile seeds in liquid culture (Murashige and Skoog medium with Gamborg's B-5 vitamins, 1% Suc, GIBCO-BRL) for 2 weeks under continuous light at 25°C. Roots (0.1–0.5 g) were harvested, rinsed with sterile water, and blotted dry. For seed analy-

ses, seeds were harvested from dry siliques and the fatty acid composition was determined from lipids extracted from pools of 50 seeds.

Fatty acids from leaf, root, or seed samples were transmethylated in 1 N methanolic HCl (80°C, 1 h) and extracted twice into hexane after addition of an equal volume of aqueous 0.9% NaCl. Fatty acid methyl esters were derivatized with bis(trimethylsilyl)trifluoroacetamide:trimethylchlorosilane (99:1, v/v) at 70°C for 30 min to obtain TMS fatty acid methyl esters of hydroxylated fatty acids. GC analysis of leaf lipids was performed in splitless mode on an HP5890 series II gas chromatograph using either an SP2330 glass column (0.53 mm i.d., 30 m [Supelco, Bellefonte, PA]) or an SP2330 fused silica capillary column (0.25 mm i.d., 30 m, Supelco). The injector and detector temperatures were 300°C; the temperature program was 100 to 160°C at 25°C/min, 160 to 240°C at 10°C/min, held at 240°C for 5 min, and then decreased to 100°C at 25 min. Seed fatty acids were resolved on an SP2340 fused silica capillary column (0.25 mm i.d., 60 m, Supelco) in splitless mode using 1 mL/min He. The injector and detector temperatures were 300°C; the temperature program was 100 to 160°C at 25°C/min, 160 to 240°C at 7°C/min, held at 240°C for 5 min, and then decreased to 100°C at 25 min. The SP2440 column was used for seed lipids because it provided better separation of hydroxy fatty acids from long-chain nonhydroxylated fatty acids than the SP2330 columns (i.e. it was difficult to separate 18:1-OH from 20:0 on SP2330). The identity of fatty acids in the samples was determined by comparing retention times and mass spectra with those of standards. A HP5971 mass spectrometer was used to confirm the identity of eluting compounds. Lipid extracts from *L. fendleri* were used as sources of 20:1-OH and 20:2-OH, whereas 18:2-OH was obtained from the seeds of *L. kathryn*.

For the analysis of the composition of individual lipid classes, lipids were extracted, as discussed by Miquel and Browse (1992). Phospholipids were separated by TLC, as discussed by Benning and Somerville (1992). After light staining with iodine, individual phospholipids were identified by their R_F and co-migration with standards (Sigma). Spots were scraped off the plate and lipids were recovered in chloroform:methanol:acetic acid (50:50:1, v/v/v). The products obtained by lipolysis of TAG were separated by TLC using hexane:ethyl ether:acetic acid (50:50:1.5, v/v/v). In all other experiments neutral lipids were separated by TLC using hexane:ethyl ether:formic acid (80:20:2, v/v/v). Neutral lipids were identified using DAG or TAG standards (Sigma), neutral lipid extracts from *L. fendleri* seeds, and castor developing endosperm, and were recovered from the plate as mentioned above. After drying the samples under nitrogen, fatty acids were transmethylated, derivatized, and analyzed as described above.

Digestion of TAG Species with *Rhizopus arrhizus* Lipase

TAG species were purified by preparative TLC as described above and digested with *R. arrhizus* lipase (Sigma) as described by Bafor et al. (1991), except that 8000 units of lipase were used for each sample. The products of the digestion reaction were separated by TLC using hexane:ethyl ether:acetic acid (50:50:1.5, v/v/v) as the mobile phase, and identified by comparison with lipids resulting from lipolysis of purified TAG from wild-type Arabidopsis, *L. fendleri*, or castor. Lipids identified as monoacylglycerol species were eluted from the TLC plate and subjected to a second round of lipolysis to establish that they could not be hydrolyzed further. The fatty acid compositions of monoacylglycerol and free fatty acid fractions were analyzed by GC, as described above. To verify that fatty acids were selectively cleaved from the *sn*-1 and *sn*-3 positions and that no significant fatty acid migration occurred on the glycerol backbone, we subjected TAG-containing hydroxylated fatty acids (from *L. fendleri*) to lipase digestion. 20:1-OH was absent from the resulting monoacylglycerol, as previously reported (Hayes and Kleiman, 1992), which confirmed that the fatty acid composition of the monoacylglycerol product of lipolysis reflected the fatty acid composition of the *sn*-2 position of the TAG fraction.

RESULTS

Production of Transgenic Plants

Twenty-one transgenic lines expressing the castor oleate hydroxylase gene driven by the 35S designated W35H lines (Table I), and 7 lines in which the hydroxylase was under the control of the napin promoter (WNH lines), were obtained by *Agrobacterium*-mediated transformation of wild-type Arabidopsis. To determine if increased availability of oleate affects accumulation of ricinoleate, one of the W35H lines producing the most hydroxy fatty acids (line W35H7) was crossed with the *fad2* mutant line JB12, which is deficient in oleate-12 desaturation (Miquel and Browse, 1992). F_2 progenies homozygous for the *fad2* mutation and expressing the castor hydroxylase were obtained (designated R35H7). In addition, several transgenic *fad2* plants were also produced by *Agrobacterium*-mediated transformation of the *fad2* mutant (designated JB35H). Kanamycin-resistant lines were tested for the presence of an intact hydroxylase gene using a set of PCR primers (HR1 and HF2) complementary to regions at the 5′ and 3′ ends of the gene, respectively. In each case DNA fragments of the expected size were amplified, confirming the presence of the hydroxylase gene (results not presented).

Table I. *Transgenic lines used in this study*

Name	Source	*fad2* Genotype	Construct	No. of Lines
WT	Wild type	+[a]	None	1
JB12	JB12	−[b]	None	1
W35H	Wild type	+	35S-fah12	21
W35H7[c]	Wild type	+	35S-fah12	1
WNH	Wild type	+	Napin-fah12	7
JB35H	JB12	−	35S-fah12	2
R35H7	W35H7 × JB12	−	35S-fah12	1

[a] +, *fad2* is functional. [b] −, *fad2* has been inactivated by mutation. [c] One of the 21 transgenic lines in the W35H series.

Accumulation of Ricinoleate in Transgenic Seeds

The fatty acid compositions of lipids extracted from samples of pooled T_2 seeds from the various transgenic lines was analyzed by GC. 18:1-OH was detected in 13 W35H lines, 4 WNH lines, 2 JB35H lines, and the R35H7 line. Typical chromatograms of seed fatty acids from untransformed wild type and a typical WNH line are shown in Figure 1. Chromatograms of the transgenic lines contained a peak (Fig. 1, R) that corresponded to the retention time of *O*-TMS methylricinoleate. In addition, chromatograms of the transgenic lines contained two additional peaks (Fig. 1, D and L) that were not present in wild-type or *fad2* mutant lines. On the basis of retention time, these peaks were tentatively designated as *O*-TMS derivatives of 18:2-OH and 20:1-OH. 18:1-OH and the two novel fatty acids constituted up to 20% of the total seed fatty acids (Table II) in the WNH lines and up to 5% in the W35H lines.

Wild-type, JB35H, and R35H7 transgenic lines did not accumulate detectable amounts of hydroxy fatty acids in the leaf or in the root (Table II). In this respect, the results with transgenic Arabidopsis lines were identical to those obtained with transgenic tobacco (van de Loo et al., 1995). The leaf and root fatty acid compositions of the transgenic plants was indistinguishable from those of untransformed plants (Table II).

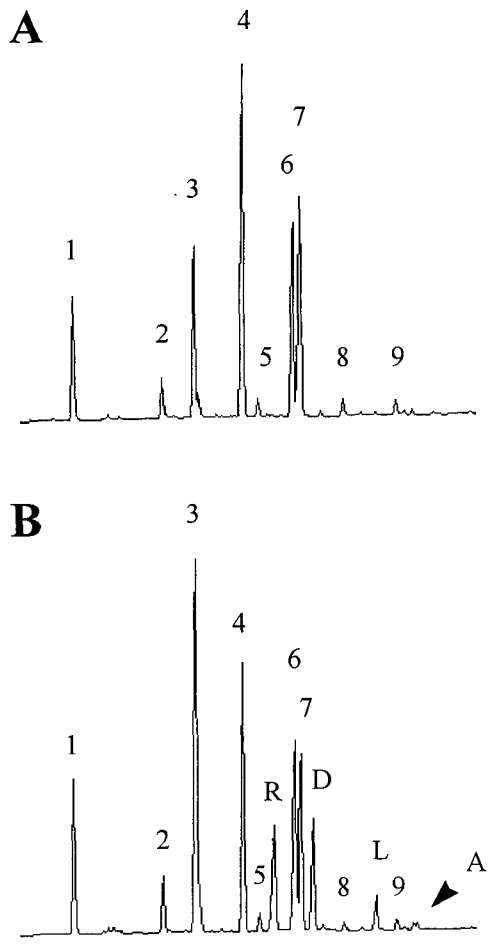

Figure 1. Gas chromatogram of fatty acid methyl esters and *O*-TMS derivatives of seed lipids from wild-type (A) and transgenic (B) Arabidopsis plants on an SP2340 column. The numbers correspond to the following fatty acids: 1, 16:0; 2, 18:0; 3, 18:1; 4, 18:2; 5, 20:0; 6, 20:1; 7, 18:3; 8, 22:1; and 9, 24:1; R, Ricinoleic acid; D, densipolic acid; L, lesquerolic acid; A, auricolic acid.

MS of Novel Fatty Acids

GC-MS analysis of the three novel compounds in extracts of the transgenic lines confirmed the identity of the three novel peaks as *O*-TMS derivatives of 18:1-OH, 18:2-OH, and 20:1-OH (Fig. 2). The three compounds had mass spectra identical to those of standards prepared from commercially available 18:1-OH or fatty acids extracted from the seeds of *L. fendleri* or *L. kathryn* (results not presented).

TMS methylricinoleate can be identified by three peaks of mass units 187 (A1), 270 (A2), and 299 (A3) (Fig. 2A). These peaks unequivocally establish the position of the hydroxyl group on carbon 12 of the fatty acid chain (van de Loo et al., 1995). A1 and A3 correspond to two fragmentation ions, and A2 is a rearrangement product (van de Loo et al., 1995). MS analysis of peaks D and L (Fig. 1) indicates that they correspond to hydroxy fatty acids related in structure to ricinoleate. The mass spectrum of peak D is shown in Figure 2C. Three major peaks of mass units 185, 270, and 299 are characteristically observed. The structures of the corresponding fragmentation ions can be inferred by comparison with A1, A2, and A3 in the mass spectrum of *O*-TMS methylricinoleate; ion C1 of peak D differs from A1 by 2 mass units due to the loss of two hydrogen atoms. The spectrum of peak D is identical to that of 18:2-OH (12-hydroxy-octadeca-9,15-dienoic acid), a derivative of ricinoleate with an additional double bond distal to the hydroxyl group.

The mass spectrum of peak L is characterized by three major ions, B1, B2, and B3, with mass units 187, 298, and 327, respectively (Fig. 2B). B1 is identical to A1 in the spectrum of *O*-TMS methylricinoleate. B2 differs from A2 of *O*-TMS methylricinoleate by addition of 28 mass units, which correspond to the masses of two carbon and four hydrogen atoms. The spectrum of peak L is identical to that of *O*-TMS methyl-20:1-OH, an elongated derivative of 18:1-OH.

Another fatty acid, peak A (Fig. 1), was barely detectable by flame ionization but produced a distinct mass spectrum (results not presented). The major ions in the mass spectrum of this peak were 185, 298, and 327 mass units. Therefore, peak A is structurally related to ricinoleate and probably results from elongation, hydroxylation, and desaturation of 18:1. Indeed, the mass spectrum of peak A is identical to a fourth hydroxy fatty acid found in *Lesquerella* species, auricolic acid (14-OH-20:2) (results not presented).

Hydroxy Fatty Acids Accumulate in Neutral Lipids

The two transgenic lines WNH1 and WNH2 accumulating the highest amounts of hydroxylated fatty acids were chosen to determine in which lipid class the unusual fatty acids accumulated. Lipids were extracted from approximately 50 mg of seeds, and the different lipid classes were

Table II. *Fatty acid composition of lipids from transgenic and wild-type Arabidopsis*
The values are the means obtained from analysis of three independent samples of each line.

Fatty Acid	Seed					Leaf		Root	
	Wild type	WNH	W35H7	R35H7	JB12	Wild type	W35H7	Wild type	W35H7
16:0	8.6	8.5	5.4	4.4	4.1	18	16.9	23.9	24.9
16:3	0	0	0	0	0	11.7	12.1	0	0
18:0	3.2	3.4	2.8	3.3	2.7	1.2	1.1	2.0	1.9
18:1	14.7	24.1	17.3	47.6	40.6	1.8	2.1	5.4	3.2
18:2	28.3	16.9	22.5	2.2	7.1	9.4	8.8	32.2	29.4
18:3	18.3	9.2	15.9	4.1	11.4	38.5	41	26.7	30.6
20:0	2.1	1.9	1.8	1.3	1.4	0	0	0	0
20:1	17.4	14.3	18.3	16.8	20.0	0	0	0	0
20:2	1.8	0.7	1.3	0.2	0.5	0	0	0	0
22:1	1.6	0.7	1.4	0.6	1.3	0	0	0	0
18:1-OH	0	7.8	1.1	3	0	0	0	0	0
18:2-OH	0	6.6	1.3	7.6	0	0	0	0	0
20:1-OH	0	2.5	0.4	0.8	0	0	0	0	0
20:2-OH	0	0.4	0.2	0.5	0	0	0	0	0

separated by TLC. Fatty acid methyl esters and *O*-TMS derivatives were prepared from PC, PE, PI, and neutral lipids and analyzed for the presence of hydroxylated fatty acids by GC-MS (Tables III and IV). Extracts from seeds of wild-type Arabidopsis, *L. fendleri*, *L. kathryn*, and castor were used as controls. A small amount of 18:2-OH was detected in PC, PE, and PI of transgenic seeds (Table III). Similarly, 18:2-OH was detected in the phospholipids from *L. kathryn*. However, none of the other hydroxy fatty acids was detected in phospholipids from transgenic plants or in either of the *Lesquerella* species (results not presented).

The TAG fraction from *L. fendleri* and the transgenic Arabidopsis lines resolved into three bands on TLC plates that we have designated TAG-1 (highest R_F value), TAG-2, and TAG-3 (lowest R_F value). By contrast, the TAG fraction from wild-type Arabidopsis migrates as a single band with the same R_F as TAG-1 (Fig. 3). The TAG-1 fraction, which was the most abundant species in transgenic Arabidopsis, did not contain detectable accumulation of hydroxy fatty acids (Table IV). Hydroxy fatty acids were detected in the TAG-2 and TAG-3 fractions from transgenic plants, which had the same R_F values as the two main TAG classes in *L. fendleri* and two minor classes in castor. Lipase digestion has shown that hydroxy fatty acids are excluded from the *sn*-2 position of *L. fendleri* TAG (Hayes and Kleiman, 1992). Thus, it appears that the TAG-1, TAG-2, and TAG-3 species correspond to populations of TAG molecules having 0, 1, or 2 hydroxylated fatty acids, respectively. By analogy, the two lipid classes in which hydroxy fatty acids were detected in transgenic Arabidopsis are likely to carry either one or two hydroxy fatty acids, like their *Lesquerella* homologs. The proportion of hydroxy fatty acids in TAG-2 and TAG-3 was close to the 33 and 66% expected (Table IV).

Comparison of the amount of 18:2-OH recovered in TAG with the amount present in total seed lipid extracts indicated that 18:2-OH was significantly underrepresented in the two hydroxylated TAG classes. In 18:2-OH-rich *Lesquerella* oils, the polyunsaturated hydroxy fatty acid is mostly present in monoestolide-containing triglycerides (Hayes et al., 1995). Such neutral lipids are more apolar than their TAG counterparts and would not be expected to have the same mobility as TAG-2 or TAG-3 on TLC plates. We did not attempt to purify estolide-containing triglycerides. However, their presence in transgenic seeds might explain the relatively low recovery of 18:2-OH in TAG-2 and TAG-3.

Analysis of Neutral Lipids by 1,3-Specific Lipolysis

The most abundant TAG species containing hydroxylated fatty acids (TAG-2, see above) was purified from transgenic seeds by preparative TLC and subjected to digestion with *R. arrhizus* lipase. The products of lipolysis were separated by TLC (Fig. 4), and the fatty acid composition of monoacylglycerol and free fatty acid fractions was determined by GC. Standards were produced by similar digests of the dihydroxylated TAG species from *L. fendleri* and the trihydroxylated species from castor. Only one lipolysis product, designated X in Figure 4, could not be identified. This product was not recovered by lipolysis of TAG from *L. fendleri* or castor lipids. We believe that it may contain estolides, since methylricinoleic acid was obtained (along with a mixture of other fatty acids) by transmethylation of this unidentified product with methanolic HCl.

Lipase treatment of the TAG-2 species from transgenic plants produced a mixture of nonhydroxylated monoacylglycerol and hydroxylated monoacylglycerol. Approximately 7% of the 18:1-OH present in seed lipids was found in the hydroxylated monoacylglycerol fraction. This is probably an underestimate of the proportion of ricinoleate at the *sn*-2 position of TAG because lipolysis was not complete and all monoacylglycerol was not recovered. However, this result suggests that most of the 18:1-OH is removed from the *sn*-2 position of the glycerol moiety after synthesis. GC analysis of the fatty acid composition of the hydroxylated monoacylglycerol revealed that, except for several minor peaks of unknown composition and origin, it contained exclusively 18:1-OH. This implies that in trans-

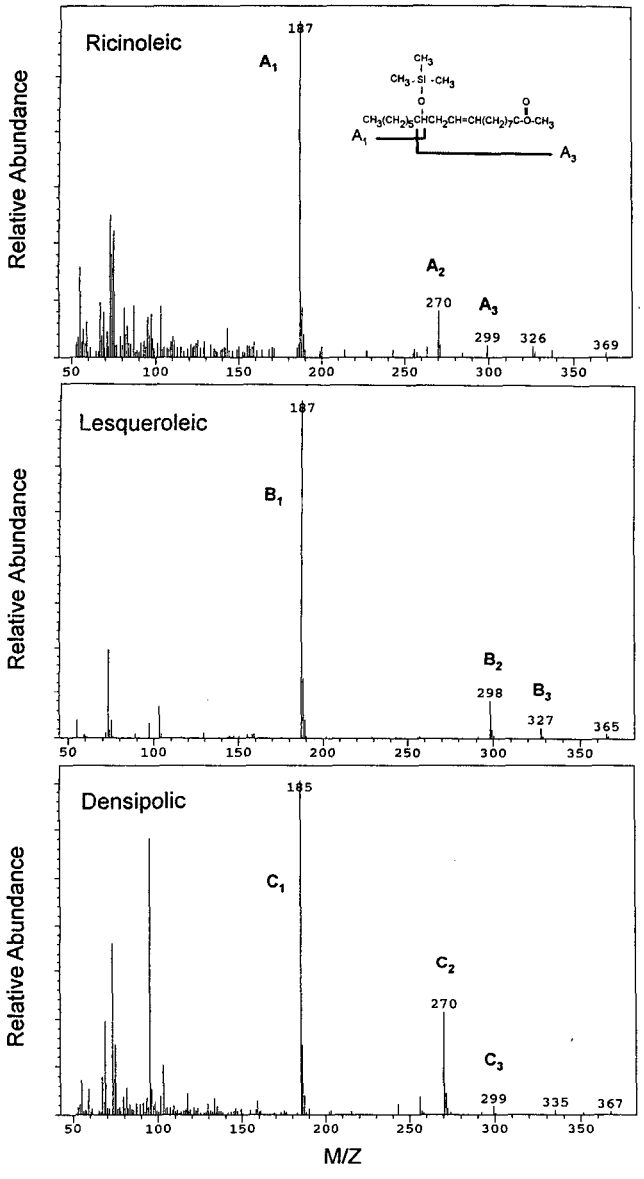

Figure 2. Mass spectra of novel fatty acids from transgenic Arabidopsis.

genic Arabidopsis, as in *L. fendleri*, 20:1-OH is excluded from the *sn*-2 position of TAG.

Effects on Levels of Desaturation

Ricinoleate accumulation in seeds of transgenic Arabidopsis plants was accompanied by an unexpected alteration of seed fatty acid composition compared with wild-type levels. In particular, 18:1 levels increased by up to 70%, whereas 18:2 and 18:3 accumulation decreased proportionately (Table II). The effect on 18:1 levels was not as dramatic in transgenic *fad2* lines, which is not unexpected, since 18:1 already accounts for about 40% of the total fatty acids in *fad2* seeds. This suggests that the presence of the castor hydroxylase in transgenic plants has a direct or indirect inhibitory effect on the oleate desaturase, resulting in oleate buildup and a decrease in the amount of 18:2 and 18:3 synthesized. In contrast to the 18-carbon fatty acids, the amounts of 20:1 were not significantly affected by the presence of hydroxylase activity in transgenic seeds.

The Arabidopsis FAD2 and castor FAH12 genes are approximately 65% identical at the DNA level. This level of sequence identity is below that normally associated with silencing of the expression of genes by introduced genes due to co-suppression (Gibson et al., 1994). Nevertheless, to address the possibility that lower levels of desaturation in transgenic plants could be due to co-suppression of the FAD2 gene, we examined the abundance of FAD2 message in different tissues of wild-type and transgenic plants (Fig. 5). It appears that the presence of high amounts of hydroxylase message in leaves and roots had no effect on FAD2 mRNA accumulation. Northern analysis of silique and seed RNA, although confirming the low activity of the 35S in this organ, also showed no difference in the abundance of FAD2 message between wild-type and transgenic plants. Therefore, we conclude that co-suppression does not account for the reduced amount of oleate desaturation in developing seeds.

Effects of Oleate Desaturase Activity on Hydroxy Fatty Acid Accumulation

The fatty acid composition of seed lipids from transgenic wild-type and *fad2* lines was compared to assess the effect of the reduced level of oleate desaturase activity caused by the *fad2* mutation on hydroxy fatty acid accumulation. In transgenic *fad2* plants, decreased oleoyl-12 desaturase activity resulted in increased levels of both oleate and hydroxylated fatty acid accumulation compared with transgenic wild-type lines (Table II). This effect was particularly clear in the R35H7 line, which accumulated about four times more hydroxy fatty acids than the W35H7 line (which contained the same transgene but lacked the *fad2* mutation). The increased levels of hydroxy fatty acid accumulation in the *fad2* lines may be due to decreased competition between the desaturase and the hydroxylase for fatty acid substrates.

The low level of oleate desaturation in *fad2* transgenic plants did not affect accumulation of the various hydroxy fatty acids equally. Accumulation of 18:1-OH and 18:2-OH increased 3- and 5-fold in the mutant background, respectively, whereas levels of 20:1-OH and 20:2-OH were about two times higher than in the transgenic wild-type lines (Table II). The high levels of 18:2-OH in the *fad2* transgenic line may result from a higher 18:1-OH to 18:2 ratio, which favors desaturation of ricinoleate rather than linoleate by the FAD3 desaturase. Higher levels of ricinoleate accumulation in *fad2* transgenic lines resulted in a roughly proportional increase in 20:1-OH levels. However, a strong increase in 18:1 levels was not accompanied by any increase in elongation to 20:1, suggesting that elongation is not limited by availability of 18:1.

DISCUSSION

We have previously reported that expression of the castor oleate hydroxylase in transgenic tobacco seeds resulted

Table III. *Fatty acid composition of seed phospholipids*

Fatty Acid	PC		PE		PI	
	Wild type	WNH	Wild type	WNH	Wild type	WNH
			mol%			
16:0	12.1	11.9	21.4	18.8	37.8	36.3
18:0	3.8	4.7	5.2	4.4	8.1	7.5
18:1	10.8	37.7	6.3	17.7	3.8	12.7
18:2	44.9	17.6	37.9	24.4	21	16.3
18:3	10.5	6.1	5.3	5.2	4.7	4.3
20:1	4.6	5.6	3.1	2.5	1.5	1.3
18:2-OH	0	1.1	0	1.5	0	1.2

in the accumulation of small amounts of 18:1-OH in seed lipids. The results presented here confirm and extend these observations by providing evidence that, in transgenic Arabidopsis plants, expression of the castor hydroxylase leads to the accumulation of several additional hydroxylated fatty acids that are found in association in several other plant species. A hypothetical pathway for the synthesis of hydroxylated fatty acids in transgenic Arabidopsis plants is presented in Figure 6. According to this hypothesis, an *n*-3 fatty acid desaturase converts 18:1-OH to 18:2-OH. Based on the known properties of the major fatty acid desaturases of Arabidopsis (Somerville and Browse, 1996), it seems likely that the ER-localized FAD3 desaturase is responsible for this conversion. We have previously reported that one or more of the *n*-3 desaturases of Arabidopsis was capable of converting $18:1^{\Delta 9}$ to $18:2^{\Delta 9,15}$, indicating that the double bond at carbon 12 was not absolutely required by the desaturase (Browse et al., 1989). In view of the fact that the amount of 18:2-OH that accumulates in the transgenic plants is similar to the amount of 18:1-OH, it appears that the presence of the 12-hydroxyl on 18:1-OH either does not interfere with or actually stimulates desaturation by the FAD3 desaturase. It should be possible to directly test the role of the FAD3 desaturase by introducing the FAH12 gene into a *fad3* mutant line.

Arabidopsis seeds normally contain substantial amounts of fatty acids of 20 carbons or longer (Table II). Studies of the substrate specificity of the castor hydroxylase indicated that the hydroxylase accepts long-chain fatty acids as substrates (Howling et al., 1972). Thus, the presence of 20:1-OH in the transgenic lines may be due to the conversion of $20:1^{\Delta 11}$ to 20:1-OH by the castor hydroxylase. Mutations at the *fae1* locus inactivate a fatty acid elongase activity and abolish accumulation of long-chain fatty acids in Arabidopsis seeds (James et al., 1995). Thus, expression of the FAH12 gene in a *fae1* mutant line would be expected to abolish accumulation of 20:1-OH (and 20:2-OH).

Hydroxylated fatty acids accumulated up to 4% of the total seed fatty acids in transgenic lines expressing the castor hydroxylase gene behind the 35S. This is approximately 50-fold higher than the amount of 18:1-OH observed in transgenic tobacco plants (van de Loo et al., 1995). We do not know the reason for this difference, but it might simply be due to poor expression of the 35S in seeds. Replacement of the 35S with the strong seed-specific napin promoter increased accumulation of hydroxy fatty acids in Arabidopsis 4- to 5-fold to approximately 17% of total fatty acids. It is not clear why the level of hydroxy fatty acid accumulation was not higher. Transgenic Arabidopsis plants accumulated significant amounts of mono- and dihydroxyacylglycerol but did not accumulate detectable

Table IV. *Fatty acid composition of seed TAGs*

Fatty Acid	TAG-1		TAG-2	TAG-3
	Wild type	WNH	WNH	WNH
		mol%		
16:0	10.6	9.9	5.8	6.0
18:0	4.0	4.1	3.3	2.7
18:1	19.4	29.2	21.9	21.0
18:2	30	24.3	6.7	3.4
18:3	17.0	16.7	3.1	2.2
20:0	1.6	1.3	0	0
20:1	16.4	13.1	19.1	7.0
22:1	1.4	0.8	0	0
18:1-OH	0	0	18.5	42.3
18:2-OH	0	0	10.4	3.7
20:1-OH	0	0	4.0	10.0

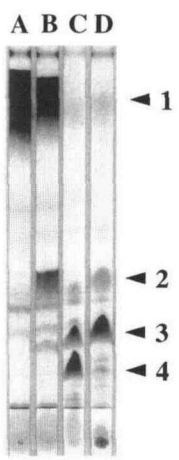

Figure 3. TLC of seed neutral lipids from transgenic Arabidopsis. A, Wild type; B, WNH; C, castor developing endosperm; D, *L. fendleri*. 1, TAG-1; 2, TAG-2; 3, TAG-3; 4, TAG with hydroxy fatty acids at *sn*-1, *sn*-2, and *sn*-3. The TLC plate was developed in hexane:ethyl ether:acetic acid (50:50:1.5, v/v/v).

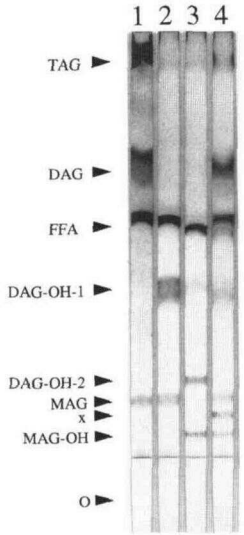

Figure 4. TLC of seed neutral lipids digested with *R. arrhizus* lipase. The TLC plate was developed using hexane:ethyl ether:formic acid (80:20:2, v/v/v). Lane 1, Wild-type Arabidopsis TAG; lane 2, *L. fendleri* TAG containing hydroxy fatty acids at the *sn*-1 and *sn*-3 positions; lane 3, *R. communis* triricinolein; lane 4, WNH TAG-2. TAG, Wild-type Arabidopsis triacylglycerol; DAG, diacylglycerol containing no hydroxy fatty acid; DAG-OH-1, diacylglycerol containing one hydroxy fatty acid; DAG-OH-2, diacylglycerol containing two hydroxy fatty acids; FFA, free fatty acids; MAG, monoacylglycerol containing no hydroxy fatty acid; MAG-OH, monoacylglycerol containing a hydroxy fatty acid; and X, unidentified lipid.

amounts of trihydroxyacylglycerol. This lack of trihydroxyacylglycerol presumably reflects the fact that the proportion of hydroxylated fatty acids on trihydroxyacylglycerol would be expected to be only about 0.5% of the total (i.e. in a plant with 17% hydroxy fatty acids, the probability that a hydroxy fatty acid will be on any one position of a TAG is 0.17 and the probability of a TAG having a hydroxy fatty acid on all three positions is 0.17^3). Thus, it may simply be that the napin promoter is not expressed at the right time during seed development, or there may be a certain amount of degradation of hydroxy fatty acids in developing seeds or some other unanticipated factor.

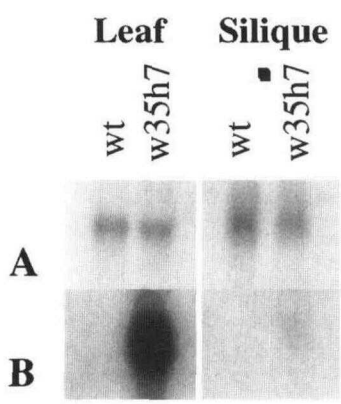

Figure 5. Northern blot of transgenic Arabidopsis RNA hybridized to two probes. A, Arabidopsis FAD2; B, FAH12.

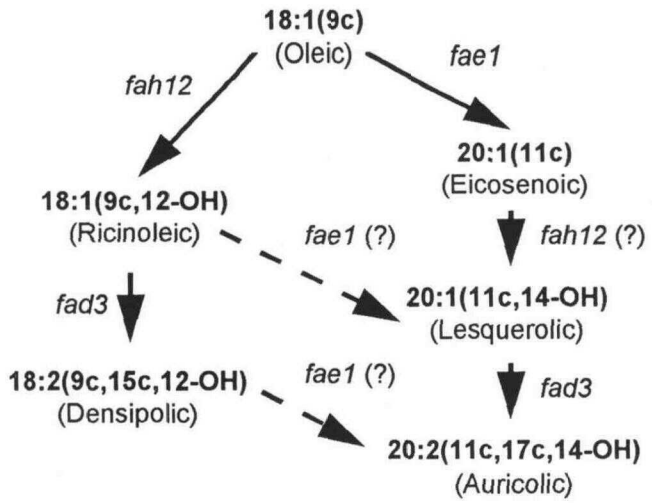

Figure 6. Hypothetical pathway for the synthesis of hydroxylated fatty acids. The name of the gene that is proposed to correspond to each step is shown adjacent to the arrows.

Whatever the case, the levels of hydroxy fatty acid accumulation obtained in the current generation of transgenic plants should be high enough to permit a detailed investigation of this and related questions.

The presence of 18:1-OH on the *sn*-2 position of TAG-1 can be interpreted in two ways. The simplest possibility is that the *sn*-2 acyltransferase accepts ricinoleoyl-CoA as a substrate. The alternative possibility is that *lyso*-phosphatidic acid acyltransferase is not involved. Instead, ricinoleoyl moieties may not be efficiently cleaved from the *sn*-2 position of PC on which they are thought to be synthesized and, therefore, the TAG pool becomes enriched with the *sn*-2 ricinoleate species by conversion of PC to TAG (via DAG). It has been proposed that fatty acid species modified on PC can be removed either by an exchange enzyme (*lyso*-PC:CoA acyltransferase), which reversibly transfers acyl moieties from the acyl-CoA pool to the *sn*-2 position of PC, or by phospholipase A2 (Bafor et al., 1991). Phospholipase A2 belongs to a family of enzymes thought to play a central role in removing oxygenated fatty acid species from membranes (Banas et al., 1992; Ståhl et al., 1995). The exchange enzyme or phospholipase A2 may have low affinity toward PC containing hydroxy fatty acids in developing seeds of Arabidopsis. However, this may be a difficult issue to resolve, since our results indicate significant presence of ricinoleoyl moieties on *sn*-1 or *sn*-3, which suggests that removal of 18:1-OH from the *sn*-2 position of PC does occur at a physiologically relevant rate. Thus, the issue is not whether ricinoleate is removed from the *sn*-2 position but rather the rate at which it is removed.

The only hydroxylated fatty acid detected on phospholipids was 18:2-OH. Presumably this reflects the fact that lipids carrying this fatty acid are less efficiently routed into the TAG biosynthetic pathway than lipids carrying the other hydroxylated fatty acids. Since the mechanism by which unusual fatty acids are routed to the TAG pool

remains a mystery, it is not possible to rationalize this effect. Since very little 18:2-OH was recovered in the TAG fraction, we do not know whether it is located on all three positions. Our data also indicate that Arabidopsis *lyso*-phosphatidic acid acyltransferase does not accept 20:1-OH as a substrate, a situation that is also found in *L. fendleri* and is reflective of the general low affinity of this acyltransferase for long-chain fatty acids in *Brassica* species.

Hydroxylated fatty acids were not observed in leaves or roots of the W35H transgenic plants. Northern blots of mRNA extracted from leaf and root tissue of the transgenic plants indicated that the FAH12 transcript was present at high levels (Fig. 5). However, we do not have parallel information concerning the accumulation of the hydroxylase polypeptide, and attempts to measure hydroxylase activity in crude leaf extracts were not successful. The simplest hypothesis is that an active enzyme is produced but the hydroxylated fatty acids are rapidly degraded. In contrast to seeds, leaves and roots normally accumulate very limited amounts of neutral lipids. As a result, hydroxy fatty acids could be more susceptible to degradation in these organs than in seeds, in which transfer to the neutral lipid pool may protect them from hydrolysis and subsequent breakdown. A similar observation was made concerning transgenic Arabidopsis plants that express a fatty acid thioesterase in both seeds and leaves (Jones et al., 1995). Although these plants accumulated high levels of short-chain fatty acids in seed lipids, short-chain fatty acids were not observed in leaf lipids. Additional studies will be required to distinguish between these and other possible explanations for the lack of hydroxylated fatty acid accumulation in the transgenic tobacco and Arabidopsis plants.

The enhanced accumulation of oleic acid in transgenic plants expressing the oleate hydroxylase is not currently understood. Hyperaccumulation of 18:1 does not appear to be due to co-suppression of FAD2 by FAH12. The DNA sequences of the two genes are only about 65% identical, and a possible instance of co-suppression was observed in only one transgenic line, in which oleate levels were increased in both leaves and seeds. All other lines accumulating hydroxy fatty acids had abnormally high oleate levels in the seeds but normal levels in their leaves and roots. In the seeds accumulation of hydroxy fatty acids indicates that the hydroxylase is active, and therefore the enzyme is targeted to its proper location in the cell, where it can compete for substrate and cofactors with the oleate desaturase. Thus, oleate buildup in transgenic seeds suggests that the presence of the hydroxylase or the hydroxylated fatty acids has direct or indirect inhibitory effects on the FAD2 desaturase. Additional experiments will be required to identify the mechanism responsible for this effect.

Recently, oil composition in species of the genus *Lesquerella* has been investigated (Hayes et al., 1995). These species, which belong to the Brassicaceae family, accumulate high amounts of hydroxy fatty acids in the seed. In *L. kathryn*, the major fatty acid is 18:2-OH, which accounts for about half of seed fatty acids. This fatty acid also accumulates in other *Lesquerella* species, such as *L. fendleri*, in which it is a minor constituent of the oil. 18:2-OH is a 12-hydroxy-octadeca-9,15-dienoic acid (12-OH-18:2). In *L. fendleri*, 18:2-OH is thought to result from desaturation of 18:1-OH. Elongation also occurs in the seeds of *L. fendleri* and drives the accumulation of its major hydroxylated fatty acid, lesquerolic acid (14-OH-20:1). The results presented here suggest that the accumulation of these and probably other fatty acids such as auricolic acid could be attributed to the action of a single hydroxylase with properties very similar to those of the oleate hydroxylase from castor. The fact that castor does not accumulate 18:2-OH suggests that the *n*-3 desaturase in castor seed is expressed at very low levels or differs from the Arabidopsis and *Lesquerella* FAD3 genes by having strict specificity for linoleic acid. These possibilities could be resolved by expressing the castor linoleate hydroxylase in a *fad3* mutant of Arabidopsis that also carries the FAH12 gene.

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