

A Condensing Enzyme from the Seeds of *Lesquerella fendleri* That Specifically Elongates Hydroxy Fatty Acids¹

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Lesquerella fendleri seed oil contains up to 60% hydroxy fatty acids, nearly all of which is the 20-carbon hydroxy fatty acid lesquerolic acid (D-14-hydroxyeicos-cis-11-enoic acid). Previous work suggested that lesquerolic acid in *L. fendleri* was formed by the elongation of the 18-carbon hydroxy fatty acid, ricinoleic acid. To identify a gene encoding the enzyme involved in hydroxy fatty acid elongation, an *L. fendleri* genomic DNA library was screened using the coding region of the Arabidopsis *Fatty Acid Elongation1* gene as a probe. A gene, *LfKCS3*, with a high sequence similarity to known very long-chain fatty acid condensing enzymes, was isolated. *LfKCS3* has a 2,062-bp open reading frame interrupted by two introns, which encodes a polypeptide of 496 amino acids. *LfKCS3* transcripts accumulated only in the embryos of *L. fendleri* and first appeared in the early stages of development. Fusion of the *LfKCS3* promoter to the *uidA* reporter gene and expression in transgenic Arabidopsis resulted in a high level of β -glucuronidase activity exclusively in developing embryos. Seeds of Arabidopsis plants transformed with *LfKCS3* showed no change in their very long-chain fatty acid content. However, when these Arabidopsis plants were crossed with the transgenic plants expressing the castor oleate 12-hydroxylase, significant amounts of 20-carbon hydroxy fatty acids accumulated in the seed, indicating that the *LfKCS3* condensing enzyme specifically catalyzes elongation of 18-carbon hydroxy fatty acids.

Hydroxy fatty acids are a component of the seed oils of a small number of diverse plant species (Badami and Patil, 1981; van de Loo et al., 1993). The most familiar of these is castor bean (*Ricinus communis*), which produces a seed rich in oil (35%–50% of seed weight) containing 85% to 90% ricinoleic acid (D-12-hydroxyoctadec-cis-9-enoic acid; 18:1-OH). This fatty acid is a valuable industrial raw material and castor is widely grown as an oil crop in countries such as India, Brazil, and China. Ricinoleic acid is formed in the developing endosperm of the castor bean by the direct hydroxylation of oleic acid (18:1 ^{Δ 9}) esterified to the *sn*-2 position of the membrane lipid phosphatidylcholine (Bafar et al., 1991). The hydroxylase responsible for this activity has been cloned (van de Loo et al., 1995) and has high homology to plant endoplasmic reticulum (ER) membrane-bound fatty acid desaturase enzymes.

In the Brassicaceae, members of the genus *Lesquerella* also produce hydroxy fatty acids in their seed oil. In most species of *Lesquerella*, the predominant hydroxy fatty acids have a chain length of 20 carbons (C20) with the hydroxy group on carbon 14 (Δ 14) and

one or two double bonds at the Δ 11 and Δ 17 positions (Hayes et al., 1995). The seeds of *L. fendleri*, for example, can contain nearly 60% lesquerolic acid (D-14-hydroxyeicos-cis-11-enoic acid; 20:1-OH) and virtually no hydroxy fatty acids with an 18-carbon chain length. However, some members of the genus do accumulate significant amounts of the C18 hydroxy fatty acids such as ricinoleic acid and densipolic acid (D-12-hydroxyoctadec-cis-9,cis-15-dienoic acid and 18:2-OH). Both of these fatty acids have the hydroxy group on carbon 12 with the first double bond between carbons 9 and 10 (Δ 9). The positions of these functional groups suggest that in *L. fendleri*, the C20 hydroxy fatty acids are formed by the elongation of C18 hydroxy precursors.

Biochemical studies conducted on developing embryos from a number of *Lesquerella* species have shown that they hydroxylate oleic acid at the Δ 12 position to form ricinoleic acid and can then desaturate and elongate this fatty acid to form densipolic, lesquerolic, and auricolc (D-14-hydroxyeicos-cis-11,cis-17-dienoic acid; 14-OH, 20:2-OH) acids (Engeseth and Stymne, 1996; Reed et al., 1997). Isolation of the gene encoding the seed-specific fatty acid hydroxylase from *L. fendleri* (Broun et al., 1998) and characterization of the gene product has demonstrated that the enzyme is a bifunctional Δ 12-oleate hydroxylase:desaturase. It is highly homologous to the hydroxylase enzyme from castor bean and plant ER Δ 12 fatty acid desaturases. Because the predominant hydroxy fatty acid accumulating in *L. fendleri* is lesquerolic acid, not ricinoleic acid, it appears that the plant very efficiently elongates C18 hydroxy fatty acid to C20.

¹ This work was supported by a grant from the Science Council of British Columbia, Canada, by a Natural Sciences and Engineering Research Council of Canada Strategic grant, and by Linnaeus Plant Sciences, Inc.

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.010544.

C20 and other very long-chain fatty acids (VLCFAs) are common components of the seed oils of members of the Brassicaceae. They are synthesized by a microsomal fatty acid elongation (FAE) system that involves four enzymatic reactions: condensation of malonyl-coenzyme A (CoA) with a long-chain fatty acyl-CoA; reduction to β -hydroxyacyl-CoA; dehydration to an enoyl-CoA; and reduction of the enoyl-CoA, resulting in acyl-CoA elongation by two carbons (von Wettstein-Knowles, 1982; Fehling and Mukherjee, 1991). Of the four enzymes of the FAE, only genes encoding condensing enzymes have been cloned and characterized. Expression of FAE1, the condensing enzyme involved in VLCFA synthesis in *Arabidopsis* seeds, in transgenic plants and yeast (*Saccharomyces cerevisiae*) has demonstrated that the substrate specificity and the extent of elongation are a function of this enzyme (Millar and Kunst, 1997; Millar et al., 1999). In *Arabidopsis*, FAE1 catalyzes the elongation of oleic acid (18:1) to eicosenoic acid (20:1) and, less efficiently, of eicosenoic acid by a further two carbons. Expression of the castor bean and *L. fendleri* oleate hydroxylases in transgenic *Arabidopsis* has shown that FAE1 also has a low level of activity with ricinoleic acid and can elongate this hydroxy fatty acid to form lesquerolic acid (Broun et al., 1998; Smith et al., 2000).

In the seed oil of *L. fendleri*, oleic acid (18:1) accounts for as much as 10% to 15% of the total fatty acids, but eicosenoic acid (20:1) content is less than 1%. Conversely, most of the hydroxy fatty acids exist as lesquerolic (20:1-OH) and auricollic (20:2-OH), whereas the ricinoleic acid (18:1-OH) content is less than 1% of the total fatty acids. This implies that only the hydroxy fatty acids are elongated in *L. fendleri*, and therefore, the condensing enzyme is highly specific for hydroxy fatty acids. Here, we report the isolation and characterization of *LfKCS3*, the gene encoding the condensing enzyme responsible for lesquerolic acid production in *L. fendleri*. In addition, we present results showing that the *LfKCS3* promoter is seed specific and active from an early stage of embryo development in transgenic *Arabidopsis*.

RESULTS

Cloning and Characterization of a Gene Encoding a VLCFA Condensing Enzyme

A genomic clone of the putative condensing enzyme was isolated by probing a genomic library of *L. fendleri* with the coding region of the *Arabidopsis* FAE1 gene (James et al., 1995). An *EcoRI* fragment subcloned into the plasmid pMHS15 was fully sequenced and a 4,313-bp consensus sequence was assembled using the GCG program (Genetics Computer Group, Madison, WI; Edelman et al., 1994). The 4,313-bp genomic DNA included a 573-bp 5'-flanking region, a 2,062-bp open reading frame, and a 1,678-bp 3'-flanking region. The nucleotide sequence of the

clone was deposited in GenBank as accession number AF367052. A sequence comparison between the *L. fendleri* genomic DNA and *Arabidopsis* FAE1 using BCM Search Launcher: Multiple Sequence Alignments (Smith et al., 1996) revealed two introns in the *L. fendleri* genomic DNA separated by 421 bp. The deduced amino acid sequence was obtained after removing the introns with the aid of a translation tool, "Translate" (<http://www.expasy.ch/tools/dna.html>). This revealed that the gene, *LfKCS3*, encoded a polypeptide (LfKCS3) of 496 amino acids with an estimated molecular mass of 55.3 kD. Sequence comparisons (Fig. 1) showed that LfKCS3 shared 80% identity with the *Arabidopsis* FAE1 (James et al., 1995), 76% identity with the oilseed rape (*Brassica napus*) FAE1 (Clemens and Kunst, 1997), and approximately 50% identity with the *Arabidopsis* CUT1/CER6 (Millar et al., 1999; Fiebig et al., 2000) and *Simmondsia chinensis* jojoba KCS (Lassner et al., 1996).

Analysis of LfKCS3 Expression and Hydroxy Fatty Acid Accumulation in *L. fendleri*

Lesquerolic acid accumulates only in the seed of *L. fendleri*. However, several condensing enzymes involved in the production of VLCFAs in vegetative tissues (Millar et al., 1999; Todd et al., 1999; Yephremov et al., 1999) also show a high degree of similarity with LfKCS3. For this reason, the expression pattern of *LfKCS3* in *L. fendleri* was examined by northern-blot analysis. A 461-bp probe was used that consisted of 367 bp of the 5' end of the coding region together with 94 bp of the adjacent 5' genomic sequence. This region was chosen because it showed the least similarity when compared with the sequences of other condensing enzymes. Northern-blot analysis revealed a single band of approximately 1.8 kb in RNA from developing embryos (Fig. 2). No detectable hybridization was observed in leaves, stems, or roots.

To examine a developmental pattern of accumulation of the very long-chain hydroxy fatty acids in the embryos of *L. fendleri*, embryos were harvested and separated into seven stages according to their size and color (Fig. 3A). Analyses of fatty acid composition of the individual embryos of different stages indicated that the C20 hydroxy fatty acids, mainly as lesquerolic acid (20:1-OH), appeared in stage three embryos, followed by a rapid increase in lesquerolic acid content in subsequent stages (Fig. 3B). This result was consistent with the data previously reported by Reed et al. (1997). In addition, there was a good correlation between the accumulation of the very long-chain hydroxy fatty acids (Fig. 3B) and *LfKCS3* transcript accumulation (Fig. 2) in *L. fendleri* embryos throughout development. *LfKCS3* transcripts were detected at low abundance in the early stages of development (group I = stages 1, 2, and 3) and abruptly increased at mid stage (group II = stages 4, 5).

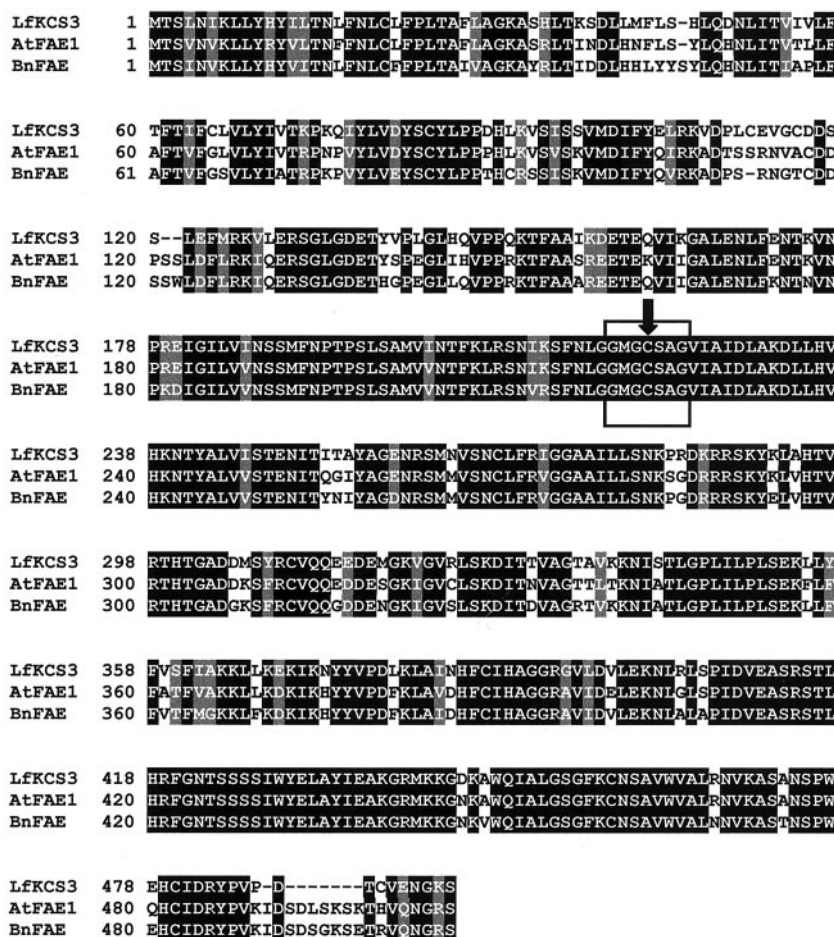


Figure 1. Alignment of deduced amino acid sequences determined for LfKCS3 and two other VLCFA condensing enzymes from members of the Brassicaceae. Identical amino acids are highlighted on a black background; similar amino acids are shown on a gray background. The boxed region with an arrow indicates a highly conserved region among all the condensing enzymes involved in VLCFA biosynthesis. The active site Cys (Ghanevati and Jaworski, 2001) is marked with an arrow. GenBank accession numbers for LfKCS3, AtFAE1, and BnFAE are AF367052, U29142, and AF009563, respectively.

The *LfKCS3* Promoter Is Seed Specific and Active from an Early Stage of Embryo Development in Transgenic Arabidopsis

To determine whether the 5'-promoter region of *LfKCS3* was sufficient to confer the tissue specificity and the developmental pattern of *LfKCS3* expression seen in *L. fendleri*, a 573-bp 5' fragment was placed upstream of the *uidA* gene encoding β -glucuronidase (GUS) and was introduced into Arabidopsis plants

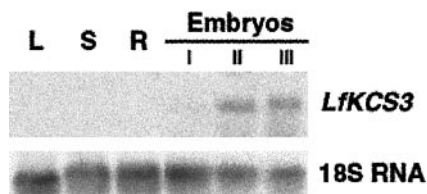


Figure 2. Northern-blot analysis of *LfKCS3* expression in *L. fendleri*. A 32 P-labeled probe corresponding to 461 bp of the 5' region of the *LfKCS3* genomic clone was hybridized to 10 μ g of total RNA from leaves (L), stems (S), roots (R), and developing embryos (Embryos). Developing embryos were divided into three stages based on their size and color: early (I), mid- (II), and late (III) developmental stages (see Fig. 3). For loading control, the same blot was stripped and rehybridized with a 32 P-labeled probe made from the Arabidopsis 18S rRNA (18S RNA; Unfried et al., 1989).

(ecotype Columbia). Histochemical staining was performed on leaves, stems, inflorescences, roots, and siliques at different stages of development for more than 30 independent primary transgenic plants. No staining was observed in any tissue other than developing embryos (data not shown) where an appearance of an intense blue color indicated that GUS was exclusively expressed in this tissue. The timing of expression directed by the *LfKCS3* promoter (*LfKCS3P*) was compared with that of the *L. fendleri* fatty acid hydroxylase (*LesqH*) promoter, a strong early seed-specific promoter (Broun et al., 1998). Histochemical staining patterns of representative Arabidopsis embryos from the *LfKCS3P*-GUS and *LesqH*-GUS lines at different stages of embryo development are shown in Figure 4. For both promoters, GUS activity was evident at the earliest stages examined (4 d after flowering), and continued to be present throughout subsequent seed development.

The *LfKCS3* Is a Condensing Enzyme That Is Specific for Hydroxy Fatty Acids

Because the deduced amino acid sequence of *LfKCS3* was highly similar to those of FAE1 and related VLCFA condensing enzymes from other

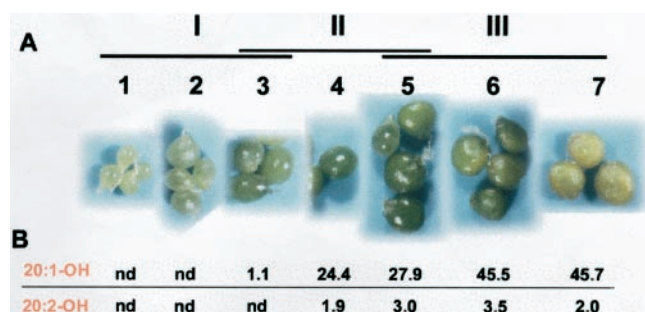


Figure 3. Development of *L. fendleri* embryos. A, Developing embryos of *L. fendleri* were harvested and divided into seven stages based on their color and size. Stages grouped for the northern-blot experiment (see Fig. 2) are indicated: I, embryos at the early stage of development; II, mid-stage; and III, late developmental stage. B, The C20 hydroxy fatty acid content (weight percentage) of the embryos at each stage was determined by gas chromatography. 20:1-OH, Lesquerolic acid; 20:2-OH, auricolic acid; nd, not detected.

plant species, we employed a transgenic approach to establish its functional identity. *LfKCS3* was introduced into *Arabidopsis* wild-type and *fad2/fae1* double-mutant plants under the control of its own promoter (*LfKCS3G* cassette) or *LesqH* promoter (*LesqH-LfKCS3C* cassette). Eighteen *fad2/fae1* transformants were obtained containing the *LfKCS3G* cassette and 23 *fad2/fae1* plants containing the *LesqH-LfKCS3C* cassette. The fatty acid composition of T_2 seeds from individual plants was determined for all transformants. Even though the *fad2/fae1* mutant accumulated approximately 85% (w/w) of total seed fatty acids as 18:1, none of the transgenic plants produced VLCFAs above the background level measured in untransformed double-mutant plants. Similarly, 32 independent transgenic lines obtained by transformation of *Arabidopsis* wild-type plants with the *LfKCS3G* cassette did not show any increase in VLCFAs levels compared with untransformed controls. These results indicated that 18:1 was not a substrate of the *LfKCS3*.

To test whether the lack of any change in fatty acid composition in the transgenic plants was due to the specificity of the *LfKCS3* condensing enzyme for hydroxy fatty acids, transgenic *fad2/fae1* mutant lines containing the *LfKCS3G* cassette or the *LesqH-LfKCS3C* cassette were crossed with transgenic *Arabidopsis* plants expressing the castor bean oleate 12-hydroxylase in the same *fad2/fae1* double-mutant background (*LesqH-CFAH12* lines). F_1 plants were grown to maturity, and fatty acid analyses were carried out on the pools of F_2 seeds harvested from individual plants. As controls, fatty acid compositions of the seeds of untransformed *fad2/fae1* double-mutant (Fig. 5A) and transgenic plants expressing the *Arabidopsis* FAE1 and the castor hydroxylase (*LesqH-FAE1* and *LesqH-CFAH12* lines) in the double-mutant background (Fig. 5B) were also analyzed. Coexpression of FAE1 and the castor hydroxylase resulted in a major increase in the levels of

eicosenoic acid (20:1) and the accumulation of hydroxy fatty acids that were not detected in untransformed seeds. These were identified as ricinoleic acid (18:1-OH) and densipolic acid (18:2-OH) (Fig. 5B). Densipolic acid has been shown to be a result of the $\Delta 15$ desaturation of ricinoleic acid catalyzed by the microsomal FAD3 desaturase (Engeseth and Stymne, 1996; Reed et al., 2000; Smith et al., 2000). In plants with the highest levels of hydroxy fatty acids (up to 4% of total seed fatty acids in this experiment), there were also traces of lesquerolic and (20:1-OH) and auricolic acid (20:2-OH). Previous results have shown that *fad2/fae1* plants expressing CFAH12 alone accumulate ricinoleic and densipolic acid with no detectable C20 hydroxy fatty acids (Smith et al., 2000), and very low levels of 20:1 (less than 1% of total seed fatty acids). Thus, the presence of 20:1 and hydroxy fatty acids in the plants transformed with FAE1 and the CFAH12 suggests that both enzymes were active and confirmed that, as reported previously (Broun and Somerville, 1997; Smith et al., 2000), the *Arabidopsis* FAE1 is able to elongate hydroxy fatty acids, although this does not appear to be an efficient process.

In the F_2 lines containing the *LfKCS3* condensing enzyme and CFAH12, ricinoleic and densipolic acid were again present. However, in contrast to the plants coexpressing FAE1 and CFAH12, these plants contained significant amounts of the C20 hydroxy fatty acids lesquerolic and auricolic acid (Fig. 5C). In the plants containing the highest amount of hydroxy fatty acids, just over 4% of total seed fatty acids were hydroxylated, and of these, 23% had a chain length of 20 carbons. Plants containing the *LfKCS3* gene under the control of its own promoter appeared to have slightly higher levels of C20-hydroxy fatty acids than those containing the *LesqH-LfKCS3C* cassette. In all transgenic plants coexpressing *LfKCS3* and CFAH12, levels of 20:1 remained virtually the same as the controls. Taken together, these results provided conclusive evidence that the *LfKCS3* was a condensing enzyme that specifically elongated hydroxy fatty ac-

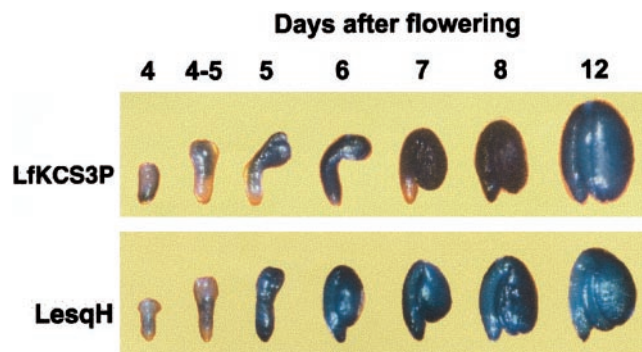
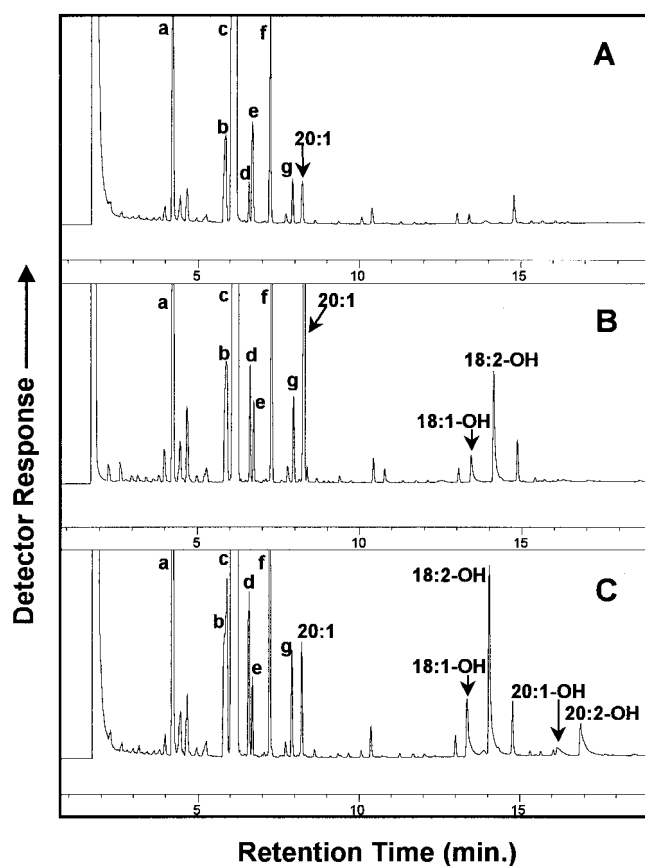


Figure 4. Histochemical staining of embryos from transgenic *Arabidopsis* expressing the GUS gene under the control of the *LfKCS3* promoter (*LfKCS3P*) or the *L. fendleri* oleate 12-hydroxylase promoter (*LesqH*; Broun et al., 1998).



D

Fatty acid	Fatty acid composition (%)		
	(A) Untransformed	(B) CFAH12 + FAE1	(C) CFAH12 + <i>LfKCS3</i>
(a) 16:0	5.8	6.2	5.6
(b) 18:0	2.4	2.3	2.8
(c) 18:1	85.9	77.8	81.6
(d) 18:2 ^{Δ9,12}	0.5	0.9	1.0
(e) 18:2 ^{Δ9,15}	1.2	0.6	0.5
(f) 18:3	2.7	3.3	3.1
(g) 20:0	0.7	0.8	0.8
20:1	0.7	6.3	0.8
18:1-OH	0.0	0.4	0.8
18:2-OH	0.0	1.4	2.2
20:1-OH	0.0	T	0.2
20:2-OH	0.0	T	0.6

Figure 5. Gas chromatographic analyses of fatty acid methyl esters prepared from mature Arabidopsis seeds. A, Untransformed *fad2/fae1* mutant. B, Transformed *fad2/fae1* double mutant expressing the castor oleate 12-hydroxylase (CFAH12) and Arabidopsis FAE1. C, An F_2 line expressing the CFAH12 and *LfKCS3*. Peak identification: a, 16:0; b, 18:0; c, 18:1; d, 18:2^{Δ9,12}; e, 18:2^{Δ9,15} (Reed et al., 2000); f, 18:3^{Δ9,12,15}; g, 20:0. D, Percentage of fatty acid composition of the seed samples shown in the chromatograms. T, Trace amount, not integrated.

ids, not the common C18 fatty acids, in the seed of *L. fendleri*.

DISCUSSION

We report here the isolation and characterization of *LfKCS3*, a gene encoding a VLCFA condensing en-

zyme expressed in the seeds of *L. fendleri*. To date, genes for several VLCFA condensing enzymes have been isolated and characterized from higher plants. They include those with seed-specific expression that are involved in VLCFA biosynthesis for seed storage lipids in Arabidopsis (James et al., 1995), jojoba (Lassner et al., 1996), and oilseed rape (Clemens and Kunst, 1997), and a few enzymes involved in cuticular wax production such as CUT1/CER6 (Millar et al., 1999; Fiebig et al., 2000) and KCS1 (Todd et al., 1999) from Arabidopsis. The seed-specific condensing enzymes exhibit a preference for oleic acid (18:1) in vivo, whereas the CUT1/CER6 appears to catalyze the elongation of C24 fatty acids or longer in the epidermal cells of Arabidopsis (Millar et al., 1999). Arabidopsis KCS1 elongates saturated fatty acids with a chain length of C20 to C26 (Todd et al., 1999). In addition, a cDNA encoding a FAE1-related polypeptide was recently identified from a collection of *Limnanthes douglasii* expressed sequence tags (Cahoon et al., 2000). Expression of the *L. douglasii* FAE1 homolog in soybean (*Glycine max*) somatic embryos indicated that this enzyme utilized mainly 16:0 as a substrate, resulting in the accumulation of saturated, principally 20:0, VLCFAs. Despite differences in their substrate specificities and the types of products made, all of the plant condensing enzymes mentioned above share a high degree of sequence identity. The alignment of the predicted amino acid sequence of *LfKCS3* with the known VLCFA condensing enzymes and the presence of a strictly conserved region between Gly218 and Gly224 (Fig. 1) in all condensing enzymes involved in VLCFA biosynthesis strongly suggested that *LfKCS3* also encoded a VLCFA condensing enzyme.

Expression in transgenic Arabidopsis clearly demonstrated that *LfKCS3* was a VLCFA condensing enzyme, and that it only catalyzed the elongation of C18 hydroxy fatty acids. When *LfKCS3* and the castor hydroxylase were expressed together in the Arabidopsis *fad2/fae1* double-mutant plants, C20 hydroxy fatty acids were synthesized that were not seen in plants expressing the hydroxylase alone. No changes were detected in the levels of non-hydroxy C20 fatty acids (less than 2%) in these plants, suggesting that the enzyme was only elongating hydroxy fatty acids. Further evidence for this specificity comes from the observation that the fatty acid composition of *fad2/fae1* seeds transformed with *LfKCS3* alone was not different from untransformed controls. Similarly, *LfKCS3* expression in wild-type plants did not result in any changes in fatty acid composition. Thus, the endogenous fatty acids of Arabidopsis do not appear to be substrates for *LfKCS3*.

For comparison we also coexpressed FAE1, a well-characterized condensing enzyme, and the castor hydroxylase in the *fad2/fae1* double-mutant plants. The introduction of FAE1 resulted in the accumulation of 20:1 in transgenic seeds, a product of 18:1 elongation.

Therefore, in contrast to LfKCS3 that only utilizes hydroxy C18 fatty acids as a substrate, FAE1 is highly active with 18:1. In plants containing the highest levels of 20:1, very low levels of C20 hydroxy fatty acids were detected in the seed. This result is in agreement with earlier reports that FAE1 was capable of catalyzing the elongation of ricinoleic acid (Broun and Somerville, 1997; Smith et al., 2000). In both previous studies, the castor hydroxylase was expressed in wild-type plants that had normal levels of FAE1 activity, and as a result, accumulated around 16% 20:1. In contrast, in the work reported here, FAE1 was introduced into a mutant lacking FAE1 activity (*fad2/fae1* mutant background). The highest level of 20:1 measured in *fad2/fae1* transgenic lines was around 7%, indicating that FAE1 activity in these plants was likely significantly lower than in a wild-type plant. Hydroxylase activity also appeared to be low, as total levels of hydroxy fatty acids in these plants never exceeded 4%. Therefore, it is not possible to comment on the relative efficiency of elongation of hydroxy fatty acids by FAE1 in comparison with LfKCS3. However, there is a clear distinction between FAE1 and LfKCS3 in that FAE1 elongates 18:1, whereas LfKCS3 does not.

The presence of the hydroxy group at the $\Delta 12$ position of a C18 fatty acyl chain is likely to give the molecule an electrophilic profile more similar to 18:2 than 18:1. Some enzymes, for example the ER $\Delta 12$ and $\Delta 15$ desaturases, can accept substrates with electrophilic groups such as hydroxy and epoxy groups in place of a double bond (Engeseth and Stymne, 1996). However, it is not known whether the same is true for condensing enzymes. To determine if LfKCS3 can elongate 18:2 in addition to hydroxy 18:1, we transformed wild-type plants, which contain around 30% 18:2, with LfKCS3. Analysis of seed fatty acids showed no significant difference between transformed and control plants (data not shown). This result is consistent with the fatty acid profile of *L. fendleri* seed, which contain around 10% 18:2 but no detectable 20:2. Thus, it appears that the LfKCS3 condensing enzyme is specific for a hydroxy group at the $\Delta 12$ position of ricinoleic acid and that the hydroxy group cannot be substituted for by a double bond. If this is correct, then the mechanism of substrate recognition by the condensing enzymes is different from that of the ER $\Delta 12$ and $\Delta 15$ desaturases. The availability of condensing enzymes with specificities toward saturated, monounsaturated, or hydroxy fatty acids from plants, and polyunsaturated fatty acids from animals (Beaudoin et al., 2000), fungi (Parker-Barnes et al., 2000), and a moss (Zank et al., 2000) will allow us to further investigate the mechanism of acyl group recognition and specificity of this class of enzymes.

The predominant hydroxy fatty acids in plants expressing LfKCS3 and the castor hydroxylase are the diunsaturated fatty acids, densipolic and auricolic

acid. Several reports (Engeseth and Stymne, 1996; Reed et al., 2000; Smith et al., 2000) have shown that densipolic acid is formed by the $\Delta 15$ (n-3) desaturation of ricinoleic acid catalyzed by the FAD3 desaturase. The mechanism of synthesis of auricolic acid is less clear, and it is still uncertain whether 20:2-OH is formed by the elongation of 18:2-OH, as suggested by Engeseth and Stymne (1996), or by the FAD3 catalyzed desaturation of 20:1-OH, as proposed by Reed et al. (1997). Isolation of a cDNA encoding LfKCS3 and in vitro analysis, for example by expression in yeast, may help answer these questions.

In the transgenic Arabidopsis plants reported here, levels of hydroxy fatty acids were generally low, reaching up to 4% of the total seed fatty acids in the best lines. There could be a number of reasons for this, including low levels of gene expression, poor incorporation of hydroxy fatty acids into triacylglycerol, or degradation of hydroxy fatty acids. However, it is interesting to note that in this and previous studies, hydroxy fatty acids of C18 and C20 chain lengths accumulated in transgenic Arabidopsis. This suggests that although enzymes of triacylglycerol synthesis such as acyltransferases tend to show specificity and selectivity biased toward the fatty acids normally present in the plant (Wiberg et al., 1994; Frentzen, 1998), they do not entirely exclude hydroxy fatty acids from storage lipids.

Analysis of fatty acid composition of developing *L. fendleri* embryos revealed that hydroxy fatty acids are not readily detectable until developmental stage 4, which seems to coincide with the beginning of seed oil deposition. C18 and C20 hydroxy fatty acids appeared at the same time, indicating that LfKCS3 condensing enzyme must be expressed at the same time as, or earlier than, LFAH12 hydroxylase in the *L. fendleri* embryos. We compared the timing of both promoter activities using the GUS reporter gene in transgenic Arabidopsis plants. Histochemical staining showed that with the LfKCS3 or the LesqH promoter (Broun et al., 1998), GUS expression was first observed in very young torpedo stage embryos 4 d after flowering, and continued throughout subsequent embryo development. Because the LfKCS3 promoter directs gene expression only in developing embryos, it will benefit a variety of seed-specific applications, especially those aimed at genetic engineering of storage lipid composition in oilseed crops.

MATERIALS AND METHODS

Screening of the Genomic DNA Library

A *Lesquerella fendleri* genomic DNA library was obtained from Dr. Chris Somerville (Carnegie Institution of Washington, Stanford, CA). The genomic library was plated on *Escherichia coli* LE392 (Promega, Madison, WI), and about 150,000 clones were screened using the entire deduced coding region of Arabidopsis FAE1 as a probe. The probe was amplified by PCR from the pGEM7-FAE1 template

(Millar and Kunst, 1997) using *FAE1* upstream primer, 5'-CCGAGCTCAAAGAGGATACATAC-3' and *FAE1* downstream primer, 5'-GATACTCGAGAACGTTGGCACTCAGATAC-3'. PCR was performed in a 10- μ L reaction containing 10 ng of the template, 2 mM $MgCl_2$, 1.1 μ M of each primer, 100 μ M of dCTP + dGTP + dTTP mix, 50 μ Ci of [α - 32 P]dATP, 1 $\times$ PCR buffer, and 2.5 units of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). Amplification conditions were 2 min of initial denaturation at 94°C, 30 cycles of 94°C for 15 s, 55°C for 30 s, 72°C for 1 min and 40 s, followed by a final extension at 72°C for 7 min. The amplified radioactive probe was purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and was denatured by boiling before addition to the hybridization solution. Hybridization was carried out overnight at 65°C in a solution containing 6 $\times$ standard saline citrate (SSC), 20 mM NaH_2PO_4 , 0.4% (w/v) SDS, 5 $\times$ Denhardt's solution, 0.1% [w/v] of Ficoll [Type 400, Pharmacia, Buckinghamshire, UK], 0.1% [w/v] polyvinylpyrrolidone, 0.1% [w/v] bovine serum albumin [Fraction V; Sigma, St. Louis]), and 50 μ g mL $^{-1}$ sonicated, denatured salmon sperm DNA (Sigma), followed by three washes for 20 min each in 2 $\times$ SSC and 0.5% (w/v) SDS at 65°C.

Plasmid Construction and Plant Transformation

After tertiary screening, nine positive clones were purified from the *L. fendleri* genomic library. The phage DNA from those nine clones was extracted using the Lambda mini kit (Qiagen) according to the manufacturer's protocol. One of them was digested with *EcoRI*, and a 4.3-kb fragment, designated LfKCS3G, was subcloned into the pGEM-7Zf(+) vector (Promega) cut with *EcoRI*, resulting in the vector pMHS15. The whole insert was sequenced in both directions with a Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, CA) using di-deoxy Big Dye chemistry (Applied Biosystems) according to the Terminator Cycle Sequencing protocol.

To prepare the promoter-GUS fusion construct, the fragment directly upstream of the *LfKCS3* coding region was amplified using the high-fidelity *Pfu* polymerase (Stratagene, La Jolla, CA) with a forward primer 5'-CGCAAGCTTGAATTCGGAATGGGCCAAG-3' and a reverse primer 5'-CGCGTCGACTGTTTGTGTTGTTGTCGGG-3'. The amplified 573-bp promoter fragment (designated LfKCS3P) was inserted upstream of the *uidA* gene in pBI101 (CLONTECH, Palo Alto, CA) cut with *HindIII* and *SalI*, resulting in the vector pLfKCS3P-GUS.

Two constructs were generated for *LfKCS3* expression in Arabidopsis. In the first, a 3.1-kb genomic fragment containing the promoter and the coding sequence was excised from pMHS15 with *EcoRI* and *HpaI* and was ligated into a binary plant transformation vector, pRD400 (Datla et al., 1992) cut with *EcoRI* and *SmaI*, resulting in the vector pLfKCS3G. For comparison, the coding region of *LfKCS3* (designated LfKCS3C) was also placed under the control of the *L. fendleri* oleate 12-hydroxylase promoter (designated LesqH) to produce the LesqH-LfKCS3C cassette. This promoter was isolated from *L. fendleri* and was reported to be highly active in developing seeds of transgenic Arabidop-

sis starting early in embryogenesis (Broun et al., 1998). To generate the LesqH-LfKCS3C cassette, the coding region of *LfKCS3* was amplified from pMHS15 using the high-fidelity Platinum *Pfx* DNA polymerase (Invitrogen), forward primer 3ApaI (5'-TTAGGGCCCCATCTCATCCC-TTAGTACCCTC-3'), and the reverse primer 3NotI (5'-TATTGCGGCCGCTTCGATCAAGCGTGCTAC-3'). The resulting fragment was cut with *ApaI* and *NotI* and was ligated in sense orientation between the LesqH promoter and nopaline synthase terminator to give the vector pMHS23. The entire cassette was then cut from pMHS23 with *EcoRI* and *SalI* and was ligated into the binary vector pRD400, cut with *EcoRI* and *SalI*, to produce the vector pLesqH-LfKCS3C.

All three plant transformation vectors, pLfKCS3P-GUS, pLfKCS3G, and pLesqH-LfKCS3C were introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90; Koncz and Schell, 1986) by heat shock and were selected for resistance to kanamycin (50 μ g mL $^{-1}$). They were then used to transform Arabidopsis Columbia-2 wild type and/or *fad2/fae1* double mutant. The *fad2/fae1* double mutant is characterized by a very high level (>80%) of oleic acid (18:1) in its seed oil due to deficiency in the activities of cytoplasmic oleate Δ 12 desaturase (FAD2) and the FAE1 condensing enzyme. Plants were grown in a growth chamber at 20°C under continuous light. Transformation of Arabidopsis was performed by the floral dip method (Clough and Bent, 1998). Screening for transformed seed was done on 50 μ g mL $^{-1}$ kanamycin as previously described (Katavic et al., 1994).

To test the activity of the *LfKCS3* with hydroxy fatty acids, *fad2/fae1* double-mutant lines transformed with the LfKCS3G or LesqH-LfKCS3C cassettes were crossed with the transgenic *fad2/fae1* Arabidopsis plants expressing the castor oleate 12-hydroxylase under the control of the LesqH promoter (LesqH-CFAH12 lines; Smith et al., 2000). For comparison, transgenic *fad2/fae1* double-mutant plants were also generated that expressed the castor oleate 12-hydroxylase and the Arabidopsis FAE1 condensing enzymes. These expression cassettes (LesqH-CFAH12 and LesqH-FAE1) contained the coding regions of each gene, under the control the LesqH promoter and nopaline synthase terminator.

GUS Assay

GUS assay was performed by immersing tissues in GUS histochemical staining solution (Jefferson, 1987) for 4 to 7 h at 37°C. The assay solution was composed of 50 mM sodium phosphate, pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, 0.05% (w/v) Triton X-100, and 0.35 mg mL $^{-1}$ 5-bromo-4-chloro-3-indolyl- β -D-glucuronide. Following staining, the samples were fixed in 70% (v/v) ethanol.

RNA Gel-Blot Analysis

For RNA gel-blot analyses, total RNA from various tissues of *L. fendleri* was extracted using the RNeasy Plant

Mini Kit (Qiagen) or a conventional phenol-chloroform method. For the phenol-chloroform method, frozen tissue was pulverized in a precooled mortar using liquid nitrogen and was transferred to a centrifuge tube containing 9 mL of NTES buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1% [w/v] SDS) and 6 mL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v). After shaking for a minimum of 10 min, the tube was centrifuged at 5,000g for 10 min at 4°C to separate the two phases. Nucleic acids were precipitated by the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of ethanol to the aqueous layer. Following centrifugation, the precipitated nucleic acids were resuspended in water. Lithium chloride was then added to a final concentration of 2 M. Following precipitation on ice for at least 3 h, total RNA was collected by centrifugation at 10,000g for 10 min at 4°C and was resuspended in water.

Ten micrograms of total RNA lane⁻¹ was separated in an 1.2% (w/v) agarose gel containing formaldehyde, blotted onto a Hybond-XL membrane (Amersham Pharmacia Biotech, Piscataway, NJ) in 20× SSC, and baked for 2 h at 80°C. The membrane was then hybridized to a 461-bp probe from the 5' region of the genomic clone that had been labeled with [α -³²P]dATP by PCR using pMHS15 as a template with the oligonucleotides 5'-ATGAAAGCAACGCACCA-CAAAACGAAGAC-3' (forward primer) and 5'-ACTC-AAGAGAAGAATCATCACAACCCACC-3' (reverse primer). The following PCR program was used: 2 min of initial denaturation at 94°C, 36 cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 5 min. The amplified radioactive probe was purified by QIAquick PCR purification kit (Qiagen), denatured by boiling, and was added to the hybridization solution. Hybridization was performed overnight at 65°C in 0.5 M phosphate buffer, pH 7.2, 7% (w/v) SDS, and 10 mM EDTA. The blots were washed three times in 2× SSC and 0.1% (w/v) SDS at 65°C for 30 min each, before exposing the x-ray film (Eastman-Kodak, Rochester, NY). To show equal loading of the RNA samples, Arabidopsis 18S rRNA (Unfried et al., 1989) was used as a probe. A ³²P-labeled rRNA probe was prepared by PCR using *Taq* DNA polymerase (Invitrogen) and the oligonucleotides 5'-CTGCCAGTAGTCATATGC-3' and 5'-ATGGATCCTCGTTAAGGG-3' with 10 ng of Arabidopsis (ecotype Columbia) DNA as a template. Amplification conditions were: 2 min of initial denaturation at 94°C, 30 cycles of 94°C for 15 s, 50°C for 30 s, 72°C for 30 s, followed by a final extension at 72°C for 7 min. After overnight hybridization at 65°C, the blot was washed three times in 0.1× SSC and 0.1% (w/v) SDS at 65°C before exposure of the x-ray film.

Analysis of Fatty Acid Composition

To determine the fatty acid composition of the seed oil, fatty acid methyl esters were prepared by refluxing the seed samples in 2 mL of 1 N methanolic-HCl for 90 min at 80°C. After cooling, 2 mL of 0.9% (w/v) NaCl solution and 150 μ L of hexane were added and the mixture was vortexed vigorously. The fatty acid methyl esters in the hexane

phase were analyzed by gas-liquid chromatography as described previously (Kunst et al., 1992). The identity of fatty acids in the samples was determined by comparing retention times with those of standards (Sigma). Lipid extracts from castor bean and *L. fendleri* were used as sources of ricinoleic (18:1-OH), densipolic (18:2-OH), lesquerolic (20:1-OH), and auricolic (20:2-OH) acid.

ACKNOWLEDGMENTS

We thank Chris Somerville and Pierre Broun (Carnegie Institution of Washington, Stanford, CA) for *L. fendleri* genomic DNA library and LesqH-GUS transgenic seeds, and David Dierig (U.S. Department of Agriculture-Agricultural Research Service Water Conservation Laboratory, Phoenix) for the *L. fendleri* plants. We also thank Tanya Hooker (University of British Columbia, Vancouver) for taking pictures of the *L. fendleri* developing embryos and Sabine Clemens (Simon Fraser University, Vancouver) for providing the developing embryos from the LesqH-GUS transgenic Arabidopsis plants.

Received June 21, 2001; returned for revision July 25, 2001; accepted September 20, 2001.

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