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Two Novel Thioesterases Are Key Determinants of the Bimodal Distribution of Acyl Chain Length of *Cuphea palustris* Seed Oil

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The seed oil of *Cuphea palustris* has an unusual fatty-acyl composition, whereby the principal fatty-acyl groups, myristate (64%) and caprylate (20%), differ by more than two methylenes. We have isolated two thioesterase (TE) cDNAs from *C. palustris*, encoding proteins designated *Cp* FatB1 and *Cp* FatB2, which, when expressed in *Escherichia coli*, have TE activities specific for 8:0/10:0- and 14:0/16:0-acyl carrier protein substrates, respectively. The specific activities of the recombinant affinity-purified enzymes indicate that *Cp* FatB2 is kinetically superior to *Cp* FatB1. This result is consistent with the predominance of 14:0 in the seed oil, despite apparently equal mRNA abundance of the two transcripts in the seed. In *C. palustris* the expression of both sequences is confined to the seed tissues. Based on these findings we propose that these two enzymes are major factors determining the bimodal chain-length composition of *C. palustris* oil. Analysis of the immature and mature seed oil by reverse-phase high-performance liquid chromatography confirmed that the principal triglycerides contain both 8:0 and 14:0. This result indicates that both fatty acids are synthesized at the same time and in the same cells at all developmental stages during oil deposition, suggesting that the two TEs act together in the same fatty acid synthesis system.

The end products of plant fatty acid synthetase activities are usually 16- and 18-carbon fatty acids (Harwood, 1988). There are, however, several plant families that store large amounts of 8- to 14-carbon (medium-chain) fatty acids in their oilseed. Several mechanisms have been proposed for the synthesis of medium-chain fatty acids in plants. A specific acyl-ACP TE might terminate fatty acid synthesis by hydrolyzing the thioester bond of a particular acyl-ACP, resulting in the release of both ACP and free fatty acid (Stumpf, 1987). Alternatively, a specific 3-ketoacyl-ACP synthase (condensing enzyme) or an acyl-ACP acyltransferase might be involved (Harwood, 1988). At the time these mechanisms were proposed, however, there were limited experimental data to support these hypotheses. More recently, studies with *Umbellularia californica* (California bay), a plant that produces seed oil rich in lauric acid, have demonstrated the existence of a medium-chain-specific isozyme of acyl-ACP TE in the seed plastids (Pollard et al., 1991). These authors were able to separate the activity of a long-chain TE from a medium-chain TE, suggesting that there is medium-chain TE involved in oil synthesis. Subsequent purification of the 12:0-ACP TE from *U. californica* by Davies et al. (1991) led to the cloning of a TE cDNA (*Uc FatB1*), which was expressed in seeds of Arabidopsis and *Brassica* (Voelker et al., 1992).

A substantial accumulation of lauric acid (12:0) was observed in the triglyceride pool of these transgenic seeds (Voelker et al., 1992; Davies and Voelker, 1993), thus confirming the chain-length-determining role of acyl-ACP TEs during de novo fatty acid biosynthesis. Several other acyl-ACP TE cDNAs and genes have been isolated from MCT-accumulating plant species, none of which is reported to have substantial activities on medium-chain acyl-ACPs (Töpfer and Martini, 1994; Jones et al., 1995).

The genus *Cuphea* has attracted much attention because of the diverse and unusually high content of medium-chain (8:0–14:0) fatty acids in the seed storage lipids (Graham et al., 1981; Graham, 1989). The fact that a series of closely related species exhibits such variation suggests that differences in the properties of only one or a few enzymes may be responsible for this biosynthetic diversity (Somerville and Browse, 1991).

To identify the key enzyme(s) involved, the biosynthesis of medium-chain lipids was studied in vivo by incubating extracts of developing *Cuphea* seeds with [^{14}C]acetate. These studies have shown that *Cuphea* seed extracts incorporate the exogenous label ([^{14}C]acetate) into medium-chain fatty acid (Slabas et al., 1982; Singh et al., 1986). In other studies, only very low levels of medium-chain TE activity were detected in extracts of developing *Cuphea* seeds (Dörmann et al., 1991). To evaluate the role that specific TEs play in determining the composition of medium-chain fatty acids, partial purification of two acyl-ACP TEs with marked differences in their substrate specificity were carried out (Dörmann et al., 1993; M. Davies, unpublished data). *Cuphea lanceolata*, a plant with 83% decanoic acid (10:0), and *Cuphea wrightii*, a plant that contains 29% decanoic acid and 54% lauric acid, were examined (Dörmann et al., 1993). In both of these *Cuphea* species, two TEs with different substrate specificities were found. One enzyme had high activity on 18:1-ACP, and the other one had a broad specificity with all of the substrates examined. Similar enzymes, one predominantly active on 18:1-ACP

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Abbreviations: ACP, acyl carrier protein; ECN, equivalent carbon number; MCT, medium-chain triglyceride; TAG, triacylglycerol; TE, thioesterase.

and the other with broad substrate specificity, were detected in seed extracts of *Cuphea hookeriana*, a species with 50% caprylate acid (8:0) and 25% decanoic acid (M. Davies, unpublished data). Based on these reports, it is difficult to be confident about the role of such a medium-chain TE in the production of specific medium-chain fatty acids in vivo.

Screening of a *C. lanceolata* library led to the isolation of a cDNA encoding an acyl-ACP TE with unknown substrate specificity (Töpfer and Martini, 1994). Recently, a TE cDNA (*Ch FatB1*) was isolated from *C. hookeriana*, a plant with 8:0 and 10:0 as its predominant seed fatty-acyl groups (Jones et al., 1995). The *Escherichia coli*-expressing *Ch FatB1* was active on 14:0- to 18:1-ACP with a strong preference for 16:0-ACP. Furthermore, overexpression of this clone in *Brassica* seed led to the production of oil rich in 16:0 fatty acids. The enzymatic specificity of *Ch FatB1* was unexpected, since *C. hookeriana* seeds do not accumulate large amounts of 16:0 fatty acids. A 16:0-ACP-specific TE clone with sequence similarity to the bay 12:0-ACP TE was obtained from Arabidopsis (Dörmann et al., 1995). This TE cDNA appears to be similar to the *C. hookeriana* TE, in that both are expressed throughout the plant.

More recently, the cloning of four TE genes from *C. lanceolata* was reported (Töpfer et al., 1995). Transformation of rapeseed lines with two of these genes resulted in an altered fatty acid profile. Seed of transgenic plants overexpressing the *Cl FatB3* gene contained 1 and 3% caprylic (8:0) and capric (10:0) acids, respectively, whereas overexpression of the *Cl FatB4* resulted in accumulation of 7% myristic (14:0) and 15% palmitic (16:0) acids in the oil. *C. lanceolata* is a species with up to 83% capric acid (10:0) in seed oil, and overexpression of neither of its TE genes in transgenic rapeseed produced a comparable amount of capric acid. Improving the promoter strength or the transformation vector or cloning of a more active TE gene may help to narrow this wide discrepancy between fatty-acyl profiles of the *C. lanceolata* seed storage lipids and that of the transgenic seed oil.

To date almost all studies on the molecular mechanisms of fatty acid biosynthesis in the genus *Cuphea* have been focused on those species containing fatty-acyl groups that differ by only two methylenes. *Cuphea palustris*, an unusual species with a bimodal chain-length specificity of 20 mol% caprylate (8:0) and 64 mol% myristate (14:0), offers a new challenge to understanding the possible regulatory role and kinetics of enzymes such as TE(s) in determining this precise chain-length specificity of fatty-acyl composition of the oilseed.

MATERIALS AND METHODS

Plant Material

Cuphea palustris plants were propagated from a cutting originally obtained from the U.S. Department of Agriculture (Ames, IA). Plants were grown at 28°C with 16 h of light and 8 h of dark until flowering, at which time the dark period was increased to 18 h and the temperature was decreased to 22°C. Tissues for RNA isolation were frozen in liquid nitrogen and kept at −80°C.

RNA Isolation and cDNA Library Construction and Screening

Total cellular RNA was isolated according to the method of Jones et al. (1995). RNA isolated from developing seed was used to prepare double-stranded cDNA for cloning. Commercial kits were used for cDNA synthesis and λZip-Lox cloning (GIBCO-BRL). Approximately 500,000 unamplified recombinant phage were plated, and the plaques were then transferred to nitrocellulose using standard methods (Maniatis et al., 1982). Filters were prehybridized for 16 h at 42°C in 30% formamide, 5× SSC, 5× Denhardt's solution, 40 mM $NaPO_4$ (pH 6.8), 0.5% BSA, 1% SDS, and 100 μg/mL sonicated denatured salmon testes DNA. Subsequent hybridization was carried out for 18 h under the same conditions as that of prehybridization, using the *Ch FatB1* clone (Jones et al., 1995) and its homologs (Dehesh et al., 1996; K. Dehesh, unpublished data) as probes. These membranes were washed under low-stringency conditions: twice for 30 min at room temperature in 2× wash solution (2× SSC, 5 mM EDTA, 1.5 mM sodium PPi, 0.5% SDS). Of the several clones obtained from each type, the longest ones, *Cp FatB1* (1.48 kb) and *Cp FatB2* (1.43 kb), were sequenced on both strands.

Northern Blot Analysis

A northern blot analysis was carried out according to the method of Colbert et al. (1985) using 20 μg of total RNA per lane. Blots were prehybridized and hybridized to the *Sal*I-*Not*I fragments corresponding to 1488 bp (*Cp FatB1*) and 1433 bp (*Cp FatB2*), under conditions similar to those used in library screening except for formamide concentration in the buffer that was increased to 50%. Blots were washed for 1 h at 65°C in 0.1× wash solution (high-stringency conditions). Autoradiography was performed at −70°C with an intensifying screen.

DNA Sequencing and Sequence Analysis

The cDNAs were sequenced completely in both directions using an automated ABI 373A sequencer (Applied Biosystems). DNA and polypeptide sequence analyses were performed using the programs of Intelligenetics, version 5.3 (Intelligenetics, Inc., Mountain View, CA).

Bacterial Expression System and Enzyme Activity Assays

Two expression systems, pUC118 driven by the *lacZ* promoter and pQE30 driven by the *Escherichia coli* phageT5 promoter with N-terminal His_6 affinity tags (QIAexpress; Qiagen Inc., Chatsworth, CA), were utilized for production of recombinant *Cp* FatB1 and *Cp* FatB2 protein. Appropriate cloning sites were designed in the synthetic oligonucleotide primers for in-frame fusions of the cDNA clones into both pUC118 and QIAexpress vectors. The mature regions of both clones (see "Results") were amplified in a standard PCR using the synthetic oligonucleotide primers representing the 5′ end sequence (*Cp FatB1*, GAATTCGCATGCAGGCCTAACATGCTCATG; *Cp FatB2*, GAATTCGCATGCAGGCCTATGCTTGACCGGAAATCT) and

M13 universal primer for the 3′ end of the clones. PCR products were either cut with *Stu*I and *Sph*I and cloned into an *Sma*I-*Sph*I linearized pUC118 vector or cloned as an *Sph*I-*Sna*BI fragment into an *Sph*I-*Sma*I site of pQE-30 vectors. These plasmids, once constructed, were sequenced and their authenticity was verified. Strain DH5α was transformed with pUC118 plasmids, and strain M15[pREP4] was transformed with the QIAexpress vector. Transformed bacteria were grown at 37°C and were induced with 0.2 mM isopropyl β-D-thiogalactopyranoside to an A_{600} of 0.7 to 0.8 for 1 h and harvested. Cells were sedimented by centrifugation, resuspended in TE assay buffer (Voelker et al., 1992), and lysed by three 5-s sonications. Debris was sedimented by a 15-min centrifugation at 14,000*g*, and supernatants were analyzed on SDS-polyacrylamide gels to verify expression and stored at −20°C for enzyme activity assay. Activity assays were carried out according to the method of Pollard et al. (1991). Protein measurements were performed using a BCA protein assay kit obtained from Pierce.

Enzyme Assays of the Affinity-Purified Recombinant *Cp* FatB1 and *Cp* FatB2 Proteins

Soluble extracts from identical cell cultures were used for both enzyme assays and for further processing to obtain affinity-purified *Cp* FatB1 and *Cp* FatB2 proteins. In all of the experimental procedures, cultures of *E. coli* cells transformed with the insertless plasmid were included. The same protocol as described above for the TE activity assays was used to obtain crude lysates, except that samples were centrifuged for 30 min. To purify the soluble recombinant protein from *E. coli* crude lysates, the supernatants from cultures with identical absorbances were affinity purified over an Ni column according to the manufacturer's recommendations. Affinity-purified recombinant proteins were assayed for enzyme activity and analyzed by SDS gel electrophoresis. Loading of the proteins on a SDS polyacrylamide gel was on a per initial crude extract volume basis. Protein concentration was determined as described in the previous section.

To compare the enzymes' specific activities, we conducted the experiments under "subsaturation" substrate concentrations. Under the conditions of our assay, both enzyme activities were dependent on the substrate concentration with 8:0-ACP, 10:0-ACP, and 14:0-ACP.

Sample Preparation for TAG Analysis

Approximately 0.6 g fresh weight of immature (9–12 d postanthesis) or mature (18–20 d postanthesis) *C. palustris* seeds were ground in a mortar and pestle at 0°C with 6 mL of *n*-hexane. After the sample was filtered through 0.2-μm nylon 66, the solvent was evaporated from the extract under a nitrogen stream at 40°C. The residual oil was stored at −60°C under nitrogen prior to analysis.

Reverse-Phase HPLC

The extracted seed oil was dissolved in methylene chloride to approximately 10 mg/mL, and 25 μL were chromatographed on a 4.6- × 250-mm Beckman Ultrasphere ODS reverse-phase column (Beckman). The mobile phase comprised methylene chloride:acetonitrile, 30:70 (v/v), and the flow rate was 1.5 mL/min. Eluted triglycerides were detected by passing 50% of the effluent stream into an evaporative light-scattering detector (Varex, Burtonsville, MD), which was operated with a drift tube temperature of 115°C and a nitrogen flowmeter reading of 50 mm/min. Effluent samples corresponding to the triglyceride peaks were collected from the remaining 50% of the solvent stream, and the solvent was evaporated from them as described above. The light-scattering signal was used exclusively for cuing peak collection and not for any quantitation. A standard mixture of 8:0, 10:0, 12:0, and 14:0 symmetrical triglycerides was used to calibrate retention time in terms of ECN.

Analysis of Triglyceride Fractions

The quantities and compositions of the triglyceride fractions from reverse-phase HPLC were determined by acidic methanolysis and capillary GC of the resulting fatty acid methyl esters essentially according to the method of Browse et al. (1986). Tri-17:0 triglyceride was included as internal standard.

RESULTS

Isolation and Sequence Analysis of *Cp FatB1* and *Cp FatB2* Clones

The 16:0 TE clone from *C. hookeriana* (*Ch FatB1*) and its homologs (Jones et al., 1995; Dehesh et al., 1996; K. Dehesh, unpublished data) were used as probes to isolate a series of clones from a cDNA library made from *C. palustris* developing seed. One class of clones strongly hybridized with this probe, and after restriction mapping and partial sequence analysis, it proved to be homologous to the nucleic acid sequence designated *Ch FatB1* (Jones et al., 1995). Members of a second class of clones, which hybridized to a lesser extent, were sequenced and found to belong to a novel class of TEs designated *Cp FatB1* and *Cp FatB2* (Fig. 1). A full-length clone was obtained for each of the *Cp FatB1* and the *Cp FatB2* classes, and they were found to encode predicted polypeptides of 422 and 421 amino acids with molecular masses of 47.1 and 46.9 kD and pIs of 9.3 and 8.9, respectively. Pairwise comparison of these clones with each other and with the *Ch FatB1* clone indicates that these sequences are equally diverged from each other with 75% amino acid identity. Hydropathy profile analysis (Fig. 2) demonstrates that, despite their scattered sequence differences, *Ch FatB1*, *Cp FatB1*, *Cp FatB2*, and *Ch FatB2*, a 8:0/10:0 TE recently cloned from *C. hookeriana* (Dehesh et al., 1996), have a very similar profile over almost the entire length of their sequences. This result suggests that the overall structure of these proteins has been conserved during evolution.

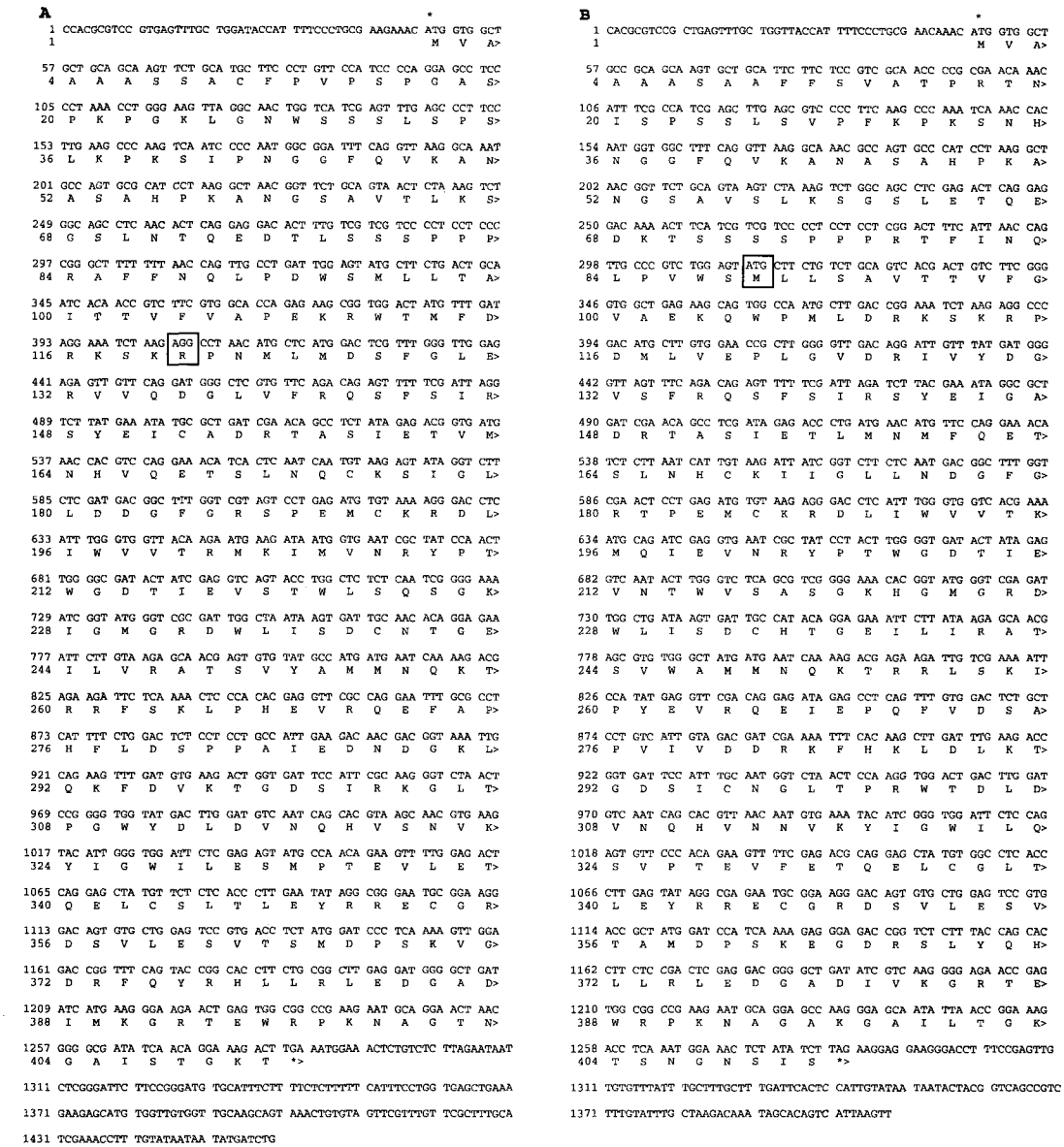

Figure 1. Nucleotide and derived amino acid sequence of *C. palustris* TEs *Cp FatB1* (A) and *Cp FatB2* (B). The amino acid sequence is presented below the nucleotide sequence in a single-letter code numbered starting at the first in-frame Met residue. The boxed nucleotides encode the first residue of each recombinant polypeptide at the fusion point with the six His residues used for affinity purification.

Cp FatB1 and *Cp FatB2* Encode TEs with Contrasting Substrate Specificities and Specific Activities

To measure the TE activity of *Cp* FatB1 and *Cp* FatB2 expressed in *E. coli*, both cDNAs were cloned into the QIAexpress plasmid, which allows high-level bacterial expression of recombinant protein with an N-terminal His_6 affinity tag. The mature portions of both *Cp* FatB1 and *Cp* FatB2 (Fig. 1), as defined by sequence homology with *Uc* FatB1 and *Ch* FatB1 (Voelker et al., 1992; Jones et al., 1995), were fused in-frame to the His_6 tag in the respective expression cassettes. Crude lysates of transformed *E. coli* strains expressing *Cp* FatB1 and FatB2 were corrected for the total soluble protein concentration and assayed for in vitro acyl-ACP hydrolytic activity (Fig. 3), according to a previously reported method (Pollard et al., 1991). These results show that *Cp FatB1* encodes an enzyme that acts predominantly on 8:0- and 10:0-ACP with 2.5-fold more activity on 8:0-ACP than on 10:0-ACP. The *Cp* FatB1 also has a low level of activity on 14:0-ACP, about 10% of that on 8:0-ACP. By contrast, *Cp FatB2* encodes an enzyme that exhibits preferred activity on a 14:0-ACP substrate with 50% of that activity on the 16:0-ACP substrate.

These two enzymes differ not only in their substrate specificity but also in their intrinsic specific activities. Rep-

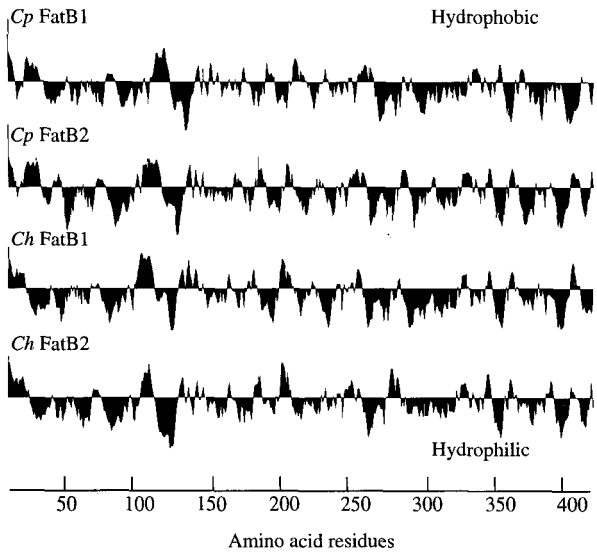

Figure 2. Hydropathy profiles of *C. palustris* (*Cp* FatB1 and *Cp* FatB2) and *C. hookeriana* (*Ch* FatB1 and *Ch* FatB2) TEs. Analysis was performed according to the method of Kyte and Doolittle (1982) using a window of seven amino acids.

licates of the *E. coli* crude lysates used in enzyme activity assays were also used to affinity purify each of the native recombinant proteins using an Ni column (Fig. 4). If we assume equal recovery of the two enzymes, the results suggest that the *Cp* FatB1 is present in the soluble fraction of *E. coli* at severalfold higher levels than the *Cp* FatB2 protein. Together, with the higher enzymatic activity measured in crude extract, the presence of lower levels of *Cp* FatB2 protein in the soluble fraction as compared with the levels of *Cp* FatB1 protein suggests that *Cp* FatB2 is an enzyme with superior kinetics to the *Cp* FatB1. To examine this possibility directly, the affinity-purified recombinant enzymes were assayed enzymatically (Fig. 5). These results clearly demonstrate that *Cp* FatB2 has a higher intrinsic specific activity with its appropriate substrate than *Cp* FatB1 with its respective acyl-ACP substrate. The data also demonstrate that the substrate specificity profiles obtained from these enzyme assays were similar to those measured in the bacterial crude lysates; thus the manipulations required for protein purification did not alter the nature or activity profile of these enzymes.

Both *Cp FatB1* and *Cp FatB2* were also fused in-frame to a *lac*Z promoter-driven expression cassette and expressed in *E. coli* (data not shown). The enzymatic substrate specificity profiles of these *C. palustris* clones were similar to those observed using the QIAexpress system. However, the level of TE activity obtained with the His-tagged clones in the latter system was 15- to 278-fold higher (depending on the enzyme) than the level of activity measured with the *lac*Z fusion. Utilization of the QIAexpress expression system enabled us to increase the detectability to the levels that allowed measurements of those activities that were otherwise below the levels of detection in crude lysates.

Levels and Pattern of *Cp FatB1* and *FatB2* Expression

Northern blot analyses performed on total RNA isolated from root, leaf, flower, and seed of *C. palustris* were used to examine relative levels and patterns of *Cp FatB1* and *FatB2* expression in these tissues. Probes of similar length and equal specific activities were used on duplicated, identical blots, allowing comparison of the levels of *Cp FatB1* and *Cp FatB2* in the tissues to be examined. The ethidium bromide staining pattern of the ribosomal bands was similar, indicating equal loading of RNA sample on each lane (results not shown). The hybridization results (Fig. 6) show that both cDNAs are expressed similarly and detectably only in seeds. The same pattern of expression was observed when a 3'-end-specific probe was used (results not shown), indicating that the coding region probe does not cross-hybridize with other mRNAs under the high-stringency hybridization and wash conditions used. This pattern of expression provides additional evidence supporting the notion that these two TEs are the principal enzymes determining the acyl composition of the *C. palustris* seed oil.

TAG Composition as an Indication of Spatial and Temporal Expression of *Cp FatB1* and *FatB2*

The oil composition of immature and mature *C. palustris* seed was analyzed by reverse-phase HPLC to examine

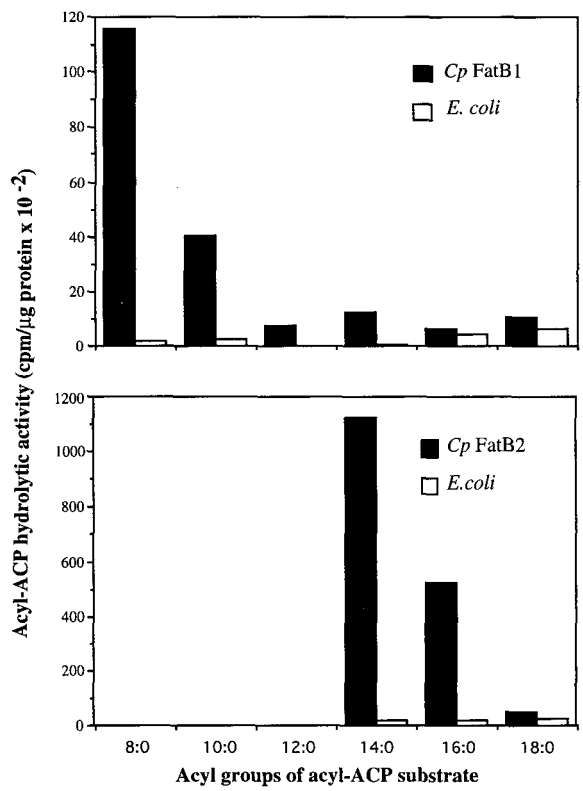

Figure 3. TE activity of *Cp* FatB1 and *Cp* FatB2 in *E. coli*. A, TE activity of *Cp* FatB1; B, TE activity of *Cp* FatB2. *Cp* FatB1 and *Cp* FatB2 were cloned into the QIAexpress plasmid. These plasmids and the insertless pQE vector, as the control, were used to transform *E. coli*. For determination of the in vitro acyl-ACP hydrolytic activities, cells growing in the logarithmic phase were induced with isopropyl β-D-thiogalactopyranoside, grown for 1 h, and frozen in −80°C. Cells were subsequently lysed by sonication, and the TE activity was assayed. The activites were adjusted to account for differences in the protein concentration. Data are mean values from two replicate experiments.

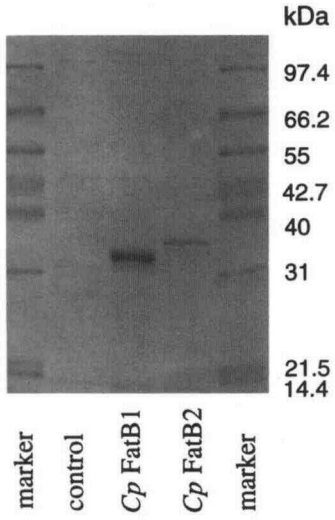

Figure 4. Silver-stained gel following SDS-PAGE of affinity-purified fraction of control, *Cp* FatB1, and *Cp* FatB2 recombinant proteins. Aliquots of the *E. coli* crude lysate used in enzyme assays were used for affinity purification of the recombinant proteins. Loading of the proteins was based on equal volume of original crude lysate.

whether 8:0 and 14:0 are synthesized with different spatial and temporal patterns. TAGs were extracted from mature and immature seeds (see "Materials and Methods"), fractionated according to ECN on reverse-phase HPLC, and subsequently analyzed for their fatty acid composition. In both immature and mature seeds, the principal TAG classes were 17 and 22 mol% 8/8/14 (ECN30) (this terminology does not imply any stereochemical designation)

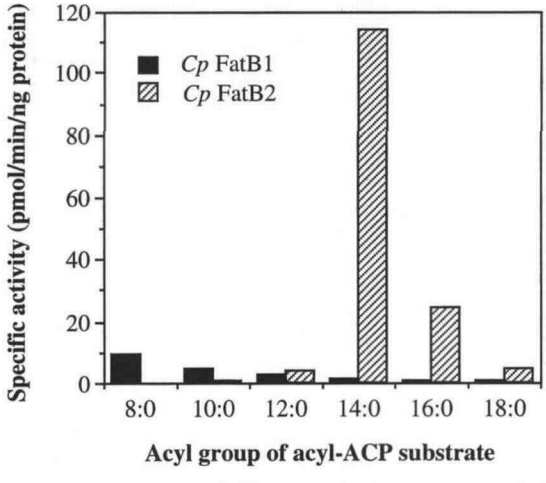

Figure 5. Specific activity of affinity-purified recombinant *Cp* FatB1 and *Cp* FatB2. The in vitro hydrolytic activities of the affinity-purified *Cp* FatB1 and *Cp* FatB2 were determined according to the same protocol described for the enzyme assays using the crude lysate. The activities measured using proteins purified from the control cultures (insertless plasmid) were identical with those obtained with assays containing no enzymes. Data are the mean values of three replicate experiments, in which background levels measured from controls were subtracted from *Cp* FatB1 and *Cp* FatB2 activities. 8:0, Caprylic acid; 10:0, decanoic acid; 12:0, lauric acid; 14:0, myristic acid; 16:0, palmitic acid; 18:0, stearic acid.

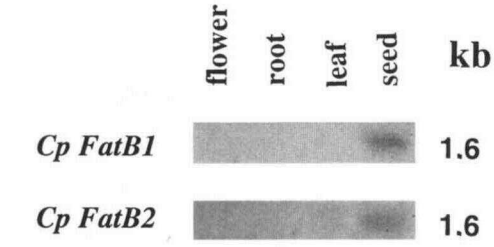

Figure 6. Northern blot analysis of *C. palustris* TE (*Cp FatB1* and *Cp FatB2*) mRNA levels in different tissues. Total RNA (20 μg per lane) was isolated from seed, leaf, root, and flower tissue. Blots were hybridized with ^{32}P-labeled DNA fragments to detect *Cp FatB1* and *FatB2*. The indicated sizes were estimated from the molecular size markers.

and 47 and 50 mol% 8/14/14 (ECN36), respectively (Table I). These values agree with those previously reported for the mature seeds (Singh et al., 1984), with the major triacylglycerides being 8/8/14 (15% mass) and 8/14/14 (59% mass). These results indicate that different growing conditions or ages of material used in the analysis may change the relative proportions of the triacylglycerides but not the co-presence of 8:0 and 14:0 in the same TAG species. Furthermore, the proportions of the TAG classes differed considerably from that which would have been expected from random acylation (Table I). This could be explained by selectivity on the part of the acyltransferases or a varying ratio of 8:0/14:0 production during seed development. Overall, the results indicate that 8:0 and 14:0 are present on the same TAG molecules and, therefore, suggest that both fatty acids are synthesized in the same cells and at the same time at all developmental stages of oil deposition.

DISCUSSION

The lipids of *Cuphea* seeds have a variety of medium-chain fatty acid compositions. Some species such as *C. palustris* have a bimodal chain-length specificity (Singh et al., 1984; Graham, 1989), and it is for this reason that we searched for substrate-specific TEs involved in chain-length determination in this species. We isolated two TE

Table I. *Triglyceride analysis of immature and mature seed oil of C. palustris by reverse-phase HPLC*

Triglycerides were extracted, fractionated on reverse-phase HPLC, and analyzed for fatty-acyl composition. Measured composition was calculated from moles of identifiable TAGs as percentages of theoretical TAG content of samples (total acyl content /3). Random composition was calculated from whole-seed fatty acid composition, assuming equal use of all fatty acids by each acyltransferase.

TAG Class[a]	Measured Composition		Random Composition	
	Immature	Mature	Immature	Mature
	mol%		*mol%*	
8/8/8	(trace)	(trace)	5.3	6.4
8/8/14	17	22	18.2	23.5
8/14/14	47	50	20.9	28.2
8/14/16	14	10	3.1	2.7
8/16/16	(trace)	(trace)	0.5	0.3
14/14/14	(trace)	(trace)	8.0	11.6

[a] No regiospecific or stereospecific composition implied.

cDNAs belonging to the *FatB* class (Jones et al., 1995), *Cp FatB1* and *Cp FatB2*, with hydrolytic specificities similar to the predominant medium-chain fatty acids of *C. palustris* seed oils (64% myristate and 20% caprylate). The results from in vitro enzyme activities of *Cp* FatB1 and *Cp* FatB2, when expressed in *E. coli*, demonstrate that both of these plant medium acyl-ACP TEs are capable of redirecting the path of fatty acid synthesis in bacteria from long-chain to medium-chain production (data not shown). In previous experiments (Voelker and Davies, 1994; Jones et al., 1995; Dehesh et al., 1996), the substrate specificities of the TEs expressed in *E. coli* predicted the oil compositions observed, subsequently, in transgenic seeds. Based on these observations, we expect that when *Cp FatB1* and *Cp FatB2* are expressed in plants the fatty acid composition of the seed will reflect the TE substrate specificity we observed in *E. coli*. The in vitro hydrolytic activity measurements of these enzymes indicate that *Cp FatB1* is an acyl-ACP TE active on both 8:0/10:0 chains with stronger preference for 8:0-ACP, whereas *Cp FatB2* is active on 14:0/16:0 chains with very strong preference for 14:0-ACP. The enzymatic activity of *Cp* FatB1 with 10:0-ACP and the activity of *Cp* FatB2 with 16:0-ACP were unexpected, since the seeds of *C. palustris* accumulate only minor amounts of 10:0 and 16:0 fatty-acyl groups. An apparent mismatch between hydrolytic activities on different medium-chain substrates relative to the proportions of acyl groups accumulated in the tissue was also reported for other plants such as camphor, bay, and coconut (Davies, 1993).

There are two intriguing aspects to the relationships between these TEs and the in vivo product composition. First, why are there only minor levels of 10:0 and 16:0 detectable in the seed oil of *C. palustris*, when *Cp* FatB1 acts on both 8:0- and 10:0-ACPs and *Cp* FatB2 acts on both 14:0- and 16:0-ACPs? Second, what is the mechanism that allows accumulation of 14:0 fatty-acyl groups to levels as high as 64 mol% in vivo, when the *Cp* FatB1 intercepts at 8:0-ACP? There may be several explanations to account for the quantitative difference between the hydrolytic specificity and the medium-chain fatty acid proportions in the tissue, as well as the mechanism(s) controlling the bimodal chain-length specificity. One possibility would be different spatial and temporal patterns of TE expression causing deposition of triglycerides having different fatty-acyl groups in different cells and stages of embryo development. Our northern blot data, however, indicate that both *Cp FatB1* and *Cp FatB2* are expressed at similar levels and are apparently confined to the seed tissues. This pattern of expression is consistent with the hypothesis that these two enzymes are major factors determining the bimodal chain-length composition of the *C. palustris* oil. Previous reports (Singh et al., 1984) of TAG analysis indicate that in the mature seeds the major molecular species are 8:0/8:0/14:0 and 8:0/14:0/14:0. From these results the authors concluded that either the mechanism for medium-chain fatty acid biosynthesis must be able to account for a bimodal chain-length specificity or there are two sites for fatty acid synthesis. Our data from the analysis of both the immature and mature seed oil by reverse-phase HPLC are consistent with those obtained from mature seeds (Singh et al., 1984), which showed that the principal triglyceride molecules contain both 8:0 and 14:0. These data suggest that the bimodal acyl specificity is a consequence of simultaneous biosynthesis in the same cell, inferring that the two TEs act together in the same fatty acid synthesis system at all seed developmental stages. Although the levels of the principal TAG classes in both mature and immature seeds are different, the quantitative profile is similar. These data exclude any possibility of differential spatial and temporal pattern of TE expression over time. Such results are also consistent with the reports suggesting that the reactions involved in oil biosynthesis are probably not restricted to a particular developmental stage and must function at the onset of seed development (Post-Beittenmiller et al., 1992).

An alternative explanation, which was previously proposed (Davies, 1993), holds that the product of fatty acid biosynthesis is determined by substrate specificities of the TEs in combination with the overall pathway kinetics. Apparently equal levels of *Cp FatB1* and *Cp FatB2* mRNAs detected on the northern blots may be an indication that these two enzymes are indeed present at similar levels in the seed. Thus, the bimodal chain-length specificity of fatty acid composition may not be explained in terms of expression levels of these two TEs. The in vitro activities of the affinity-purified recombinant enzymes show that *Cp* FatB2 is an enzyme with superior kinetics to the *Cp* FatB1 enzyme. Therefore, the requirement that 14:0 fatty acid should be made at a higher rate can be satisfied by different kinetics of these two enzymes. However, this difference in the kinetics of TE enzymes does not entirely account for the quantitative difference between hydrolytic specificity of the recombinant enzyme and fatty acid composition in *Cuphea* seed oil. There are reports implying that TEs are not the only determinants of fatty acid chain length and that β-ketoacyl-ACP synthases (condensing enzymes) are involved in the control of chain-length termination (Fuhrmann and Heise, 1993). Although there are no reports of the isolation of a clone encoding a condensing enzyme with altered substrate specificity, it is tempting to speculate that the existence of such an enzyme could contribute to the composition of medium-chain fatty acids in *C. palustris*. Obviously, it is impossible in a bimodal oil-producing species to envisage a special β-ketoacyl-ACP synthase action without the two TEs described here. Such a condensing enzyme may have superior kinetics to *Cp* FatB1, enabling it to compete with *Cp* FatB1 for extension of 10:0-ACP to 14:0-ACP. The 14:0-ACP would then be hydrolyzed to 14:0 by *Cp* FatB2, an enzyme with higher specific activity than *Cp* FatB1 and the other synthases involved in further elongation of 14:0-ACP. The fatty-acyl composition of a given seed would therefore be determined by the overall kinetics of both TEs and synthases competing for the same acyl-ACP substrates.

The pairwise comparison of *Cp FatB1* and *Cp FatB2* full-length clones with each other and with *Ch FatB1* (Jones et al., 1995) shows that these sequences are equally diverged from each other, with 75% amino acid identity. Sequences of all of the above *Cuphea* TE clones have also been compared with a *C. hookeriana* TE (*Ch FatB2*) homolog that is specific for 8:0/10:0 substrates (Dehesh et al., 1996). The *Ch FatB2* sequence is

more related to *Cp FatB1* (83% amino acid sequence identity) than to either *Ch FatB1* or *Cp FatB2* (75% identity). It appears that the sequences of TEs specific for the same acyl-ACP chain length from two different *Cuphea* species are more closely related to each other than the TEs of different substrate specificity within the same species (*Cp FatB1* versus *Cp FatB2* and *Ch FatB1* versus *Ch FatB2*). These data are consistent with the idea that the FatB genes diverged early in the evolution of *Cuphea*, most likely prior to the divergence of different species within this genus. Despite the overall divergence in sequence identity, the similarity of hydropathy profiles among all of these FatB clones suggests that the major structural features of the TE molecule within the FatB class have been conserved during evolution.

The chain lengths and composition of fatty acids determine the physical and chemical properties that ultimately dictate the utility of an oil. Among the MCTs, 8:0 and 10:0 fatty acids are important constituents of certain foods, pharmaceutical products such as dietary fats as sources of energy for patients deficient in fat resorption, and biodegradable lubricants, whereas 14:0 acid is used in detergents, cosmetics, and several other applications. The current sources of MCTs are tropical coconut and palm kernel oils. Production of these MCTs in an established oil crop such as rapeseed could provide an abundant and economical source of these compounds that may also have an impact on widening their applications.

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