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Lysophosphatidic Acid Acyltransferase from Coconut Endosperm Mediates the Insertion of Laurate at the *sn-2* Position of Triacylglycerols in Lauric Rapeseed Oil and Can Increase Total Laurate Levels

Deborah S. Knutzon, Thomas R. Hayes, Annette Wyrick, Hui Xiong, H. Maelor Davies[1], and Toni A. Voelker*

Calgene, 1920 Fifth Street, Davis, California 95616

Expression of a California bay laurel (*Umbellularia californica*) 12:0-acyl-carrier protein thioesterase, bay thioesterase (BTE), in developing seeds of oilseed rape (*Brassica napus*) led to the production of oils containing up to 50% laurate. In these BTE oils, laurate is found almost exclusively at the *sn-1* and *sn-3* positions of the triacylglycerols (T.A. Voelker, T.R. Hayes, A.C. Cranmer, H.M. Davies [1996] Plant J 9: 229–241). Coexpression of a coconut (*Cocos nucifera*) 12:0-coenzyme A-preferring lysophosphatitic acid acyltransferase (D.S. Knutzon, K.D. Lardizabal, J.S. Nelsen, J.L. Bleibaum, H.M. Davies, J.G. Metz [1995] Plant Physiol 109: 999–1006) in BTE oilseed rape seeds facilitates efficient laurate deposition at the *sn-2* position, resulting in the acccumulation of trilaurin. The introduction of the coconut protein into BTE oilseed rape lines with laurate above 50 mol % further increases total laurate levels.

In most plant seeds, a fraction of the stored carbon consists of TAGs and is commonly called the seed oil. Vegetable oils are a large renewable resource, harvested at a rate of about 60 million tons annually (Padley et al., 1994). Seed oils normally contain predominantly C18 unsaturated fatty acids; saturated fatty acids represent only a minor fraction (Hilditch and Williams, 1964). Interestingly, the saturated acyl groups are normally found only in the *sn-1* and *sn-3* positions of TAGs (Frentzen, 1998; Padley et al., 1994).

There are many angiosperm oilseed species that produce TAGs with elevated levels of saturated fatty acids. Highly saturated oils from different plants fall into two classes with respect to TAG structure. An example of the first class is cocoa butter, which contains approximately 60 mol % saturates, predominantly 16:0 and 18:0. These saturated fatty acids are not found randomly distributed between all three positions at the glycerol backbone, but reside almost exclusively in positions *sn-1* and *sn-3* of the TAGs. Such saturated-unsaturated-saturated TAGs have very specific melting characteristics that make them suitable for a variety of specialized applications (Padley et al., 1994). There is a large body of evidence that this fatty acyl distribution results from the respective specificities of the acyltransferases involved in the biosynthesis of TAGs. The transferases responsible for the esterification at *sn-1* and *sn-3*, glycerol-3-P acyltransferase and diacylglycerol acyltransferase, respectively, appear to accept saturated and unsaturated acyl-CoA substrates (Frentzen, 1998). In contrast, LPAAT, which catalyzes the esterification at *sn-2*, appears to discriminate against saturates in most oilseeds (Sun et al., 1988). Based on biochemical evidence it was proposed that this enzyme might be responsible for the production of such structured TAGs (Bafor et al., 1990; for review, see Frentzen, 1998).

In contrast to cocoa butter, the oils of the second class are from natural, medium-chain (C8–C14)-producing species, and include many palms, Lauraceae, Myristicaceae, and *Cuphea* spp. These oils contain almost exclusively saturated fatty acids (Hilditch and Williams, 1964); coconut (*Cocos nucifera*) oil, for example, has 92% saturates, predominantly laurate, and most of its TAGs are trisaturated. Laurate is found enriched at *sn-2* (Padley et al., 1994), which indicates that a laurate-CoA-preferring LPAAT is active during endosperm maturation. Davies et al. (1995) were able to detect such an enzyme from this tissue, which allowed Knutzon et al. (1995) to perform the protein purification and cloning of a cDNA encoding the 299-amino acid CLP protein from coconut. When expressed in *Escherichia coli*, and using 12:0-LPA as an acceptor, this enzyme preferred medium-chain CoAs over 18:1-CoA as acyl donors. This is direct evidence that in coconut endosperm, not only had the common fatty acid biosynthesis pathway been modified to produce almost entirely saturated medium chains, but at least one enzyme of lipid biosynthesis (LPAAT) had been modified.

Canola varieties of *B. napus* produce seed oil that contains only about 7% saturates, mostly 16:0 and 18:0. Expression of BTE, a 12:0-ACP thioesterase cDNA derived from seeds of California bay laurel (*Umbellularia californica*), in canola seeds resulted in a highly saturated canola oil (BTE canola), with saturated levels up to about 60%. Laurate alone was found to represent up to 48 mol % of the total fatty acid composition (Voelker et al., 1996). More detailed

[1] Present address: Tobacco and Health Institute, Cooper and University Drives, Lexington, KY 40546–0236.

* Corresponding author; e-mail toni.voelker@monsanto.com; fax 1–530–792–2453.

Abbreviations: ACP, acyl-carrier protein; BTE, bay thioesterase; CLP, coconut 12:0-CoA preferring lysophosphatitic acid acyltransferase; LPA, lysophosphatidic acid (1-acyl-*sn*-glycerol-3-P); LPAAT, LPA acyltransferase; TAG, triacylglycerol.

analysis of high-laurate canola oil showed that 12:0 was found almost exclusively at the *sn-1* and *sn-3* positions. For example, at 48 mol % total laurate, *sn-2* laurate was only 4% and almost no trilaurin accumulated (Voelker et al., 1996). These transgenic seeds provided in vivo confirmation of the previously accumulated biochemical evidence that in conventional oilseeds, the resident LPAAT discriminates highly against saturates at the second step of the TAG assembly, whereas the other two transferases apparently accept this novel, saturated substrate (Frentzen, 1998). High-laurate canola, therefore, falls into the class of cocoa-butter-like oils and, indeed, it can be used in applications where structured TAGs are preferred (Del Vecchio, 1996). Stearate-containing, structured TAGs accumulated after antisense suppression of the resident delta 9 desaturase (Knutzon et al., 1992; G. Thompson, personal communication) or expression of a specialized thioesterase (Hawkins and Kridl, 1998; G. Thompson, personal communication). In summary, the metabolic engineering demonstrated that the redirection of fatty acid biosynthesis to saturates results in the production of cocoa-butter-like structured TAGs.

We wanted to find the minimum number of enzyme modifications required for the reprogramming of a conventional oilseed such as canola to the production of trisaturate TAGs, which can be found in coconut oil. We therefore crossed BTE canola with transgenic canola plants expressing a CLP cDNA. Seed oil from lines harboring both transgenes (BTE/CLP plants) showed a drastic increase of laurate at *sn-2*. In addition, we present evidence that CLP can boost the overall laurate levels in the resulting BTE/CLP seed oil.

MATERIALS AND METHODS

Plants

Canola-type oilseed rape (*Brassica napus*) plants transformed with BTE, a 12:0-ACP thioesterase from California bay laurel (*Umbellularia californica*) that is driven by a napin promoter, were derived from the original transformant pCGN3828-23, as described previously (Voelker et al., 1996). This plant had 37 mol % of laurate in pools of segregating seeds, and its genome contained approximately 11 to 14 copies of the transgene on at least four to five segregating loci (not shown). Single seeds had up to 56 mol % laurate. After several generations of self-pollination and crossing to other canola varieties, homozygous lines were obtained and contained up to 58 mol % laurate. Line DH22 (full designation [A112X3828-23-198]-14-DH22-125) contained 51 mol % laurate; line DH63 (full designation LA30056-5-DH63-14-2) contained 59 mol % laurate.

Expression of CLP in Transgenic Plants

To facilitate cloning into plant expression vectors, the CLP cDNA clone pCGN5503 (Knutzon et al., 1995) was modified by PCR to insert *Sal*I and *Bam*HI restriction sites immediately upstream of the start and downstream of the termination codons, respectively. The CLP-coding region was inserted as a *Sal*I/*Bam*HI fragment into the seed-specific napin expression cassette pCGN3223 (Kridl et al., 1991), which had been digested with *Sal*I and *Bgl*II, creating pCGN5509. The *Hin*dIII fragment of pCGN5509 containing the napin 5′-regulatory region, the CLP-coding region, and the napin 3′-regulatory region was inserted into the *Hin*dIII site of the binary vector pCGN1578PASS (McBride and Summerfelt, 1990) to create pCGN5511. pCGN5511 was transferred to *Agrobacterium tumefaciens* EHA101 and used to transform *B. napus* as described by Radke et al. (1988). Two different canola varieties were used for transformation: LP004, a low-linolenic line, and Quantum, a line with a standard canola composition. Plant cultivation and sample harvesting have been described previously (Voelker et al., 1996). Dihaploid plants were generated by the procedure of Eickenberry (1994).

Enzyme Assays and Total Fatty Acyl Composition

For LPAAT assays, developing seed pools at the mid-maturation stage (approximately 30 d after pollination) were harvested. Crude (P2) membrane fractions were prepared and assayed using radiolabeled acyl-CoA substrates, as described previously (Davies et al., 1995). Total fatty acid composition was determined via quantitative GC analysis of methyl esters, as described previously (Browse et al., 1986).

sn-2 Analysis of Triglycerides

Procedures for the extraction of seed oil, for analysis of the *sn-2* composition of oil using *Rhizopus arrhizus* lipase and for reversed-phase HPLC of seed oil TAGs, were modifications of Voelker et al. (1996). For *sn-2* determinations, *R. arrhizus* lipase was diluted to approximately 600,000 units/mL and held on ice. The reaction mixture contained 2.0 mg of oil, 200 μL of 0.1 M Tris-HCl, pH 7.4, 40 μL of 2.2% (w/v) $CaCl_2 \cdot 2H_2O$, and 100 μL of 0.05% (w/v) bile salts. After 5 min of sonication to disperse the oil, 20 μL of dilute lipase was added and the mixture was vortexed continuously for 1 min at room temperature. The reaction was stopped with 100 μL of 6 M HCl. Subsequently, 500 μL of $CHCl_3$:MeOH (2:1, v/v) was added. The organic extract was removed, and the aqueous phase was re-extracted with 300 μL of $CHCl_3$. The organic extracts were combined and held on ice to minimize acyl migration before HPLC separation. The digestion products were dried down in chloroform to approximately 200 μL, and 60 μL was used for HPLC analysis.

The HPLC system was equipped with an evaporative light-scattering detector (Varex ELSD IIA, Alltech, Deerfield, IL) with the tube temperature set at 105°C and the nitrogen gas flow at 40 mL/min, an autosampler (model 712 Wisp, Waters), three solvent-delivery modules (model 114M, Beckman), a controller (model 421A, Beckman), a pneumatically actuated stream splitter (Rheodyne L.P., Rohnert Park, CA), and a microfractionator (Gilson USA, Middleton, WI). The chromatography column was a 220- × 4.6-mm, 5-μm, normal-phase silica cartridge (Brownlee Precision Co., Santa Clara, CA). The mobile phases were hexane:toluene, (1:1, v/v) (solvent A), toluene:ethyl acetate,

(3:1, v/v) (solvent B), and 5% (v/v) formic acid in ethyl acetate (solvent C). At a flow rate of 2.0 mL/min, an isocratic gradient of 10% solvent B and 2% solvent C for 2 min were applied, followed by a linear gradient of 2% to 25% solvent C over 6 min and then an isocratic gradient of 25% C for 8 min and a linear gradient of 25% to 2% solvent C over 1 min. A chromatographic standard mixture was prepared in hexane:toluene (1:1) containing the following: 0.2 mg/mL tri-16:0, 2.0 mg/mL 16:0 FFA, 0.2 mg/mL di-16:0 mixed isomers, 0.2 mg/mL 3-mono-16:0, and 0.2 mg/mL 2-mono-16:0. For each sample, the fraction containing the 2-monoacylglycerol peak was collected automatically by controlled timed events relays, the fractions were evaporated at room temperature overnight, and the fatty acyl composition was obtained as described by Browse et al. (1986).

Triglyceride Chromatography

Silver-phase HPLC resolution of TAGs was performed with the chromatograph described for the *sn-2* analysis. A lipid column (250 × 0.6 mm; Chrompack, Raritan, NJ) fitted with a cation-exchange guard column was held at 30°C. The detector's drift tube temperature and nitrogen flow were maintained at 130°C and 40 mL/min, respectively. The mobile phase was composed of 1:1 hexane: toluene (solvent A), 3:1 toluene:ethyl acetate (solvent B), and 500:0.12 toluene:formic acid (solvent C). The mobile phase gradient was programmed as isocratic at 90% solvent A and 10% solvent B for 1.5 min, a linear gradient to 100% solvent B from 1.5 to 1.75 min, isocratic at 100% solvent B for 7.5 min, a step change to 100% solvent C at 9.25 min, followed by a step change to the initial conditions at 9.99 min, and an equilibration delay of 6 min. The flow rate was 2 mL/min and the stream splitter was set at 60/40 (fraction/detector). Seeds were crushed and extracted overnight with 100 μL of toluene containing tri-11:0 as an internal standard at 0.5 mg/mL. Whole-sample extracts were filtered (0.2 μm) and injected (25 μL) onto the silver-phase column. The trisaturated TAG peak fraction including the tri-11:0 was collected and dried at room temperature overnight, and methyl esters were produced by the addition of 50 μL of toluene and 105 μL of 0.5 N NaOH, incubation at 90°C for 10 min, and addition of 500 μL of acetic acid and 60 μL of heptane. After GLC analysis, the trilaurin content was calculated.

Oil Level Determination

Seed oil levels were determined by NMR. Mature seeds (1–2 g) were dried at 130°C for 2 h and equilibrated to room temperature for 12 h before NMR analysis (model 4000, Oxford Instruments, Concord, MA) with an R_F level of 20 μA, an AF gain of 500, and a gate width of 1.0 G. The time of analysis was two times for 30 s (averaged signal/mass). Samples were run in triplicate in 2.0-mL Nessler cylinders of identical weight against 0% and 100% oil calibration controls.

PCR

To detect the CLP gene in transformants, leaf DNA was isolated (Bernatzky and Tanksley, 1986) and coconut (*Cocos nucifera*) sequences were amplified with the primers TGTGGAACATGATCATGCTGATTTTGCTCC and ATCGAGTACCCTCTGGAAAAATGATCAGCG using standard procedures.

RESULTS

Expression of CLP Alters the LPAAT Substrate Specificity Profile of Transgenic Canola Seeds

Knutzon et al. (1995) identified a coconut cDNA encoding CLP, a 299-amino acid protein with LPAAT activity. Expression of this cDNA in *Escherichia coli* conferred upon these cells a novel, laurate-preferring LPAAT activity whose substrate specificity profile matched that of the coconut enzyme. We were interested in evaluating whether this CLP enzyme would be capable of competing with the endogenous LPAAT activity in transgenic canola and facilitate the incorporation of laurate into the *sn-2* position of TAG. To obtain information on the expression of CLP in canola, the cDNA was expressed in seeds under the control of a seed-specific napin promoter. Seeds at mid-stage maturation were collected and assayed for LPAAT activity using 12:0-LPA and a variety of acyl-CoA donors. As shown in Figure 1 for one transgenic event, the activity profile of the transgenic seeds differed considerably from that of control, nontransformed seeds. 12:0-CoA was the preferred substrate in the transgenic seeds. Overall, the profile matches that of CLP expressed in *E. coli* or of a coconut endosperm extract (Davies et al., 1995; Knutzon et al., 1995) overlayed on the endogenous 18:1-CoA activity of the canola control.

Expression of CLP in Canola Results in Incorporation of Laurate into the *sn-2* Position of TAG

Since the activity of the CLP was highest using 12:0-LPA and 12:0-CoA, and since the activity of the endogenous

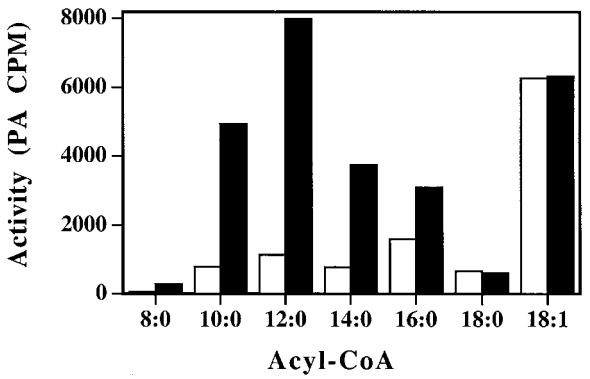

Figure 1. LPAAT substrate specificity of CLP-expressing canola seeds. A pool of mid-maturation seeds from a control plant (white bars) and from a transgenic plant, pCGN5511-LP004–5 (black bars), were assayed for LPAAT substrate specificity using 12:0-LPA and various ^{14}C-labeled acyl-CoAs. PA, Phosphatitic acid.

LPAAT was low, those substrates were used to evaluate CLP activity in maturing seeds of all of the pCGN5511-transformed *B. napus* LP004 lines. All of the transgenic lines showed increased 12:0-LPAAT activity compared with control (*x* axis of Fig. 2). However, since these transformants produce no 12:0-CoA, it was not possible to directly observe the effects of the CLP in planta on altered oil composition. To evaluate CLP in a laurate-containing background, each pCGN5511 primary transformant was crossed with DH22, a homozygous BTE line that contains 51 mol % laurate. Since the BTE parent was homozygous, each resulting F_1 seed should have a complement of BTE alleles. As predicted, all F_1 seeds from these crosses contained laurate, albeit at a lower level then the homozygous parent. Seed-to-seed laurate levels ranged from 30% to 40% (not shown). Since the CLP parents were the primary hemizygous transformants, CLP alleles were expected to segregate in the F_1 population and, depending on the genetic loci of the CLP in the parents, up to 50% of these seeds were expected not to contain any CLP allele (nulls).

Twenty F_1 seeds from each of these crosses were pooled and the *sn-2* fatty acid composition determined. These values are shown plotted against the 12:0 LPAAT activity, as previously determined with the respective primary CLP transformants (Fig. 2). In all cases, laurate levels at *sn-2* were greater than 5%, with maximum levels at 30%. Canola oil from BTE-only transformants with 30% to 40% laurate contains less than 5% laurate at the *sn-2* position (Voelker et al., 1996). Clearly, the combination of BTE and CLP increased this value, in some cases drastically, demonstrating the efficient competition of CLP with the canola LPAAT in planta. The plot indicates only a weak correlation between 12:0-LPAAT activity of the CLP parent and the resulting invasion of 12:0 into *sn-2* in F_1 seeds. We attribute this imperfect correlation to several factors. The LPAAT assay is only semiquantitative, and a variability of up to 2-fold has been observed when repeatedly assaying developing seeds from the same transformants. This was compounded by the fact that the respective maturing seed pools were segregating for the respective LPAAT alleles (homozygous, heterozygous, and null seeds), and we could not ensure that we always had a representative sample.

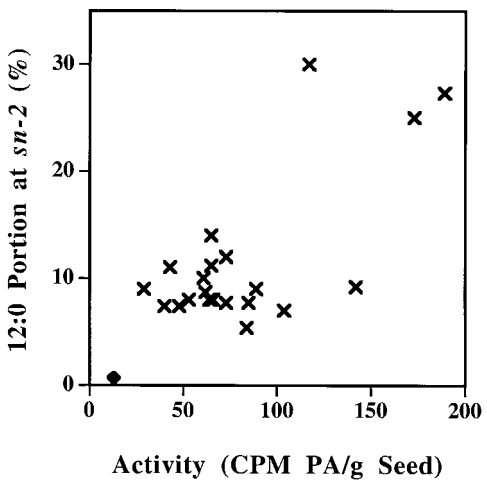

Figure 2. Correlation of 12:0-LPAAT activity with 12:0 accumulation at *sn-2*. LPAAT activity using 12:0-CoA and 12:0-LPA was determined in membrane fractions derived from developing untransformed, control canola seeds, as well as from independent CLP LP004 transformants. In addition, the proportion of 12:0 at *sn-2* was measured in 20-seed pools of mature F_1 seeds derived from crosses of the same set of CLP plants with a homozygous BTE-containing line, DH22. The figure correlates these two determinations. ♦, Control; ×, individual CLP × BTE F_1 seed lots. PA, Phosphatitic acid.

Coconut LPAAT Induces the Accumulation of Trilaurin

The large amounts of laurate produced by the BTE in developing canola seeds are deposited almost exclusively in the *sn1* and *sn3* positions of TAGs. Even at 47 mol % laurate, trilaurin represents only 2.68 weight % of all TAGs (Voelker et al., 1996). We wanted to study whether the laurate invasion into *sn-2* catalyzed by CLP boosted the trilaurin fraction.

In a first experiment, F_1 plants from crosses of several LPAAT transformants with the 50 mol % laurate-containing BTE homozygous line DH22 were grown and CLP-homozygous F_2 lines selected in the next generation. Since the laurate trait was carried by several loci in the BTE parent, we observed a wide range of laurate levels in F_2 plants. Seeds from canola lines harboring the BTE alone served as controls. Extracted oil was analyzed by reversed-phase HPLC. Under these conditions, TAGs with an equal total number of unsaturations in their fatty acyl moieties migrated with almost the same mobility, which was nearly independent of the total carbon number (Hammond, 1993; Voelker et al., 1996).

In Figure 3 the overall laurate proportion of the respective oil samples is plotted against their trilaurin fraction. As reported earlier, a BTE transformant line with 47 mol % of laurate accumulated only a few percent of TAGs as trilaurin (Voelker et al., 1996). This was not unexpected, since the canola LPAAT does not accept 12:0-CoA (Bafor et al., 1990). However, when CLP is present, the trilaurin fraction at a given laurate level is drastically higher. Even at 30% of total laurate, more than 5% of the TAGs are trilaurin. The trilaurin fraction increased to up to 15% when total laurate was at 50%. In summary, at all laurate levels measured, the trilaurin fraction was significantly higher with the CLP present than what is found in lines harboring BTE alone; it was even higher than calculated values that assume random distribution for laurate at all three glycerol positions. This result demonstrated that CLP is active in vivo and that during TAG biosynthesis it facilitates the entry of a significant portion of laurate into position 2. Since the seeds look normal and germinate, it also demonstrates that the invasion of laurate into the *sn-2* position of glycerolipids is not harmful to the canola seeds.

CLP Can Channel Laurate Preferentially to *sn-2*

Since we had previously found that measuring LPAAT activity in maturing seeds of primary transformants was a poor predictor of induced phenotype (Fig. 2), we initiated a second transformation (this time in a conventional canola

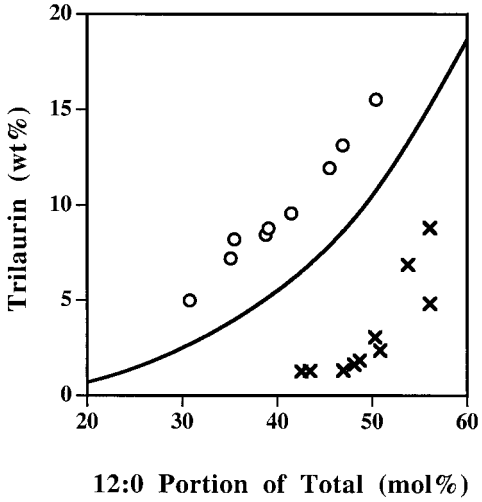

Figure 3. Relationship between total laurate content and trilaurin. Seed oil was extracted from transformed canola plants and the total laurate content of the oil and proportion of trilaurin compared with other TAGs in the oil were determined. Each symbol reflects a sample derived from a seed pool from one dihaploid plant as described. ×, Canola lines transformed with BTE alone; ○, seeds from F_2 plants resulting from crosses of several different Q04 CLP transformants with the homozygous BTE plant DH22. The line was calculated by assuming that laurate was positioned randomly at all three positions of the triglyceride.

variety with higher agronomic yield than QO4) and screened the resulting CLP transformants by measuring the in vivo impact on triglyceride assembly. To achieve this goal, all primary CLP transformants were crossed to a homozygous BTE plant. Assuming Mendelian segregation, we could predict that all resulting F_1 seeds had an identical BTE gene dosage, namely one copy of each BTE parent locus. Because of the nature of the plant transformation, all CLP parents were hemizygous for CLP, and therefore the CLP transgene should segregate in the cross according to locus number. In the resulting F_1 seeds, the impact of the different CLP transgenic alleles on laurate deposition at *sn-2* was measured. Since the direct determination of laurate at *sn-2* in single cotyledons was not feasible, we choose to measure trilaurin levels. F_1 seeds with highest levels of trilaurin were considered to harbor the highest CLP-expression alleles and were selected for the establishment of the BTE/CLP lines.

For this experiment, the canola var Quantum was transformed with pCGN5511, and 20 independent transformants were generated. Each original transformant was crossed with the homozygous BTE line DH63, which featured 59 mol % laurate in the seeds. In this BTE line the laurate phenotype was caused by three or four genetic loci (not shown). After seed maturation, one cotyledon each of several F_1 seeds from each cross was analyzed for trilaurin and the total laurate fraction. Total laurate levels were between 50 mol % and 60 mol %, and the trilaurin fraction ranged from very low to up to 25% (not shown). For further study, we selected F_1 lines that harbored the coconut LPAAT at one genetic locus and had induced the highest trilaurin levels in single F_1 seeds.

Since in such lines BTE and CLP together were expected to segregate in four to five genetic loci, breeding to complete homozygosity would have required a multigeneration breeding program. For a more efficient strategy, microspores were grown from the pollen of F_1 plants, and approximately 100 dihaploid plants were generated (Eickenberry, 1994). Since genetic segregation during the generation of dihaploid plants is at the haploid level, the probability for any given combination of four loci is 1 in 32. We therefore calculated that 100 independent dihaploids should contain all possible combinations of these four loci with a high probability. Because of the nature of dihaploid plants, they are exclusively in the homozygous state.

In the resulting population of 100 dihaploid plants, the CLP transgene was detected via PCR of leaf tissue extracts. In addition, the total fatty acid composition was determined in mature seed pools. The laurate fraction ranged from 0 mol % to 67 mol %. We also analyzed the proportion of laurate at *sn-2* of the seed oil TAGs in a subset of dihaploids. In Figure 4 the total laurate levels for each selected line is plotted against the laurate at *sn-2* for both populations. The graph demonstrates that in BTE-only plants with less than 45 mol % total laurate, very little laurate accumulates at *sn-2*, as was observed for the two lines in Voelker et al. (1996). However, at laurate levels above 45 mol %, *sn-2* laurate unexpectedly rose rapidly to significant levels. When CLP was present, however, laurate *sn-2* infusion was much higher at any given total laurate

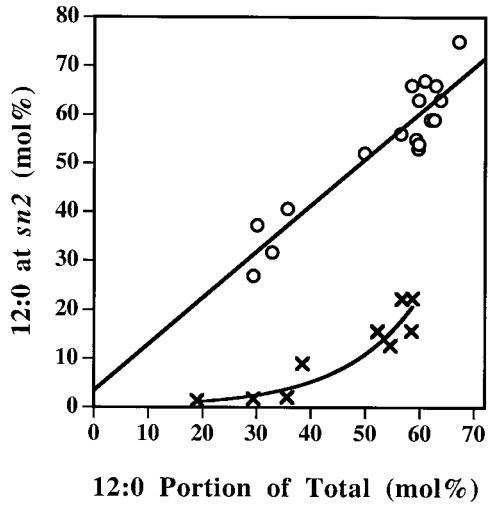

Figure 4. Laurate proportion at *sn-2* is dependent on total laurate levels and coconut LPAAT. The primary CLP transformants (pCGN5511 in var Quantum) were crossed with the homozygous BTE line DH63. A F_1 plant harboring CLP and BTE alleles was grown. Independently segregating F_2 microspores derived from this plant were made diploid and grown into (homozygous) dihaploid plants as described. The presence or absence of the coconut LPAAT gene in the individual dihaploid plants was determined via PCR of leaf tissue. All plants were selfed, and oil was extracted from the resulting seeds. The *sn-2* analysis of seed oil was executed using *R. arrhizus* lipase. Each symbol represents a seed pool derived from one dihaploid plant. ○, CLP-positive plant; ×, CLP-negative plant. For this analysis, we selected the top 12 laurate producers of the generated dihaploids, as well as randomly chosen plants throughout the laurate range.

level. In the very highest laurate plants, laurate makes up to 75% of the acyl groups in this position, indicating that in this plant *sn-2* is the preferred position for this saturated fatty acid.

CLP Can Boost Laurate Levels in Seeds

There are several lines of evidence indicating that in developing seeds of BTE canola that accumulate more than 45% laurate, a fraction of newly synthesized laurate might enter a futile cycle during seed development (Voelker et al., 1996; Eccleston and Ohlrogge, 1998). It was reasoned that in such seeds, since the *sn-2* position in the TAG could not be used efficiently and the *sn-1* and *sn-3* positions were nearly filled with laurate, a fraction of the laurate output of the plastid could not be utilized and was subsequently degraded. The introduction of the CLP was therefore predicted to not only allow access of laurate to position *sn-2*, but also to increase total laurate levels when expressed in very high laurate lines.

To test this hypothesis, we determined the absence or presence of the coconut LPAAT gene in all 100 dihaploid plants used in this study. As predicted by the underlying genetics, the dihaploid population was split equally into CLP positives and CLP negatives. In Figure 5, the data are sorted by total laurate levels and by the absence or presence of CLP. Plants without CLP have oil with up to 59 mol % laurate, and the BTE parent used for the cross had 59 mol % of laurate; therefore, it was expected that a dihaploid (homozygous) plant with all of the BTE loci reassembled after the cross should also have about the same laurate levels. However, plants with the coconut LPAAT gene had up to 67 mol % laurate. Altogether, we obtained about 12 BTE/LPAAT plants that had more laurate than any of their BTE-only siblings (up to almost 10% more). It is also interesting to note that a disproportionate number of BTE plants had laurate levels between 56 mol % and 58 mol % laurate. Detailed genomic analysis revealed no trend of higher BTE gene dosage in CLP-positive versus CLP-negative plants (not shown).

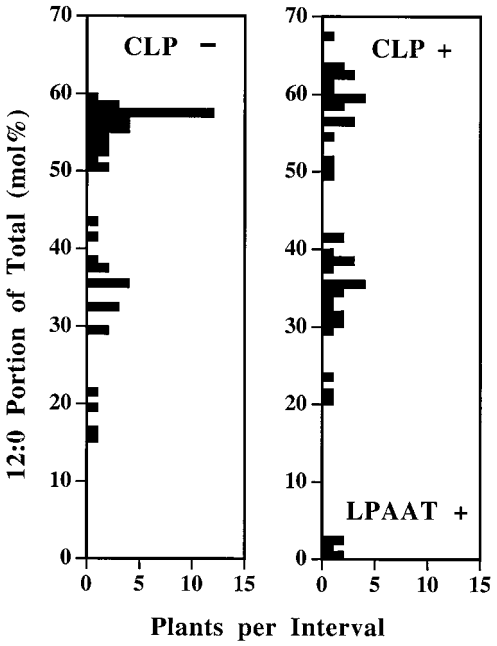

Figure 5. Coconut LPAAT can boost laurate levels. Each symbol represents a seed-pool analysis of an individual dihaploid plant. All dihaploid plants resulting from crosses described in Figure 4 were sorted via PCR into a CLP-containing (CLP +) and a CLP-free (CLP −) populations. Plants of both populations were grouped into 1% laurate intervals.

We conclude that the presence of CLP allows a more efficient laurate TAG deposition in very high BTE-expressing plants. We also wanted to determine whether the presence of CLP leads to more total lipid per seed. Therefore, we determined the total oil as a percentage of dry weight in mature seeds of all dihaploid plants. In Figure 6, these data are shown correlated with laurate levels. It is obvious that there was wide plant-to-plant variability, which is common for greenhouse-grown plants, but for both subgroups the distribution is similar. The average oil percentage for plants with laurate levels less than 40 mol % laurate was 34% to 35%, but the average of plants with more than 50 mol % laurate was only 30% to 32%.

DISCUSSION

We used CLP, a LPAAT from coconut endosperm, to increase the proportion of trilaurin in laurate-producing canola plants previously transformed with a 12:0-ACP thioesterase. Our results demonstrate that fatty acid biosynthesis and lipid biosynthesis can be redirected simultaneously by introduced enzymes from different species. In BTE-transformed canola lines, laurate accumulated predominantly at the *sn-1* and *sn-3* positions and was underrepresented at the *sn-2* position of the seed triglycerides. Only at total laurate levels above 50% did the proportion at *sn-2* begin to increase (Fig. 4). In such plants, with *sn-1* and *sn3* positions nearly saturated with laurate, cytoplasmic 12:0-CoA levels might have risen to sufficiently high levels that the 12:0-CoA could compete with the long-chain CoA substrates for the canola LPAAT. As shown in Figure 1, in developing canola seeds, LPAAT activity on 12:0 substrates is low relative to long-chain CoAs, but the discrimination is not complete (Sun et al., 1988). This low activity could explain the observed trend.

In the presence of CLP, however, laurate is accepted at the *sn-2* position, and for the ranges tested the correlation between total laurate levels and the *sn-2* laurate proportions was essentially linear (Fig. 4). This was also reflected in the drastic increase of trilaurin in the resulting oil (Fig. 3). In BTE/CLP dihaploids with 60% laurate, we found oils with up to 40% of trilaurin, a value higher than expected if random assembly occurs at all positions (21.6%). The induced CLP can effectively compete with the endogenous LPAAT, providing in vivo proof that a LPAAT specialized for saturated substrates can drastically alter the stereochemical composition of triglycerides during biosynthesis. This effect was demonstrated previously with a different LPAAT. Lassner et al. (1995) isolated a LPAAT cDNA from

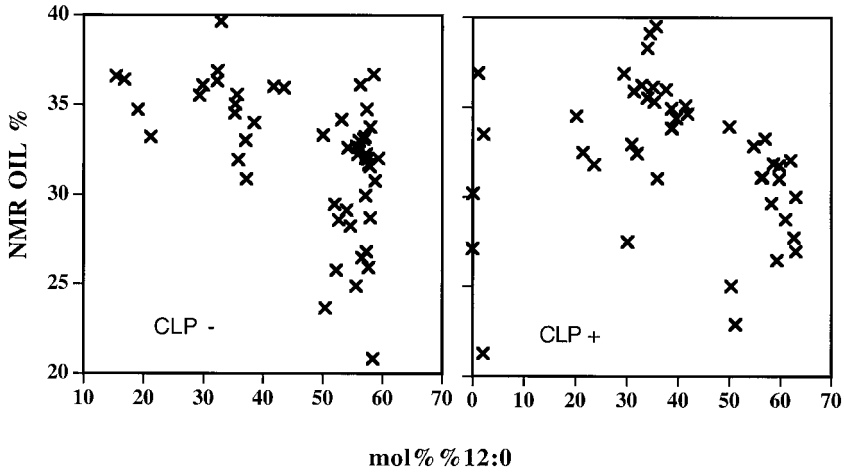

Figure 6. Correlation of oil levels with BTE and CLP. The total seed oil mass as a percentage of dry weight of the dihaploid plants described in Figure 4 were determined by NMR. The data are shown separately for CLP-free (CLP−) and CLP-containing (CLP+) populations, with the oil percentage plotted against total laurate levels.

meadowfoam seeds, a plant that accumulates seed oil rich in triglycerides with very-long-chain (≥20 carbons) monounsaturates in *sn-2*. Subsequently, this cDNA was expressed the seeds of a high erucic acid (22:1) canola variety containing approximately 40% of 22:1, almost all of which is excluded from *sn-2* (Sun et al., 1988). Meadowfoam LPAAT transformants contain up to 22% of this fatty acid at *sn*-2, demonstrating that the transgenic, specialized LPAAT from meadowfoam competed efficiently with the endogenous enzyme for substrates. We conclude from these results and from the results of the present study that a specialized LPAAT is probably present when unusual fatty acids are found at *sn-2* in a plant oil.

As reported previously (Voelker et al., 1996), the correlation between BTE enzyme activity during seed maturation and laurate levels in mature seeds is approximately linear between 0 and 35 mol % laurate. Above 35%, however, the linear relationship is lost even though thioesterase activity can still be increased. It was reasoned therefore that in seeds in which *sn-1* and *sn-3* are almost completely filled with laurate, not all of the 12:0-CoA could be deposited in triglycerides and would therefore be recycled by the cells. This hypothesis was examined in detail using BTE canola transformants with 60 mol % laurate in seed lipids (Eccleston and Ohlrogge, 1998). When developing embryos from these BTE plants were fed radioactive acetate, only 50% of the tracer accumulated as lipids; the remainder accumulated predominantly as Suc and malate. In control embryos more than 90% of the radioactivity was channeled to lipids. In addition, it was found that in developing BTE-canola embryos, fatty-acid-biosynthesis enzymes were significantly elevated and enzymes of β-oxidation were drastically induced. Altogether, this study provides evidence that in these developing seeds a coordinated induction of the fatty acid synthesis pathway occurred, presumably to compensate for the lauric acid lost through β-oxidation.

We reasoned that, since the introduction of CLP in 60% laurate BTE lines leads to the efficient use of the *sn-2* position, much higher laurate levels might be attained than with BTE alone. Indeed, when we sorted the dihaploid lines resulting from a BTE/CLP cross with respect to the absence or presence of CLP (Fig. 5), we observed a clustering around 57 ± 2 mol % for plants without CLP; no plant had more than 59 mol % laurate. In contrast, 12 CLP-containing plants had more than 59 mol % laurate, with the highest one containing 67 mol %. We conclude that the presence of CLP allowed a more efficient deposition of laurate into TAG in very high BTE-expressing cells.

In summary, the addition of CLP to 60 mol % laurate BTE canola leads to an approximately 5% laurate increase of the mature seed lipid composition, indicating that in such BTE-only seeds as much as 10% of the de novo laurate might be recycled, probably by β-oxidation. This is in contrast to the embryo-feeding data, where in developing embryos 50% of acetate label was found to be incorporated into nonlipids (Eccleston and Ohlrogge, 1998), indicating a much higher rate of β-oxidation. We cannot explain this discrepancy, but it could be that the predominant source of recycled laurate during development might not be TAGs, but laurate-containing phospholipids. Indeed, during the phase of active laurate deposition, laurate is found in all phospholipid classes. For example, developing embryos of BTE laurate canola, which had 48 mol % laurate in TAGs, had about 30 mol % laurate in PC, but after cessation of fatty acid production, the laurate proportion in PC was reduced to 5% (Wiberg et al., 1997). The addition of CLP to such plants did not lead to a reduced infusion of laurate into phospholipids, as might have been expected (E. Wiberg and S. Stymne, personal communication). It was also observed (Lassner et al., 1995) that the expression of a 22:1-preferring LPAAT from meadowfoam in transgenic canola, although it had a drastic effect on *sn-2* levels of 22:1, did not lead to the elevation of 22:1 in the total oil composition. We interpret this as evidence that in this canola variety, LPAAT is not a limiting factor for total 22:1 deposition.

Crossing a BTE canola line with CLP expressors transformed the cocoa-butter type high-laurate canola to a predominantly trisaturate oil. This type of oil resembles seed oil deposited by natural medium-chain producers such as coconut or *Cuphea* species, and it provides further evidence that in the evolution of such oil plants only a very few key enzymes needed to be modified to achieve such drastically

different oil compositions and triglyceride species. Certain natural medium chain producers accumulate just one triglyceride. For example, *Actinodaphne hookeri* seeds contain 95% trilaurin, nutmeg oil contains more than 80% tri-14:0, and it has been suggested that "pure" triglycerides produced in commercial crops could become important feed stocks for a wide range of industries (Shukla and Blicher-Mathiesen, 1993). This study demonstrates a next step toward the development of such crops by genetic engineering.

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