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Lysophosphatidic Acid Acyltransferase from Meadowfoam Mediates Insertion of Erucic Acid at the *sn*-2 Position of Triacylglycerol in Transgenic Rapeseed Oil

Michael W. Lassner*, Charlene K. Levering, H. Maelor Davies, and Deborah S. Knutzon

Calgene Inc., 1920 Fifth Street, Davis, California 95616

Lysophosphatidic acid acyltransferase acylates the *sn*-2 hydroxyl group of lysophosphatidic acid to form phosphatidic acid, a precursor to triacylglycerol. A cDNA encoding lysophosphatidic acid acyltransferase was isolated from developing seeds of meadowfoam (*Limnanthes alba alba*). The cDNA encodes a 281-amino acid protein with a molecular mass of 32 kD. The cDNA was expressed in developing seeds of transgenic high-erucic-acid rapeseed (*Brassica napus*) using a napin expression cassette. Erucic acid was present at the *sn*-2 position of triacylglycerols from transgenic plants but was absent from that position of seed oil extracted from control plants. Trierucin was present in the transgenic oil. Alteration of the *sn*-2 erucic acid composition did not affect the total erucic acid content. These experiments demonstrate the feasibility of using acyltransferases to alter the stereochemical composition of transgenic seed oils and also represent a necessary step toward increasing the erucic acid content of rapeseed oil.

Rapeseed (*Brassica napus*) and meadowfoam (*Limnanthes* sp.) accumulate fatty acids longer than 18 carbons (VLCFAs) in their seed TAGs. Rapeseed amasses 22:1 and 20:1 to make up to 65% of its oil composition (Stefansson, 1983). VLCFAs are found in the *sn*-1 and *sn*-3 positions of the TAGs but are excluded from the *sn*-2 position (Mattson and Volpenhein, 1961; Norton and Harris, 1983). This *sn*-2 exclusion limits the 22:1 content of rapeseed oil and prevents the synthesis of trierucin. In contrast, most meadowfoam seed oils are composed of more than 90% VLCFAs (Miller et al., 1964). The *sn*-2 position of at least one species, *Limnanthes douglasii*, has a 22:1 content that is enriched relative to total oil composition (Phillips et al., 1971).

Seed storage TAGs are synthesized from acyl-CoA and glycerol-3-phosphate by microsomal membrane proteins of the Kennedy pathway (reviewed by Stymne and Stobart, 1987; Browse and Somerville, 1991; Frentzen, 1993). In this pathway, three different enzymes catalyze successive acylation of the *sn*-1, *sn*-2, and *sn*-3 positions of glycerol moieties. The substrate specificities of these acyltransferases in conjunction with the acyl-CoA pool sizes are thought to determine the fatty acyl compositions of seed oils. LPAAT (EC 2.3.1.51; lysophosphatidic acid acyltransferase), the second enzyme of the pathway, acylates the *sn*-2 hydroxyl group of lysophosphatidic acid to form phosphatidic acid.

*Corresponding author; e-mail lassner@calgene.com; fax 1–916–753–1510.

In vitro assays of extracts of developing rapeseed embryos showed that LPAAT does not incorporate erucic acid (22:1) into the *sn*-2 position of lysophosphatidic acid (Sun et al., 1988; Bernerth and Frentzen, 1990; Cao et al., 1990). These experiments suggested that the substrate specificity of this enzyme is responsible for the exclusion of 22:1 from the *sn*-2 position of TAGs. In contrast, LPAAT present in meadowfoam seed extracts efficiently incorporated 22:1 into the *sn*-2 position of lysophosphatidic acid (Cao et al., 1990; Laurent and Huang, 1992; Löhden and Frentzen, 1992). Based on these results, it has been suggested that the expression of the meadowfoam LPAAT in transgenic rapeseed could alter the stereochemical composition of rapeseed oil such that 22:1 would be incorporated into the *sn*-2 position of TAGs (Sun et al., 1988; Bernerth and Frentzen, 1990; Cao et al., 1990; Laurent and Huang, 1992; Löhden and Frentzen, 1992).

Recently, Davies et al. (1995) reported the solubilization of LPAAT from coconut endosperm. This led to the partial purification and cloning of a cDNA that encodes an LPAAT that utilizes lauryl-CoA as the acyl donor substrate (Knutzon et al., 1995). Hanke et al. (1995) isolated a cDNA from meadowfoam that complements an *Escherichia coli* strain deficient in LPAAT activity (Coleman, 1990). The LPAAT activity of *E. coli* expressing the meadowfoam cDNA mirrored the substrate specificity of meadowfoam seed extracts.

In this paper, we report the isolation of a cDNA from meadowfoam that encodes a seed-expressed LPAAT. We also describe the modification of transgenic rapeseed oil resulting from the expression of the meadowfoam LPAAT in maturing rapeseed embryos.

MATERIALS AND METHODS

Plant Materials

Limnanthes alba alba seeds were obtained from the Western Regional Plant Introduction Station (Pullman, WA). Developing seeds used in this study were harvested 13 to 19 d after pollination and frozen at −70°C. High-22:1 rapeseed (*Brassica napus* cv reston) was used for transformation studies.

Abbreviations: LPAAT, acyl-CoA:1-acyl-*sn*-glycerol-3-phosphate acyltransferase; TAG, triacylglycerol; VLCFA, very-long-chain fatty acid; 20:1, eicosenoic acid; 22:1, erucic acid.

cDNA Isolation and Analysis

RNA was isolated from developing seeds of meadowfoam as described by Jones et al. (1995) and was further purified on an RNeasy column (Qiagen Inc., Chatsworth, CA). Total RNA (2.5 μg) was used in 20-μL first-strand cDNA reactions using Superscript reverse transcriptase (GIBCO-BRL) according to the manufacturer's protocol; the oligonucleotide CAUCAUCAUCAUAAGCTTCTGCAGGAGCTCTTTTTTTTTTTTTTTT (5'RACESYN) was used as a primer. After first-strand cDNA synthesis, the reaction volume was increased to 40 μL by the addition of 20 μL of water, and unincorporated nucleotides and small cDNA synthesis products were removed by chromatography on MicroSpin S-400 columns (Pharmacia). A 3' rapid amplification of cDNA ends PCR (Frohman et al., 1988) was performed in 50-μL reactions containing 1 μL of the purified first-strand cDNA, the primers CAUCAUCAUCAUGAATTCAAGCTTYCCNGARGGNACNMG and 5'RACESYN, and other standard reaction components as specified by the manufacturer (Perkin-Elmer). The PCR reactions were carried out in a Perkin-Elmer PCR thermal cycler (model 9600). The reactions were heated to 96°C for 5 min and then reduced to 72°C for 5 min (during which time the *Taq* polymerase was added). The reaction temperature was reduced to 50°C over a period of 10 min and raised to 72°C for 5 min. This was followed by 35 cycles of 94°C for 15 s, rapid reduction of the temperature to 65°C, slow reduction of the temperature to 42°C with a 3-min ramp time, and 72°C for 60 s. The PCR products were digested with *Eco*RI and *Pst*I and cloned into *Eco*RI- and *Pst*I-digested pBS− (Stratagene).

The partial cDNA isolated by PCR was used as a probe to screen a cDNA library to isolate a clone encoding the entire protein. Library construction and screening were as described by Knutzon et al. (1995). DNA was sequenced using an ABI 373A automated sequencer (Perkin-Elmer). Protein sequences were aligned using MEGALIGN software (DNASTAR Inc., Madison, WI). Hydropathy analysis and potential transmembrane domain analysis were performed using TOPPREDII software (Claros and von Heijne, 1994).

Expression of Meadowfoam LPAAT in Transgenic Rapeseed

The coding region of the meadowfoam LPAAT cDNA clone was PCR amplified using the primers CAUCAUCAUCAUGTCGACAATGGCCAAAACTAGAACTAGCT and CUACUACUACUAGTCGACGGATCCTCACTTTGAGCGATTTGTGCT. The primers introduced *Sal*I and *Bam*HI cloning sites to the 5' and 3' ends, respectively, of the open reading frame of the meadowfoam LPAAT cDNA. The PCR product was cloned into pAMP1 (GIBCO-BRL). The clone was sequenced to ensure that no mutations were introduced by PCR, and the *Sal*I-*Bam*HI fragment was cloned into the napin cassette of pCGN3223 (Kridl et al., 1991) digested with *Sal*I and *Bgl*II. The *Hin*dIII fragment containing the napin/meadowfoam LPAAT gene fusion was cloned into the binary plant transformation vector pCGN1559PASS (McBride and Summerfelt, 1990) to yield pCGN7695. pCGN7695 was transferred to *Agrobacterium tumefaciens* EHA101 and used to transform the rapeseed cv Reston as described by Radke et al. (1987).

Lipid Analysis

Oil was extracted from mature seeds by grinding seeds in 10 mL/g hexane. The solvent was removed by evaporation under N_2. For pooled seed analysis, a minimum of 0.2 g of seeds (approximately 40 seeds) was used. *Sn*-2 analysis was modified from the protocol of Luddy et al. (1964). The oil was digested with *Rhizopus arrhizus* lipase (Sigma) for 1.5 min. (with vigorous mixing) at room temperature using 0.28 mL reaction buffer/μL oil. The reaction buffer was 0.071 M Tris-HCl, 0.16% $CaCl_2$, 0.009% bile salts, 25,000 units lipase/mL, pH 7.0. The reaction was stopped by the addition of 6 M HCl (0.05 mL/μL oil), and the organic phase was extracted with chloroform:methanol (2:1, v/v). After concentration by evaporation under N_2, the *sn*-2

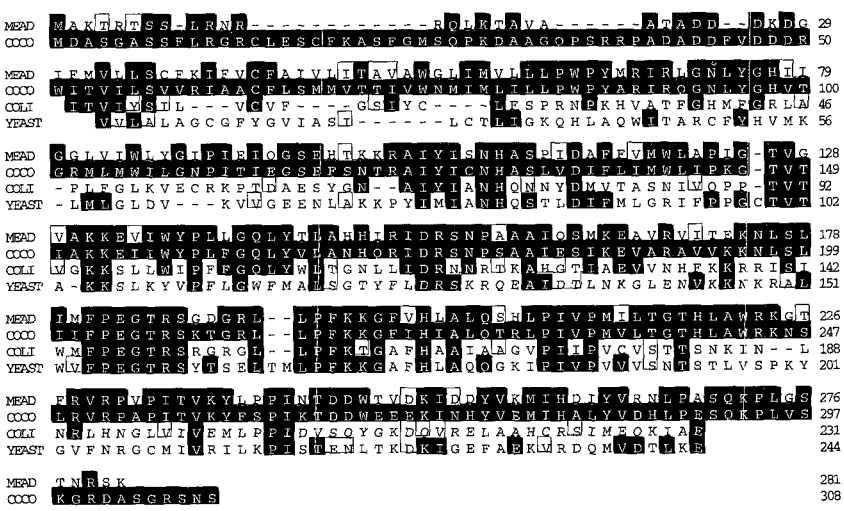

Figure 1. Predicted amino acid sequence of the meadowfoam LPAAT (MEAD) aligned with the coconut LPAAT (COCO, Knutzon et al., 1995), portions of the *E. coli* LPAAT (COLI; Coleman, 1992), and yeast *sn*-2 acylglyceride fatty acyltransferase (YEAST; Nagiec et al., 1993). Amino acids identical with those in coconut LPAAT are surrounded by black boxes, and conservative amino acid changes are surrounded by clear boxes.

monoacylglycerols were purified by TLC on silica G plates developed in diethyl ether:acetic acid (100:1, v/v). The monoacylglycerols were visualized with rhodamine 6G and subjected to acidic methanolysis, and their fatty acyl composition was determined by GLC (Browse et al., 1986).

The HPLC resolution of TAGs by argentation chromatography was conducted on a 250- × 4.6-mm ChromSphere Lipids column (Chrompack, Raritan, NJ). Solvent A was hexane:toluene (1:1, v/v), solvent B was toluene:ethyl acetate (3:1, v/v), and solvent C was toluene:99% formic acid (500 mL:40 μL). The column was eluted at a flow rate of 1.5 mL/min using the following program: 0 to 3 min, 98% solvent A/2% solvent B; 3 to 6 min, ramp to 10% solvent B; 6 to 12 min, ramp to 50% solvent B; at 16 min, change to 100% solvent C; and at 21 min, change to 100% solvent A. The eluted TAGs were detected using a Varex evaporative light-scanning detector (Alltech, Deerfield, IL).

RESULTS

Alignment of the coconut (Knutzon et al., 1995) and *E. coli* (Coleman, 1992) LPAAT sequences with a yeast *sn*-2 acylglyceride fatty acyltransferase (Nagiec et al., 1993) revealed several conserved peptides (Fig. 1). An oligonucleotide encoding the peptide FPEGTR was used for 3′ rapid amplification of cDNA ends (Frohman et al., 1988) to isolate a seed-expressed meadowfoam cDNA clone. The cloned PCR product was used as a probe in screening a library to isolate cDNAs encoding the entire protein. The largest cDNA isolated was 1108 nucleotides. It encoded a 281-amino acid protein with a molecular mass of 32 kD. Hydropathy analysis revealed several potential membrane-spanning domains, which would be expected for an integral membrane protein (Fig. 2).

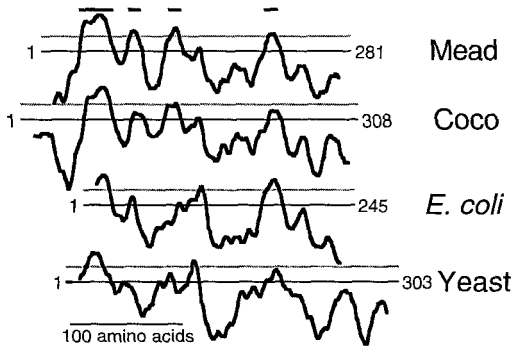

Figure 2. Hydropathy analysis of LPAAT. Hydropathy profiles of LPAAT from meadowfoam (Mead), coconut (Coco), and *E. coli* and the yeast *sn*-2 (Yeast) acylglyceride fatty acyltransferase were determined with TOPPREDII software (Claros and von Heijne, 1994) using the Goldman, Engelman, Steitz scale. Numbers indicate amino acid positions in the protein sequences. The solid line through each profile indicates hydropathy scores of 0, and the dotted line through each profile indicates hydropathy scores of 1. The bars above the meadowfoam profile indicate "likely" transmembrane domains. Regions where proteins have scores higher than 1 are "likely" transmembrane domains, and regions with scores above 0.5 are "possible" transmembrane domains.

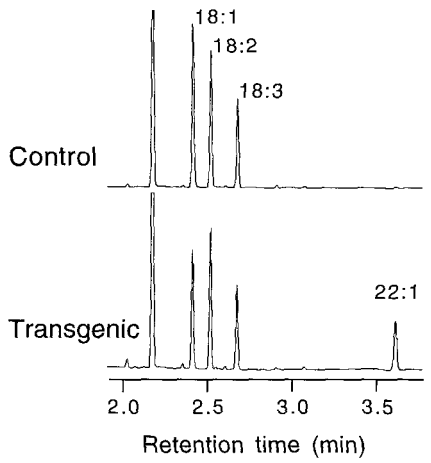

Figure 3. Analysis of the *sn*-2 fatty acyl composition in control and transgenic rapeseed oil by GLC of methyl esters. In the control plant (reston), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids predominate in the *sn*-2 position. The transgenic plant (7695–6) oil contains significant quantities of 22:1 in addition to the C_{18} fatty acids. The unlabeled peak present in both samples (retention time of 2.2 min) is a 17:0 internal standard.

To verify its identity, the meadowfoam cDNA was expressed in maturing embryos of transgenic rapeseed under the control of a napin expression cassette (Kridl et al., 1991). Pooled seed samples were analyzed to determine the *sn*-2 composition of their oils. Oil from control plants contained predominantly 18:1, 18:2, and 18:3 at the *sn*-2 position, with 22:1 composing less than 0.5 mol% of the *sn*-2 fatty acids. Oil from all four of the transgenic plants analyzed contained significant quantities of 22:1 in that position (Fig. 3; Table I). There was no significant difference in the total quantity of 22:1 in the transgenic and control seed oils (Table I). These experiments demonstrate that the meadowfoam cDNA encodes an LPAAT capable of incorporating 22:1 into the *sn*-2 position during TAG synthesis.

Silver-phase HPLC analysis of intact TAGs showed that the control plant did not contain detectable trierucin, whereas the transgenic plants contained significant quantities (Fig. 4, trienoic C_{69}). The control seeds contained a small quantity of trienoic C_{67} TAGs, presumably containing 24:1/18:1/22:1 and 22:1/18:1/24:1. The trienoic C_{67} TAG content of the transgenic oils was significantly increased because incorporation of 22:1 in the *sn*-2 position enabled the synthesis of 22:1/22:1/20:1 and 20:1/22:1/22:1. The increase of trienoic C_{67} in the transgenic oil reflected the abundance of 20:1 (a constituent of the transgenic oil C_{67} TAG) relative to 24:1 (a constituent of the control oil C_{67} TAG) in rapeseed oil. Reston oil is composed of approximately 10 mol% 20:1 and less than 1 mol% 24:1. GLC analysis of intact TAGs confirmed the results obtained by silver-phase HPLC analysis (data not shown).

Since the pooled seeds used in the previous analyses were segregating for the presence of the meadowfoam LPAAT transgene, single seeds were analyzed to determine the effect of different gene dosages. Figure 5 shows that the *sn*-2 composition of TAGs in 20 individual transgenic seeds

Table I. *sn-2 Fatty acyl compositions of control and transgenic seed oils (mol%)*

Pooled seed samples from four transgenic (7695) and two control (reston) plants were analyzed.

Sample	Acyl Group																			
	sn-2 fatty acids										Total seed oil fatty acids									
	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:0	22:1
Reston	1.1	0.4	40.2	34.1	23.0	0.0	0.8	0.0	0.0	0.3	3.6	1.3	19.7	12.6	8.7	0.7	9.8	0.5	1.0	39.0
Reston	2.9	1.5	41.7	32.6	20.1	0.0	1.2	0.0	0.0	0.0	3.3	1.2	23.3	13.9	9.4	0.0	11.2	0.0	0.0	37.7
7695–1	1.0	0.7	28.4	33.6	20.4	0.0	0.4	0.0	0.0	15.1	3.7	1.3	16.5	13.6	9.0	0.6	9.8	0.7	1.3	37.5
7695–2	2.9	2.2	30.9	41.5	17.5	0.0	0.0	0.0	0.0	4.1	6.4	2.6	14.9	12.6	6.1	1.1	9.0	1.8	1.0	41.0
7695–3	0.7	0.3	32.8	40.6	19.1	0.2	0.7	0.0	0.0	5.4	3.6	1.2	18.3	13.6	8.3	0.7	10.3	0.6	0.7	40.0
7695–6	2.1	0.9	28.3	33.5	20.8	0.0	0.8	0.0	0.0	13.3	3.5	1.4	17.5	13.5	9.0	0.7	9.9	0.7	1.5	38.0

ranged from 12.4 to 22.3 mol% 22:1, whereas the 10 control seeds contained less than 0.5% 22:1 at the *sn*-2 position. Incorporating 22:1 in the *sn*-2 position of the seed oil did not markedly affect the total 22:1 content (Fig. 5). In control rapeseed oil, the total 22:1 content was approximately 40%. Since 22:1 is excluded from the *sn*-2 position of the TAGs, the average *sn*-1 and *sn*-3 compositions were approximately 60% 22:1. In transgenic seed oils containing more than 20% 22:1 at the *sn*-2 position of triglycerides 22:1 content ranged from 35.5 to 37.9%. Thus, the average 22:1 compositions at the *sn*-1 and *sn*-3 positions ranged from 42 to 47%. Expression of the meadowfoam LPAAT in transgenic rapeseed resulted in the accumulation of 22:1 at the *sn*-2 position of TAGs, with a concomitant decrease in the 22:1 content at the *sn*-1 and *sn*-3 positions.

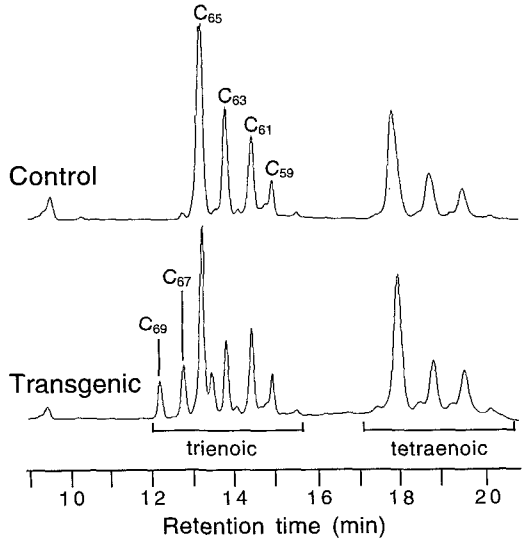

Figure 4. Silver-phase HPLC analysis of intact TAGs from control and transgenic rapeseed oil. Control oil was extracted from untransformed reston, and the transgenic oil was extracted from plant 7695–6. The major constituent of trienoic C_{65} TAG is 22:1/18:1/22:1. Trierucin (trienoic C_{69}) and trienoic C_{67} are discussed in the text. The retention times of trienoic C_{57}, C_{63}, and C_{69} TAGs were determined by chromatography of standards. The retention times of the other labeled peaks were estimated by interpolation.

DISCUSSION

We have used LPAAT from meadowfoam to incorporate 22:1 into the *sn*-2 position of rapeseed TAGs, and we have demonstrated that the transgenic rapeseed can synthesize trierucin. These experiments establish the feasibility of using acyltransferases with unusual substrate specificities to alter the stereochemical composition of transgenic seed oil. At present, no commercial processes are available to synthesize economically important "structured" TAGs.

We used the homology between coconut and *E. coli* LPAATs and a yeast *sn*-2 acyltransferase to design a cloning strategy enabling us to isolate the meadowfoam LPAAT cDNA. There are several peptide sequences that are conserved in all of these *sn*-2 acyltransferases, and 62% of the primary sequence of the meadowfoam LPAAT is identical with that of the coconut LPAAT (Fig. 1). All four proteins have similar hydropathy profiles (Fig. 2). Two of the four potential transmembrane domains found in the meadowfoam LPAAT are less hydrophobic in the coconut, *E. coli*, and yeast acyltransferases. Thus, the LPAATs are

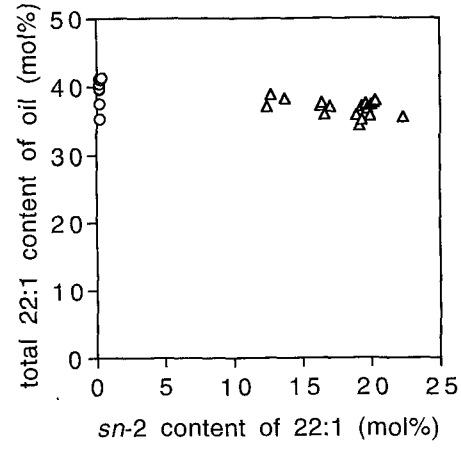

Figure 5. Total seed-oil 22:1 versus *sn*-2 22:1 content in oil extracted from single seeds of control and transgenic rapeseed plants. Oil was extracted from single seeds and was analyzed for *sn*-2 and total oil fatty acyl compositions. Ten control (reston) seeds and 20 transgenic (7695–1) seeds were analyzed. The total 22:1 content was plotted against the *sn*-2 value. ○, Control seeds; △, transgenic seeds.

likely to contain between two and four transmembrane segments.

Meadowfoam and coconut represent widely diverged taxa, Dicotyledonae and Monocotyledonae, and their LPAATs have very different substrate specificities. Thus, peptide sequences that are conserved between the two enzymes are likely to be conserved among a wide variety of plant LPAATs and should be useful in designing strategies to isolate clones encoding LPAATs from other taxa.

Reston seed oil is made up of approximately 10 mol% 20:1. Studies of the substrate specificities of meadowfoam LPAAT (Löhden and Frentzen, 1992) showed that the enzyme was capable of using 20:1-CoA and 22:1-CoA as acyl donors. Oil extracted from transgenic rapeseed, which contained 22:1 at the *sn*-2 position, was not enriched in 20:1 at that position. This may reflect the relatively low levels of 20:1-CoA present in the developing rapeseed embryos. Alternatively, there might be differences in the substrate specificities of the LPAATs from the meadowfoams used in the previous study (*Limnanthes douglasii*) and this report (*L. alba alba*).

The highest level of *sn*-2 22:1 observed in transgenic seed oils was 22.3%. This reflects incorporation by both the introduced meadowfoam transgene and endogenous *Brassica* LPAAT. From the oil composition data, it is difficult to assess the relative activities of the two LPAATs because meadowfoam LPAAT efficiently incorporates both 18:1 and 22:1 into the *sn*-2 position of phosphatidic acid (Cao et al., 1990; Laurent and Huang, 1992; Löhden and Frentzen, 1992). However, it is likely that the meadowfoam LPAAT activity is similar in magnitude to the endogenous *Brassica* LPAAT activity in plants 7695–1 and 7695–6.

At the expression levels achieved in this study, the meadowfoam LPAAT did not increase 22:1 production in transgenic reston. Reston seed oil contains approximately 40 mol% 22:1, whereas other cultivars contain more than 50%. Expression of meadowfoam LPAAT in rapeseed cultivars with higher 22:1-CoA pool sizes (in maturing embryos) may lead to an increase in the overall 22:1 content of their seed oils. Recently, the genes encoding condensing enzymes involved in the elongation of fatty acids to chain lengths greater than 18 carbons have been isolated and used to increase the 22:1 content of canola oil (James et al., 1995; Lassner et al., 1996). Expression of these genes in conjunction with seed-specific expression of meadowfoam LPAAT may lead to the development of transgenic rapeseed cultivars showing dramatically increased levels of 22:1.

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