

Modification of Seed Oil Content and Acyl Composition in the Brassicaceae by Expression of a Yeast *sn*-2 Acyltransferase Gene

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A putative yeast *sn*-2 acyltransferase gene (*SLC1-1*), reportedly a variant acyltransferase that suppresses a genetic defect in sphingolipid long-chain base biosynthesis, has been expressed in a yeast *SLC* deletion strain. The *SLC1-1* gene product was shown in vitro to encode an *sn*-2 acyltransferase capable of acylating *sn*-1 oleoyl-lysophosphatidic acid, using a range of acyl-CoA thioesters, including 18:1-, 22:1-, and 24:0-CoAs. The *SLC1-1* gene was introduced into *Arabidopsis* and a high erucic acid-containing *Brassica napus* cv Hero under the control of a constitutive (tandem cauliflower mosaic virus 35S) promoter. The resulting transgenic plants showed substantial increases of 8 to 48% in seed oil content (expressed on the basis of seed dry weight) and increases in both overall proportions and amounts of very-long-chain fatty acids in seed triacylglycerols (TAGs). Furthermore, the proportion of very-long-chain fatty acids found at the *sn*-2 position of TAGs was increased, and homogenates prepared from developing seeds of transformed plants exhibited elevated lysophosphatidic acid acyltransferase (EC 2.3.1.51) activity. Thus, the yeast *sn*-2 acyltransferase has been shown to encode a protein that can exhibit lysophosphatidic acid acyltransferase activity and that can be used to change total fatty acid content and composition as well as to alter the stereospecific acyl distribution of fatty acids in seed TAGs.

INTRODUCTION

There is interest in modifying seed oil fatty acid composition and content by molecular genetic means to provide a source of super high erucic acid rapeseed oil for use as an industrial feedstock (Taylor et al., 1993). A similar interest exists for producing other strategic nonedible oils (e.g., seed oils high in hydroxy, epoxy, and short- and medium-chain fatty acids) in traditional oilseed crops (Murphy et al., 1994; MacKenzie, 1995).

There are no published demonstrations of increases in oil content by transgenic approaches, although yield increases by traditional breeding and selection continue to bring about incremental improvements. In contrast, increases in the proportions of some strategic fatty acids have been achieved by the introduction or modulated expression of various plant fatty acid biosynthesis genes in oilseeds (Knutzon et al., 1992; Voelker et al., 1992, 1996; Hitz et al., 1995; Lassner et al., 1996). In addition, the use of plant acyltransferase transgenes from *Limnanthes* spp has resulted in altered seed oil

proportions of *sn*-2 erucic acid in rapeseed (Lassner et al., 1995; Brough et al., 1996). However, the overall proportions of 22:1 in the seed oil did not increase, and there was no report of increased total fatty acid or increased oil content.

There is little understanding of how the Kennedy pathway is regulated in oilseeds. Recently, we demonstrated that an *Arabidopsis* seed mutant, which accumulated lower amounts of triacylglycerols (TAGs) than did the wild type and had dramatically altered fatty acid composition, also exhibited reduced diacylglycerol acyltransferase activity (Katavic et al., 1995). However, it is clear that additional studies are necessary before we can achieve directed increases in seed oil content and changes in TAG composition in oilseed plants.

The *SLC1* (for sphingolipid compensation) gene was cloned originally from a yeast (*Saccharomyces cerevisiae*) mutant lacking the ability to make sphingolipids (Lester et al., 1993). The suppressor allele, designated *SLC1-1* (in which nucleotide 131 is a T residue rather than an A residue, resulting in amino acid 44 being a leucine rather than a glutamine), encodes a protein that suppresses the genetic defect in sphingolipid long-chain base biosynthesis (Nagiec et al., 1993). Preliminary analyses of the *SLC1-1* mutant, which

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was capable of normal growth without the need to supply a long-chain base, indicated that it was able to synthesize novel phosphatidylinositols having long-chain saturated fatty acyl groups in the *sn*-2 position. The working hypothesis has been that the *SLC1-1* suppressor allele encodes an acyltransferase with an altered substrate specificity, enabling it to use a very-long-chain fatty acid (VLCFA; 26:0) to acylate the *sn*-2 position of inositol-containing glycerolipids (Nagiec et al., 1993). The *SLC1* sequence is somewhat similar (27% amino acid identity) to the *Escherichia coli* *PLSC* gene, which has been claimed to encode a lysophosphatidic acid acyltransferase (LPAT; EC 2.3.1.51) (Coleman, 1990, 1992). The *SLC1* gene complemented the growth defect in JC201 (a temperature-sensitive *E. coli* strain mutated in *PLSC*). Based on these findings, it was reported that *SLC1* encodes a yeast *sn*-2 acyltransferase. However, the authors were unable to detect LPAT activity in the complemented *E. coli* JC201 mutant (Nagiec et al., 1993). To date, no conclusive evidence of the activity encoded by *SLC1-1* or *SLC1* has been reported.

Based on our interest in modifying the VLCFA content of Brassicaceae (Taylor et al., 1993), we have performed yeast transformation studies to determine the nature of the *SLC1-1* gene product. We have also expressed the *SLC1-1* suppressor allele gene in the model oilseed *Arabidopsis* and in a high erucic acid cultivar of *Brassica napus*. We report that the yeast *SLC1-1* gene product is indeed an *sn*-2 acyltransferase that can enhance seed LPAT activity and alter the oil content and oil composition of plant seed lipids.

RESULTS

Analysis of the *SLC1-1* Gene Product

To confirm the function of *SLC* genes, we have cloned *SLC1-1* and *SLC1* encoding regions under the control of the *Gal1* promoter in the yeast expression vector pYEura3. The

yeast strain YMN5, an *SLC1* null mutant, was used for the overexpression of *SLC* genes. As shown in Table 1, after a 6-hr galactose induction period, lysates of both the *SLC1* and *SLC1-1* transformants of YMN5 exhibited 18:1-CoA:LPAT activity, which was several-fold higher than the basal LPAT activity residing in the native YMN5 strain and/or YMN5 strain transformed with pYEura3 only. In addition, both the *SLC1-1* and *SLC1* gene products displayed LPAT activities with 22:1-CoA and 24:0-CoA, whereas neither YMN5 transformed with pYEura3 only (Table 1) nor nontransformed YMN5 (data not shown) was capable of using these very-long-chain acyl-CoAs. Furthermore, the *SLC1-1* gene product appeared to have a higher relative activity with the very-long-chain acyl-CoA donors (22:1-CoA and 24:0-CoA) than did the corresponding *SLC1* gene product; the longer the chain length of the fatty acyl moiety, the greater the difference in relative enzyme activity.

The trends in relative LPAT activity were consistent for two different induction periods (6 or 24 hr). In the *SLC1-1* YMN5 transformant, the relative LPAT activity in the presence of 22:1-CoA and 24:0-CoA was ~23 to 26% and 0.5 to 1.1%, respectively, of the activity observed when 18:1-CoA was supplied (Table 1). The *SLC1* YMN5 transformant exhibited LPAT activities of only 9 to 15% and 0.1 to 0.2% in the presence of 22:1-CoA and 24:0-CoA, respectively, compared with that observed in the presence of 18:1-CoA. Similar trends were observed in experiments in which another *SLC* deletion strain (YMN6-2C; *slc1Δ1::URA3 leu2*) was transformed with YEp351 (a multicopy vector with an *LEU2* marker; this was also supplied by M.M. Nagiec and R.C. Dickson, University of Kentucky, Lexington) containing either an *SLC1* or *SLC1-1* gene insert (data not shown). Our results suggest that *SLC1* and *SLC1-1* encode yeast *sn*-2 acyltransferases and are in good agreement with the previous suggestion (Nagiec et al., 1993) that the point mutation in the *SLC1-1* gene results in a change in substrate specificity.

Because it had been suggested that the *SLC1-1* suppressor allele gene product may be capable of acylating the *sn*-2 position of inositol-containing glycerolipids with C₂₆ fatty acids

Table 1. LPAT Activity in Lysates of the YMN5 *SLC* Deletion Strain after Transformation with YEpUra3 (Control), YEpUra3:*SLC1*, or YEpUra3:*SLC1-1* and Either a 6- or 24-Hr Induction Period in the Presence of Galactose

Strain:Transformation	Induction Period (hr)	LPAT Activity		
		18:1-CoA:LPAT ^a (pmol/min/mg protein)	22:1-CoA:LPAT ^a (pmol/min/mg protein)	24:0-CoA:LPAT (pmol/min/mg protein)
YMN5:YEpUra3 control (without <i>SLC</i> insert)	6	3300 (200)	0	0
	24	2500 (200)	0	0
YMN5:YEpUra3 <i>SLC1</i>	6	8400 (1900)	760 (150)	10
	24	9200 (1600)	1365 (215)	20
YMN5:YEpUra3 <i>SLC1-1</i>	6	9800 (300)	2280 (130)	50
	24	8200 (300)	2140 (245)	92

^a Values within parentheses represent \pm SD for which at least three determinations were performed.

(Lester et al., 1993; Nagiec et al., 1993), we decided to test the capacity of the *SLC* genes to express novel *sn-2* acyltransferase activities when supplied with lysophospholipids other than lysophosphatidic acid (LPA). Lysates of the YMN5 strain, or YMN5 transformed with pYEUra3 only, appeared to exhibit a basal capacity to produce ^{14}C -18:1–radiolabeled phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) when *sn-1* acyl lysophosphatidylinositol (LPI), *sn-1* acyl lysophosphatidylcholine (LPC), or *sn-1* acyl lysophosphatidylethanolamine (LPE) were supplied in the presence of ^{14}C -labeled 18:1-CoA. These activities depended on the addition of exogenous acceptor and were always at least 10-fold higher than control reactions (without exogenous lysophospholipid). Lysates of the YMN5 transformants containing the *SLC1* and *SLC1-1* genes exhibited 10 to 20% increases in these apparent activities. However, in all cases, the level of radiolabeled PI, PC, or PE observed was always two to three times lower than the level of radiolabeled phosphatidic acid (PA) produced in the corresponding LPAT reaction.

Because these conversions were measured in cell-free lysates, it is possible that the observed activities could be artifacts arising from phospholipase D acting on the exogenous LPC, LPE, or LPI, converting these to LPA, which could then in turn act as a substrate in an LPAT reaction. Indeed, the reaction mixtures in which LPI was supplied always produced low levels of radiolabeled PA, which were approximately twofold higher than in the control reactions (without LPI). To observe this LPAT activity, the radiolabeled products were separated by thin-layer chromatography in a solvent system that resolved radiolabeled PA. Furthermore, neither the *SLC1-1* nor the *SLC1* gene products were able to produce appreciable radiolabeled PI when lysates were incubated in the presence of *sn-1* acyl LPI and either ^{14}C -labeled 22:1-CoA or 24:0-CoA. Hence, it is unlikely that the *SLC* genes encode LPI-, LPC-, or LPE-specific acyltransferases, although we cannot rule out the possibility that the *SLC* gene products can use various lysophospholipids, if available in vivo. With this information, and our interest in modifying seed VLCFA content, we chose to conduct our plant transformation experiments using the mutated *SLC1-1* gene encoding an *sn-2* acyltransferase able to insert VLCFAs.

Generation and Molecular Characterization of Arabidopsis and *B. napus* *SLC1-1* Transgenic Plants

We constructed a plant transformation vector (p*SLC1-1*/pRD400) with the *SLC1-1* gene under the control of a tandem cauliflower mosaic virus 35S promoter (Datla et al., 1993). We reasoned that with a constitutive promoter, the effect of the *SLC1-1* gene, if any, could be investigated in developing seeds as well as in vegetative tissues.

Transformation of Arabidopsis (ecotype Columbia) was performed by wound inoculation (Katavic et al., 1994) or

vacuum infiltration (Bechtold et al., 1993) methods and yielded >50 transgenic lines. Transformation of *B. napus* cv Hero utilized hypocotyl explants and a modified method of DeBlock et al. (1989), producing 10 transgenic lines. The presence of the *SLC1-1* gene in kanamycin-resistant transformants was verified by polymerase chain reaction (PCR) and by DNA gel blot hybridization analyses (Southern, 1975). Restriction digests with EcoRI and HindIII performed with genomic DNA isolated from Arabidopsis transformants indicated that most of the transgenic lines have multiple inserts per genome (data not shown). Similar analyses of *B. napus* cv Hero transformants digested with EcoRI showed only one strong hybridization signal in lines 3, 5, 7, and 8, suggesting that there is probably only one T-DNA insert in these transgenic lines.

Although the yeast acyltransferase was expressed under the control of a constitutive (tandem 35S) promoter, there were no discernible phenotypic effects on vegetative growth or on development. Plant morphology was generally unaffected, with many transgenic plants indistinguishable from nontransformed plants. Seed germination, flowering, and seed development occurred at the same pace as it did in wild-type plants. Plant fertility and seed set were not perturbed by the *SLC1-1* transgene. For example, in the T_2 generation of the Arabidopsis transgenic plants, counting the number of siliques per 15-cm segment of stem from the top of the main bolt revealed that wild-type plants had 20.9 ± 2.4 siliques, whereas *SLC1-1* transgenic lines 42 and 52 contained 19.1 ± 2.0 and 19.6 ± 2.3 siliques, respectively. Wild-type plants had 49.5 ± 5.3 seeds per silique, whereas the corresponding values for transgenic lines 42 and 52 were 47.1 ± 5.8 and 51.9 ± 7.7 , respectively.

Gel blot hybridization analyses of RNA isolated from Arabidopsis T_3 seeds at middevelopment confirmed the expression of the *SLC1-1* gene in all lines tested. As shown in Figure 1, the highest level of transcript among T_3 seeds was observed in lines 42-1 and 42-4. Similar analyses confirmed the presence of the *SLC1-1* transcript in developing *B. napus* T_3 seed (data not shown). There was no detectable signal after RNA gel blot analyses of developing seed from nontransformed Arabidopsis (Figure 1) or *B. napus* (data not shown).

LPAT Analyses of Arabidopsis and *B. napus* *SLC1-1* Transformants

Table 2 shows the results of LPAT analyses of developing seed from *SLC1-1* transgenic plants and from nontransformed control plants. In Arabidopsis developing seed, those *SLC1-1* transgenic lines showing high levels of *SLC1-1* expression (42-1 and 42-4) and one line showing relatively low expression (42-5; cf. Figure 1) exhibited 18:1-CoA and 20:1-CoA:LPAT activities that were elevated, ranging from 17 to 130% higher than in the nontransformed control. Similarly,

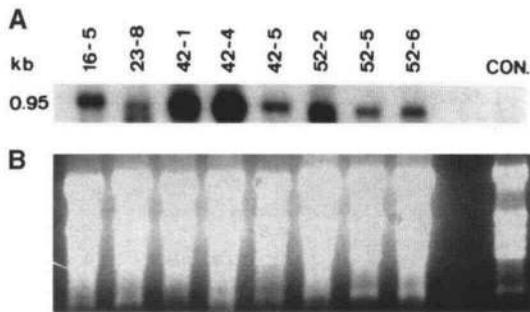


Figure 1. RNA Gel Blot Analysis of *SLC1-1* Gene Expression in Transgenic Arabidopsis.

(A) Gel blot analysis of RNA isolated from seeds of Arabidopsis *SLC1-1* transgenic lines 16-5, 23-8, 42-1, 42-4, 42-5, 52-2, 52-5, and 52-6 and a nontransformed wild-type control plant (CON.). Total RNA was isolated by the method of Lindstrom and Vodkin (1991) from T_3 midcotyledonary embryos. RNA samples were denatured with formaldehyde, and 10 μ g of RNA was loaded per lane and resolved on a 1.2% formaldehyde-agarose gels. The RNA was transferred to a Zeta probe nylon membrane (Bio-Rad) and hybridized according to the manufacturer's protocol, using the random primer labeled 0.95-kb *SLC1-1* fragment. The intensity of signal varied among lines, with the strongest signal in lines 42-1 and 42-4. No signal was detected in the nontransformed wild-type control.

(B) The amount of RNA loaded per lane was calibrated by the relative ethidium bromide staining of the rRNA bands.

the LPAT activities of several *B. napus* transgenic plants were highly elevated, with 18:1-CoA:LPAT activity increased by two- to fivefold. 22:1-CoA:LPAT activity in these transgenic lines was markedly increased compared with the negligible activity present in developing seed of nontransformed controls.

Lipid Analyses of Arabidopsis *SLC1-1* Transformants

Many Arabidopsis *SLC1-1* transgenic T_2 seed lines (21 of 48) showed significantly increased oil content (an 11 to 49% increase in micrograms of total fatty acids per 50 or 100 seeds) compared with nontransformed controls and pBI121 or pRD400 transgenic controls (without the *SLC1-1* insert, but with a kanamycin resistance gene as a selectable marker).

Table 3 shows data for several of the Arabidopsis *SLC1-1* T_2 generation seed lines that, in addition to increased oil content, also exhibited significant increases in total VLCFA content, especially eicosenoic (20:1) and erucic acids (22:1), as well as increased overall proportions of VLCFAs. The processes of transformation and kanamycin selection alone had no significant effect on seed oil content or fatty acid composition, because these values were not significantly

different in seed from the nontransformed wild type and from pBI121 or pRD400 control transformants (both without *SLC1-1* insert). Within the total lipid extract, the proportions of polyunsaturated C_{18} fatty acids in nontransformed wild-type controls and in pBI121 and pRD400 control transformants were 46.4 ± 2.3 , 46.1 ± 2.3 , and 43.5 ± 1.0 weight %, respectively, and the corresponding proportions of VLCFAs were also not significantly different (Table 3). Those *SLC1-1* T_2 transformants showing the greatest increases in seed oil and VLCFA content were selected, and individual seeds were planted to give T_3 progeny lines. To maintain valid comparisons within each experiment or generation, nontransformed control plants or control transformants (e.g., pBI121) were always grown in the same growth chamber at the same time as the *SLC1-1* transformants.

As shown in Figure 2, analyses of T_3 seed from several independent *SLC1-1* lines indicated that total lipid content (expressed as micrograms of fatty acids per 100 seeds) was increased by 35 to 110% compared with pBI121 control T_3 seed, and this was correlated with increased TAG content in the *SLC1-1* transgenic plants (data not shown). These trends were equally valid when the oil content was expressed on a dry weight basis. The oil content of pBI121 control T_3 seeds ($n = 5$ plants) was $31.8 \pm 1.1\%$ dry weight, similar to previous studies with nontransformed Arabidopsis ecotype Columbia (Taylor et al., 1995a). The oil contents (on a dry weight basis) of T_3 seeds of *SLC1-1* transgenic lines 16-5, 23-8, 42-1, 42-4, 42-5, 52-2, 52-5, and 52-6 were 42.0, 42.9, 37.8, 49.8, 42.5, 43.0, 40.7, and 44.1%, respec-

Table 2. Relative LPAT Activities in Homogenates Prepared from Seeds at Middevelopment from Nontransformed Control Plants and Selected *SLC1-1* T_3 Transgenic Lines of Arabidopsis or T_2 Transgenic Lines of *B. napus* cv Hero

Line	LPAT Activity/ 14 C-Labeled Acyl-CoA Provided	
	(pmol/min/mg protein)	(pmol/min/mg protein)
Arabidopsis	18:1-CoA	20:1-CoA
n-Control ^a	2000	350
42-1	2400	410
42-4	2600	600
42-5	3650	800
<i>B. napus</i> cv Hero	18:1-CoA	22:1-CoA
n-Control	880	tr ^b
Hero 3-1	5250	105
Hero 5-4	ND ^c	15
Hero 7-6	3085	81
Hero 8-6	2075	31

^an-Control, nontransformed control.

^btr, trace; very low activity (< 0.5 pmol/min/mg of protein) with 22:1-CoA in nontransformed control *B. napus* cv Hero.

^cND, not determined.

Table 3. Total Fatty Acid, Eicosenoic and Erucic Acid, and Total VLCFA Content of Seed from Nontransformed Wild-Type Controls and T₂ Seed from pBI121 and pRD400 Controls (without the *SLC1-1* Insert) and *SLC1-1* Transgenic Lines of Arabidopsis^a

Line	Total Fatty Acids (μg)	20:1 (μg)	22:1 (μg)	Total VLCFAs	
				(μg)	Weight %
Nontransformed wild-type controls ^b	745.0 (37.2)	149.6 (12.8)	16.6 (1.4)	205.6 (20.2)	27.6 (1.0)
pBI121 controls ^c	720.4 (36.0)	147.6 (4.6)	14.0 (0.6)	193.4 (6.8)	26.8 (1.3)
pRD400 controls ^c	714.0 (23.8)	152.8 (1.6)	13.6 (0.4)	200.0 (3.6)	28.0 (0.5)
16	896.0	192.6	21.8	262.6	29.3
20	1002.4	226.8	23.0	300.8	30.0
23	884.2	190.2	20.2	258.4	29.2
42	912.0	207.2	34.8	300.6	33.0
52	863.6	205.0	22.4	274.2	31.8
54	912.4	214.6	20.8	283.4	31.1

^a Values are reported for 100-seed samples or as weight %. For controls, values within parentheses represent standard errors of the means of three to five determinations performed with samples pooled from several plants. For *SLC1-1* transgenic seeds, values are the average of two determinations.

^b ±SE; *n* = 5.

^c ±SE; *n* = 3.

tively. However, given the small size and weight of samples of even substantial numbers of transgenic seeds of Arabidopsis and the fact that the number of siliques and seeds per plant is not significantly affected, we believe it more accurate to express the data on the basis of analyzing 50 to 100 seed samples carefully counted under a dissecting microscope. The availability of more transformed seed and fewer lines to analyze allowed replicates of the larger 100-seed sample size to be analyzed in the T₃ generation.

In addition to increases in oil content, T₃ seeds from several *SLC1-1* transformants exhibited increases in the amounts of VLCFAs (micrograms per 100 seeds) and levels of VLCFA-containing C₅₆ to C₆₀ TAGs, as shown in Table 4. Thus, the traits of elevated oil and VLCFA contents originally obtained in the segregating T₂ generation were heritable.

As shown in Table 5, stereospecific analyses of T₃ seed TAGs from selected independent *SLC1-1* lines contained increased proportions of VLCFAs (e.g., eicosenoic acid, 20:1) at the *sn*-2 position. This trend was consistent, regardless of whether the data were expressed as the proportion of total 20:1 found at the *sn*-2 position in TAGs (Table 5, footnote b) or as the proportion, among all *sn*-2 position fatty acids in TAGs, which is represented by 20:1 (Table 5, footnote c). Furthermore, the increase in proportions of VLCFAs (e.g., eicosenoic acid) at the *sn*-2 position of TAGs was generally correlated with a decrease in the proportions of C₁₈ polyunsaturated fatty acids at this position. After propagating these selected Arabidopsis T₃ seed lines, analyses of the T₃ plantlets indicated that leaf fatty acid content (on a dry weight basis) and composition were not significantly different from that

observed in leaves of pBI121 control transformants and in nontransformed wild-type plants.

Lipid Analyses of *B. napus* cv Hero *SLC1-1* Transformants

Several *B. n.* Hero *SLC1-1* T₂ transgenic seed lines exhibited significantly increased erucic acid content, as shown in Table 6. The overall proportions of VLCFAs, including erucic acid, were also increased in most of these seed lines. The proportions of 22:1 in *SLC1-1* transgenic seeds ranged from 49.1 to 56.2 weight %, compared with 45.2 ± 0.5 weight % for nontransformed controls. Analyses of TAG classes by gas chromatography showed that Hero *SLC1-1* transformant lines had increased proportions of C₆₂ to C₆₆ TAGs (containing two or more C₂₂ fatty acids), primarily at the expense of TAGs containing two (C₅₆) or three (C₅₄) C₁₈ fatty acids (data not shown). In the *SLC1-1* Hero transgenic lines, the proportions of C₆₂ to C₆₆ TAGs ranged from 47.2 to 59.6 mol%, compared with nontransformed Hero, where the C₆₂ to C₆₆ TAGs constituted 38.1 ± 1.5 mol% (*n* = 9). The proportion of C₆₆ TAGs increased from 0.10 mol% in nontransformed controls to 0.25 to 0.40 mol% in several *SLC1-1* lines (including lines 5-4, 8-4, and 8-6).

Lipase-based stereospecific analyses of TAGs isolated from several *SLC1-1* transformants showed small increases in the proportions of erucic acid and total VLCFAs at the *sn*-2 position. In general, these *sn*-2 increases were correlated with increases in overall proportions of erucic acid or VLCFAs in the seed oil. For example, in the seed oil of *SLC1-1*

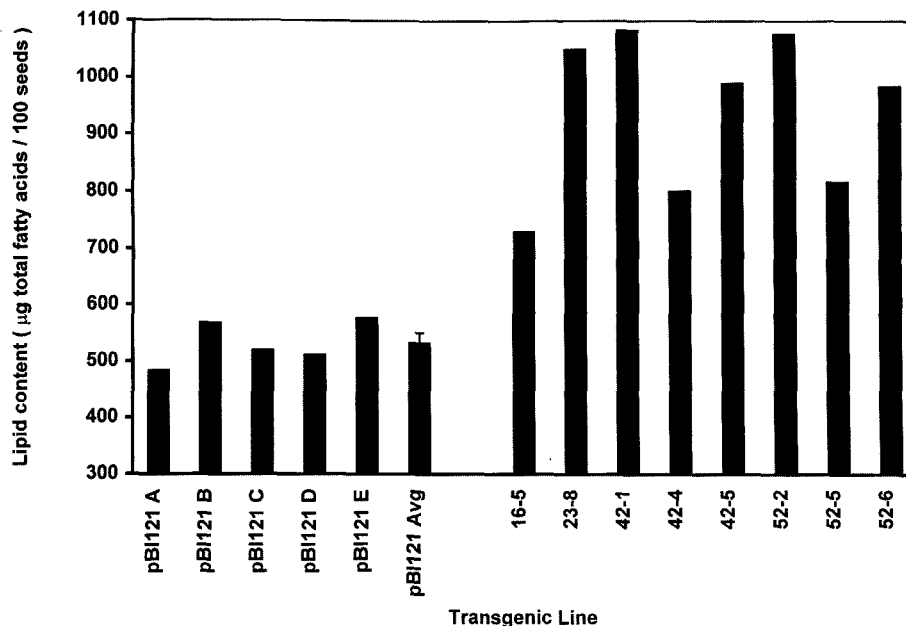


Figure 2. Oil Content in pBI121 Control and *SLC1-1* Transgenic T₃ Seeds of Arabidopsis Ecotype Columbia.

Duplicate determinations were performed with seed samples from five independent pBI121 (without the *SLC1-1* insert) control transgenic plants, designated A to E. The average pBI121 control value is designated pBI121 Avg, and the error bar represents the standard error of the mean for plants A to E. For *SLC1-1* transgenic plants, values are the average of two or three determinations. Oil content is given as micrograms of fatty acids per 100 seeds.

transgenic lines 3-1 and 8-6, the *sn-2* erucic proportions were 4.1 and 3.5 weight %, respectively, and the respective total proportions of erucic acid were 56.2 and 50.5 weight %, whereas in nontransformed seed, erucic acid typically constituted <1 weight % of the *sn-2* fatty acids, and the total proportion of erucic acid in TAGs was ~45 weight %. The finding of negligible levels of erucic acid at the *sn-2* position in nontransformed Hero is consistent with our previous work with this cultivar (Taylor et al., 1994).

The oil content of several *SLC1-1* transgenic lines (expressed on a dry weight basis) was elevated by 8 to 22% over that observed in the nontransformed controls (Table 6). Values for oil content in the *SLC1-1* transgenics were consistently higher than were those in the nontransformed controls, whether the data were expressed on the basis of percentage of dry weight or micrograms of total fatty acids per seed. Furthermore, estimates of relative oil content by a nondestructive ¹H-nuclear magnetic resonance (¹H-NMR) method generally correlated with measurements made by a destructive (transmethylation-based) method (Table 6). The one exception to this general trend was line 3-1, which had an oil content comparable to the controls but the highest proportions of erucic acid and VLCFAs.

As in the Arabidopsis transgenic plants, analyses of leaf lipids in T₁ *B. napus* cv Hero plants showed no significant changes in fatty acid content and only minor changes in acyl

composition, with proportions of 16:0 decreasing and proportions of 24:0 increasing slightly. The average proportions of 16:0 and 24:0 in nontransformed Hero leaves were 19.6 ± 1.7 weight % and 0.6 ± 0.2 weight %, respectively, whereas the corresponding values for the average of seven independent T₁ lines (3-1, 7-3, 7-6, 7-9, 8-4, 8-6, and 8-10) were 17.0 ± 0.6 weight % (16:0) and 1.5 ± 0.3 weight % (24:0).

DISCUSSION

We have provided strong evidence that the yeast *SLC* gene products encode enzymes that possess *sn-2* acyltransferase activity and can exhibit LPAT activity in vitro. The expression of multiple copies of the *SLC1* or *SLC1-1* genes in the *SLC* deletion mutant resulted in a 2.5- to threefold increase in 18:1-CoA:LPAT activity after galactose induction, compared with the activity indigenous to the YMN5 strain (Table 1). Furthermore, both the *SLC1* and *SLC1-1* gene products were able to use a range of C₁₈ to C₂₄ acyl-CoAs when LPA was supplied as the acyl acceptor. The capacity to use 22:1-CoA or 24:0-CoA in the LPAT reaction was unique to the *SLC* transformants and was not found in the YEpUra3 (without *SLC* insert) transformant or in the nontransformed YMN5 deletion mutant. Taken together, the

yeast expression data indicate that both *SLC* genes encode *sn-2* acyltransferases capable of performing the LPAT reaction, using a broad range of acyl-CoA donors. The fact that 22:1-CoA and 24:0-CoA:LPAT activities were observed only in the *SLC* transformants indicates that these genes actually encode *sn-2* acyltransferases and minimizes the possibility that they encode regulatory proteins. Compared with the *SLC1* gene product, the single base change of nucleotide 131 from an A to a T residue, which results in an amino acid change at position 44 from glutamine to leucine, gives the *SLC1-1* gene product an improved capacity to utilize very-long-chain acyl-CoAs in an LPAT reaction.

The *SLC* deletion mutant YMN5 (Nagiec et al., 1993) was found to possess a low level of *sn-2* acyltransferase activity. The known mechanism(s) for *de novo* synthesis of membrane phospholipids in yeast, and indeed all eukaryotes, necessarily involves an acylation of the *sn-2* position of LPA to give PA (Carman and Zeimet, 1996). One might expect that the inability to perform such a reaction would prove lethal. Because the *SLC* deletion mutant was viable without a functional *SLC* gene, the presence of another gene product capable of acylating the *sn-2* position of LPA was therefore expected.

Thus far, we have no evidence that the *SLC* genes express *sn-2* acyltransferase activity other than LPAT. The increased proportions of 26:0 found at the *sn-2* position of novel PIs in the *SLC1-1* mutant, as reported in a previous study by Nagiec et al. (1993), are probably the result of the *SLC1-1* LPAT's capacity to incorporate very-long-chain saturated fatty acids into the *sn-2* position of PA. PA could then

be converted to cytidine diphosphate-diacylglycerol (CDP-DAG) and then to PI, via the enzymes CDP-DAG synthase and PI synthase, respectively (Carman and Zeimet, 1996).

Because the *SLC1-1* gene product exhibits LPAT activity and is capable of using a range of acyl-CoAs, we decided to test its effect when expressed in oilseed plants containing varying levels of VLCFAs in their seed oils. The expression of a yeast *SLC1-1 sn-2* acyltransferase in both *Arabidopsis* and *B. napus* resulted in dramatic increases in seed oil content and changes in seed oil composition.

The higher levels of LPAT activity exhibited in developing seed of the *SLC1-1* transgenic plants correlate well with the yeast expression data and provide additional evidence that the gene does indeed encode a variant *sn-2* acyltransferase that is capable of utilizing LPA as an acceptor. The fact that developing seed of the *B. napus* cv Hero transgenic plants contained significant levels of 22:1-CoA:LPAT activity, whereas the nontransformed control was essentially devoid of this activity (see Table 2), further confirms that the *SLC1-1* gene encodes an acyltransferase with an altered specificity compared with the native plant gene.

Because the yeast *sn-2* acyltransferase is able to accept a wide range of acyl-CoAs as substrate, it is perhaps not surprising that the seeds of *SLC1-1* transgenic plants were shown to contain oils with increased proportions of VLCFAs at the *sn-2* position. Similar and even more dramatic results have been reported in experiments in which meadowfoam (*Limnanthes* spp) LPAT transgenes were expressed in *B. napus* (Lassner et al., 1995; Brough et al., 1996), resulting in elevated *sn-2* proportions of erucic or lauric acid, respectively. However, to our knowledge, the current *sn-2* acyltransferase transgenic experiments are novel in that they result in increased overall proportions or amounts of VLCFAs in seed oils. The best *Arabidopsis* and *B. napus* transformant lines exhibited increases in proportions of seed VLCFAs of 6 and 20 weight %, respectively. Hero transgenic line 3-1 had in excess of 56 weight % erucic acid and almost 80 weight % total VLCFAs in T_2 seed oil, which to our knowledge is the highest proportion of VLCFAs achieved in *B. napus* to date.

Of even greater significance, however, is the fact that the expression of the yeast *sn-2* acyltransferase resulted in increased seed oil content in many transgenic plants. Until now, increases in seed oil content of this magnitude by plant transformation have not been reported. This effect was consistent, regardless of how the data were expressed. Indeed, noninvasive $^1\text{H-NMR}$ estimates of increased oil content in *B. napus* cv Hero *SLC1-1* transgenic plants were also generally correlated with increased seed weights in many of the transgenics (increases of 9 to 38%) and suggested that the increased seed dry weights were at least partially attributable to increased oil, with negligible contribution from seed water (absence of broad water resonance between the $\text{CH}_2\text{OCO-}$ and CHOCO- chemical shifts; Rutar, 1989). Seed weight increases of 10 to 80% were also observed in many of the *Arabidopsis* transgenic plants.

Table 4. VLCFA Content and C_{56} , C_{58} , and C_{60} TAG Content of Mature T_3 Seed from pBI121 Control Plants and Selected *SLC1-1* Transgenic Lines of *Arabidopsis*^a

Line	VLCFAs (μg)	C_{56} TAGs (nmol)	C_{58} TAGs (nmol)	C_{60} TAGs (nmol)
pBI121 control	133 (15) ^c	145 (9)	31 (3)	— ^b
16-5	200	252	71	6
23-8	311	409	107	8
42-1	314	402	129	12
42-4	254	366	128	14
42-5	309	359	113	10
52-2	311	ND ^d	ND	ND
52-5	244	ND	ND	ND
52-6	270	323	86	6

^aAverages are based on 100-seed samples. For pBI121 control seeds (without the *SLC1-1* insert), values within parentheses represent the standard errors of the means of determinations for samples from five or six plants. For *SLC1-1* transgenic seeds, values are the average of two determinations.

^b—, not detected.

^c \pm SE; $n = 5$ or 6.

^dND, not determined.

Table 5. Proportions of Total 20:1 and VLCFAs That Are Found at the *sn*-2 Position of TAGs and the Proportion (among All *sn*-2 Position Fatty Acids in TAGs) That Is Represented by 20:1, VLCFAs, and 18:2 Plus 18:3 in Mature T₃ Seed of pBI121 Control Transgenic Plants and Selected *SLC1-1* Transgenic Lines of Arabidopsis^a

Transgenic Line	Weight % of Total 20:1 Found at <i>sn</i> -2 Position ^b	Weight % of Total VLCFAs Found at <i>sn</i> -2 Position ^b	Weight % <i>sn</i> -2 20:1 ^c	Weight % <i>sn</i> -2 VLCFAs ^c	Weight % <i>sn</i> -2 [18:2 + 18:3] ^c
pBI121 controls ^d	2.2 ± 0.7	1.8 ± 0.8	1.1 ± 0.3	1.3 ± 0.5	81.1 ± 1.3
16-5	16.3	17.8	4.2	6.2	72.1
23-8	12.0	13.3	7.5	10.9	61.7
42-1	8.5	8.9	5.1	7.4	72.5
42-4	12.8	14.5	7.9	11.1	65.9
42-5	8.7	9.4	5.3	7.7	72.1
52-2	10.0	10.6	6.2	8.8	69.8
52-5	9.7	11.8	5.8	10.3	66.2
52-6	12.0	13.4	7.5	10.9	64.1

^a Analyses were performed with oil isolated from 100-seed samples.

^b Weight % (wt%) of total 20:1 (or VLCFAs) that are found at the *sn*-2 position of TAGs = [(wt% in *sn*-2) ÷ (3 × wt% total 20:1 (or VLCFAs) in TAGs) × 100].

^c Weight %, among all *sn*-2 position fatty acids, that is represented by 20:1, VLCFAs, or 18:2 + 18:3 (based on lipase treatment and analysis of *sn*-2 monoacylglycerols, according to the method of Christie [1982]).

^d Mean determinations were made with seed from four individual plants (±SE).

The one exception to the general trend of increased oil content and seed weight was line 3-1; the total oil content in this line (33.5% dry weight) was not significantly different than it was in seeds of the Hero controls (Table 6), but seed weight was decreased by up to 40%. As cited above, this line also had the highest proportion of VLCFAs (~80%). It is possible that the accumulation of very high proportions of seed VLCFAs can affect seed development/size so that oil deposition (and perhaps other processes) is somewhat inhibited. Although data on such effects have not been documented, it is interesting that transgenic *B. napus* containing a meadowfoam LPAT resulted in significant increases in seed oil *sn*-2 erucic acid but that there were no reports of seeds with increased oil content or higher total proportions of erucic acid; average seed weights and plant vigor were not addressed (Lassner et al., 1995; Brough et al., 1996).

In transgenic Arabidopsis and *B. napus* cv Hero seeds, 60 to 75% of the increase in oil content could be accounted for by increases in the proportions and absolute amounts of VLCFAs, particularly the respective increases in eicosenoic and erucic acids. Twenty-five to 40% of the increment could be accounted for by the substantial increases in the accumulation of other non-VLCFAs, such as palmitate, oleate, linoleate, and linolenate. This suggests that it is reasonable to predict that similar increases in oil content or improvements in composition could be achieved in oilseed crops that do not accumulate appreciable VLCFAs, such as canola-quality *B. napus*, soybean, sunflower, flax, maize, and cotton.

It is known that some metabolic pathways are controlled at multiple levels. The regulation is spread over several steps, and the cumulative contributions determine the overall flux

through the metabolic process (Ohlrogge and Browse, 1995). Thus far, regulation of the Kennedy pathway in developing oilseeds has been poorly understood. Our results, obtained in a plant transformation approach using a heterologous yeast acyltransferase, suggest that the reactions catalyzed by *sn*-2 acyltransferases (e.g., LPAT) may constitute one of the regulatory steps in the biosynthesis of TAGs in developing oilseeds.

In previous experiments in which meadowfoam or coconut LPAT genes were expressed in *B. napus*, no increase in overall oil content was reported (Lassner et al., 1995; Brough et al., 1996). However, such results do not exclude and indeed may suggest the possibility that plant LPATs are tightly controlled in vivo. The regulation of enzyme activity could be achieved at several levels. For example, mammalian acyl-CoA:cholesterol acyltransferase activity is primarily controlled by protein phosphorylation (Gavey et al., 1983). A possible reason for the significant increase in oil content in the *SLC1-1* transgenic Brassicaceae may be the result of differences in *sn*-2 acyltransferase regulation. The overall homology between the yeast *sn*-2 acyltransferase and the plant *sn*-2 acyltransferases from meadowfoam and coconut (Brown et al., 1995; Knutzon et al., 1995; Lassner et al., 1995) is only 24% and is restricted mainly to the C termini of the proteins. Indeed, there was no cross-hybridization of a labeled *SLC1-1* probe with RNA isolated from nontransformed Arabidopsis (see Figure 1) or *B. napus* seeds at middevelopment, and it was not possible to isolate plant LPATs by using the *SLC1-1* gene as a probe (J.-T. Zou, V. Katavic, and D.C. Taylor, unpublished result). In contrast, the meadowfoam and coconut acyltransferases have much greater overall homol-

ogy to each other (62% amino acid identity), and regions of homology extend throughout these sequences.

It may be that the yeast *SLC1-1* transgene is not as tightly regulated as are plant LPAT transgenes. In this context, it is interesting that both the meadowfoam and coconut LPAT sequences possess putative tyrosine phosphorylation sites. The characteristic motif consisting of an arginine at position -7, tyrosine at position 0, and glycine at position +5 (Cooper et al., 1984) is present at amino acids 68 (arginine), 75 (tyrosine), and 80 (glycine), respectively, in the meadowfoam sequence (Brown et al., 1995; Lassner et al., 1995) and at amino acids 89 (arginine), 96 (tyrosine), and 101 (glycine), respectively, in the coconut sequence (Knutzon et al., 1995). However, this motif is absent in the yeast *SLC1-1* gene. Thus, although further concrete experimental evidence is needed, the increased oil content after *SLC1-1* expression may be the result of unregulated *sn-2* acyltransferase activity, creating a new sink for lipid deposition. The channeling of more fatty acids into PA, via increased LPAT activity, could result in a larger flux through the Kennedy pathway from PA to DAG and ultimately to TAG. Our results also imply that fatty acid biosynthesis is capable of keeping up with substantial increases in demand from the expanded sink capacity.

Analogous results were obtained in attempts to alter the deposition of starch in plant tissues (Stark et al., 1992). By

expression of a mutant *E. coli glgC16* gene encoding an ADP-glucose pyrophosphorylase in potato tubers, an increase in starch accumulation was achieved. The increase was observed only by using the mutated form of the gene (encoding an ADP-glucose pyrophosphorylase enzyme with a single substitution of aspartate for glycine at position 336), and this result was attributed to reduced interference by the complex allosteric regulation of the wild-type *E. coli* gene.

However, given its overall effect on increases in seed weight, only part of which can be accounted for by increased oil content, it is perhaps too simplistic to attribute the overall effects of the yeast transgene solely to increased LPAT activity. It is equally plausible to speculate that in addition to its role as an *sn-2* acyltransferase, the expression of *SLC1-1* in the developing seed might result in a perturbation of steady state levels of diacylglycerol, PC, or inositol phospholipids, which may trigger signal transduction mechanisms involved in cell growth and differentiation during embryogenesis. The *SLC1-1* gene product has a relatively loose substrate specificity and therefore could also function as a "scavenger *sn-2* acyltransferase enzyme," which could channel various *sn-1* acyl-glycerophospholipids into the diacylglycerol pool and then into TAG.

Whereas the presence of the *SLC1-1* gene resulted in increased TAG content in many transgenic plants, the extent

Table 6. Oil Content, Erucic Acid Content, and Proportions of Erucic Acid and All VLCFAs in Seeds of Nontransformed Controls and T₂ Seeds of Selected Transgenic Lines of *B. napus* cv Hero^a

<i>B. n.</i> Hero Line	Oil Content ^b		Total Fatty Acids per Seed (μg)	22:1 Content ^c		22:1 Weight % of Total Fatty Acids	VLCFAs Weight % of Total Fatty Acids
	% of Seed Dry Weight ^d	NMR Response		μg/mg DW	μg/Seed		
n-CON ^e	33.9 (1.3) ^f	1.00	966 (102)	144 (6)	438 (49)	45.2 (0.5)	58.2 (0.8)
5-1	38.4 (0.4)	1.52	1396 (35)	173 (5)	687 (21)	50.2 (1.0)	62.2 (0.7)
5-4	41.2 (0.3)	ND ^g	1267 (36)	186 (9)	613 (17)	49.1 (0.4)	61.6 (0.5)
7-3	37.8 (0.2)	1.27	1220 (58)	174 (7)	594 (29)	49.6 (0.9)	62.1 (0.6)
7-4	36.8 (0.7)	ND	1475 (37)	171 (8)	717 (69)	49.4 (0.8)	61.6 (0.7)
7-6	39.3 (0.6)	1.39	1352 (68)	182 (7)	668 (32)	50.2 (0.9)	62.2 (0.6)
7-9	37.9 (0.3)	1.33	1397 (74)	173 (6)	681 (35)	49.4 (0.7)	61.5 (0.4)
8-4	37.3 (0.5)	1.57	1430 (36)	171 (6)	701 (17)	49.6 (0.6)	61.7 (0.4)
8-6	41.3 (0.7)	1.53	1492 (62)	193 (10)	739 (40)	50.5 (1.0)	62.5 (0.7)
8-7	38.6 (0.6)	1.48	1300 (33)	172 (5)	635 (17)	49.6 (0.7)	62.1 (0.5)
8-10	40.3 (0.8)	1.63	1477 (24)	180 (3)	723 (16)	50.5 (0.9)	62.7 (0.5)
3-1	33.5 (0.5)	ND	600 (48)	180 (9)	325 (12)	56.2 (2.1)	79.0 (0.9)

^a Values are the mean of three or four determinations performed with seeds sampled from individual plants. All analyses (except ¹H-NMR) used 12 randomly sampled seeds for each determination. ¹H-NMR was performed on 35-seed samples, and responses for resonances assigned to liquidlike oil (Rutar, 1989) are reported relative to the response for a nontransformed control sample set at 1.00.

^b Expressed as percentage of dry weight of seed, relative ¹H-NMR response, or micrograms of fatty acid per seed.

^c Expressed as micrograms per milligram dry weight (DW) of seed or micrograms per seed.

^d Calculated by determining the total fatty acid content and correcting for the fact that 3 mol of fatty acid are esterified to the glycerol backbone of every mole of TAG.

^e n-CON, nontransformed control.

^f The value in parentheses is the standard error of the mean of three or four determinations.

^g ND, not determined.

to which oil content was augmented was not always linearly correlated with the level of *SLC1-1* transcript or increased expression of LPAT activity measured *in vitro*. For example, among *Arabidopsis* lines 42-1, 42-4, and 42-5, the oil content on a dry weight basis was highest in line 42-4 (49.7%), followed by 42-5 (42.9%) and 42-1 (34.0%). Whereas the level of *SLC1-1* transcript was equally high in lines 42-1 and 42-4 and somewhat lower in line 42-5 (see Figure 1), the augmented LPAT activity measured *in vitro* was highest in line 42-5 (cf. Table 2). In comparing three *B. napus* cv Hero *SLC1-1* transgenic lines (3-1, 7-6, and 8-6), the highest augmented LPAT activity measured *in vitro* was in line 3-1 (cf. Table 2), yet it was line 8-6 that exhibited the highest increase in oil content (cf. Table 6); rather unexpectedly, there was no oil content increase in line 3-1. Such differences in levels of transcript or activity of gene product among independent transgenic events are not uncommon. These results suggest that expression of even small amounts of the *SLC1-1* gene may be sufficient to overcome limitations in PA levels during TAG biosynthesis. It is also possible that *in vivo*, the supply of LPA or acyl-CoAs may become rate limiting, causing variations in the actual level of TAG accumulating in each transgenic seed. In contrast, *in vitro* assays of LPAT activity are performed with cell-free extracts and in the presence of probable excesses of both LPA and acyl-CoA substrates.

The use of the yeast *SLC1-1* gene to achieve the effects described herein may also present an opportunity to further improve seed oil composition and content in a way not possible when the initial transformation is performed with a plant acyltransferase. In effect, the limited homology between plant LPATs and the yeast *sn-2* acyltransferase is low enough to allow strategies to downregulate the host plant LPAT by conventional means (e.g., antisense RNA technology or a cosuppression phenomenon; Mol et al., 1990; Van Blokland et al., 1993; De Lange et al., 1995) without a concomitant negative impact on the expression of the yeast transgene or on plant seed development. Thus, the yeast transgene strategy has a distinct advantage over that in which another plant transgene is introduced into a host plant in which there is a highly homologous, indigenous seed LPAT.

Although the yeast acyltransferase was expressed under the control of a constitutive (tandem 35S) promoter in the current experiments, there were no significant effects on fatty acid content or composition in the vegetative portions of the transgenic plants. Because the *SLC1-1* transcript levels were generally stronger in leaves of the transgenic plants than in seeds, these results suggest that the pools of available LPA and/or acyl-CoAs may be more tightly regulated in leaves (source) that accumulate little TAG compared with the cotyledons of developing seeds constituting a strong storage lipid sink. Although it is tempting, given the demonstrated capacity of the *SLC1-1* acyltransferase to utilize very-long-chain saturated fatty acyl-CoAs, it is difficult to assign a high level of significance to the doubling in propor-

tions of 24:0 observed in leaves of the Hero transgenic plants. The wild-type 24:0 proportion is very small (0.6 weight %), and the selection of rapidly expanding leaves is difficult to normalize.

In summary, the current study demonstrates that the yeast *SLC1-1* gene encodes an *sn-2* acyltransferase, which, when expressed in plants, is capable of significantly increasing seed oil content, altering the fatty acid composition of seed TAGs, and increasing LPAT activity in the developing seed. Such findings strongly suggest that in developing oilseed embryos, plant *sn-2* acyltransferases may be tightly regulated, playing some larger role in controlling the overall flux of acyl moieties through the Kennedy pathway. Recently, we also obtained similar increases in oil content and VLCFA proportions in T_2 seeds of transgenic *B. napus* cv Reston, and in a *B. carinata* breeding line C90-1163 (both are high erucic acid *Brassica* spp), after transformation with the *SLC1-1* gene under the control of the tandem 35S promoter. In Reston, the oil content in seed from four *SLC1-1* transgenic lines was increased by 21 to 26% compared with the nontransformed control, and proportions of erucic acid increased from 34.7 weight % in nontransformed controls to 37.4 and 41.1 weight % in the two best transgenic lines. *B. carinata* seed oil content in two transgenic lines increased by 11 and 19% compared with nontransformed controls, whereas erucic acid proportions rose from ~42 weight % in nontransformed *B. carinata* to 50 weight % in the best transgenic line. Clearly, the results obtained thus far are similar in a wide variety of *Brassica* spp.

We conclude that the increases in overall proportions and amounts of VLCFAs in the *SLC1-1* transgenic *Brassica* seeds are probably the cumulative result of an increased flux of fatty acids through the Kennedy pathway, combined with a significantly improved capacity to incorporate VLCFAs into the *sn-2* position. Admittedly, although the relative specificity of the yeast *sn-2* acyltransferase for erucoyl-CoA versus oleoyl-CoA measured *in vitro* is apparently lower than that of the LPAT from developing meadowfoam seeds (Taylor et al., 1993; Lassner et al., 1995; Brough et al., 1996), the overall effects of the yeast transgene appear superior. In a previous study (Katavic et al., 1995), we demonstrated that an *Arabidopsis* mutant exhibiting decreased diacylglycerol acyltransferase activity accumulated less TAG and synthesized much reduced proportions and amounts of VLCFAs, particularly 20:1. Clearly, the assembly of TAGs and the flux of VLCFAs into Kennedy pathway intermediates can have a profound effect on the levels of VLCFAs synthesized by elongation. Studies with *Arabidopsis* have shown that in the elongase complex, the level of the fatty acid elongation (*FAE1*) gene product for the condensing enzyme is rate limiting to the accumulation of VLCFAs (Kunst et al., 1992; Millar and Kunst, 1997). Thus, it would be interesting to attempt to boost the level of VLCFAs in *Arabidopsis* or *B. napus* by combining the *FAE1* and *SLC1-1* transgene effects. New information concerning factors that may limit VLCFA accumulation may come from additional analyses of *B. napus* cv Hero

SLC1-1 lines 3-1 versus 8-6 or other transgenic *B. napus* plants containing the meadowfoam LPAT gene.

There are other important issues to be addressed beyond the scope of the current study. For example, the effects (both quantitative and qualitative) of *SLC1-1* expression on other seed components, such as storage proteins, oleosins, minor lipid constituents, phytate, and glucosinolates, need to be examined. Utilizing these transgenic oilseed lines, it is now possible to develop homozygous lines and thereby investigate the relative importance of the *sn-2* acyltransferase transcript and protein levels on increased TAG synthesis and changes in acyl composition as well as its effects on many of these other important seed components.

Because the Kennedy pathway is common to all oilseeds, it is reasonable to assume that the effect of the *SLC1-1* transgene on oil content will be generic. Accordingly, additional experiments are in progress to express the *SLC1-1* gene under the control of seed-specific promoters and in a variety of other oilseed crops. It is hoped that these transgenic plants will exhibit equally dramatic increases in oil content and changed lipid composition.

METHODS

General Molecular Biology Techniques

Unless stated otherwise, all molecular biological techniques (polymerase chain reaction [PCR], DNA and RNA gel blot analyses, plasmid preparations, and restriction digestion) were performed by methods generally prescribed by Sambrook et al. (1989) or Ausubel et al. (1995).

Construction of the *SLC1-1* Vector for Plant Transformation

The DNA and amino acid sequences for the coding region of the *SLC1-1* gene have GenBank, EMBL, and DDBJ accession number L13282. Plasmid p411ΔB/C (kindly provided by R.C. Dickson; Nagiec et al., 1993) harboring the suppressor allele of the *SLC* gene (*SLC1-1*) was used as a template in a PCR with two primers, OM087 and OM088. Primer OM087 (5'-AGAGAGAGGGATCCATGAGTGTGATAGGTAGG-3') and primer OM088 (5'-GAGGAAGAAGGATCCGGGTC-TATATACTACTCT-3') have 5' BamHI restriction site extensions and were designed according to the 5' and 3' end sequences, respectively, of the *SLC1* gene. This reaction yielded an *SLC1-1* PCR fragment with a BamHI site at both ends. The *SLC1-1* PCR fragment therefore represents the suppressor allele of the *SLC1* gene, with nucleotide T substituting for nucleotide A at position 131, resulting in an amino acid residue change from glutamine to leucine at residue 44. The fragment was digested with BamHI and ligated into the BamHI cloning site located between the tandem cauliflower mosaic virus 35S promoter and nopaline synthase terminator in vector pBI524 (obtained from R. Datla, National Research Council of Canada, Plant Biotechnology Institute, as described by Datla et al. [1993]) to give vector *SLC1-1*-pBI524. The orientation of *SLC1-1* in the vector

SLC1-1-pBI524 was verified by restriction digestion with BglIII, which cuts *SLC1-1* at nucleotide 377 from the 5' end and immediately downstream of the tandem 35S promoter in vector pBI524. The translation initiation codon of *SLC1-1* is maintained, and hence the construct is a transcriptional fusion. The HindIII and EcoRI fragment containing a tandem 35S promoter, alfalfa mosaic virus enhancer, *SLC1-1* coding sequence, and nopaline synthase terminator was freed from *SLC1-1*-pBI524 and cloned into the EcoRI-HindIII site of vector pRD400 (also obtained from R. Datla; Datla et al., 1992). The final vector p*SLC1-1*/pRD400 was introduced into *Agrobacterium tumefaciens* GV3101 (bearing helper plasmid pMP90; Koncz and Schell, 1986) by electroporation.

Construction of *SLC1-1* and *SLC1* Multicopy Vectors and Transformation of Yeast *SLC* Deletion Strain

Genomic DNA of *Saccharomyces cerevisiae* was used as a template to amplify the coding region of *SLC1* with two primers, OM089 (5'-TGA-ATTCCGATCCGTTAAC AATGAGTGTGATAGG-3') and OM088 (described above). The coding regions of the PCR-amplified alleles *SLC1* and *SLC1-1* (described previously) were confirmed by sequencing and demonstrated that the expected point mutation (a T at nucleotide 131) exists in *SLC1-1*. The PCR fragments were digested with BamHI and cloned into the corresponding site in vector pYEUra3 (Clontech, Palo Alto, CA). The orientation of the inserts was checked using XbaI and BglIII double digestion. Constructs with *SLC* transcription under the control of the *GAL1* promoter released a fragment of ~600 bp. Because both *SLC1* and *SLC1-1* fragments have their own initiating ATG codons, the products expressed are not fusion proteins.

An *SLC* deletion strain (YMN5; *slc1Δ2::LEU2 ura3*) was kindly provided by M.M. Nagiec and R.C. Dickson (Nagiec et al., 1993). Yeast transformation was performed according to Elble (1992). Nontransformed YMN5 (*SLC* deletion mutant) cells and transformants containing only vector were used as controls. Single colonies were cultured overnight in 20 mL of SD medium (synthetic dextrose medium with glucose and without uracil, as described by Ausubel et al. [1995]) on a rotary shaker (270 rpm) at 28°C. Cells were pelleted from the overnight culture and resuspended in 50 mL of either SD medium containing glucose and without uracil (noninduction medium) or medium for induction of expression (SD medium containing galactose and without uracil). Cells were reincubated at 28°C, with shaking at 270 rpm, and they were harvested after 6 or 24 hr.

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Columbia was obtained originally from G. Haughn (University of Saskatchewan, Saskatoon, Canada). *Brassica napus* cvs Hero (Scarath et al., 1991) and Reston were obtained from the Plant Science Department of the University of Manitoba (Winnipeg, Canada). *B. carinata* breeding line C90-1163 was obtained courtesy of G. Rakow (Agriculture and Agri-Food Canada Research Centre, Saskatoon).

All *Arabidopsis* control and transgenic plants were grown at the same time in controlled growth chambers, under continuous fluorescent illumination (150 to 200 $\mu\text{E m}^{-2} \text{sec}^{-1}$) at 22°C, as described by Katavic et al. (1995). All control and transgenic *B. napus* and *B. carinata* plants were grown simultaneously in the Kristjansson Biotechnology Complex greenhouses (Saskatoon) under natural light

conditions supplemented with high-pressure sodium lamps with a 16-hr photoperiod (16 hr of light and 8 hr of darkness) at 22°C and a relative humidity of 25 to 30%.

Plant Transformation

Agrobacterium bearing the *SLC1-1/RD400* construct was used to transform *Arabidopsis* by wound inoculation or vacuum infiltration methods. In high erucic acid *B. napus* cv Hero and Reston and in *B. carinata* breeding line C90-1163, transformation was by cocultivation of cotyledonary petioles and hypocotyl explants.

Wild-type *Arabidopsis* ecotype Columbia plants were grown in soil. Transformation was performed by wound inoculation (Katavic et al., 1994) or vacuum infiltration (Bechtold et al., 1993) with a bacterial suspension (which had been cultured overnight) of *Agrobacterium* strain GV3101 bearing helper nopaline synthase plasmid pMP90 (disarmed Ti plasmid with intact *vir* region acting in *trans* with gentamycin and rifampicin selection markers; Koncz and Schell, 1986) and binary vector p*SLC1-1/pRD400* containing a kanamycin selection marker. After inoculation or infiltration, plants were grown to set seeds (T_1). Mature T_1 seeds were harvested in bulk and screened on selective medium with 50 mg/L kanamycin. After 2 to 3 weeks on selective medium, seedlings were transferred to soil. Leaf DNA was isolated from kanamycin-resistant T_1 plants and analyzed by PCR amplification of the *SLC1-1* fragment. Developing leaves from T_1 plants as well as mature T_2 seeds were used for lipid and biochemical analyses. Developing leaves and mature seeds from nontransformed wild-type Columbia plants and from plants transformed with either pB1121 (binary vector pB1121 containing the kanamycin selection marker and the β -glucuronidase reporter gene; Jefferson et al., 1987) or pRD400 (containing the kanamycin selection marker; Datla et al., 1992), both without the *SLC1-1* insert, were used as controls in analyses of seed lipids. Based on these analyses, T_2 seeds of lines exhibiting changed acyl composition and/or lipid content were grown on selective medium (to eliminate homozygous wild-type segregants) and then transferred to soil to yield T_3 seeds.

Transformation experiments performed with *B. napus* cv Hero, using *Agrobacterium* bearing the *SLC1-1/RD400* construct, utilized hypocotyl explants and a modified method of DeBlock et al. (1989). To optimize transformation conditions, modifications of the hypocotyl-explant transformation method (DeBlock et al., 1989) included (1) preculture of explants on agar-solidified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with the growth regulators 2,4-D and kinetin; (2) cocultivation of hypocotyl explants with *Agrobacterium* in Petri dishes on sterile filter paper with the same medium as that used for preculture; (3) after cocultivation, a 7-day explant recovery period on medium with 2,4-D and kinetin, and with timentin for *Agrobacterium* elimination; (4) regeneration of transgenic shoots on MS medium with the cytokinins benzyladenine and zeatin, the ethylene inhibitor silver nitrate, and the antibiotics timentin (for *Agrobacterium* elimination) and kanamycin (for transformed-cell and shoot selection). For transformation of *B. napus* cv Reston and *B. carinata* plants, cotyledonary petiole explants were used, and the method of Moloney et al. (1989) was modified to include the introduction of a 7-day explant recovery period after cocultivation on MS medium supplemented with benzyladenine and timentin.

Green shoots were rooted and transferred to soil. Genomic DNA was isolated from developing leaves, and PCR and gel blot analyses (Southern, 1975) were performed. Seeds (T_1) from transgenic plants were harvested, and from each transgenic line, 10 T_1 plants were

grown in soil. Mature T_2 seeds from these plants were harvested and subjected to lipid and biochemical analyses.

Lipid Substrates, Chemicals, and Biochemicals

$1\text{-}^{14}\text{C}$ -labeled 18:1, 22:1, and 24:0 free fatty acids (50 to 55 mCi/mmol) were purchased from Amersham Canada (Oakville, Ontario), Du Pont Canada (Mississauga, Ontario), and American Radiolabeled Chemicals (St. Louis, MO), respectively. $1\text{-}^{14}\text{C}$ -labeled 20:1 was synthesized as described previously (Taylor et al., 1992). $1\text{-}^{14}\text{C}$ -labeled acyl-CoA thioesters were then synthesized by the method of Taylor et al. (1990). *sn*-1-18:1-lysophosphatidic acid (18:1-LPA), *sn*-1-18:1-lysophosphatidylcholine (18:1-LPC), *sn*-1-18:1-lysophosphatidylethanolamine (18:1-LPE), *sn*-1-acyl lysophosphatidylinositol (LPI), CoA, ATP, and all other biochemicals were purchased from Sigma. Silica G and silica H were purchased from Merck (Darmstadt, Germany). All solvents were of HPLC grade or better and were purchased from BDH (Toronto, Ontario).

Lipid Analyses

Mature seeds were harvested from *SLC1-1* transgenic plants, nontransformed wild-type control plants, or control transgenic plants transformed with pB1121 or pRD400 without the *SLC1-1* insert. For leaf lipid assays, leaves at midexpansion were chosen from control and transgenic plants. In the *Arabidopsis* leaf analyses, two to three whole leaves were utilized, whereas in the case of *B. napus* transformants, 1-cm-diameter leaf discs were sampled from several leaves and pooled. In the case of *Arabidopsis* plants, because of the extremely small seed size and weight, analyses were performed on 50- or 100-seed replicates that were carefully counted under a dissecting microscope. When dry weights per sample were required, 400-seed equivalents were counted and weighed, and the values were used to calculate the equivalent lipid content for a 100-seed sample. Seed samples were ground using a polytron in chloroform-isopropanol (2:1 [v/v]) containing 0.2% (w/v) butylated hydroxytoluene and triptadecanoic as an internal standard. All other conditions for the isolation and gas chromatography analyses of seed and leaf lipids for total fatty acid content and fatty acid composition (expressed as weight % of total fatty acids) were as described previously (Taylor et al., 1992, 1995b; Katavic et al., 1995). Triacylglycerol (TAG) species were analyzed by high-temperature gas chromatography, as described by Katavic et al. (1995), and stereospecific analyses of TAGs were performed using pancreatic lipase on intact seed lipids (chiefly TAGs), as described by Christie (1982).

sn-2 Acyltransferase Assays

Cells of yeast transformants and control cultures (induced with galactose) and noninduced cultures (with glucose) were harvested by centrifugation at 5000 rpm for 5 min and resuspended in 100 mM Hepes-NaOH, pH 7.4, containing 1 mM EDTA and 1 mM DTT. Cell lysates were prepared using acid-washed glass beads as described by Ausubel et al. (1995), and assays of lysophosphatidic acid acyltransferase (LPAT) activity in lysate protein (30 to 50 μg) were conducted using the protocols described below.

For *Arabidopsis*, 25 to 30 siliques were harvested at 15 to 18 days postanthesis to isolate midcotyledonary T₃ embryos from both controls (the nontransformed wild type and the pBI121 control transformant) and selected transgenic plants. *B. napus* T₂ embryos at the midcotyledonary stage of development were harvested from three to six siliques of nontransformed control and selected transgenic plants. All plant material was frozen immediately in liquid nitrogen and stored at -70°C until homogenized. Homogenates of developing seed tissues were prepared as described by Taylor et al. (1995b).

LPAT assays were conducted at pH 7.4, with shaking at 100 rpm in a water bath at 30°C for 15 to 30 min. Assay mixtures (0.5 mL final volume) contained homogenate or lysate protein (10 to 200 µg), 90 mM Hepes-NaOH, 0.5 mM ATP, 0.5 mM CoA, 1 mM MgCl₂, 45 µM *sn*-1-oleoyl-LPA, and 18 µM 1-¹⁴C-labeled 18:1-CoA, 1-¹⁴C-labeled 20:1-CoA, 1-¹⁴C-labeled 22:1-CoA, or 1-¹⁴C-labeled 24:0-CoA (each at a specific activity of 10 nCi/nmol) as the acyl donor. Control reactions were conducted in the absence of protein and in the absence of an exogenous acyl acceptor. All other conditions for the measurement of LPAT activity were as detailed previously (Taylor et al., 1995b). Protein in yeast lysates or seed homogenates was measured using the Bradford (1976) assay.

LPI, LPC, and LPE acyltransferase activities were assayed under conditions identical to those described for LPAT, except that 45 µM *sn*-1-acyl-LPI, *sn*-1-oleoyl-LPC, or *sn*-1-oleoyl-LPE was substituted for LPA as the acyl acceptor. ¹⁴C-labeled phosphatidylinositol (PI), ¹⁴C-labeled phosphatidylcholine (PC), or ¹⁴C-labeled phosphatidylethanolamine (PE) was resolved by thin-layer chromatography on 0.3-mm silica H plates developed fully in CHCl₃-MeOH-acetic acid-water (25:15:4:2 [v/v]); Christie, 1982). The ¹⁴C-labeled PI (R_f ≈ 0.5), PC (R_f ≈ 0.25), and PE (R_f ≈ 0.75) were identified by cochromatography with authentic standards, and the regions were scraped from the thin-layer chromatography plate and counted as described previously (Taylor et al., 1995b).

¹H-Nuclear Magnetic Resonance Analyses of Mature Seeds

¹H-nuclear magnetic resonance (¹H-NMR) analyses for relative oil yield (Rutar, 1989) were conducted with intact seeds of control and *SLC1-1*-transformed *B. napus* cv Hero, using a Bruker AM wide-bore spectrometer (Bruker Analytische Masstechnik GHBH, Silberstreifen, Germany) operating at 360 Mhz. To reduce anisotropic line broadening, the seeds (35 per sample) were rotated at 1 kHz in a zirconium rotor oriented 54.7° to the magnetic field (magic angle sample spinning; Rutar, 1989).

ACKNOWLEDGMENTS

We thank Drs. Robert C. Dickson and M. Marek Nagiec (University of Kentucky, Lexington) for kindly supplying plasmid p411ΔB/C, the *SLC* deletion strains YMN5 and YMN6-2C, and yeast transformation vector YEp351 and for helpful discussions during the course of this work. We also gratefully acknowledge Drs. Peter B.E. McVetty and Rachael Scarth (Department of Plant Science, University of Manitoba, Winnipeg) for supplying seed of *B. n.* Hero and Reston; Dr. Gerhard Rakow (Agriculture and Agri-Food Canada, Saskatoon) for supplying seed of *B. carinata* C90-1163; Dr. Lawrence Pelcher, Barry Panchuk, and Don Schwab (Plant Biotechnology Institute, DNA Technologies Unit) for DNA sequencing and construction of PCR

primers; Brock Chatson for performing the ¹H-NMR analyses; Vivian Babic for assistance with *B. carinata* transformation; and Drs. Adrian J. Cutler and Fawzy Georges for critical reviews of the manuscript. The final vector used in this study (pSLC1-1/pRD400) has been submitted under the terms of the Budapest Treaty to the American Type Culture Collection (No. ATCC 97545). This investigation was partially supported by the National Research Council Biotechnology Contributions Agreement No. GC103-3-2024 awarded to CanAmera Foods (Oakville, Ontario). This is National Research Council of Canada Publication No. 40709.

Received February 3, 1997; accepted March 26, 1997.

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