

Seed-Specific Heterologous Expression of a Nasturtium *FAE* Gene in *Arabidopsis* Results in a Dramatic Increase in the Proportion of Erucic Acid¹

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The fatty acid elongase [often designated *FAE* or β -(or 3-) ketoacyl-CoA synthase] is a condensing enzyme and is the first component of the elongation complex involved in synthesis of erucic acid (22:1) in seeds of garden nasturtium (*Tropaeolum majus*). Using a degenerate primers approach, a cDNA of a putative embryo *FAE* was obtained showing high homology to known plant elongases. This cDNA contains a 1,512-bp open reading frame that encodes a protein of 504 amino acids. A genomic clone of the nasturtium *FAE* was isolated and sequence analyses indicated the absence of introns. Northern hybridization showed the expression of this nasturtium *FAE* gene to be restricted to the embryo. Southern hybridization revealed the nasturtium β -ketoacyl-CoA synthase to be encoded by a small multigene family. To establish the function of the elongase homolog, the cDNA was introduced into two different heterologous chromosomal backgrounds (*Arabidopsis* and tobacco [*Nicotiana tabacum*]) under the control of a seed-specific (napin) promoter and the tandem 35S promoter, respectively. Seed-specific expression resulted in up to an 8-fold increase in erucic acid proportions in *Arabidopsis* seed oil, while constitutive expression in transgenic tobacco tissue resulted in increased proportions of very long chain saturated fatty acids. These results indicate that the nasturtium *FAE* gene encodes a condensing enzyme involved in the biosynthesis of very long chain fatty acids, utilizing monounsaturated and saturated acyl substrates. Given its strong and unique preference for elongating 20:1-CoA, the utility of the *FAE* gene product for directing or engineering increased synthesis of erucic acid is discussed.

Very long chain fatty acids (VLCFAs) with 20 carbons or more are widely distributed in nature. In plants, they are mainly found in epicuticular waxes and in the seed oils of a number of plant species, including members of the Brassicaceae, Limnantheceae, Simmondsia, and Tropaeolaceae (Harwood, 1996; Post-Beittenmiller, 1996; Ghanevati and Jaworski, 2001). A strategic goal in oilseed modification is to genetically manipulate high erucic acid germ plasm of the Brassicaceae to increase the content of erucic acid (22:1 Δ 13) and other strategic VLCFAs in the seed oil for industrial niche market needs (Puyaubert et al., 2001; Taylor et al., 2001). Erucic acid and its derivatives are feedstocks in manufacturing slip-promoting agents, surfactants, plasticizers, nylon 1313, and surface coatings; more than 1,000 patents have been issued (Sonntag, 1991, 1995; Leonard, 1994). The current

market for high erucate oils exceeds \$120 million U.S./annum. Worldwide erucic acid demand is predicted to increase from about 40 million pounds (M pds) in 1990 to about 80 M pds by the year 2010. Similarly, demand for the derivative, behenic acid, is predicted to triple to about 102 M pds by 2010 (Sonntag, 1995). In recent years, production has increased to meet market needs, and high erucic acreage in western Canada is currently at a record high (D. Males, Saskatchewan Wheat Pool, personal communication). A Brassica cultivar containing erucic acid levels approaching 80% would significantly reduce the cost of producing erucic acid and its derivatives, and could meet the forecast demand for erucic and behenic acids as renewable, environmentally friendly industrial feedstocks (Leonard, 1994; Taylor et al., 2001).

VLCFAs are synthesized outside the plastid by a membrane-bound fatty acid elongation complex (elongase) using acyl-CoA substrates. The first reaction of elongation involves condensation of malonyl-CoA with a long chain substrate producing a β -ketoacyl-CoA. Subsequent reactions are reduction of β -hydroxyacyl-CoA, dehydration to an enoyl-CoA, followed by a second reduction to form the elongated acyl-CoA (Ghanevati and Jaworski, 2001). The β -ketoacyl-CoA synthase (KCS) catalyzing the con-

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denation reaction plays a key role in determining the chain length of fatty acid products found in seed oils and is the rate-limiting enzyme for seed VLCFA production (Millar and Kunst, 1997; Katavic et al., 2001). The composition of the fatty acyl-CoA pool available for elongation and the presence and size of the neutral lipid sink are additional important factors influencing the types and levels of VLCFAs made in particular cells (Zou et al., 1997; Lardizabal et al., 2000; Han et al., 2001).

Our knowledge of the mechanism of elongation and properties of FAE1 and other elongase condensing enzymes is, in part, limited by their membrane-bound nature—as such they are more difficult to isolate and characterize than soluble condensing enzymes (Ghanevati and Jaworski, 2001; Katavic et al., 2002). The genes encoding FAE1 and its homologs have been cloned from *Arabidopsis* (James et al., 1995), *jojoba* (*Simmondsia chinensis*; Lassner et al., 1996), and *Brassica napus* (Barret et al., 1998).

Ghanevati and Jaworski (2001) have performed site-directed mutagenesis experiments on the *Arabidopsis* FAE1 to decipher the importance of Cys and His as residues in condensing enzyme catalysis; their results have shown that Cys-223 and four His residues are essential for the enzyme activity.

In this study, we selected garden nasturtium (*Tropaeolum majus*) as a source of the elongase involved in VLCFA synthesis based on the fact that this plant is capable of producing significant amounts of erucic acid (70%–75% of total fatty acid; Pollard and Stumpf, 1980) and accumulates trierucin as the predominant triacylglycerol (TAG) in its seed oil (Taylor et al., 1992a). Here, we report the isolation of a nasturtium FAE gene and demonstrate the involvement of its encoded protein in the elongation of saturated and especially monounsaturated fatty acids.

RESULTS

Acyl Composition of Nasturtium cv Dwarf Cherry Rose

The acyl composition of the TAG fraction of this cultivar was typical in that it had highly enriched proportions of VLC monounsaturated fatty acids, particularly 22:1 (77.5%) and 20:1 (16.0%) with a trace of 24:1 (1.5%), and a low proportion of total C₁₈ fatty acids (2.5%), primarily oleate (18:1 Δ⁹; 2.4%). The predominant TAG species were trierucin followed by 22:1/20:1/22:1 (Taylor et al., 1992a, 1993).

Substrate Specificity of Nasturtium Embryo Elongases in Vitro

Although there has been considerable debate regarding the acyl substrate for elongase activity in developing oilseeds (Hlousek-Radojic et al., 1995), recent studies of developing seeds of *B. napus* have revealed the presence of two types of elongation activity in vitro: an acyl-CoA-dependent activity and

an ATP-dependent activity that apparently utilizes an endogenous acyl primer (Domergue et al., 1999). A 15,000g particulate fraction was isolated from nasturtium embryos collected at mid-development (at 14–17 d after pollination), the stage which exhibited the highest enrichment in acyl-CoA-dependent elongase activity.

It has been shown that while ATP is necessary for acyl-CoA-dependent elongation in vitro, too high a concentration of ATP strongly inhibited elongase activity (Murphy and Mukherjee, 1989; Domergue et al., 1999). In addition, elongase enzyme activity has been reported to be stimulated by the presence of 10 μM CoA-SH (Barret and Harwood, 1997). In order to optimize reaction conditions, we assessed the roles of these two cofactors. Elongase activity was measured in vitro in the 15,000g particulate fraction from nasturtium embryos under different ATP concentrations (0–5 mM) in the presence of 10 μM CoA-SH with 18 μM [1-¹⁴C]18:1-CoA and 200 μM malonyl-CoA. The highest activity was found at cofactor concentrations of 0.75 mM for ATP and 10 μM for CoA-SH (data not shown). These were the reaction conditions used to examine activity with a range of [1-¹⁴C]acyl-CoA substrates of different chain length and degree of saturation. Our results indicate that in a developing nasturtium embryo particulate fraction, acyl-CoA-dependent elongases have the capacity to elongate a wide range of saturated (C₁₆–C₂₀) and monounsaturated (C₁₈ and C₂₀) fatty acyl moieties (Fig. 1). Of the [1-¹⁴C]-labeled acyl-CoA series (16:0-CoA, 18:0-CoA, 18:1-CoA, 20:0-CoA, 20:1-CoA, 22:1-CoA), tested in vitro, elongase(s) from mid-developing nasturtium embryos exhibited the highest activity with 18:1-CoA and 20:1-CoA (102 and 95 pmol min⁻¹ mg⁻¹ protein, respectively). These elongase activity

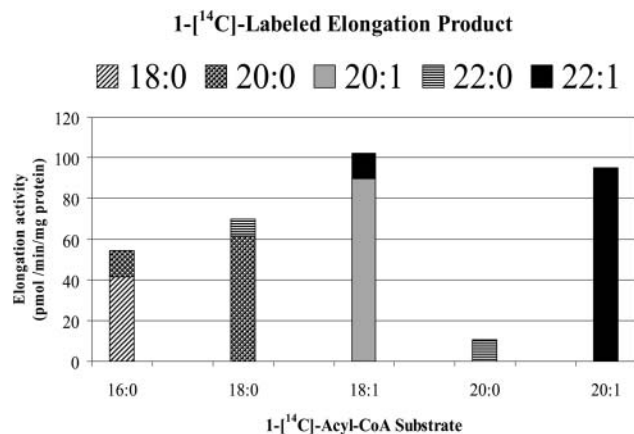


Figure 1. Substrate specificity of elongase(s) from mid-developing nasturtium embryos. A total of 200 μg of protein from a 15,000g particulate fraction was used in the elongase assay. Reaction conditions were as described in “Materials and Methods.” Results represent the average of three replicates. For each [1-¹⁴C]acyl-CoA substrate, the relative proportional distribution of radiolabeled fatty acid elongation product(s) is demarcated.

rates are of the same order of magnitude as that reported for acyl-CoA elongase(s) in a similar particulate fraction from developing rapeseed embryos (Puyaubert et al., 2001). The particulate fraction was also able to elongate, in order of specificity, the saturated substrates 18:0-CoA, 16:0-CoA, and, to a much lesser extent, 20:0-CoA. In general, regardless of the [^{14}C]acyl-CoA substrate supplied in vitro, the major labeled fatty acyl product was the C_2 extension of its respective precursor (about 80%–90%), with the next respective C_4 extension product being present in proportions of about 10% to 20% (Fig. 1). The one critical exception to this trend was the production solely of radiolabeled erucic acid from its respective [^{14}C]eicosenoyl-CoA precursor. There was no detectable elongation of [^{14}C]-labeled 22:1-CoA to 24:1 (data not shown), even though the latter is found in trace amounts in nasturtium seed oil.

Isolation of Nasturtium FAE Homolog

Based on sequence homology among plant fatty acid elongase genes, a full-length cDNA clone was amplified by PCR using a degenerate primers approach and the sequence submitted to GenBank (accession no. AY082610). The nucleotide sequence had an open reading frame of 1,512 bp. Subsequently, 3 partial clones of about 0.6 kb, representative of the AY082610 FAE clone, were found among 2,800 expressed sequence tags surveyed (about 0.1% representation) from a nasturtium embryo subtracted cDNA library.

The nasturtium FAE cDNA encodes a polypeptide of 504 amino acids that is most closely related to an FAE2 from roots of maize (*Zea mays*; 69% amino acid identity; Schreiber et al., 2000; Fig. 2). The nasturtium FAE polypeptide also shared strong identity with FAEs from *Limnanthes douglasii* (67%; Cahoon et al., 2000; Marillia et al., 2002) and from seeds of jojoba (63%; Lassner et al., 1996). Homology of the nasturtium FAE to two Arabidopsis β -KCSs AraKCS (Todd et al., 1999) and AraCUT1 (Millar et al., 1999) involved in cuticular wax synthesis was on the level of 57% and 53%, respectively. These homologs all exhibit the capability to elongate saturated fatty acids to produce saturated VLCFAs. The FAE1 polypeptides involved in the synthesis of VLCFAs in Arabidopsis (James et al., 1995) and Brassica seeds (Clemens and Kunst, 1997; Katavic et al., 2002) showed approximately 52% to 54% identity with the nasturtium FAE. The nasturtium FAE protein was predicted to have a theoretical pI value of 9.3 using the algorithm of Bjellqvist et al. (1993, 1994) and a molecular mass of 56.8 kD, which are similar to the respective values reported for the *B. napus* CE7 and CE8 FAE homologs (Barret et al., 1998) as well as those from *Brassica rapa* (*campestris*) and *Brassica oleracea* (Das et al., 2002).

Alignment of the amino acid sequence of the nasturtium FAE with other plant condensing enzymes

revealed the presence of six conserved Cys residues (Fig. 2). Further sequence analysis showed that one out of the four conserved His residues suggested to be important for Arabidopsis FAE1 activity (Ghanevati and Jaworski, 2001) was substituted with Tyr at residue position 429 in the nasturtium FAE polypeptide.

A hydropathy analysis (Kyte-Doolittle) of the amino acid sequence of the nasturtium FAE revealed several hydrophobic domains (Fig. 3A). Protein analyses with the TMAP algorithm (Persson and Argos, 1994) predicted two N-terminal transmembrane domains, the first corresponding to amino acid residues 35 to 55 and the second spanning residues 68 to 88 (Fig. 3B). Like other elongase condensing enzymes, the nasturtium FAE lacks N-terminal signal sequences typically found for plastidial or mitochondrial-targeted plant enzymes (Emanuelsson et al., 2000). It also lacks a KXXXX or KKXX motif (X = any amino acid) often found at the C terminus of proteins retained within endoplasmic reticulum membranes (Jackson et al., 1990). Rather, it is a type IIIa protein, typically present on endoplasmic reticular membranes (Nakai and Kanehisa, 1992; Das et al., 2002).

An analysis of the nucleotide sequence of the corresponding nasturtium FAE genomic clone (data not shown) revealed the absence of intron sequences. A similar absence of introns was observed in homologs from Arabidopsis FAE1 (Millar and Kunst, 1997), rapeseed CE7 and CE8 (Barret et al., 1998), and high and low erucic lines of *B. oleracea*, *B. rapa*, *B. napus* cv Westar, and *B. napus* cv Hero (Das et al., 2002; Katavic et al., 2002).

Tissue-Specific Expression and Copy Number Estimate of Nasturtium FAE

Northern-blot analyses were performed to investigate the expression profile of the FAE gene. Total RNA was isolated from different nasturtium tissues, including roots, leaves, floral petals, and mid-developing embryos. A strong hybridization signal with FAE-specific probe was observed only with RNA isolated from developing embryos (Fig. 4A).

A Southern-blot hybridization was performed with nasturtium genomic DNA digested with several restriction enzymes, including *EcoRI*, *AccI*, *NcoI*, and *HindIII*. The FAE gene has no internal *EcoRI*, *AccI*, or *NcoI* sites, while one internal *HindIII* site exists. Autoradiography revealed the presence of one strongly hybridizing fragment in all cases except with *HindIII*, for which two strongly hybridizing fragments were evident (Fig. 4B). In addition, a minimum of four weakly hybridizing fragments were detected. After washing under high stringency conditions, the number of hybridizing fragments was unchanged. Thus, we have concluded that nasturtium FAE belongs to a multigenic family consisting of four to six members. A similar multigenic family has been found for a rapeseed FAE1 gene member (Barret et al., 1998).

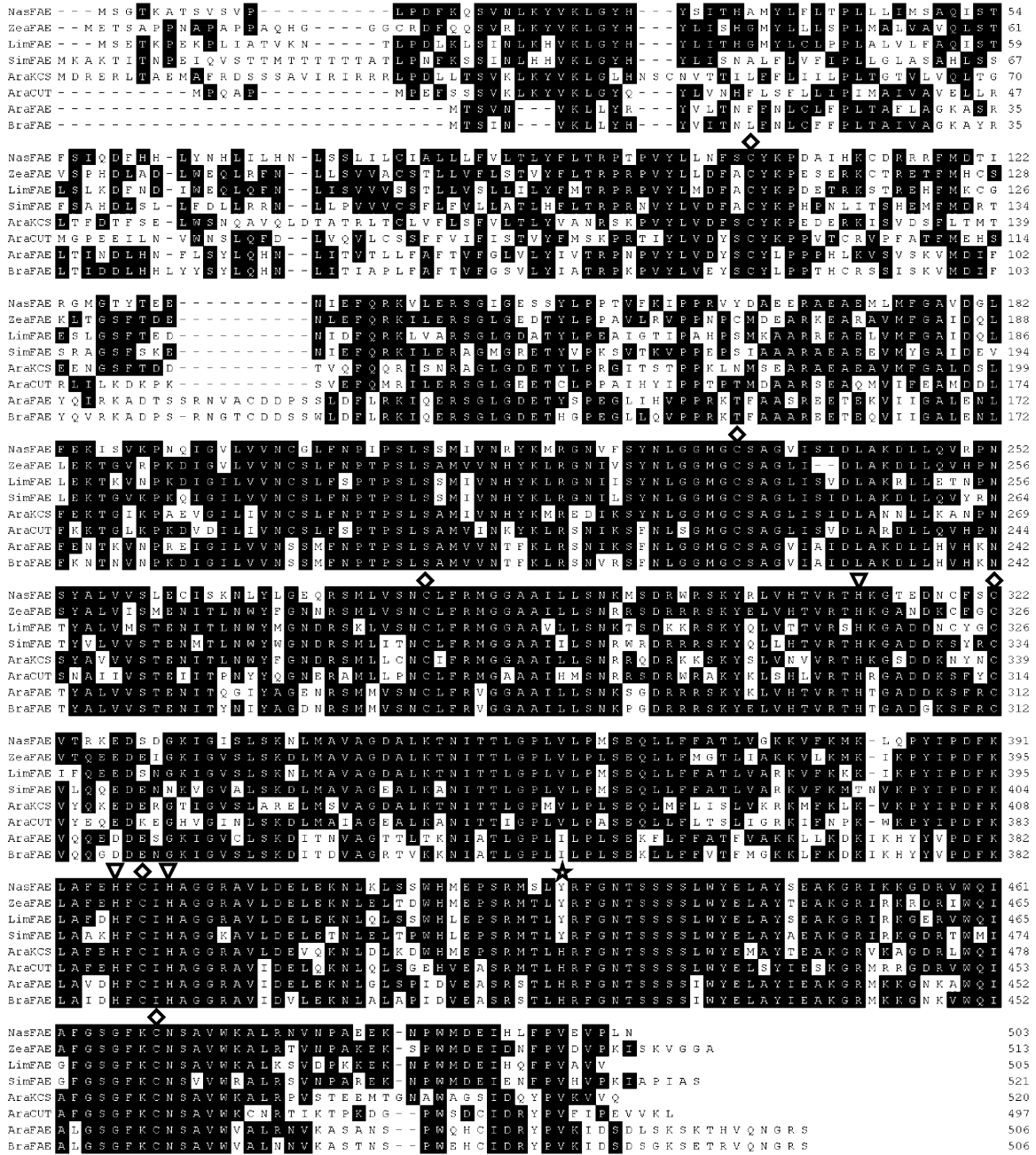


Figure 2. Comparison of the amino acid sequences of the nasturtium FAE homolog (NasFAE, GenBank accession no. AY0826190) with FAE1 and related β -KCSs from other plant species. The alignment contains the sequences of the maize (ZeaFAE, GenBank accession no. AJ292770), Limnanthes (LimFAE, GenBank accession no. AF247134), jojoba (SimFAE, GenBank accession no. U37088), Arabidopsis (AraFAE, GenBank accession no. U29142), Brassica (BraFAE, GenBank accession no. AF009563), and two Arabidopsis β -KCSs associated with wax synthesis (AraKCS, GenBank accession no. AF053345, and AraCUT, GenBank accession no. AF129511). Conserved Cys and His residues are labeled with diamonds and triangles, respectively. Tyr at residue position 429 in the nasturtium FAE polypeptide is indicated by a star.

Heterologous Expression of the Nasturtium FAE in Yeast

To study the function of the protein encoded by the nasturtium FAE, the coding region was linked to the Gal-inducible GAL1 promoter in the expression vector pYES2.1/V5-His-TOPO and transformed into yeast

cells: strain Inv Sc1 (Invitrogen) and two mutant strains *elo2* Δ and *elo3* Δ (Oh et al., 1997). None of the transgenic yeast strains harboring the nasturtium FAE showed any difference in fatty acid composition in comparison to yeast cells transformed with empty vector (data not shown). To study the elongase activity, microsomal proteins from yeast mutant strains *elo2* Δ

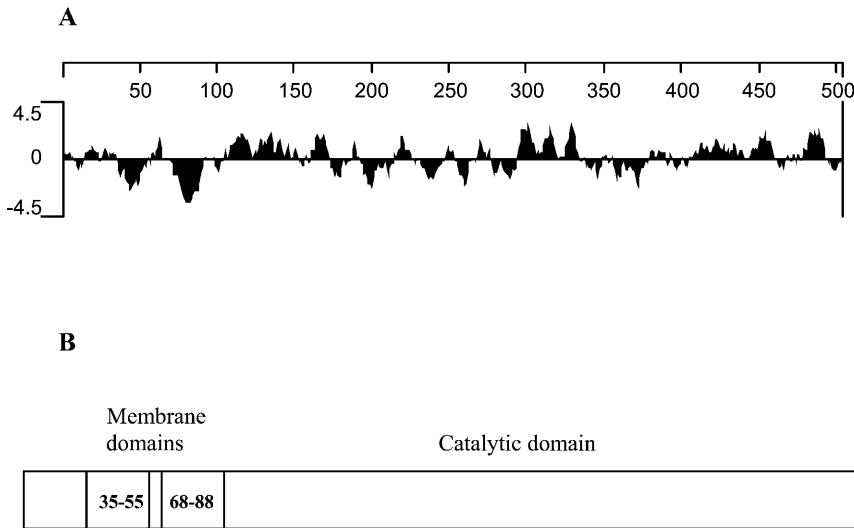


Figure 3. Hydropathy analysis of nasturtium FAE. A, Hydropathy plot of FAE indicating the presence of several hydrophobic regions. B, Schematic representation of the putative transmembrane domains of nasturtium FAE amino acid sequence as predicted by TMAP analysis. Numbers shown in the boxes correspond to the residues of each domain in the FAE polypeptide.

harboring nasturtium FAE were incubated with a range of the [^{14}C]-labeled acyl-CoA substrates (16:0-CoA, 18:0-CoA, 18:1-CoA, 20:1-CoA, 22:1-CoA). Nasturtium FAE did not show any elongase activity in vitro with the [^{14}C]-labeled acyl-CoA substrates, while a high

activity for Arabidopsis FAE1 with 18:1-CoA substrate was found (data not shown). Western-blot analyses performed with anti-His (C-terminal) FAE antibody did not detect any nasturtium FAE/V5-His fusion protein, while in the control, a strong band of about 61 kD corresponding to Arabidopsis FAE1/V5-His was observed (data not shown). The reason for the inability to functionally express the nasturtium FAE in these various yeast strains is not known. As described above, a *Limnanthes* and a maize FAE showed the highest homology to the nasturtium FAE polypeptide. Therefore, in the current context it is interesting to note that similar difficulties with expression of the *Limnanthes* and maize FAEs in yeast cells have been experienced by other researchers (E. Cahoon and R. Lessire, respectively, personal communications).

As indicated earlier, a comparison of the predicted amino acid sequence of the nasturtium FAE with other plant condensing enzymes (Fig. 2) showed that one of the four conserved His residues, suggested to be important for Arabidopsis FAE1 activity in yeast cells (Ghanevati and Jaworski, 2001), was substituted with Tyr in the nasturtium FAE polypeptide. To study the importance of His residue for enzyme activity, we used a site-directed mutagenesis approach to replace the Tyr-429 residue with His. This mutated version of nasturtium FAE was expressed in yeast cells. Analyses of fatty acid composition of transformed yeast cells showed that His at position 429 did not restore enzyme activity (data not shown). Therefore, we decided to study the function of nasturtium FAE in plant heterologous chromosomal backgrounds.

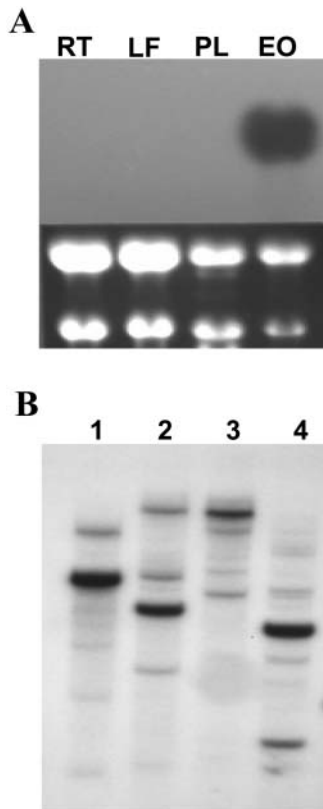


Figure 4. Northern and Southern analyses of nasturtium FAE. A, Northern analysis of FAE gene expression in nasturtium. Total RNA was isolated from roots (RT), leaves (LF), petals (PL), and embryos (EO). B, Southern-blot analysis of the FAE gene in nasturtium. Genomic DNA was digested with restriction enzymes *EcoRI* (lane 1), *Accl* (lane 2), *NcoI* (lane 3), and *HindIII* (lane 4).

Expression of Nasturtium FAE in Tobacco Plants

To establish functional identity, the cDNA for the FAE-related polypeptides was expressed in tobacco (*Nicotiana tabacum*) plants under the control of the tandem 35S constitutive promoter. In addition, to assess the importance of His residues for enzyme

activity, the Tyr at position 429 in the nasturtium FAE was replaced with His and subsequently used to prepare a plant transformation vector under the control of the tandem 35S promoter. Integration of the 35S/*FAE*/Nos expression cassette into tobacco plants was confirmed by PCR amplification on genomic DNA (data not shown). Fatty acid composition was determined in callus, leaves, and seeds of transgenic tobacco plants.

Expression of the nasturtium *FAE* homolog in tobacco callus resulted in an increase in proportions of VLCFAs from 3.7% in the wild-type background to as high as 8.6% (a 132% increase) in transgenic lines (Table I). In particular, the increase in proportions of saturated VLCFAs (22:0, 24:0, 26:0) was most pronounced. The fact that the synthesis of the saturated VLCFAs occurs at the expense of 16:0 and 18:0 suggests that the nasturtium *FAE* is able to elongate C₁₆ and C₁₈ fatty acids. Expression of the mutated version of the nasturtium *FAE* (SMF) resulted in a slight increase in the VLCFA content in tobacco callus, on average 18.5% in comparison to the wild-type background. Increased proportions of VLCFAs at the expense of LCFAs were observed in leaves of transgenic tobacco plants carrying either the nasturtium *FAE* or its mutated form (Table II). Comparison of fatty acid composition in tobacco tissues upon expression of the nasturtium *FAE* and its mutated version revealed that Tyr at position 429 is likely important to achieve full activity of the enzyme. A decreased proportion of 18:3 in leaves of transgenic tobacco lines in comparison to the wild-type (empty vector) background suggests that the metabolic pull of the elongation pathway may be somewhat stronger than that of the competing desaturation pathway.

Expression of nasturtium *FAE* in tobacco seeds resulted in a 50% increase in proportions of VLCFAs from 0.6% in the wild-type background to 0.9% in transgenic plants (data not shown). The relatively low proportions of VLCFAs in tobacco leaves and calli (Tables I and II) may be an indication that (1) *in vivo*, saturated fatty acids are not present at high concentrations; therefore, even a 50% increase in relative proportions does not result in high levels of VLC

saturated fatty acids accumulating in glycerolipids; (2) expression of the nasturtium *FAE* when under the control of the 35S promoter is relatively weak.

Expression of Nasturtium *FAE* in Arabidopsis Plants: Constitutive Expression

Since expression of nasturtium *FAE* did not result in a high accumulation of VLCFA in tobacco seeds, we decided to study the effect of expressing it in Arabidopsis. Using a vacuum-infiltration method, 18 kanamycin-resistant plants were obtained. The fatty acid composition of T₂ seeds was determined. A significant increase was observed only in the content of erucic acid (22:1 Δ13). On average, the level of erucic acid increased up to 3.2% (a 50% increase) in transgenic seeds compared to 2.1% in a wild-type background (data not shown). In the best transgenic lines, the content of erucic acid increased up to 4.0% (a 90% increase).

Expression of Nasturtium *FAE* in Arabidopsis Plants: Seed-Specific Expression

Constitutive tandem 35S-driven *FAE* expression did not result in a strong increase in VLCFA proportions in tobacco and Arabidopsis seeds. Thus, we decided to use the seed-specific promoter napin to study *FAE* expression in an Arabidopsis seed background in which total VLCFA proportions in wild-type seed oils are of the order of about 28%. From vacuum-infiltration experiments, 25 kanamycin-resistant T₁ plants were selected. The T₂ progeny were collected individually from each plant and the fatty acid composition determined. Significant changes in fatty acid composition in comparison to the wild type (empty vector) were found. On average, the proportion of erucic acid (22:1 Δ13) increased from 2.1% in wild type to 9.6% in T₂ transgenic seeds at the expense of 20:1 Δ11 (Table III). Homozygous T₃ lines were analyzed to examine the range of VLCFA proportional redistribution induced by expression of the nasturtium *FAE* gene. The 12 best T₃ lines are shown in Figure 5, A and B. The erucic acid content was increased by up to 7- to 8-fold

Table I. Fatty acid composition of transformed tobacco calli

Results represent the average (±SE) of 10 measurements using independent calli. Constructs: RD, Control (plasmid-only) transgenic calli; SF, 35S: nasturtium *FAE* transgenic calli; SMF, 35S: mutated nasturtium *FAE* transgenic calli.

Construct	Fatty Acid Composition							LCFA	VLCFA
	16:0	18:0	20:0	22:0	24:0	26:0			
	% (w/w) of total fatty acids [% increase] ^a								
RD	20.38 ± 0.12	7.99 ± 0.26	1.32 ± 0.03	0.59 ± 0.03	0.70 ± 0.03	0.89 ± 0.16	96.28 ± 0.31	3.72 ± 0.31	
SF	18.01 ± 0.42	5.23 ± 0.41	1.58 ± 0.54	1.32 ± 0.16	1.93 ± 0.27	1.31 ± 0.20	91.37 ± 0.84	8.63 ± 0.84	
			[19.7]	[123.9]	[175.7]	[147.2]		[131.9]	
SMF	19.48 ± 0.34	7.12 ± 0.19	1.30 ± 0.02	0.57 ± 0.03	0.73 ± 0.04	1.01 ± 0.32	95.59 ± 0.40	4.41 ± 0.40	
								[18.5]	

^aRelative to value for calli from RD, the tobacco control (plasmid-only) calli, set at 100%.

Table II. Fatty acid composition of transformed tobacco leaves

Results represent the average (\pm SE) of 10 measurements using leaf discs from 10 independent transgenic plants. Constructs: RD, Control (plasmid-only) transgenic leaves; SF, 35S: nasturtium FAE transgenic leaves; SMF, 35S: mutated nasturtium FAE transgenic leaves.

Construct	Fatty Acid Composition								
	16:0	18:0	18:3	20:0	20:1 Δ 11	22:0	24:0	LCFA	VLCFA
	% (w/w) of total fatty acids [% increase] ^a								
RD	16.32 \pm 0.14	3.94 \pm 0.11	53.30 \pm 0.72	0.53 \pm 0.02	1.18 \pm 0.00	0.27 \pm 0.01	2.74 \pm 0.09	93.77 \pm 0.29	6.23 \pm 0.29
SF	15.83 \pm 0.14	3.35 \pm 0.12	47.02 \pm 0.66	0.91 \pm 0.12	2.34 \pm 0.12	0.42 \pm 0.02	4.14 \pm 0.15	88.64 \pm 0.35	11.36 \pm 0.35
				[71.7]	[98.3]	[55.6]	[51.1]		[82.3]
SMF	15.53 \pm 0.17	4.00 \pm 0.12	47.25 \pm 0.85	0.98 \pm 0.16	2.61 \pm 0.02	0.30 \pm 0.01	3.24 \pm 0.08	90.05 \pm 0.28	9.95 \pm 0.28
				[84.5]	[121.2]		[18.2]		[59.7]

^aRelative to value for leaves from RD, the tobacco control (plasmid-only) plants, set at 100%.

in lines 15-2, 15-10, 16-1, 20-1, and 23-8. Small increases in the proportions of 24:1 Δ 15 were also observed (Table III). There was also a relatively significant increase in the proportions of the saturated VLCFAs 22:0 and 24:0 at the expense of 18:0 and 20:0. In both the case of the VLC monounsaturated fatty acids (Fig. 5A) and the VLC saturated fatty acids (Fig. 5B), the highest proportional increases in erucic and in [behenic + lignoceric] acids were generally correlated with the concomitant reduction in the proportion of their corresponding elongase primers, eicosenoic and [stearate + arachidic] acids, respectively.

DISCUSSION

We have isolated a cDNA clone from nasturtium, which exhibits high similarity to the sequences of β -KCSs from various plant species. Our in vitro studies have determined biochemically the range of acyl-CoA specificities of the nasturtium FAE family using experimental conditions that correctly exclude selective conditions fueling the currently heated debate regarding the acyl substrate for elongase activity. Our findings suggest that the FAE proteins in a 15,000g nasturtium particulate fraction have a broad acyl-CoA preference, with the ability to elongate both monounsaturated and saturated C₁₈-CoA and C₂₀-CoA substrates. In like manner, a partially purified joboba FAE1 showed maximal activity with

monounsaturated and saturated C₁₈- and C₂₀-CoAs in vitro (Lassner et al., 1996). However, it is important to note that the particulate elongation activity reported in this study most likely represents the cumulative effect of expression of more than one member of this small gene family. Thus, from this experiment one can only conclude that the capacity to elongate both monounsaturated and saturated acyl moieties is represented in this nasturtium particulate fraction.

While genetic analyses and homology assessments might predict that the isolated nasturtium FAE gene encodes an enzyme that prefers to elongate saturated acyl-CoAs, transgenic experiments in tobacco callus, tobacco leaves, and Arabidopsis seed collectively confirmed that the heterologously expressed nasturtium FAE can elongate monounsaturated and, to a lesser extent, saturated acyl moieties.

Constitutive expression of the nasturtium FAE resulted in a low increase in the content of erucic acid in transgenic Arabidopsis seeds in comparison to seed-specific expression. It is possible that higher VLCFA accumulation in seeds could not be achieved using the 35S promoter. A similar observation was made by Millar and Kunst (1997) in analyzing seed oils from Arabidopsis plants constitutively expressing FAE1. More recently, while trying to overexpress a borage Δ -6 desaturase in evening primrose plants, Mendoza de Gyvest et al. (2004) reported that the 35S promoter was similarly ineffective in seeds.

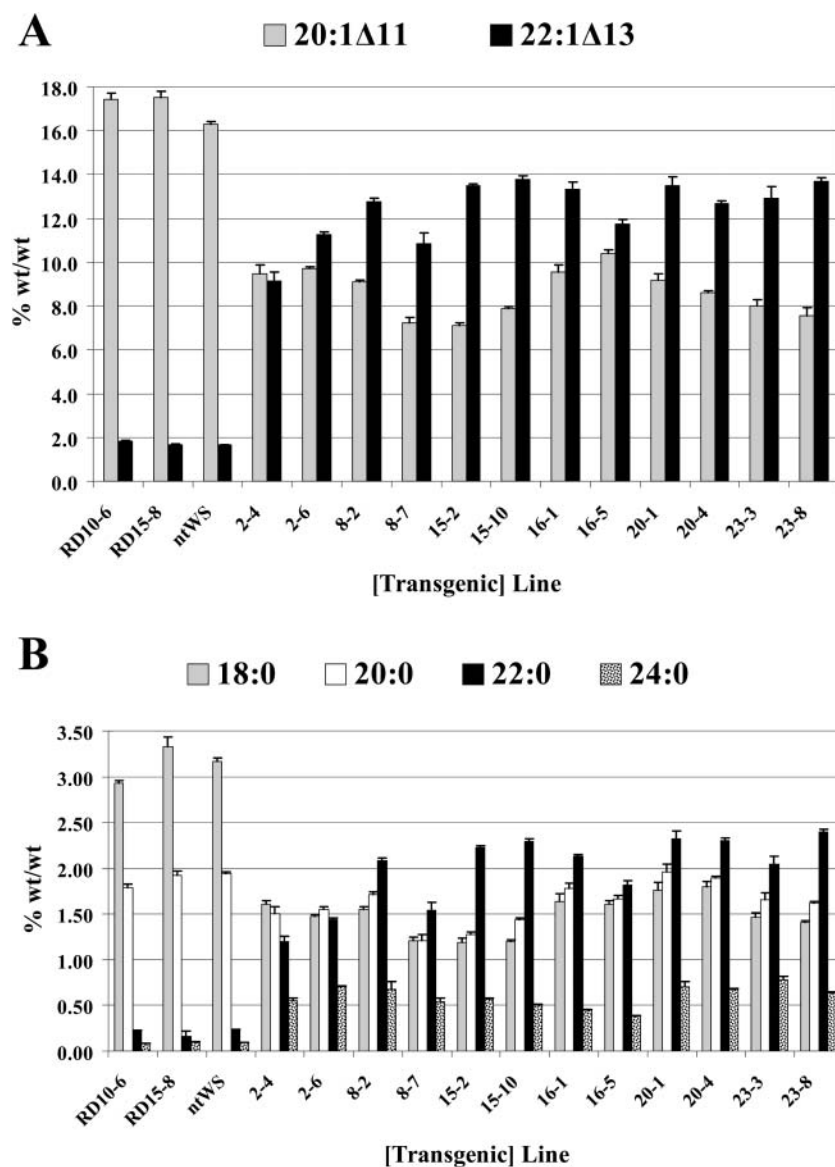
Table III. Fatty acid composition of transgenic Arabidopsis T₂ seed oils

Results represent the average (\pm SE) of triplicate measurements using 200 seeds from 25 independent Arabidopsis transgenic lines. Constructs: RD, Control (plasmid-only) transgenic seeds; NF, Napin: nasturtium FAE transgenic seeds.

Construct	Fatty Acid Composition							LCFA	VLCFA
	18:0	20:1 Δ 11	22:0	22:1 Δ 13	24:0	24:1 Δ 15			
	% (w/w) of total fatty acids [Range] [% increase] ^a								
RD	3.72 \pm 0.07	19.87 \pm 0.26	0.30 \pm 0.01	2.12 \pm 0.05	0.11 \pm 0.01	0.19 \pm 0.01	70.15 \pm 0.22	29.85 \pm 0.22	
	{3.35–4.03}	{17.97–20.86}	{0.27–0.34}	{1.88–2.28}	{0.09–0.15}	{0.15–0.24}	{69.35–71.36}	{28.64–30.65}	
NF	2.57 \pm 0.10	12.78 \pm 0.42	1.57 \pm 0.12	9.63 \pm 0.59	0.46 \pm 0.03	0.46 \pm 0.03	68.77 \pm 0.47	31.30 \pm 0.47	
	{1.58–3.31}	{8.87–16.85}	{0.66–2.78}	{4.43–15.57}	{0.24–0.65}	{0.29–0.70}	{65.53–72.06}	{27.94–34.47}	
			[423.3]	[354.2]	[318.2]	[142.1]		[4.8]	

^aRelative to value for seeds from RD, the Arabidopsis control (plasmid-only) plants, set at 100%.

Figure 5. Fatty acid composition of transgenic *Arabidopsis* seeds. A, Proportions of 20:1 Δ 11 and 22:1 Δ 13 in seed oils from nontransformed *Arabidopsis* ecotype Wassilewskija (ntWS), 2 plasmid-only transgenic control lines (RD10-6 and RD15-8), and the 12 best *Arabidopsis* T₃ homozygous transgenic lines expressing the nasturtium *FAE* gene under control of the napin promoter. B, Proportions of 18:0, 20:0, 22:0, and 24:0 in seed oils from nontransformed *Arabidopsis* ecotype Wassilewskija (ntWS), 2 plasmid-only transgenic control lines (RD10-6 and RD15-8), and the 12 best *Arabidopsis* T₃ homozygous transgenic lines expressing the nasturtium *FAE* gene under control of the napin promoter. The values are the average \pm SD of three determinations performed on 200-seed lot.



In comparison, when we introduced the nasturtium *FAE* (under the control of napin promoter) into *Arabidopsis* plants, the erucic acid content in seed oils increased by almost an order of magnitude (8-fold) at the concomitant expense of 20:1 Δ 11. Indeed eicosenoic acid (20:1 Δ 11) proportions fell from about 18% in the plasmid-only control transformants to 7% to 10% in the best T₃ transgenics harboring the nasturtium *FAE*. In a biotechnological context, it is noteworthy that there was no significant increase in the proportion of the next predicted extension product 24:1 Δ 15. Thus, the acyl composition of the transgenic *Arabidopsis* seed oil was reproporioned, such that erucic acid generally surpassed eicosenoic acid as the predominant VLCFA. This suggests a high specificity of the nasturtium elongase for extending 20:1-CoA to yield 22:1 as its primary product. In fact, in a transgenic *Arabidopsis* background, the nasturtium *FAE* was

much stronger than the previously reported jojoba β -KCS in its ability to increase the level of 22:1: Introducing the jojoba cDNA into *Arabidopsis* seeds only resulted in an increase in 22:1 proportions from about 2% in the control to 4% in the transgenic jojoba *FAE* plants (Lassner et al., 1996).

As would be expected, there was some variation in the proportions of 22:1 that accumulated (Fig. 5A), possibly due to positional effects from nasturtium *FAE* transgene insertion at different sites in the *Arabidopsis* genome. Similar variations were observed in the expression of a castor fatty acid hydroxylase gene (*CEAH12*) in the *Arabidopsis fad2/fae1* double mutant (Rossak et al., 2001).

The ability of the nasturtium *FAE* protein to elongate 18:1-CoA and, preferentially, 20:1-CoA is consistent with the observed acyl composition of nasturtium seed oil, which consists primarily of very long chain

and, specifically, erucoyl moieties. Thus, the nasturtium FAE homolog described herein may have a larger engineering impact when strongly expressed in a seed-specific manner in high erucic acid Brassicaceae (e.g. *B. napus* or *Brassica carinata*), wherein 18:1 $\Delta 9$ [and 18:2/18:3] and 20:1 $\Delta 11$ represent a potential acyl-CoA elongation substrate pool totaling almost 40% over and above the existing 40% to 45% 22:1 $\Delta 13$ content. Given that the pathway for 18:1 elongation has a somewhat stronger metabolic pull over 18:1 desaturation in developing seeds, the resulting erucoyl pool may become quite large. Clearly, the current studies indicate that the nasturtium FAE expression could also be combined with other genetic modifications we have made (e.g. oleoyl-desaturase [*FAD 2*] silencing) to enhance the VLCFA content of high erucic acid Brassicaceae (Katavic et al., 2001; Taylor et al., 2001; E.-F. Marillia, A. Jadhav, E.M. Giblin, V. Katavic, D.L. Barton, C. Sonntag, V. Babic, and D.C. Taylor, unpublished data) and the proportions of erucic acid, in particular, to provide an industrial feedstock oil of high value and broad applicability.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All experimental lines propagated in the greenhouse were grown at the Kristjanson Biotechnology Complex greenhouses, Saskatoon, Canada, under natural light conditions supplemented with high-pressure sodium lamps with a 16-h photoperiod (16 h of light and 8 h of darkness) at 22°C and a relative humidity of 25% to 30%. Garden nasturtium (*Tropaeolum majus*) plants (cv Dwarf Cherry Rose) were grown in the greenhouse, and flowers were hand-pollinated. Seeds at various stages of development were harvested, their seed coats were removed, and embryos were frozen in liquid nitrogen and stored at -80°C. Tobacco (*Nicotiana tabacum*) plants were grown under sterile conditions on Murashige and Skoog medium (Murashige and Skoog, 1962) as well as under normal greenhouse conditions. Arabidopsis plants were grown in a growth chamber at 22°C with photoperiod of 16 h light (120 $\mu\text{E m}^{-2} \text{s}^{-1}$) and 8 h dark.

Nasturtium Embryo Protein Preparations and Elongase Assays

A 5,000 to 15,000g particulate fraction enriched in elongase activity was isolated essentially as described previously (Löhden and Frentzen, 1992). Briefly, embryos (2–3 g) were ground in a mortar under liquid nitrogen and then 10 mL of isolation buffer (80 mM HEPES, pH 7.2, containing 2 mM dithiothreitol, 320 mM Suc, and 5% polyvinylpyrrolidone) per g fresh weight was added. The homogenate was filtered through Miracloth and spun for 5 min at 5,000g in a Sorvall refrigerated centrifuge at 5°C, and the supernatant retained and recentrifuged at 15,000g for 25 min. The resulting pellet was resuspended in 80 mM HEPES containing 20% glycerol and 2 mM dithiothreitol. The concentration of protein was determined as described by Bradford (1976). This subcellular fraction was either used directly to determine enzymatic activities or stored at -80°C until used.

The 15,000g particulate preparation was used to perform elongation assays as described by Taylor et al. (1992b) with the following modifications. The assay mixture consisted of 80 mM HEPES-NaOH, pH 7.2, containing 0.75 mM ATP, 10 μM CoA-SH, 0.5 mM NADH, 0.5 mM NADPH, 2 mM MgCl_2 , 200 μM malonyl-CoA, 18 μM [^{14}C]acyl-CoA (0.37 GBq mol^{-1}), and nasturtium protein in a final volume of 500 μL . The reaction was started by the addition of 200 μg of protein and incubated in a shaking water bath at 30°C, 100 rpm for 0.5 h. [^{14}C]Radiolabeled acyl-CoAs were synthesized from the corresponding free fatty acids as described (Taylor et al., 1990). Elongase reaction assays were stopped with 3 mL of 100 g/L KOH in methanol. Fatty acid methyl esters

(FAMES) were prepared and quantified by radio-HPLC as described (Taylor et al., 1992b).

Lipid Analyses

The total fatty acid content and acyl composition of tobacco plant lipids and Arabidopsis seed oils were determined by gas chromatography of the FAMES with 17:0 FAME as an internal standard as described (Zou et al., 1997; Katavic et al., 2001; Taylor et al., 2001).

Isolation of FAE cDNA by a Degenerate Primers Approach

Degenerate primers were designed for amino acid sequences conserved among Arabidopsis KCS1 (GenBank accession no. AF053345), *Brassica napus* FAE1 (GenBank accession no. AF009563), *Limnanthes douglasii* FAE (GenBank accession no. AF247134), and jojoba (*Simmondsia chinensis*) FAE (GenBank accession no. U37088). Single-stranded cDNA template for reverse transcription-PCR was synthesized at 42°C from embryo poly(A) RNA with PowerScript (CLONTECH, Palo Alto, CA). A 50- μL PCR reaction contained single-stranded cDNA derived from 40 ng of poly(A) RNA, 20 μM of each primer: 5'-TCT(A/T)GG(A/T)GG(C/A)ATGGGTG-3' [LGGMGC], and 5'-T(G/A)TA(T/C)GC(C/T)A(A/G)CTC(A/G)TACC-3' [WYELAY] and 2.5 units of Taq DNA Polymerase (Amersham Biosciences, Baie d'Urfe, Canada) under standard conditions. An internal part of the elongase sequence was amplified in a thermocycler during 30 cycles of the following program: 94°C for 30 s, 48°C for 30 s, and 72°C for 1 min. The sequence of a 650-bp PCR product was used to design a primer to amplify the 5' and 3' ends of the cDNA using the SMART RACE cDNA Amplification kit (CLONTECH). After assembly to determine the full-length sequence of the cDNA, the open reading frame without a stop codon was amplified using the primers 5'-ACCATGT-CAGGAACAAAAGC-3' and 5'-ATTAAATGGAACCTCAACCG-3', and subsequently cloned into the pYES2.1/V5-His-TOPO expression vector (Invitrogen, Burlington, Canada). Omitting the stop codon allows for the PCR product to be expressed as a fusion to the C-terminal V5 epitope and polyhistidine tag for protein detection and purification.

Expression of Nasturtium FAE in Yeast

The nasturtium FAE in YES2.1/V5-His-TOPO plasmid was transformed into *Saccharomyces cerevisiae* strain Inv Sc1 (Invitrogen) and two mutant strains *elo2 Δ* and *elo3 Δ* (Oh et al., 1997) using the S.c. EasyComp transformation kit (Invitrogen). Yeast cells transformed with pYES2.1/V5-His-TOPO plasmid only and with Arabidopsis FAE1 were used as controls. The transformants were selected and grown as described previously (Katavic et al., 2002). Fatty acid elongase activity of the microsomal membrane preparation was measured essentially as described by Katavic et al. (2002), with modifications described above. For western-blot analyses, 100 μg of microsomal protein were separated on 15% SDS-PAGE Ready Gel (Bio-Rad) and electrotransferred to PVDF membrane (Hybond^P; Amersham Biosciences). Recombinant proteins were detected with anti-His (C-terminal) antibody using WesternBreeze kit (Invitrogen) according to manufacturer's protocol.

Site-Directed Mutagenesis of FAE

A site-directed mutagenesis experiment was performed essentially as described (Katavic et al., 2002). The desired mutation (Tyr at position 429 is replaced with His) was introduced into the FAE coding region by PCR using primers 5'-TCGAGGATGTCGCTTACCGGATTTGGAAACAC-3' and 5'-GTTTCCAAATCGGTGAAGCGACATCCTCGATGG-3'. These primers were complementary to the opposite strands of pYES2.1/V5-His-TOPO containing the nasturtium FAE gene.

cDNA Library Construction

To construct the nasturtium developing cDNA library, immature seeds were collected 17 d after pollination. Total RNA was extracted from embryos as described by Lindstrom and Vodkin (1991), then poly(A) RNA was isolated using Dynabeads Oligo(dT)₂₅ (Dynal Biotech, Lake Success, NY). Copy DNA synthesis was performed on 1 μg of poly(A) RNA using SMART PCR cDNA synthesis kit (CLONTECH) according to manufacturer's protocol.

The cDNA population was then subtracted with 12S and 2S seed storage protein cDNA clones using PCR-Select cDNA subtraction kit (CLONTECH). The subtracted embryo cDNA population was cloned and then sequenced as described by Jako et al. (2002).

Sequence Handling

Sequence analyses were performed using Lasergene software (DNASTar, Madison, WI). Sequence similarity searches and other analyses were performed using BLASTN, BLASTX (Altschul et al., 1990), and PSORT (Nakai and Kanehisa, 1992) programs.

Northern Analysis

Total RNA from nasturtium plant material was isolated as described by Lindstrom and Vodkin (1991). A total of 20 μ g of RNA was fractionated on a 1.4% formaldehyde-agarose gel, and the gels were then stained with ethidium bromide to ensure that all lanes had been loaded equally (Sambrook et al., 1989). The RNA was subsequently transferred to Hybond N⁺ membrane (Amersham Biosciences) and hybridized with the ³²P-labeled *FAE* DNA probe, prepared using the Random Primers DNA labeling kit (Gibco-BRL, Cleveland). Membranes were hybridized at 60°C overnight.

Plant Transformation Vectors

The coding regions of the nasturtium *FAE* (natural and mutated versions named SF and SMF, respectively) were amplified by PCR with primers 5'-taggatcATGTCAGGAACAAAAGC-3' (lowercase shows restriction site for *Bam*HI) and 5'-tagagctcTTAATTTAATGGAACCTCAACC-3' (lowercase shows restriction site for *Sac*I enzyme) and subsequently cloned as a *Bam*HI and *Sac*I fragment behind the constitutive tandem 35S promoter in binary vector pBI121 (CLONTECH).

The coding region of the nasturtium *FAE* was cloned behind the seed-specific napin promoter as follows. A *Bam*HI site was introduced in front of the start codon and behind the stop codon of *FAE* by PCR with primers 5'-taggatcATGTCAGGAACAAAAGC-3' and 5'-taggatcTTAATTTAATGGAACCTCAACC-3' (lowercase shows the restriction site for *Bam*HI). The *B. napus* napin promoter (Josefsson et al., 1987) was cloned in *Hind*III/*Xba*I sites of the pUC19 (MBI Fermentas, Burlington, Canada), and the nos terminator (Bevan, 1983) was introduced as an *Eco*RI/*Bam*HI fragment. The resulting vector was named pDH1. The napin promoter/nos terminator cassette was excised from pDH1 by *Hind*III/*Eco*RI digestion and subsequently cloned into the respective sites of pRD400 (CLONTECH), resulting in pVK1. The coding region of *FAE* was then cloned into the *Bam*HI site of pVK1 behind the napin promoter, and the resulting vector was named NF. Sense orientation of the *FAE* coding region with respect to promoter was confirmed by restriction analyses with *Xba*I.

The final binary vectors (SF, SMF, and NF) were electroporated into *Agrobacterium tumefaciens* cells strain GV3101 containing helper plasmid pMP90 (Koncz and Schell, 1986). Plasmid integrity was verified by DNA sequencing following its reisolation from *A. tumefaciens* and transformation into *Escherichia coli*.

Plant Transformation and Genetic Analysis

Tobacco (cv Xanthi) was transformed using a leaf disc transformation procedure (Horsch et al., 1985). Shoots that rooted in the presence of 50 mg/L kanamycin were considered to be transgenic. Transgenic plants were transferred to soil and grown in the greenhouse.

Arabidopsis (ecotype Wassilewskija) were transformed by vacuum infiltration method (Clough and Bent, 1998). Transgenic plants were selected and analyzed essentially as described by Jako et al. (2001).

Molecular Analysis of Transgenic Plants

DNA was isolated from 2 to 3 g of tobacco or 150 mg of Arabidopsis leaf material using a urea-phenol extraction method (Chen et al., 1992) with the following minor modification: Tissue extraction was performed for 15 min at room temperature, and 400 mM ammonium acetate, pH 5.2, was used for the first two precipitation steps. Stable integration of the napin:*FAE*:nos cassette into the genome of transgenic plants was checked by PCR amplification on genomic DNA as described by Katavic et al. (2001).

Southern analyses were performed to further confirm and select those transformants containing single or multiple copies of the inserted fragments. Fifteen micrograms of tobacco or 1 μ g of Arabidopsis genomic DNA was digested with the restriction enzyme *Sac*I, and the resulting fragments were separated on a 0.9% (w/v) agarose gel, transferred to Hybond N⁺ nylon membrane (Amersham Biosciences) via an alkali blotting protocol. A 1.5-kb probe containing the coding sequence of *FAE* was generated by PCR using primers 5'-ATGTCAGGAACAAAAGC-3' and 5'-TAATTTAATGGAACCTCAACCG-3' and subsequently radioactively labeled with ³²P as described above. Hybridization was performed at 65°C. The filters were washed once in 1 \times SSPE, 0.1% SDS for 15 min, and 0.1 \times SSPE, 0.1% SDS for 5 to 10 min at the temperature of hybridization. The blots were exposed to X-OMAT-AR film (Kodak, Rochester, NY).

To estimate the number of *FAE* isoforms in the nasturtium genome, 15 μ g of genomic DNA was digested with restriction enzymes *Eco*RI, *Acc*I, *Nco*I, and *Hind*III. Blotting and hybridization conditions were essentially as above except that filters were washed at low stringency with 1 \times SSPE, 0.1% SDS for 15 min, autoradiographed and then washed subsequently with 0.1 \times SSPE, 0.1% SDS, and re-exposed.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY082610.

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