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Cloning and functional characterization of the *fatty acid elongase 1* (*FAE1*) gene from high erucic *Crambe abyssinica* cv. Prophet

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Summary

A genomic *fatty acid elongation 1* (*FAE1*) clone was isolated from *Crambe abyssinica*. The genomic clone corresponds to a 1521-bp open reading frame, which encodes a protein of 507 amino acids. In yeast cells expression of *CrFAE* led to production of new very long chain monounsaturated fatty acids such as eicosenoic (20 : $1^{\Delta 11}$) and erucic (22 : $1^{\Delta 13}$) acids. Seed-specific expression in *Arabidopsis thaliana* resulted in up to a 12-fold increase in the proportion of erucic acid. On the other hand, in transgenic high-erucic *Brassica carinata* plants, the proportion of erucic acid was as high as 51.9% in the best transgenic line, a net increase of 40% compared to wild type. These results indicate that the *CrFAE* gene encodes a condensing enzyme involved in the biosynthesis of very long-chain fatty acids utilizing monounsaturated and saturated acyl substrates, with a strong capability for improving the erucic acid content.

Introduction

Very long chain fatty acids (VLCFA) are those that contain more than 18 carbon atoms. They are common components of plant waxes and seed oils in a number of plant families including *Cruciferaceae*, *Limnantheceae*, *Simnodsia* and *Tropaeolaceae* (Lassner *et al*., 1996; Mietkiewska *et al*., 2004; Salas *et al*., 2005). Erucic acid (*cis*-13 docosenoic acid, 22 : 1) is the major VLCFA in the seed oil from HEAR (high erucic acid rapeseed) *Brassica napus* cultivars, accounting for 45–55% of the total fatty acids (Han *et al*., 2001). HEAR cultivars are of high interest for industrial purposes because 22 : 1 is a valuable feedstock with more than 1000 potential or patented industrial applications (Scarth and Tang, 2006). Currently the major derivative of erucic acid is erucamide, which is used as a surface-active additive in coatings and in the production of plastic films as an antiblock or slip agent. Many other applications are foreseen for erucic acid and its hydrogenated derivative behenic acid, e.g. in lubricants, detergents, film processing agents and coatings, as well as in cosmetics and pharmaceuticals (Leonard, 1993; Derksen *et al*., 1995; McVetty and Scarth, 2002; Puyaubert *et al*., 2005b). For many of these industrial uses, the economics are limited by the proportion of 22 : 1 in the seed oil, approximately 45%. To compete with petroleum-based products, it is desirable to increase the 22 : 1 proportion as high as possible in order to reduce the cost of purification (Scarth and Tang, 2006). In addition, the engineering of HEAR *Brassicaceae* to produce seed oils containing substantial trierucin would lend the oil to a wide range of new applications (Sonntag, 1995).

VLCFAs are synthesized by a microsomal fatty acid elongation (FAE) complex using acyl-CoA substrates from a cytoplasmic pool maintained by *de novo* lipid biosynthesis in plastids. Each cycle of fatty acid elongation adds two carbon units to the acyl chain and involves four reactions: first, malonyl-CoA and long chain acyl-CoA are condensed by a 3-ketoacyl-CoA synthase (KCS, often designated fatty acid elongase, FAE): the resulting 3-ketoacyl-CoA is then reduced by the action of a 3-ketoacyl-CoA reductase resulting in the synthesis of a 3-hydroxyacyl-CoA. Subsequently 3-hydroxyacyl-CoA is dehydrated to 2-enoyl-CoA, which is then reduced by second reductase to form the elongated acyl-CoA (Barret *et al*., 1998; Rossak *et al*., 2001; Blacklock and Jaworski, 2002; Salas *et al*., 2005).

Over the past decade, progress in understanding VLCFA biosynthesis has been achieved by cloning KCS genes from different plants and performing functional expression studies (James *et al*., 1995; Lassner *et al*., 1996; Han *et al*., 2001; Das *et al*., 2002; Fofana *et al*., 2004). These studies have provided evidence that KCS is the rate-limiting enzyme for seed VLCFA production (Millar and Kunst, 1997; Mietkiewska *et al*., 2004). Due to the membrane-bound nature of the KCS (FAE) protein, our knowledge of the properties, and regulation of this enzyme are still limited (Puyaubert *et al*., 2005a).

Absence of erucic acid (22 : $1^{\Delta 13}$) in low erucic acid rapeseed (LEAR) was shown to be correlated with a lack of acyl-CoA elongation activity (Roscoe *et al*., 2001). Comparison of FAE1 protein sequences from HEA and LEA *Brassicaceae* revealed one crucial amino acid difference: the serine at position 282 of the HEA FAE1 sequences is substituted by phenylalanine in LEA *B. napus* cv. Westar (Katavic *et al*., 2002). Subsequently it has been demonstrated that the activity of non-functional FAE1 of *B. napus* can be restored by substituting Phe282 with serine. Thus, the low erucic acid trait in canola *B. napus* can be attributed to a single amino acid substitution that prevents the biosynthesis of eicosenoic and erucic acids.

A strategic goal of our research is to modify seed oil composition to increase the proportion of erucic acid (22 : $1^{\Delta 13}$) in *Brassicaceae*. Looking for new sources of strategic KCS genes we selected *Crambe abyssinica*. The seed oil of *C. abyssinica* is distinct from other *Cruciferae* because of its very high proportion of erucic acid, which is up to 60% (Leonard, 1993). In this study we describe the isolation and functional characterization of a *C. abyssinica FAE* homologue.

Results

Isolation and characterization of *C. abyssinica FAE* homologue

Based on the sequence homology among plant fatty acid elongase genes, a genomic clone of the *FAE* gene from *C. abyssinica* was amplified by PCR, and the sequence submitted to GenBank (accession no. AY793549). The *C. abyssinica FAE* (*CrFAE*) open reading frame of 1521 bp encodes a polypeptide of 507 amino acids which is most closely related to FAE1s from *Brassicaceae* (data not shown): *Brassica juncea* (97% identity, GenBank accession no. AJ558198), *Brassica oleracea* (96% identity, GenBank accession no. AF490460), *B. napus* (96% identity, GenBank accession no. AF490459) and *Brassica rapa* (96% identity, GenBank accession no. AF49041). The *Arabidopsis thaliana* FAE1 (GenBank accession no. U29142) polypeptide showed 84% identity with the CrFAE. A previously isolated *Tropaeolum majus* FAE (GenBank accession no. AY082610) showed 54% identity with the CrFAE. The CrFAE protein was predicted to have a molecular mass of 56.4 kDa and a theoretical pI value of 9.29.

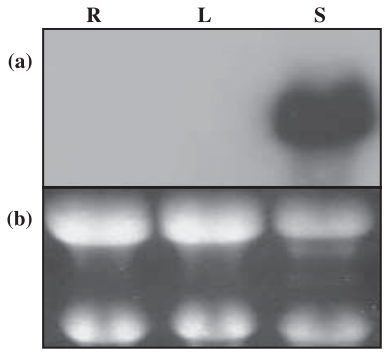

Figure 1 Northern analysis of *CrFAE* gene. (a) Total RNA was isolated from roots (R), leaves (L) and developing seeds (S), blotted and probed with ^{32}P-labelled 1.5 *CrFAE* fragment. (b) The amount of RNA loaded per line was calibrated by the relative ethidium bromide staining of the ribosomal RNA bands.

Comparison of the amino acid sequence of CrFAE with other plant FAEs revealed the presence of six conserved cysteine and four conserved histidine residues (data not shown). As reported by Ghanevati and Jaworski (2002) all histidine residues were essential for *A. thaliana* FAE1 activity. CrFAE also showed the presence of a conserved serine residue at the position 282. It has been shown that serine at position 282 is essential for FAE activity in high erucic acid (HEA) *Brassica* cultivars (Katavic *et al*., 2002).

A hydropathy analysis (Kyte-Doolittle) of the amino acid sequence of the CrFAE revealed several hydrophobic domains. Protein analyses with the TMAP algorithm (Persson and Argos, 1994) predicted two *N*-terminal transmembrane domains, the first corresponding to amino acid residues 9–31 and the second domain spanning residues 51–73 (data not shown).

Northern blot analyses performed on total RNA isolated from *C. abyssinica* roots, leaves and mid developing seeds showed that the *FAE* homologue was expressed only in developing seeds (Figure 1).

Functional heterologous expression of the *C. abyssinica FAE* in yeast cells

To confirm the function of the protein encoded by the *CrFAE*, the coding region was linked to the *GAL1*-inducible promoter in the yeast expression vector pYES2.1/V5-His-TOPO and transformed into *S. cerevisiae* strain Inv Sc1 cells. As shown in Figure 2, yeast cells transformed with the plasmid containing the *CrFAE* open reading frame were found to produce

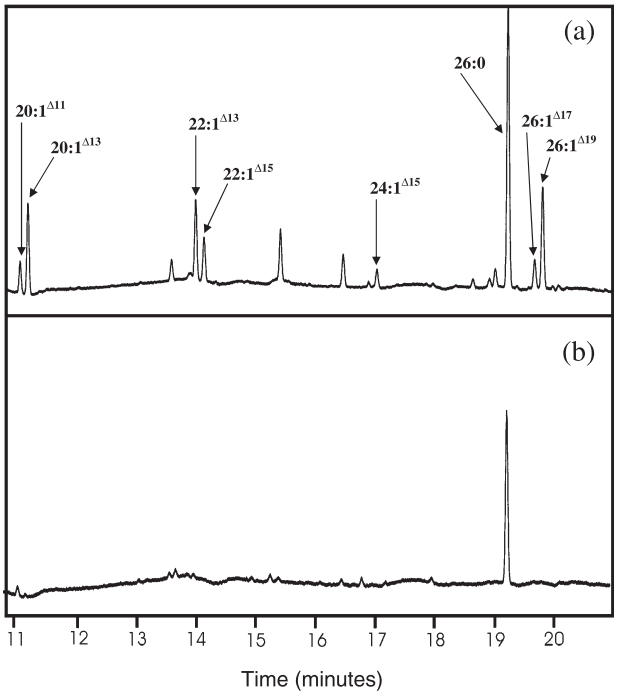

Figure 2 Gas chromatographic analyses of fatty acid methyl esters produced in transformed yeast cells. Fatty acid methyl esters were prepared from yeast cell lysates expressing *CrFAE* gene (a) and a pYES2.1/V5-TOPO plasmid-only negative control (b).

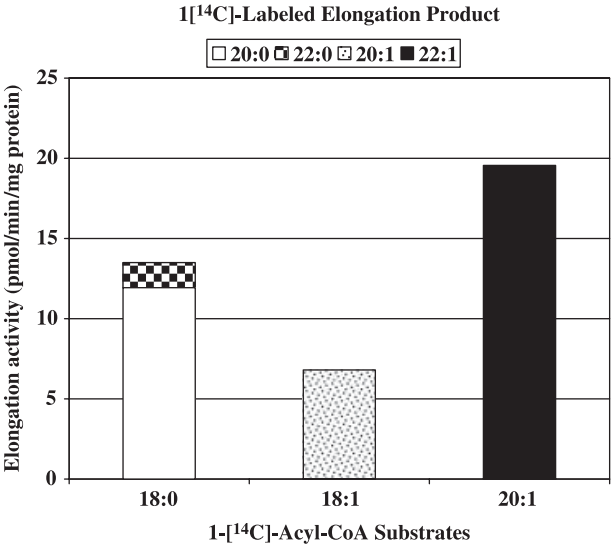

Figure 3 Elongase activity assayed in lysates from yeast cells expressing the *CrFAE*. Reaction conditions were as described in the Experimental procedures. Results are reported as the total elongation products produced in each [1-^{14}C]acyl-CoA reaction (pmol/min/mg protein) and are the average of three repeats.

20 : $1^{\Delta 11}$, 20 : $1^{\Delta 13}$, 22 : $1^{\Delta 13}$, 22 : $1^{\Delta 15}$ and 26 : $1^{\Delta 19}$. These fatty acids are not present in wild-type yeast cells. To determine substrate of preference for CrFAE activity in yeast cells (mutant strain *elo*2Δ; Oh *et al.* 1997) we performed elongase activity assays with a range of radiolabelled 1-[^{14}C]-acyl-CoAs in conjunction with malonyl-CoA as cosubstrate. The yeast cell homogenates expressing *CrFAE* showed elongase activity with monounsaturated as well as saturated acyl-CoA substrates. As shown in Figure 3, the highest elongase activity with the recombinant CrFAE was detected with 20 : 1-CoA as a substrate and erucic acid was synthesized as the major product. This was about threefold higher than the activity observed with oleoyl-CoA as substrate. Comparatively high elongase activity was also found with 18 : 0-CoA.

Seed-specific over-expression of *C. abyssinica FAE* in *A. thaliana* plants

To identify the function of the CrFAE homologue, the coding region was linked to a strong seed-specific promoter and expressed in *A. thaliana*. In order to establish substrate specificity of the CrFAE *in vivo*, we selected two *A. thaliana* backgrounds with dramatically different oil profiles: wild-type Columbia with 18–20% 20 : 1 and a *fae1* mutant line AC56 (Kunst *et al*., 1992) with less than 1% 20 : 1 in their seed oils, respectively. From vacuum infiltration we recovered 44 T_1 plants in Columbia background and 33 T_1 plants in the AC56 mutant background. The fatty acid composition of T_2 seeds from individual plants was determined for all transformants. Significant changes in fatty acid composition in T_2 transgenic seeds were found. On average, the proportion of erucic acid increased from about 1.8% in the control (empty vector in Columbia background) to 11.3% in T_2 segregating transgenic seeds at the expense of 20 : $1^{\Delta 11}$ reduced (by 42%) (Table 1). In mutant line AC56, seed-specific over-expression of *CrFAE* resulted in increased proportions of 20 : $1^{\Delta 11}$ and 22 : $1^{\Delta 13}$ at the expense of oleic acid 18 : $1^{\Delta 9}$ reduced (by 33%). On average the proportion of 20 : $1^{\Delta 11}$ and $22^{\Delta 13}$ increased from 0.4 and 0% in the AC56 mutant control to as high as 6.0% and 10.0%, respectively, in T_2 *CrFAE* transgenic AC56 lines.

Because the preliminary analysis of fatty acid composition was performed on T_2 segregating seeds for the presence of the transgene(s), we anticipated that T_3 homozygous seeds of *A. thaliana* would contain higher proportions of VLCFAs. Therefore, seeds from the T_2 lines with the highest proportion of erucic acid were sown and grown in order to obtain the T_3 seed generation. As shown in Figure 4(a) the proportion of erucic acid increased from 1.6% in wild-type to as high as 19–20% in the best homozygous transgenic lines, 22–6, 22–9 and 35–7. The increased erucic acid was correlated with a concomitant reduction in the proportions of its corresponding elongase primers: 20 : $1^{\Delta 11}$ and 18 : $1^{\Delta 9}$. A significant increase in the proportion of the saturated VLCFAs 22 : 0 and 24 : 0

Table 1 Fatty acid composition of transgenic *Arabidopsis thaliana* T_2 seed oils

	Fatty acid composition								
	18 : 0	18 : $1^{\Delta 9}$	20 : 0	20 : $1^{\Delta 11}$	22 : 0	22 : $1^{\Delta 13}$	24 : 0	24 : $1^{\Delta 15}$	VLCFA
Construct	Percentage (w/w) of total fatty acids {Range} [% increase]*								
NCRA/Col	2.9 ± 0.3 {2.5–3.5}	15.4 ± 1.7 {12.1–20.2}	1.9 ± 0.2 {1.5–2.3}	10.7 ± 1.5 {8.4–13.3}	0.94 ± 0.22 {0.46–1.51} [147]	11.3 ± 2.6 {4.8–17.5} [547]	0.34 ± 0.05 {0.22–0.48} [62]	0.42 ± 0.06 {0.28–0.59} [110]	29.1 ± 2.9 {23.0–35.0} [5]
RD/Col	3.4 ± 0.1 {3.2–3.5}	15.8 ± 0.8 {14.5–16.7}	2.5 ± 0.1 {2.3–2.7}	18.6 ± 0.3 {18.3–19.2}	0.38 ± 0.02 {0.35–0.41}	1.8 ± 0.1 {1.7–2.0}	0.21 ± 0.01 {0.20–0.22}	0.20 ± 0.01 {0.19–0.22}	27.6 ± 0.3 {27.2–28.1}
NCRA/AC56	3.0 ± 0.3 {2.4–3.5}	19.6 ± 2.7 {15.6–28.4}	1.4 ± 0.2 {0.92–1.65}	6.0 ± 0.9 {2.5–7.2} [1400]	0.87 ± 0.21 {0.41–1.48} [222.2]	10.1 ± 2.7 {2.4–16.0} [906.0]	0.35 ± 0.05 {0.20–0.45} [75.0]	0.40 ± 0.06 {0.21–0.53} [185.7]	20.7 ± 4.2 {7.2–28.7} [941]
RD/AC56	3.9 ± 0.2 {3.6–4.2}	29.2 ± 0.8 {28.1–29.7}	0.92 ± 0.03 {0.89–0.95}	0.40 ± 0.02 {0.38–0.42}	0.27 ± 0.03 {0.24–0.30}	0.0 ± 0.0	0.20 ± 0.08 {0.13–0.30}	0.14 ± 0.03 {0.11–0.18}	2.0 ± 0.2 {1.9–2.3}

Results represent the average ± SD from 44 NCRA/Col and 33 NCRA/AC56 independent *A. thaliana* transgenic lines. Construct: RD, control (plasmid-only) transgenic seeds; NCRA, Napin: *CrFAE* in *A. thaliana* ecotype Columbia (/Col) and in *fae1 A thaliana* mutant (/AC56) background.
*Relative to value for seeds from RD, the *A. thaliana* control (plasmid-only) plants, set at 100%.

at the expense of 18 : 0 and 20 : 0 was also observed (data not shown). In the *fae1* mutant AC56 background, the two step elongation of 18 : $1^{\Delta 9}$ moieties was observed (Figure 4b). In the best T_3 homozygous AC56 *CrFAE* transgenic lines, the proportion of eicosenoic and erucic acids increased from 0.5% and 0% to as high as 6–7% and 17–19%, respectively. A dramatic redistribution of fatty acid proportions was observed; the proportion of total VLCFAs increasing from 2.5% in the mutant line to as high as 36% in the best *CrFAE* transgenic line.

Seed-specific over-expression of *C. abyssinica FAE* in *Brassica carinata* plants

In order to stimulate synthesis of erucic acid in high erucic acid *B. carinata* seeds we transformed these plants with a gene construct carrying the *CrFAE* coding region under the control of the napin promoter. Mature seeds were collected from self-pollinated plants and subjected to fatty acid composition analysis of the total seed lipids. The results of the analyses of seed oil of the T_1 *B. carinata FAE* transgenic lines and wild-type control are shown in Figure 5. The proportion of erucic acid was increased from 35.5% in the wild-type background to as high as 47.4% in the best T_1 transgenic line. There was also a relatively significant increase in the proportion of the saturated VLCFA 22 : 0 at the expense of 16 : 0. Elongation of long chain fatty acids caused by seed-specific expression of *CrFAE* resulted in increased proportion of total VLCFAs from 49% in the wild-type control to as high as 60% in the best transgenic line.

The nine best transgenic lines were selected and grown in the greenhouse to the next generation. Results from analyses of these lines are shown in Figure 6. In the T_2 generation stable expression of *CrFAE* was observed. The proportion of erucic acid was as high as 51.9% in the best transgenic line 1H-3. Increased proportions of 22 : 1 were correlated with decreases in the proportion of 20 : 1 and to a lesser extent, 18 : 1 and 18 : $2^{\Delta 9,12}$. A significant increase at the content of the saturated VLCFAs was observed at the expense of long chain fatty acids (16 : 0 and 18 : 0). To analyse the pattern of transgene integration, we performed Southern hybridization analyses, which showed that line 12D had a single insert, lines 11 A and 11B a double insert, while the rest of the lines had multiple inserts of the *CrFAE* transgene in their genome (data not shown).

Discussion

We have isolated the genomic clone corresponding to the *FAE1* coding region from *C. abyssinica*. Introns were not present in the genomic clone, which now appears to be a common feature of most plant *FAE1* genes in the *Brassicaceae* (Katavic *et al*., 2002; Mietkiewska *et al*., 2004). However, unlike *CrFAE*, two introns were identified in the open reading frame of the *Lesquerella fendleri FAE* (Moon *et al*., 2001). Several lines of evidence suggested that the *CrFAE* encodes a KCS. The polypeptide showed high similarity with other condensing enzymes involved in the synthesis of VLCFAs such as: *B. juncea* FAE, *B. oleracea* FAE (Katavic *et al*., 2002), *B. napus* FAE (Katavic *et al*., 2002), *A. thaliana* FAE (James *et al*., 1995), *Simmondsia chinensis* FAE (Lassner *et al*., 1996),

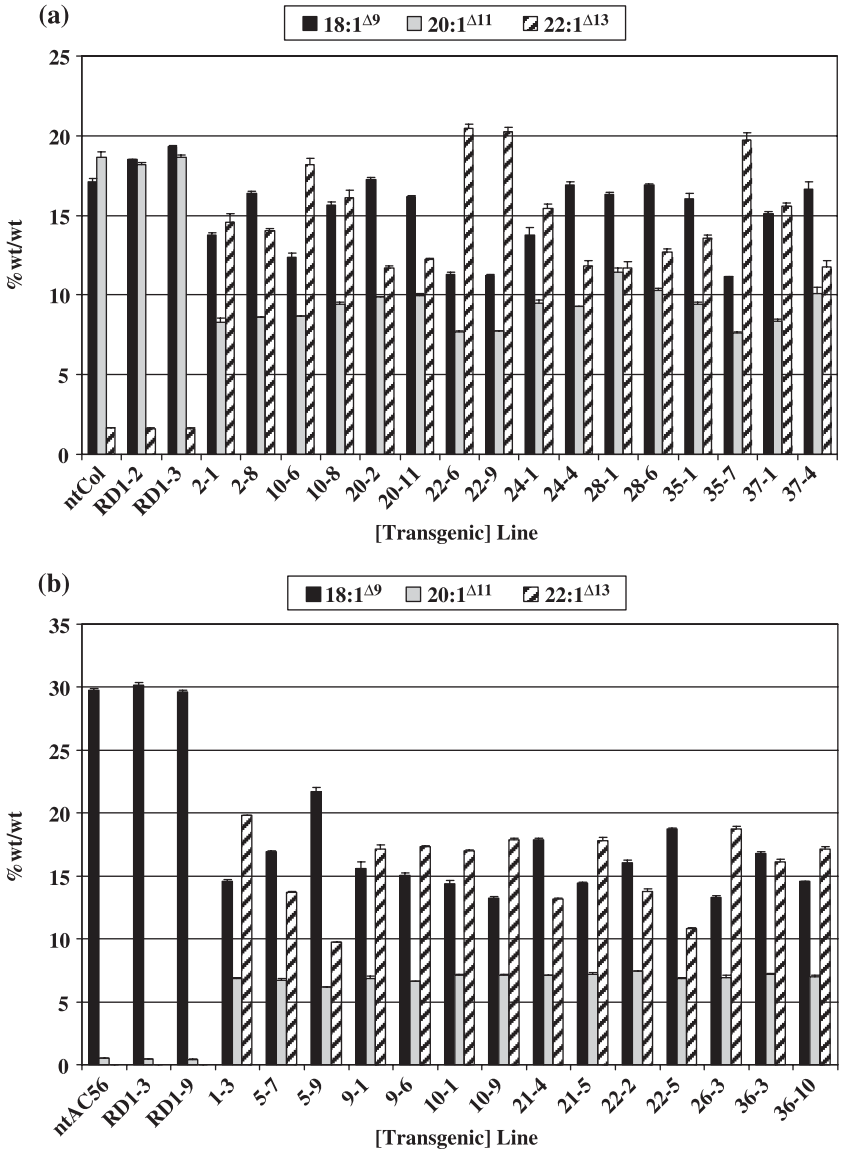

Figure 4 Fatty acid composition of transgenic *Arabidopsis thaliana* seeds. (a) Proportion of 18 : $1^{\Delta 9}$, 20 : $1^{\Delta 11}$ and 22 : $1^{\Delta 13}$ in seed oils from non-transformed *A. thaliana* ecotype Columbia (ntCol), 2 plasmid-only transgenic control lines (RD1-2 and RD1-3) and the 16 best *A. thaliana* T_3 homozygous transgenic lines transformed with the *CrFAE*. (b) Proportion of 18 : $1^{\Delta 9}$, 20 : $1^{\Delta 11}$ and 22 : $1^{\Delta 13}$ in seed oils from non-transformed *A. thaliana fae1* mutant line AC56 (ntAC56), mutant line AC56 transformed with empty vector (RD1-3, RD1-9) and the 14 best *A. thaliana* T_3 homozygous transgenic line transformed with the *CrFAE* gene. The values are the average ± SD of three determinations.

Limnanthes douglasii FAE (Cahoon *et al*., 2000) and *T. majus* FAE (Mietkiewska *et al*., 2004). Detailed sequence analyses of the CrFAE confirmed the presence of conserved regions for all VLCFA FAEs including the cysteine active site essential for enzyme activity (Ghanevati and Jaworski, 2001).

Previous work on FAE1 indicates that the yeast expression system is a predictive model of elongase condensing enzyme activity in plants (Millar and Kunst, 1997; Moon *et al*., 2001). Expression of *CrFAE* in yeast cells (Figure 2) resulted in accumulation of novel fatty acids such as 20 : 1, 22 : 1 and 26 : 1 clearly demonstrating that the isolated FAE homologue is involved in the biosynthesis of VLCFAs. The transgenic yeast cells expressing the *A. thaliana* gene were found to form higher proportions of 20 : 1 than 22 : 1 (Millar and Kunst, 1997), while those expressing the *CrFAE* also produced fatty acids longer than 20 : 1. These data suggest that CrFAE, like the *B. napus* FAE (Han *et al*., 2001), elongates 20 : 1 more efficiently than that of *A. thaliana*. Indeed the latter difference in selectivity is the probable reason for the distinctly high proportion of 20 : 1 found in *A. thaliana* seed oil.

Using the yeast mutant strain *elo*2Δ as a host (this strain is devoid of elongases of key importance to synthesis of sphingolipid fatty acyl moieties (up to 24 : 0), recombinant yeast *CrFAE* transformant lysates were assayed for elongase activity with a range of radioactive substrates. Using this mutant host, there is no *in vitro* interference from the background elongase activity that is present in wild-type yeast host strains. The CrFAE exhibited a preference for longer monounsaturated acyl moieties, showing higher elongase activity of with 20 : 1-CoA than with 18 : 1-CoA (Figure 3). This is

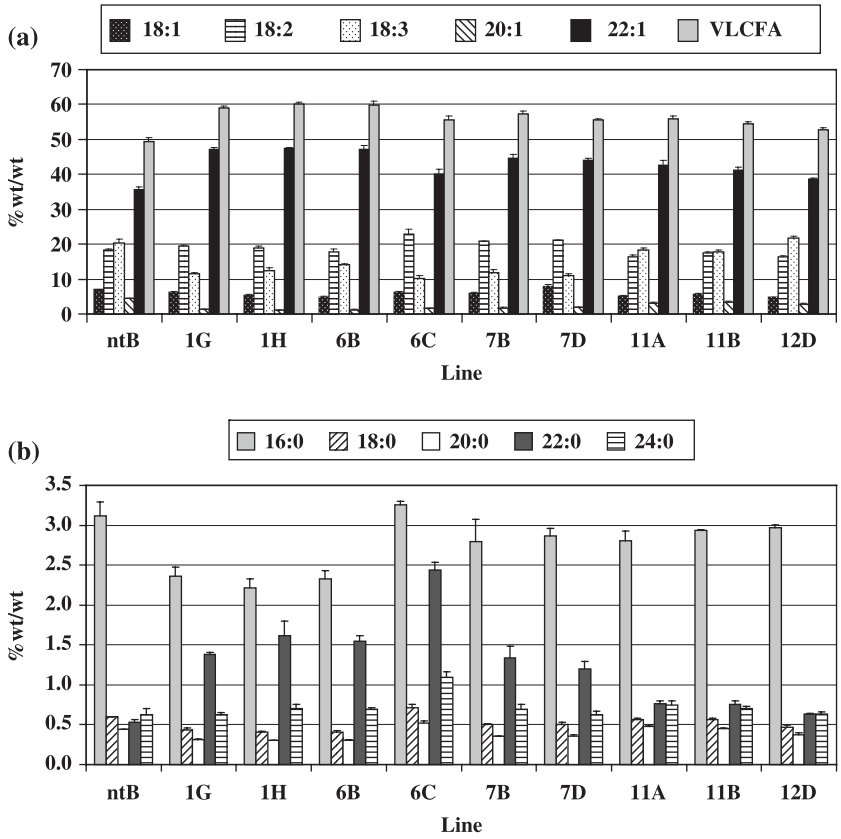

Figure 5 Fatty acid composition of mature seed of *B. carinata* (ntB) and T_1 seeds of transgenic *Brassica carinata* lines expressing the *CrFAE* gene under the control of the napin promoter. The values are the average ± SD of three determinations. (a) Proportions of 18 : $1^{\Delta 9}$, 18 : $2^{\Delta 9,12}$, 18 : $3^{\Delta 9,12,15}$, 20 : $1^{\Delta 11}$, 22 : $1^{\Delta 13}$, 24 : $1^{\Delta 15}$ and total VLCFAs (very long chain fatty acids) in seed oils. (b) Proportion of 16 : 0, 18 : 0, 20 : 0, 22 : 0 and 24 : 0 in seed oils.

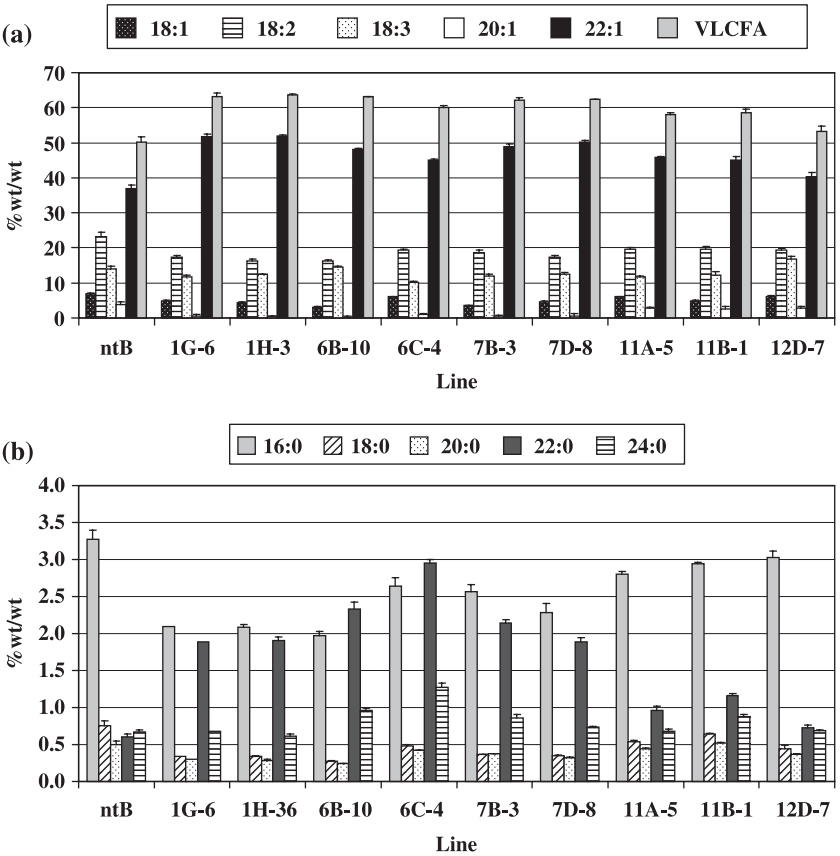

Figure 6 Fatty acid composition of mature seed of *Brassica carinata* (ntB) and T_2 seeds of transgenic *B. carinata* lines expressing the *CrFAE* gene under the control of the napin promoter. The values are the average ± SD of three determinations. (a) Proportions of 18 : $1^{\Delta 9}$, 18 : $2^{\Delta 9,12}$, 18 : $3^{\Delta 9,12,15}$, 20 : $1^{\Delta 11}$, 22 : $1^{\Delta 13}$, 24 : $1^{\Delta 15}$ and total very long chain fatty acids (VLCFAs) in seed oils. (b) Proportion of 16 : 0, 18 : 0, 20 : 0, 22 : 0 and 24 : 0 in seed oils.

consistent with the VLCFA composition of transgenic *CrFAE A. thaliana* as well as native and transgenic *B. carinata* (Figures 4a and 6a). Similar results were reported previously for *T. majus* FAE (Mietkiewska *et al*., 2004) and FAEs from high erucic *Brassicaceae* (Han *et al*., 2001; Katavic *et al*., 2001).

Seed-specific over-expression of *CrFAE* in *A. thaliana* plants resulted in up to a 12.5-fold increase in the proportion of erucic acid with a concomitant decrease of corresponding acyl precursors. The proportion of eicosenoic acid and oleic was decreased by up to 2.5- and 1.5-fold compared to the wild-type background, respectively. Compared to other *FAE* genes heterologously expressed in *A. thaliana* under the control of the napin promoter, the current expression of the *CrFAE* gene has resulted in the highest increase in the erucic acid proportions observed thus far in *A. thaliana* seeds. For instance, introducing the *S. chinensis FAE* or *T. majus FAE* into *A. thaliana* resulted in an increase in 22 : 1 proportion up to two- to three-fold and seven- to eight-fold in the transgenic seed compared to control lines, respectively (Lassner *et al*., 1996; Mietkiewska *et al*., 2004). Expression of *CrFAE* in wild-type *A. thaliana* with a high 18 : 1 and 20 : 1 background showed a strong preference for elongation of 20 : 1 (Figure 4a), while expression in the low VLCFA *fae1 A. thaliana* mutant background showed that the CrFAE could efficiently catalyse 2 successive elongation steps to form 22 : 1 (Figure 4b). This indicates that the CrFAE is not discriminatory as to which substrate it elongates; rather, it will elongate whichever precursor is predominantly present; in the case of wild-type *A. thaliana*, that precursor is 20 : 1, while in the *fae1* mutant, it is 18 : 1.

Analysis of seed oil from nine T_2 transgenic *B. carinata* lines expressing the *CrFAE* gene, showed a large increase (of up to 40%) in the proportion of erucic acid compared to the wild-type control. Unlike *B. juncea FAE1* transgenic plants (Kanrar *et al*., 2006), in the current study, *B. carinata* plants carrying the *CrFAE* (Figures 5a and 6a) also showed an increase in the proportion of total VLCFAs (by 10–30%). The synthesis of erucic acid in transgenic *B. carinata* plants was probably in part, limited by the smaller microsomal pool of eicosenoyl-(4–5%) and oleoyl-(7–8%) moieties available for elongation. As pointed out previously by Bao *et al*. (1998), the flux of 18 : 1 through distinct intermediate lipid pools before elongation might be a factor that limits the availability of 18 : 1 for elongation, while data of Domerque *et al*. (1999) suggest that the cytosolic pool of malonyl-CoA can limit VLCFA synthesis. Thus, additional intervention must be considered in order to increase the pool of substrate acyl moieties, for instance silencing of microsomal *FAD2* desaturase or over-expression of a heterologous stearoyl-ACP desaturase gene. Unlike *BjFAE* transgenic plants (Kanrar *et al*., 2006), in the present study, the very high proportion of 22 : 1 in *CrFAE* transgenic seed oil was correlated with lower proportions of 18 : 2 in T_2 seeds. This could possibly be explained by the presence of the new heterologous CrFAE, which is significantly more efficient than, e.g. the BjFAE in elongating 18 : 1 to 22 : 1 (Figures 4b and 6a), and that this might limit the pool of 18 : 1 available for desaturation. In our study the highest proportions of 22 : 1 were achieved with a higher number of *FAE* transgene inserts in the *B. carinata* genome. Similar results were reported for high erucic *B. napus* plants over-expressing *A. thaliana FAE1* (Katavic *et al*., 2001). Theoretically, an increase of transgene copy number results in an increase in transgene expression level (Butaye *et al*., 2005). However, multiple copy integration patterns often seem to be associated with low-level transgene expression, especially complex integration patterns such as tandem repeats (Jorgensen *et al*., 1996; Wang and Waterhouse, 2000) and inverted repeat (IR) structures (Muskens *et al*., 2000).

In conclusion, the ability of the CrFAE protein to elongate monounsaturated acyl chains and, preferentially, 20 : 1-CoA, is consistent with the observed acyl composition of *C. abyssinica* seed oil which consists primarily of very long chain- and specifically, erucoyl moieties. Thus, the *CrFAE* homologue described herein, may have a larger engineering impact when strongly expressed in a seed-specific manner in HEA *Brassicaceae* (e.g. *B. napus* or *B. carinata*) wherein 18 : $1^{\Delta 9}$ [and 18 : 2/18 : 3] and 20 : $1^{\Delta 11}$ represent a potential acyl-CoA elongation substrate pool totalling almost 40% over and above the existing 40–45% 22 : $1^{\Delta 13}$ proportions. 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 *CrFAE* 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 HEAR *Brassicaceae* (Katavic *et al*., 2001; Taylor *et al*., 2001; Jadhav *et al*., 2005) and the proportions of erucic acid in particular, to provide an industrial feedstock oil of high value and broad applicability.

Experimental procedures

Plant materials and growth conditions

All experimental lines propagated in the greenhouse were grown at the Kristjanson Biotechnology Complex greenhouses, Saskatoon, 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 25–30%. *C. abyssinica* plants (cultivar Prophet) 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. *B. carinata* plants were grown under sterile conditions on MS medium (Murashige and Skoog, 1962) during transformation and tissue culture; transgenic *B. carinata* plants were grown under normal greenhouse conditions. *A. thaliana* plants were grown in a growth chamber at 22 °C with photoperiod of 16 h light (120 μE/m^2/s) and 8 h dark.

Cloning of *C. abyssinica FAE* and heterologous expression in yeast

Based on *FAE1* sequences from *A. thaliana* and *B. napus*, the forward primer: 5′-GCAATGACGTCCATTAACGTAAAG-3′ and the reverse primer: 5′-TTAGGACCGACCGTTTTGGGC-3′ were designed and used to isolate the *FAE* coding region from *C. abyssinica*. Genomic DNA isolated from leaves according to urea-phenol extraction method (Chen *et al*., 1992) was used as a template for PCR amplification with Vent DNA polymerase (New England Biolabs, Mississauga, ON, Canada) in a thermocycler during 30 cycles of the following programme: 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min. A 1.5-kb PCR product was cloned into the pYES2.1/V5-His-TOPO expression vector and subsequently sequenced. The *CrFAE* in pYES2.1/V5-His-TOPO was transformed into *Saccharomyces cerevisiae* strain Inv Sc1 (Invitrogen, Burlington, ON, Canada) and a mutant strain *elo*2Δ (Oh *et al*., 1997) using the *S. c*. EasyComp transformation kit (Invitrogen). Yeast cells transformed with pYES2.1/V5-His-TOPO plasmid only were used as a control. The transformants were selected and grown as described previously (Katavic *et al*., 2002; Mietkiewska *et al*., 2004). Fatty acid methyl esters (FAME) from yeast cultures were extracted and analysed as described by Katavic *et al*. (2002).

Preparation of yeast homogenates

Yeast homogenates were prepared essentially as described by Katavic *et al*. (2004). Briefly, cells were harvested and washed with 10 mL of ice-cold isolation buffer (80 mM HEPES-NaOH, pH 7.4, 5 mM EGTA, 5 mM EDTA, 10 mM KCl, 320 mM sucrose, 2 mM dithiothreithol), pelleted by centrigugation and resuspended in 500 μL of isolation buffer. Cells were broken using three 60-s pulses with a Mini-Beadbeater™ (Biospec product, Bartlesville, OK, USA) using 0.5 mm glass beads. The homogenate was collected and briefly centrifuged to remove unbroken cells. The protein concentration of each lysate preparation was determined using the Bio-Rad method (Bradford, 1976).

Fatty acid elongase assay

Fatty acid elongase assays of yeast homogenates were performed as described previously (Katavic *et al*., 2004). The assay mixture consisted of 80 mM HEPES-NaOH, pH 7.4, 1 mM ATP, 0.5 mM NADH, 0.5 mM NADPH, 1 mM malonyl-CoA, 2 mM $MgCl_2$, 1 mM CoA-SH and 18 μM [1-^{14}C] acyl-CoA (0.37 GBq/mol) in a final volume of 500 μL. The reaction was started by addition of 500 μg of protein and incubated for 1 h at 30 °C. Elongase reactions were stopped with 3 mL of 100 g/L KOH in methanol. FAMEs were prepared and quantified by radio-HPLC as described previously (Taylor *et al*., 1992; Mietkiewska *et al*., 2004).

Lipid analyses

The total fatty acid content and acyl composition of *A. thaliana* and *Brassica* seed oils were determined by gas chromatography of the FAMEs with 17 : 0 FAME as an internal standard as described (Katavic *et al*., 2001; Taylor *et al*., 2001; Marillia *et al*., 2002).

Crambe abyssinica FAE sequence handling

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

Plant transformation vector

The coding region of the *CrFAE* was amplified by polymerase chain reaction with primers: forward: 5′-tatctagaATGACGTCCATTAACGTAAAG-3′ (lower case- restriction site for *Xba*I) and reverse: 5′-atggtaccTTAGGACCGACCGTTTTGG-3′ (lower case shows restriction site for *Kpn*I) and subsequently cloned behind the napin promoter in the respective restriction sites of the pSE vector (Jako *et al*., 2001).

The final binary vector (napin/*CrFAE*) was electroporated into *Agrobacterium tumefaciens* cells strain GV3101 containing helper plasmid pMP90 (Koncz and Schell, 1986). Plasmid integrity was verified by DNA sequencing following its re-isolation from *A. tumefaciens* and transformation into *E. coli*.

Plant transformation and genetic analysis

The binary vector was used to transform *A. thaliana* ecotype Columbia and *fae1* mutant line AC56 (Katavic *et al*., 1995) plants by the vacuum infiltration method (Clough and Bent, 1998) and *Brassica carinata* plants by the method of Babic *et al*. (1998). Transgenic plants were selected and analysed essentially as described by Mietkiewska *et al*. (2004).

Northern and Southern analyses

Total RNA from *C. abyssinica* plant material was isolated as described by Lindstrom and Vodkin (1991). Twenty micrograms of RNA was fractionated on a 1.4% (w/v) 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, Baie d'Urfe, Canada). A 1.5-kb probe containing the coding sequence of *FAE* was generated by PCR using primers 5′-ATGACGTCCATTAACGTAAAG-3′ and 5′-GGACCGACCGTTTTGGGC-3′ and subsequently radioactively labelled with ^{32}P using a Random Primers labelling kit (Invitrogen). Membranes were hybridized at 60 °C overnight. The filters were washed once in 1× SSPE, 0.1% SDS for 15 min and in 0.1× SSPE, 0.1% SDS for 5–10 min at the temperature of hybridization. The blots were exposed to X-OMAT-AR film (Kodak, Rochester, NY, USA).

Twenty micrograms of *B. carinata* genomic DNA was digested with the restriction enzyme *Xba*I, and the resulting fragments were

separated on a 0.9% (w/v) agarose gel and transferred to Hybond N^+ nylon membrane via an alkali blotting protocol. Hydridization was performed at 65 °C overnight as described above.

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