Plant Molecular Biology **46:** 717–725, 2001.

Expression of the *FAE1* gene and *FAE1* promoter activity in developing seeds of *Arabidopsis thaliana*

Maren Rossak, Mark Smith and Ljerka Kunst*
*Department of Botany, University of British Columbia, 6270 University Boulevard, Vancouver, BC V6T 1Z4, Canada (*author for correspondence; e-mail kunst@interchange.ubc.ca*

Received 7 March 2001; accepted in revised form 24 May 2001

Key words: Arabidopsis, FAE1 expression, fatty acid elongase, 3-ketoacyl-CoA synthase, seed oil deposition, seed-specific promoter

Abstract

Plant fatty acid elongase which catalyzes very-long-chain fatty acid (VLCFA) biosynthesis is a membrane-bound multienzyme complex. It is composed of four enzymes, a 3-ketoacyl-CoA synthase (condensing enzyme), a 3-ketoacyl-CoA reductase, a 3-hydroxyacyl-CoA dehydrase, and an enoyl-CoA reductase required for completion of each step of 2-carbon elongation of fatty acids. To improve our understanding of the overall regulation of the fatty acid elongase, we investigated the spatial and temporal expression of its key component, the FAE1-condensing enzyme, and examined the activity of the promoter of the *FAE1* gene in *Arabidopsis*. *In situ* hybridization results revealed that *FAE1* transcripts were found exclusively in the embryo. RNA blot analysis and histochemical analysis of GUS activity in *pFAE1::GUS* transgenic *Arabidopsis* lines demonstrated that the *FAE1* gene was already transcribed in the early torpedo stage embryos 4–5 days after flowering, with transcription reaching its peak 9–11 days after flowering. VLCFA deposition closely paralleled *FAE1* transcript accumulation. *FAE1* promoter was highly active and embryo-specific. Because its timing coincides with the period of major storage lipid accumulation, and because its *in vivo* activity in *Arabidopsis* is superior to the napin promoter, *FAE1* promoter may be ideal for genetic engineering of seed oil composition.

Abbreviations: X:Y, a fatty acyl group containing X carbons with Y double bonds

Introduction

Very-long-chain fatty acids (VLCFAs, fatty acids with chain lengths $>$ 18 carbons) are components of the seed oil of a number of plant species, including members of the Brassicaceae, and species in the genera *Limnanthes*, *Simmondsia* and *Tropaeolum*. The presence of VLCFAs in vegetable oils markedly affects their use. For example, rapeseed oil is naturally high in erucic acid (22:1; 45–60% of total seed fatty acids), which represents an important industrial feedstock for the production of high-temperature lubricants, cosmetics, pharmaceuticals and plasticizers (Johnson and Fritz, 1989). Thus, there is a great demand to further increase erucic acid content in industrial varieties of rapeseed. On the other hand, erucic acid is undesirable in edible oils because it has detrimental nutritional effects (Beare-Rogers, 1992). As a result, canola varieties of rapeseed devoid of erucic acid have been developed for edible oil production (Stefansson *et al*., 1961). These canola rapeseed varieties appear to have mutations in two genes that encode 3-ketoacyl-CoA synthases, involved in the elongation of C18 and C20 fatty acids (Lassner *et al*., 1996; Barret *et al*., 1998). These examples illustrate that the ability to manipulate VLCFA metabolism in a desired fashion is important for agriculture.

VLCFA are synthesized in the cytosol by extension of 18-carbon fatty acids generated in the plastid. Each cycle of fatty acid elongation adds two carbons to the acyl chain and involves four reactions, condensation of malonyl-CoA with an acyl primer, followed by a re-

duction, dehydration and a second reduction. The four enzymes catalyzing VLCFA biosynthesis are known as the fatty acid elongase (von Wettstein-Knowles, 1982). They are membrane-bound and thought to be associated in a functional complex (Whitfield *et al*., 1993). The 3-ketoacyl-CoA synthase (KCS) catalyzing the condensation reaction plays a key role in determining the chain length of fatty acid products found in seed oils (Lassner *et al*., 1996, Millar and Kunst, 1997) and is the rate-limiting enzyme for seed VLCFA production (Millar and Kunst, 1997).

In addition to the activity of the KCS, the composition of the fatty acyl-CoA pool available for elongation (Millar and Kunst, 1997) and the presence and size of a neutral lipid sink are important factors influencing the types and levels of VLCFAs made in a particular cell. For instance, reduced accumulation of storage triacylglycerols in a mutant of *Arabidopsis* deficient in diacylglycerol acyltransferase activity results in a severe repression of VLCFA accumulation (Katavic *et al*., 1995). Conversely, the creation of a large neutral lipid sink for VLCFAs appears to significantly increase the VLCFA content in *Arabidopsis* seeds. A good example are *Arabidopsis* seeds engineered to produce high levels of storage waxes (Lardizabal *et al*., 2000). These waxes are esters of monounsaturated VLCFA and fatty alcohols (Miwa, 1971), thus VLCFA are required for their formation. Analyses of seed oil from several independent transgenic lines indicate that there is a linear relationship between wax content and the accumulation of VLCFAs, which is maintained even in lines with the highest wax levels.

Despite recent progress in understanding the biochemistry of fatty acid elongation, relatively little is known about the expression of the enzymes comprising the elongase. In this study, our goal was to examine the temporal and spatial expression of the 'fatty acid elongation 1' (FAE1) condensing enzyme encoded by the *FAE1* gene in *Arabidopsis*. The *FAE1* gene was cloned by transposon tagging (James *et al*., 1995) and shown to be expressed in developing *Arabidopsis* seeds, but not in leaves. Our results presented here reveal that the *FAE1* transcript accumulates in the seed exclusively in the developing embryo at the time of VLCFA production. In addition, we cloned the 5′-upstream region of the *FAE1* gene, and we demonstrate that the isolated *FAE1* promoter fragment efficiently directs the seed-specific expression of foreign genes during embryogenesis.

Materials and methods

RNA isolation and hybridization

Total RNA was isolated from siliques by a method adapted from Kay *et al*. (1987). Thirty siliques of selected developmental stages were harvested, frozen in liquid nitrogen, and ground in a mortar with 2 ml extraction buffer (100 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS). The tissue was transferred into a tube containing 2.5 ml phenol/chloroform (1:1), shaken for at least 10 min, then centrifuged for 20 min at 5000 × g at 4 °C. The aqueous layer was removed and re-extracted twice with phenol/chloroform (1:1). High-molecular-weight RNA was precipitated by adding an equal volume of 5 M lithium acetate to the final supernatant followed by an overnight incubation on ice. The RNA was collected by centrifugation at 10 000 × *g* for 20 min at 4 °C and washed with ethanol. The pellet was dried and dissolved in water to a final concentration of 0.5 μg/μl. Total RNA (10 μg) was separated in each lane of a 1.2% w/v agarose gel containing formaldehyde. The RNA was subsequently transferred onto a Hybond-NX membrane (Amersham) according to the manufacturer's protocol. RNA blot hybridization was performed in 0.5 M sodium phosphate pH 6.8, 7% SDS, and 10 mM EDTA solution using PCR-amplified ^{32}P-labeled probes. The *FAE1* probe was prepared by PCR with pGEM7-FAE1 (Millar and Kunst, 1997) as a template with *FAE1* upstream primer 5′-CCGAGCTCAAAGAGGATACATAC-3′ and *FAE1* downstream primer 5′-GATACTCGAGAACGTTGGC ACTCAGATAC-3′. PCR was performed in a 10 μl reaction containing 10 ng of the template, 2 mM $MgCl_2$, 1.1 μM of each primer, 100 μM of (dCTP + dGTP + dTTP) mix, 50 μCi of [α-^{32}P]dATP, 1× PCR buffer and 2.5 units of *Taq* DNA polymerase (Life Technologies). PCR amplification conditions were: 2 min of initial denaturation at 94 °C, 30 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 100 s, followed by a final extension at 72 °C for 7 min. For the PCR amplification of the 18S rRNA probe (Unfried *et al*., 1989), 10 ng of *Arabidopsis thaliana* (ecotype Columbia) DNA was used as a template with *Taq* DNA polymerase (Life Technologies) and oligonucleotide primers 5′-CTGCCAGTAGTCATATGC-3′ and 5′-ATGGATCCTCGTTAAGGG-3′. Conditions used for PCR amplification were: 2 min of initial denaturation at 94 °C, 30 cycles of 94 °C for 15 s, 50 °C for 30 s, 72 °C for 30 s, followed by a final extension at

72 °C for 7 min. Blots were washed twice in 2× SSC for 5 min, once in 1× SSC for 15 min and twice in 0.1× SSC for 15 min at 65 °C. Membranes were then exposed to X-ray film.

In situ *hybridization*

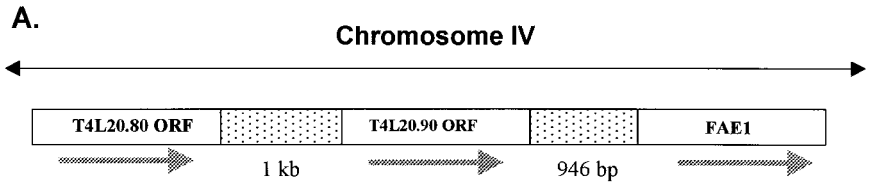
DNA templates for making *FAE1* sense and antisense probes were prepared by polymerase chain reaction using *FAE1* cDNA and *FAE1* primers with an added T7 polymerase binding site. The primer sequences were as follows: for the antisense probe forward primer 5′-ATGACGTCCGTTAAC-3′ and reverse primer 5′-GATAATACGACTCACTATAGGG CACTCAGATACATC-3′, and for the sense probe forward primer 5′-GATAATACGACTCACTATAGGAT GACGTCCGTTAAC-3′ and reverse primer 5′-GCAC TCAGATACATC-3′. PCR amplification conditions were: 2 min of initial denaturation at 94 °C, 30 cycles of 94 °C for 15 s, 65 °C for 30 s, 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. The PCR products were purified using the QIAquick PCR purification kit (Qiagen) and RNA probes were transcribed from the PCR-generated DNA templates with T7 RNA polymerase. The probes were DIG-labeled with the Boehringer Mannheim nucleic acid labeling kit according to the manufacturer's specifications, and then cleaved to ca. 150 bp pieces by alkaline hydrolysis at 60 °C in 0.2 M sodium carbonate buffer pH 10.4. Fixation and embedding of the plant material as well as *in situ* hybridization and detection of the signal were performed according to Samach *et al*. (1997).

Isolation of the FAE1 *promoter region from* Arabidopsis

Sequencing and annotation of the BAC clone T4L20 (GenBank accession number AL023094) from chromosome 4 which contains the *FAE1* gene revealed that 946 bp upstream of the *FAE1* coding region lies another open reading frame (T4L20.90) (Figure 1). Thus, the 946 bp *FAE1* 5′ genomic fragment directly upstream of the FAE1 coding region likely contains the 3′-untranslated region of the T4L20.90 gene and, because *FAE1* has no introns, all the regulatory elements required to direct correct temporal and spatial expression of the *FAE1* gene. Due to the lack of information concerning the exact length of the *FAE1* promoter, we PCR-amplified the 934 bp genomic fragment upstream of the *FAE1* coding sequence and used it in the experiments described below. For PCR amplification, the forward primer 5′-CTAGTAGATTGGTTGGTTGGTTTCC-3′ was used in combination with the reverse primer 5′-TGCTCTGTTTGTGTCGGAAAATAATGG-3′ (Figure 1). PCR conditions used were: 2 min of initial denaturation at 94 °C, 30 cycles of 94 °C for 15 s, 65 °C for 30 s, 72 °C for 45 s, followed by a final extension at 72 °C for 7 min. The amplified product was subcloned in the *Hin*cII site of the plasmid pT7T3-18U (Pharmacia), followed by complete sequence determination of both strands to verify the fragment identity.

Construction of transformation vectors and plant transformation

To make the promoter-GUS fusion construct, the 934 bp 5′ *FAE1* promoter fragment was cleaved out of pT7T3-18U vector with *Hin*dIII and *Xba*I and directionally subcloned into the corresponding sites of the binary Ti plasmid pBI101 (Clontech) upstream of the *uidA* reporter gene encoding β-glucuronidase (GUS). For comparison of the *FAE1* and napin promoters, two constructs were prepared: *pNapin-CFAH12* and *pFAE1-CFAH12*. To generate *pNapin-CFAH12* construct, the coding region of the castor hydroxylase (CFAH12; van de Loo *et al*., 1995) and the *nos* terminator from the vector pJD330 (Shaul and Galili, 1992) were ligated together behind the *Arabidopsis* napin promoter obtained from Dr M. Moloney (University of Calgary, Canada) in the vector pGEM-3zf(−) (Promega) to give plasmid pMS12. The entire cassette was then cut from pMS12 with *Eco*RI and *Sal*I and ligated into the binary vector pRD400 (Datla *et al*., 1992). For the *pFAE1-CFAH12* construct the *FAE1* promoter fragment was cut from the vector pT7T3-18U (Pharmacia) with *Pst*I and *Xba*I and subcloned into *Pst*I/*Xba*I sites of pBluescript KS+ (Stratagene) to give pMS128. This vector was then cut with *Xba*I, recessed 3′ ends were filled in using Klenow, and the promoter was released by cutting with *Eco*RI. The promoter fragment was ligated into *Eco*RI and *Sma*I sites of pMS18 (CFAH12-nos in pGEM-3zf(−)) to form pMS133. The entire expression cassette was cut from pMS133 with *Eco*RI and *Sal*I, and cloned into the binary vector pRD400 (Datla *et al*., 1992). All constructs in transformation vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90; Koncz and Schell, 1986) by heat-shock and selected for resistance to kanamycin (50 μg/ml). *A. thaliana* (L.) Heynh. ecotype Columbia-2 or *fae1/fad2* double mutant of *A. thaliana* were transformed with the con-



B.

```
 950                  CTAG TAGATTGGTT GGTTGGTTTC CATGTACCAG
-900 AAGGCTTACC CTATTAGTTG AAAGTTGAAA CTTTGTTCCC TACTCAATTC
-850 CTAGTTGTGT AAATGTATGT ATATGTAATG CGTATAAAAC GTAGTACTTA
-800 AATGACTAGG AGTGGTTCTT GAGACCGATG AGAGATGGGA GCAGAACTAA
-750 AGATGATGAC ATAATTAAGA ACGAATTTGA AAGGCTCTTA GGTTTGAATC
-700 CTATTCGAGA ATGTTTTTGT CAAAGATAGT GGCGATTTTG AACCAAAGAA
-650 AACATTTAAA AAATCAGTAT CCGGTTACGT TCATGCAAAT AGAAAGTGGT
-600 CTAGGATCTG ATTGTAATTT TAGACTTAAA GAGTCTCTTA AGATTCAATC
-550 CTGGCTGTGT ACAAAACTAC AAATAATATA TTTTAGACTA TTTGGCCTTA
-500 ACTAAACTTC CACTCATTAT TTACTGAGGT TAGAGAATAG ACTTGCGAAT
-450 AAACACATTC CCGAGAAATA CTCATGATCC CATAATTAGT CAGAGGGTAT
-400 GCCAATCAGA TCTAAGAACA CACATTCCCT CAAATTTTAA TGCACATGTA
-350 ATCATAGTTT AGCACAATTC AAAAATAATG TAGTATTAAA GACAGAAATT
-300 TGTAGACTTT TTTTTGGCGT TAAAGGAAGA CTAAGTTTAT ACGTACATTT
-250 TATTTTAAGT GGAAAACCGA AATTTTCCAT CGAAATATAT GAATTTAGTA
-200 TATATATTTC TGCAATGTAC TATTTTGCTA TTTTGGCAAC TTTCAGTGGA
-150 CTACTACTTT ATTACAATGT GTATGGATGC ATGAGTTTGA GTATACACAT
-100 GTCTAAATGC ATGCTTTGCA AAACGTAACG GACCACAAAA GAGGATCCAT
 -50 GCAAATACAT CTCATAGCTT CCTCCATTAT TTTCCGACAC AAACAGAGCA
   1 ATG
```

Figure 1. Schematic representation of the position of *FAE1* on chromosome 4 of *A. thaliana* and sequence of the *FAE1* 5′ promoter fragment. A. Position of the *FAE1* gene and the *FAE1* 5′ promoter region *pFAE1* with respect to the upstream annotated genes T4L20.80 and T4L20.90 within the BAC clone T4L20. B. Nucleotide sequence of the promoter fragment directly upstream of the *FAE1* coding region. The nucleotides corresponding to the primers used for amplifying the *FAE1* promoter fragment are underlined. The nucleotide sequence of the *FAE1* 5′ promoter region was deposited in GenBank as accession number AF355982. The translation initiation codon is boxed.

structs using the floral dip method (Clough and Bent, 1998). Screening for transformed seed was done on 50 μg/ml kanamycin as described previously (Katavic *et al*., 1994). Plants were grown in Terra-lite Rediearth (W.R. Grace Canada, Ajax, Ontario, Canada) at 20 °C under continuous light.

Histochemical β-glucuronidase (GUS) assays

Histochemical localization of GUS activity in transgenic plants was carried out essentially as described (Jefferson, 1987). Plant tissue was incubated in 50 mM sodium phosphate pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, 0.05% w/v Triton X-100, and 0.35 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) for 4 to 7 h at 37 °C. After staining the samples were fixed in 70% ethanol and photographs were taken under a dissecting microscope.

Fatty acid analysis

For the determination of the fatty acid composition in seeds, 40 seeds of each developmental stage were harvested and fatty acid methyl esters were prepared according to Browse *et al*. (1986). Briefly, transmethylation was carried out in methanolic-HCl (Supelco) at 80 °C for 2 h, followed by extraction with hexane and analysis by gas-liquid chromatography (Kunst *et al*., 1992). Quantification of fatty acids was done by comparison to the C17 methyl ester (Sigma), which was added to each sample as an internal standard.

Results

Expression of FAE1 is embryo-specific and begins early in embryo development

The *FAE1* expression during seed development in wild type *Arabidopsis* was followed by RNA blot hybridization using the entire *FAE1* coding sequence as a probe (Figure 2). For this analysis, total RNA was isolated from wild type siliques, harvested at 2-day intervals starting at one day after flowering (DAF) up to 15 DAF. Under our growth conditions *Arabidopsis* seeds were considered mature at 17 DAF. *FAE1* mRNA was not detected in wild-type siliques prior to 7 DAF (Figure 2A). After that *FAE1* transcript levels increased rapidly to reach their maximum between 9 and 11 DAF. The level of *FAE1* mRNA declined after 13 DAF as the seed approached maturity.

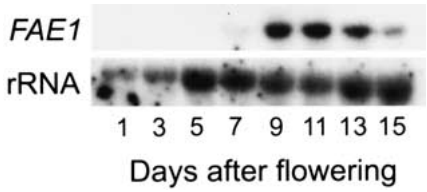


Figure 2. RNA gel blot analysis of *FAE1* mRNA accumulation during seed development in wild-type *Arabidopsis*. For the indicated time points 1–15 days after flowering (DAF), 30 siliques were harvested and total RNA extracted. RNA blot (10 μg/lane of total RNA) was hybridized with the *FAE1* probe or 18S ribosomal RNA as a loading control.

To analyze the spatial distribution of *FAE1* mRNA in seeds in detail, antisense transcripts were used as probes for *in situ* hybridizations to tissue sections of 9 DAF siliques. As shown in Figure 3, the transcripts were found exclusively in the embryo. They were most abundant in the cotyledons, the hypocotyl, and the upper parts of the root.

VLCFA accumulation during embryogenesis in Arabidopsis

VLCFAs, predominantly eicosenoic acid (20:1; ca. 21% w/w of total fatty acids) and erucic acid (22:1; ca. 2% w/w of total fatty acids), accumulate in the seed but not in other tissues of *Arabidopsis* (Lemieux *et al*., 1990). Accordingly, the *FAE1* transcript was found only in immature seed (James *et al*., 1995). To establish a time course of VLCFAs accumulation during seed development, the fatty acid composition of total lipids isolated from 40 green seeds between 3 and 15 DAF, as well as mature seeds at 17 DAF was determined (Figure 4). The synthesis of all major fatty acids in developing embryos increased dramatically around 5 DAF, with the peak occurring between 7 and 13 DAF, marking the phase of rapid oil deposition. VLCFAs followed a similar deposition pattern. They were first detected in developing embryos at 7 DAF. After that their rate of accumulation was rapid until 13 DAF. VLCFA synthesis was essentially complete at 15 DAF as the seed approached maturity.

A comparison of the RNA blot and fatty acid compositional analyses results indicated that VLCFAs accumulation mirrored the *FAE1* mRNA accumulation pattern during early to mid embryogenesis (7–11 DAF). However, around 13 DAF the *FAE1* transcript levels started to decline, while the VLCFA content leveled off (13–15 DAF) as oil biosynthesis ended.

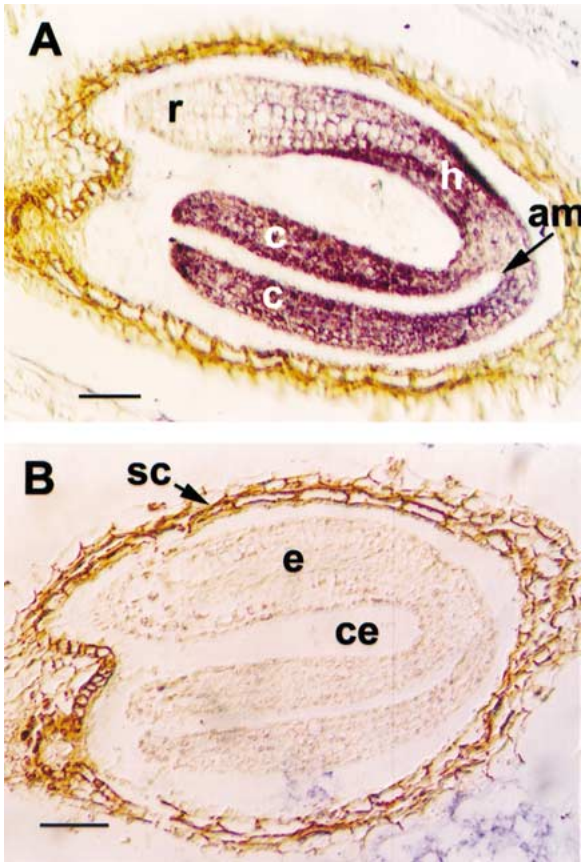


Figure 3. In situ detection of *FAE1* mRNA in developing embryos of *Arabidopsis*. Embryos were harvested at 9 DAF. 8 μm sections were incubated with 5 ng of (A) *FAE1* antisense RNA or (B) sense RNA. am, apical meristem; c, cotyledons; ce, cellular endosperm; e, embryo; h, hypocotyl; r, radicle; sc, seed coat. Bar = 0.1 mm.

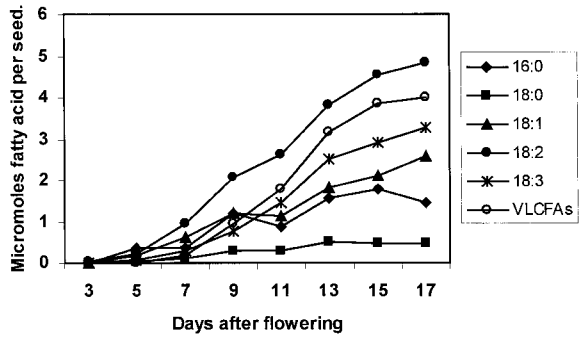


Figure 4. Accumulation of major fatty acids in developing seeds of wild-type *Arabidopsis*. The fatty acid content of 40 seeds between 3 and 15 DAF and in mature seeds 17 DAF was determined by GC analysis of fatty acid methyl esters after the addition of C17 standard. Each time point represents triplicate measurements. VLCFAs = 20:0 + 20:1 + 22:1.

The FAE1 promoter is a strong embryo-specific promoter useful for the expression of genes involved in seed lipid metabolism

The finding that *FAE1* gene expression is restricted to the embryo at the time of storage oil deposition prompted us to evaluate the utility of its promoter for genetic engineering of seed oil composition. At present, the napin storage protein promoter of *Brassica napus* is widely used to control expression of lipid biosynthetic genes in transgenic plants. However, storage protein accumulation in oilseeds seems to be delayed relative to that of oil (Murphy *et al*., 1989), suggesting that the timing of expression of the napin promoter may not be optimal for seed oil modification experiments. Thus, we examined the tissue-specific and developmental regulation of the *FAE1* promoter, and assessed its capacity to drive the expression of foreign genes in transgenic plants in comparison to the napin promoter.

To determine the developmental pattern of the *FAE1* promoter activity, we generated a *pFAE1-GUS* transcriptional fusion and transformed the construct into wild-type *Arabidopsis*. Histochemical analyses were performed on leaves, stems, siliques and developing embryos of three independent transgenic lines. Consistent with the RNA blot data obtained using a *FAE1*-specific probe (James *et al*., 1995), no GUS staining was observed in vegetative tissues of transgenic *Arabidopsis* (data not shown). In contrast, high levels of *FAE1* promoter activity, as evidenced by strong blue GUS staining, were detected in developing embryos (Figure 5). The *FAE1* promoter fragment was active as early as the torpedo stage embryo (4-5 DAF) and its activity persisted throughout subsequent embryo development.

In an attempt to compare the *in vivo* activity of the *FAE1* promoter to the napin promoter, we expressed the castor bean hydroxylase gene *CFAH12* (Broun and Somerville, 1997) behind either the *FAE1* promoter or the napin promoter in *Arabidopsis fad2/fae1* double mutant. The *fad2/fae1* double mutant, deficient in the activities of both the cytoplasmic oleate Δ-12 desaturase and the FAE1 condensing enzyme, contains seed oil with >80% of oleic acid (18:1), which is the substrate for the hydroxylase. The levels of hydroxylated fatty acids accumulating in a large number of independent transgenic lines were used to estimate the relative strength of each promoter. As shown in Figure 6, the two populations of transgenic plants accumulated levels of hydroxylated fatty acids, ranging from 0.2% to

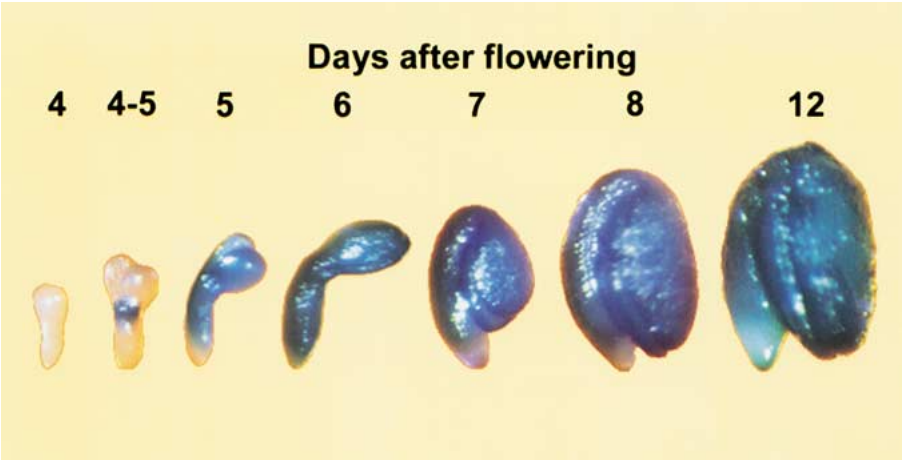


Figure 5. Histochemical staining of embryos at different developmental stages from transgenic *Arabidopsis* plants expressing GUS gene under the control of the *FAE1* promoter.

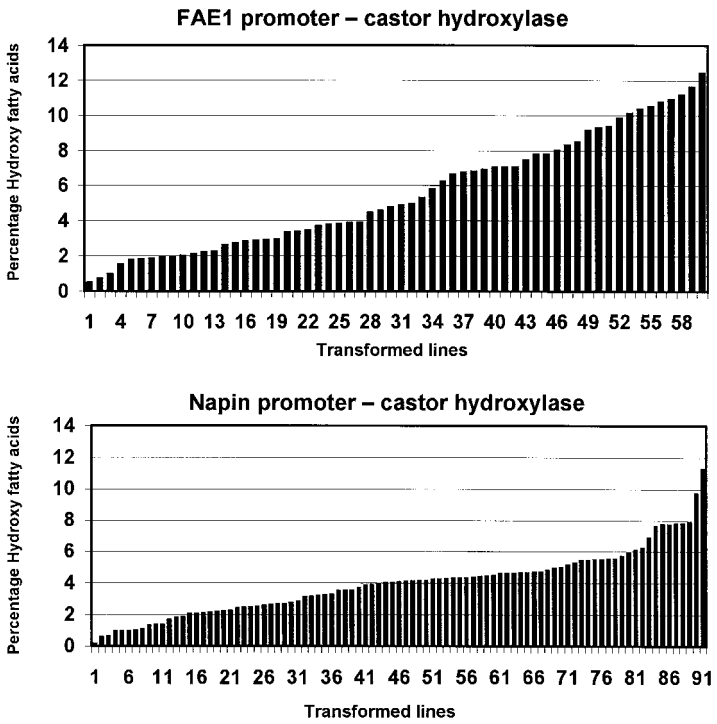


Figure 6. Hydroxy fatty acid accumulation in the seeds of transgenic *Arabidopsis*. *Arabidopsis fad2/fae1* double-mutant plants were transformed with the castor bean oleate hydroxylase cDNA under the control of the *FAE1* promoter, or the *Arabidopsis* napin promoter. Primary transformants were grown to maturity and seeds collected. Pools of 40–60 seeds from individual transformants were used for fatty acid analysis by gas chromatography. Hydroxy fatty acid levels are shown as the weight percentage of total seed fatty acids. *FAE1* promoter-castor hydroxylase, $n = 60$; range 0.5–12.4% ; average 5.5% . Napin promoter-castor hydroxylase, $n = 91$; range 0.1–11.3% ; average 3.9%.

about 11–12% of total fatty acids, with the levels being on average slightly higher in *pFAE1-CFAH12* lines. Similarly, the best *pFAE1-CFAH12* plant accumulated just over 12% w/w of hydroxylated fatty acids (as a percentage of total FAs), whereas the best *pNapin-CFAH12* plant produced 10.8% w/w of hydroxylated fatty acids. These results indicate that the *FAE1* promoter is highly active in transgenic *Arabidopsis* and that its *in vivo* activity may be superior to the napin promoter in *Arabidopsis* seeds.

Discussion

We have examined the temporal and spatial expression of the *FAE1* gene required for VLCFA biosynthesis in *Arabidopsis* seed. It encodes a seed-specific condensing enzyme, which controls the overall extent of fatty acid elongation in the embryo, as well as the chain lengths of VLCFA products made in the seed. We have also identified the *FAE1* promoter, and demonstrated that it can efficiently direct the expression of foreign genes in developing *Arabidopsis* seeds.

In situ hybridizations using antisense *FAE1* mRNA as a probe demonstrated that within the seed *FAE1* is expressed exclusively in the developing embryo, with the highest levels of expression in the cotyledons, the hypocotyl, and the top of the root (Figure 3). The *FAE1* transcript could be clearly detected as early as 7 DAF (Figure 2), after which it accumulated rapidly to around 11 DAF, followed by a gradual decline in the maturing seeds. Similarly, VLCFAs were also first detectable in developing seeds at 7 DAF, followed by a rapid increase in VLCFA levels between 7 and 13 DAF (Figure 4). The onset of VLCFA accumulation (middle to late embryogenesis) and general pattern of VLCFA accumulation throughout embryogenesis closely paralleled that of major storage lipid deposition in *Arabidopsis* (Mansfield and Briarty, 1992).

The *FAE1* expression in developing *Arabidopsis* seeds during the phase of oil production suggested that its 5′ regulatory region might be suitable for directing gene expression aimed at seed oil modification. The expression of a reporter gene by a promoter has been shown to accurately reflect the intrinsic regulatory properties of the promoter in many systems (Benfey and Chua, 1989; Bevan *et al*, 1989). Thus, we generated *FAE1* promoter-GUS fusions and evaluated the tissue specificity and developmental regulation of the *FAE1* promoter in transgenic *Arabidopsis*. Histochemical assays for β-glucuronidase activity indicated that a 934 bp *FAE1* promoter fragment could drive high levels of GUS expression in an embryo-specific manner. GUS staining was first detected at 4–5 DAF (early torpedo stage) in the hypocotyl (Figure 5). In addition to the hypocotyl, at 5 DAF GUS activity was observed in the cotyledons and the root. By 6 DAF intense blue GUS staining was already present throughout the embryo, and persisted throughout subsequent embryo development. Thus, the activity of the *FAE1* promoter coincides with storage oil accumulation in *Arabidopsis*.

In order to evaluate the activity of the *FAE1* promoter in vivo, the castor bean CFAH12 gene encoding an oleate 12-hydroxylase (Broun and Somerville, 1997) was fused to the 934-bp *FAE1* 5′ promoter fragment and expressed in *Arabidopsis*. The levels of hydroxy fatty acids were measured in seeds of transgenic plants and compared to the levels accumulating in plants transformed with the pNapin-CFAH12 construct. As expected, considerable variation was observed in the absolute levels of hydroxy fatty acids in various transformed lines in both populations, probably due to positional effects from insertion of the transgene at different sites in the genome. To overcome this problem we analyzed a large number of transformants in each case (Figure 6). Our results demonstrate that significant levels of hydroxy fatty acids can be produced in *Arabidopsis* seeds using both promoter sequences. However, the average levels of hydroxy fatty acids measured in pFAE1-CFAH12 plants were slightly higher than those in pNapin-CFAH12 transgenic lines (5.5% versus 3.9%), suggesting that the activity of the *FAE1* promoter may be greater than that of the napin promoter. Thus, we anticipate that the *FAE1* promoter will be useful for a variety of seed-specific applications, including genetic engineering of seed oil composition.

Acknowledgements

We thank Dr Sunita Chowrira and Tanya Hooker for critical reading of the manuscript. We are also grateful to Tanya Hooker for her help with the figures. This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to L.K. and a Deutscher Akademischer Austauschdienst (DAAD) postdoctoral fellowship to M.R.

References

Barret, P., Delourme, R., Renard, M., Domergue, F., Lessire, R., Delseny, M. and Roscoe, T.J. 1998. A rapeseed *FAE1* gene is linked to the E1 locus associated with variation in the content of erucic acid. Theor. Appl. Genet. 96: 177–186.

Beare-Rogers, J. 1992. Influences of findings in nutrition on the fats and oils industry. In: S.L. MacKenzie and D.C. Taylor (Eds.) Seed Oils for the Future, AOCS Press, Champaign, IL, pp. 9–13.

Benfey, P.N. and Chua, N.-H. 1989. Regulated genes in transgenic plants. Science 244: 174–181.

Bevan, M., Shufflebottom, D., Edwards, K., Jefferson, R. and Schuch, W. 1989. Tissue- and cell-specific activity of a phenylalanine ammonia-lyase promoter in transgenic plants. EMBO J. 8: 1899–1906.

Broun, P. and Somerville, C.R. 1997. Accumulation of ricinoleic, lesquerolic, and densipolic acids in seeds of transgenic Arabidopsis plants that express a fatty acyl hydroxylase cDNA from castor bean. Plant Physiol. 113: 933–942.

Browse, J., McCourt, P. and Somerville, C.R. 1986. Fatty acid composition of leaf lipids determined after combined digestion and fatty acid methyl ester formation from fresh tissue. Anal. Biochem. 152: 141–145.

Clough, S.J. and Bent, A.F. 1998. Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16: 735–743.

Datla, R.S.S., Hammerlindl, J.K., Panchuk, B., Pelcher, L.E., and Keller, W. 1992. Modified binary plant transformation vectors with the wild-type gene encoding NPTII. Gene 211: 383–384.

James, D.W. Jr., Lim, E., Keller, J., Plooy, I., Ralston, E., and Dooner, H.K. 1995. Directed tagging of the Arabidopsis FATTY ACID ELONGATION (FAE1) gene with the maize transposon Activator. Plant Cell 7: 309–319.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol. Biol. Rep. 5: 387–405.

Johnson, R.W., and Fritz, E. 1989. Fatty Acids in Industry. Marcel Dekker, New York.

Katavic, V., Haughn, G.W., Reed, D., Martin, M. and Kunst, L. 1994. *In planta* transformation of *Arabidopsis thaliana*. Mol. Gen. Genet. 245: 363–370.

Katavic, V., Reed, D.W., Taylor, D.C., Giblin, E.M., Barton, D.L., Zou, J., MacKenzie, S.L., Covello, P.S. and Kunst, L. 1995. Alteration of seed fatty acid composition by an ethyl methanesulfonate-induced mutation in Arabidopsis thaliana affecting diacylglycerol acyltransferase activity. Plant Physiol. 108: 399–409.

Kay, R., Chan, A., Daly, M. and McPherson, J. 1987. Duplication of CaMV 35S promoter sequences creates a strong enhancer for plant genes. Science 236: 1299–1302.

Koncz, C. and Schell J. 1986. The promoter of T_L-DNA gene 5 controls the tissue-specific expression of chimaeric genes by a novel type of *Agrobacterium* binary vector. Mol. Gen. Genet. 204: 383–396.

Kunst, L., Taylor, D.C. and Underhill, E.W. 1992. Fatty acid elongation in developing seeds of *Arabidopsis thaliana*. Plant Physiol. Biochem. 30: 425–434.

Lardizabal, K.D., Metz, J.G, Sakamoto, T. Hutton, W.C., Pollard, M.R. and Lassner M.W. 2000. Purification of a jojoba embryo wax synthase, cloning of its cDNA, and production of high levels of wax in seeds of transgenic Arabidopsis. Plant Physiol. 122: 645–655.

Lemieux, B., Miquel, M., Somerville, C. and Browse, J. 1990. Mutants of *Arabidopsis* with alterations in seed lipid fatty acid composition. Theor. Appl. Genet. 80: 234–240.

Lassner M.W., Lardizabal, K. and Metz J.G. 1996. A jojoba β-ketoacyl-CoA synthase cDNA complements the canola fatty acid elongation mutation in transgenic plants. Plant Cell 8: 281–292.

Mansfield, S.G. and Briarty, L.G. 1992. Cotyledon cell development in Arabidopsis thaliana during reserve deposition. Can. J. Bot. 70: 151–164.

Millar, A.A. and Kunst, L. 1997. Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. Plant J. 12: 121–131.

Murphy, D.J., Cummins, I. and Kang, A.S. 1989. Synthesis of the major oil-body membrane protein in developing rapeseed (*Brassica napus*) embryos. Biochem. J. 258: 285–293.

Samach, A., Kohalmi, S.E., Motte, P., Datla, R. and Haughn, G.W. 1997. Divergence of function and regulation of class B floral organ identity genes. Plant Cell 9: 559–570.

Shaul, O., and Galili, G. 1992. Increased lysine synthesis in tobacco plants that express high levels of bacterial dihydrodipicolinate synthase in their chloroplasts. Plant. J. 2: 203–209.

Stefansson, B.R., Hougen, F.W. and Downey, R.K. 1961. Note on the isolation of rape plants with seed oil free from erucic acid. Can. J. Plant Sci. 41: 218–219.

Unfried I., Stocker U., Gruendler P. 1989. Nucleotide sequence of the 18S rRNA gene from *Arabidopsis thaliana* Col0. Nucl. Acids Res. 17: 7513.

van de Loo, F.J., Broun , P., Turner, S. and Somerville, C.R. 1995. An oleate 12-hydroxylase from *Ricinus communis* L. is a fatty acyl desaturase homolog. Proc. Nat. Acad. Sci. USA 92: 6743–6747.

von Wettstein-Knowles, P.M. 1982. Elongase and epicuticular wax biosynthesis. Physiol. Vég. 20: 797–809.

Whitfield, H.V., Murphy, D.J. and Hills, M.J. 1993. Sub-cellular localization of fatty acid elongase in developing seeds of Lunaria annua and Brassica napus. Phytochemistry 32: 255–258.