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Acyl-CoA elongase expression during seed development in *Brassica napus*

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Abstract

The *Bn-FAE1.1* and *Bn-FAE1.2* genes encode the 3-ketoacyl-CoA synthase, a component of the elongation complex responsible for the synthesis of very long chain monounsaturated fatty acids (VLCMFA) in the seeds of *Brassica napus*. *Bn-FAE1* gene expression was studied during seed development using two different cultivars: Gaspard, a high erucic acid rapeseed (HEAR), and ISLR4, a low erucic acid rapeseed (LEAR). The mRNA developmental profiles were similar for the two cultivars, the maximal expression levels being measured at 8 weeks after pollination (WAP) in HEAR and at 9 WAP in LEAR. Differential expression of *Bn-FAE1.1* and *Bn-FAE1.2* genes was also studied. In each cultivar the same expression profile was observed for both genes, but *Bn-FAE1.2* was expressed at a lower level than *Bn-FAE1.1*. Secondly, VLCMFA synthesis was measured using particulate fractions prepared from maturating seeds harvested weekly after pollination. The oleoyl-CoA and ATP-dependent elongase activities increased from the 4th WAP in HEAR and reached the maximal level at 8 WAP, whereas both activities were absent in LEAR. In contrast, the 3-hydroxy dehydratase, a subunit of the elongase complex, had a similar activity in both cultivars and reached a maximum from 7 to 9 WAP. Finally, antibodies against the 3-ketoacyl-CoA synthase revealed a protein of 57 kDa present only in HEAR. Our results show: (i) that both genes are transcribed in HEAR and LEAR cultivars; (ii) that they are coordinately regulated; (iii) that *Bn-FAE1.1* is quantitatively the major isoform expressed in seeds; (iv) that the *Bn-FAE1* gene encodes a protein of 57 kDa responsible for the 3-ketoacyl-CoA synthase activity.

Keywords: Low erucic acid rapeseed; Seed development; Lipid synthesis; Very long chain fatty acid; 3-Ketoacyl-CoA synthase; *Fatty acid elongation 1*; *Brassica napus*

1. Introduction

In many higher plants, C16:1 and C18:1 fatty acids are the major components of the seed storage triacylglycerols (TAG). High erucic acid rapeseed (HEAR) oil is different since erucic acid (C22:1, Δ13) represents from 45 to 60% of the total fatty

Abbreviations: HEAR, high erucic acid rapeseed; LEAR, low erucic acid rapeseed; VLCMFA, very long chain monounsaturated fatty acid(s); LPAAT, lysophosphatidic acid acyltransferase; TAG, triacylglycerol(s); WAP, week after pollination

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acids. Erucic acid has many industrial uses, for instance in lubricants, plastics, etc. [1], and research programs attempting to increase the erucic acid content in rapeseed by genetic manipulations have been undertaken.

In rapeseed, it has been demonstrated that lysophosphatidic acid acyltransferase (LPAAT) is a key enzyme since it does not allow the esterification of the *sn*-2 position of the glycerol backbone by erucoyl-CoA in rapeseed [2,3]. Transgenic rapeseeds expressing the LPAAT from *Limnanthes alba*, which are able to esterify erucic acid at the *sn*-2 position, synthesize trierucin but the overall content of erucic acid in the seed remains unchanged [4]. This result is generally interpreted as reflecting a low erucoyl-CoA pool available for TAG biosynthesis [4]. Thus, elongases which are responsible for erucoyl-CoA synthesis are also key enzymes in trierucin biosynthesis. In higher plants, the existence of at least two types of elongases, the ATP-dependent elongase and the acyl-CoA elongase, has been demonstrated [5,6]. ATP-dependent elongase elongates an unknown endogenous substrate, whereas the acyl-CoA elongase, a multienzymatic and membrane-bound complex [6], uses exogenous acyl-CoA and does not require ATP. The situation is further complicated as it has been shown in rapeseed microsomes that conditions that allow maximum oleoyl-CoA elongation partially inhibit ATP-dependent elongation, and at maximum rates ATP-dependent elongation decreases oleoyl-CoA elongation [6]. These results strongly suggest that the two elongation systems cannot operate in vitro at the same time or in the same cell compartment.

The acyl-CoA elongase has been extensively studied and partly purified from many sources [5,7–9], including *Brassica napus* seeds [10]. This elongation complex comprises four different proteins with apparent molecular masses ranging from 54 to 65 kDa [8–10]. Four successive reactions are involved in the elongation process: condensation of C18:1-CoA to malonyl-CoA to form a 3-ketoacyl-CoA; reduction of the 3-ketoacyl-CoA; dehydration of the resulting 3-hydroxyacyl-CoA and reduction of the *trans*-(2,3)-enoyl-CoA [11–13]. The gene encoding the first enzyme, the 3-ketoacyl-CoA synthase, has been characterized in *Arabidopsis thaliana* (FAE1) [14] and jojoba [15] and two homologous sequences (*Bn-FAE1.1* and *Bn-FAE1.2*) have been isolated from embryos of *B. napus*. *Bn-FAE1.2* encodes a protein of 505 amino acids and *Bn-FAE1.1*, a protein of 506 amino acids, both proteins sharing 98.2% identity [16].

It has also been demonstrated that very long chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme [17]. In *B. napus*, the understanding of the role of *Bn-FAE1.1* and *Bn-FAE1.2* and their regulation during the development of the seed is necessary to implement strategies to increase the erucic acid content. Despite numerous investigations, fatty acid regulation at the molecular level is far from being understood. The pioneering work by Norton and Harris [18] reported that changes in fatty acid composition in developing rapeseed occurred in three consecutive phases, and that erucic acid accumulation took place in the last phase. To date, most of the studies have been carried out using microspore-derived cell cultures [19]. It has been shown that: (i) the fatty acid synthesis and acyltransferase activities increase from the 2nd to the 4th week of growth, and then decrease; (ii) the diacylglycerol acyltransferase (DAG-AT) activity and the oleosin gene expression increase when the sucrose concentration in the culture medium is raised from 2 to 22% (w/v) [20,21]; (iii) abscisic acid (ABA) and temperature stimulate erucic acid biosynthesis by different mechanisms [22] leading to an accumulation of very long chain monounsaturated fatty acids (VLCMFA) correlated with a stimulation of the elongase activity [23]. The expression of *Bn-FAE1* transcripts is induced by 10 μM ABA within 1 h and is further increased up to 6 h while, during the same time, the VLCMFA content doubles [24].

Canola or low erucic acid rapeseed (LEAR) varieties, characterized by a near-absence of VLCMFA, were created by the introduction of recessive alleles at two loci that control the elongation of fatty acids [25]. Elongase activities are present in HEAR embryos but absent from embryos of LEAR varieties [26], as is the case with the *FAE1* (fatty acid elongation) mutants of *Arabidopsis* [27], that exhibit a similar phenotype to LEAR. The *Arabidopsis FAE1* gene encodes a protein that shares homologies with con-

densing enzymes [14]. The jojoba homologue of *FAE1* encoding a 3-ketoacyl-CoA synthase restored elongation activity to developing embryos of LEAR, leading to the conclusion that the mutations that gave rise to the LEAR phenotype are associated with either the structural gene encoding 3-ketoacyl-CoA synthase or with genes regulating its expression [15]. In rapeseed, the two elongation steps from oleoyl-CoA to erucic acid are each controlled by alleles at two loci, E1 and E2, which exhibit additive gene action [28]. A gene encoding the rapeseed 3-ketoacyl-CoA synthase, *Bn-FAE1.1*, was shown by Barret et al. [16] to be tightly linked to the E1 locus and the homologous gene, *Bn-FAE1.2*, was assigned to the E2 locus.

Preliminary experiments indicate that *Bn-FAE1* could also be transcribed in LEAR embryos [29]. Since this observation was obtained using seeds harvested 9 weeks after pollination (WAP), no data concerning a post-transcriptional degradation, modification or regulation of this protein during seed development are available. Therefore, we investigated the enzymatic activities and regulation of *Bn-FAE1.1* and *Bn-FAE1.2* gene expression during rapeseed development using HEAR and LEAR cultivars.

2. Material and methods

2.1. Plant material

Two cultivars of *B. napus* L. (Gaspard and ISLR4) were grown outdoors under the same conditions at INRA (Le Rheu, France). Gaspard is a HEAR and ISLR4 a LEAR. Seeds from both cultivars were collected every week from 1 to 12 WAP and stored at −80°C.

2.2. Chemicals

All chemicals were from Sigma. [2-^{14}C]Malonyl-CoA (57 Ci/mol) came from NEN. *trans*-2,3-Eicosanoyl-CoA was prepared and purified according to the method of Lucet-Levannier et al. [30] and 3-[1-^{14}C]hydroxyeicosanoyl-CoA was synthesized as reported by Lessire et al. [31].

2.3. Microsome preparation

Seeds (0.5 g) were ground in a mortar in 10 ml of 80 mM HEPES buffer (pH 7.2) containing 10 mM β-mercaptoethanol, 0.32 mM sucrose and 5% polyvinylpyrrolidone (w/v). The homogenate was filtered through two layers of Miracloth and centrifuged at $10\,000\times g$ for 5 min. The resulting supernatant was then centrifuged at $100\,000\times g$ for 15 min. The microsomal pellet was resuspended in 500 μl of 80 mM HEPES buffer (pH 7.2) containing 10 mM β-mercaptoethanol and centrifuged again for 15 min at $100\,000\times g$. The final pellet was resuspended in 500 μl of the same buffer and used as enzyme source. Protein contents were determined using Bradford's method [32].

2.4. Acyl-CoA elongase assay

The acyl-CoA elongase was optimized as a function of malonyl-CoA and oleoyl-CoA concentrations (data not shown). The reaction conditions subsequently were: 20 μg of protein were incubated for 1 h at 30°C in the presence of 200 μM [2-^{14}C]-malonyl-CoA (45 mCi/mmole), 0.5 mM NADH, 0.5 mM NADPH, 1 mM $MgCl_2$, 2 mM DTT, 30 μM C18:1-CoA in a HEPES 80 mM buffer (pH 7.2). The final volume was 100 μl. The reaction was stopped with 100 μl of KOH 5 N/methanol 10% (v/v) and the samples were saponified for 1 h at 70°C. The solution was acidified with 100 μl 10 N H_2SO_4/10% malonic acid (w/v) and the fatty acids were extracted with 2 ml of chloroform. The chloroformic extract was washed three times with 2 ml of water and the radioactivity was measured.

2.5. ATP-dependent elongase assay

This assay was optimized for ATP and malonyl-CoA concentrations (data not shown) and allowed the following reaction conditions to be adopted. 20 μg of protein were incubated for 1 h at 30°C in a HEPES 80 mM buffer (pH 7.2) containing 200 μM [2-^{14}C]malonyl-CoA (45 mCi/mmole), 0.5 mM NADH, 0.5 mM NADPH, 1 mM $MgCl_2$, 2 mM DTT, 0.25 mM ATP in a final volume of 100 μl. The reaction was stopped and the fatty acids were extracted as described above.

2.6. Fatty acid analysis

Total fatty acids were converted into methyl esters using a 30 min incubation at 70°C in the presence of 1 ml methanol containing 10% of boron trifluoride. After addition of 1.5 ml of 2.5% aqueous NaCl, the methyl esters were extracted with hexane (2×2 ml) and analyzed by reverse phase TLC using RP-18 HPTLC plates (Merck, Darmstadt, USA) eluted by acetonitrile/tetrahydrofuran (80:20, v/v). In the latter case, radioactive bands were detected using a PhosphorImager and quantified with ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, USA).

2.7. 3-Hydroxyacyl-CoA dehydratase assay

The reaction mixture (final volume: 100 μl) contained in a HEPES 80 mM buffer (pH 7.2): 10 μg of microsomal proteins, 11.4 μM 3-[1-^{14}C]hydroxyeicosanoyl-CoA (52 mCi/mmole), 1 mM $MgCl_2$, 2 mM DTT, 75 μM Triton X-100. This incubation was run for 15 min at 30°C. Fatty acids were extracted as described above and layered on an HPLC plate (60F 254, Merck). The plate was developed with hexane/diethyl ether/acetic acid (75:25:1, v/v/v). The radioactivity recovered in the *trans*-2,3 C20:1-CoA and 3-OH C20:0 was quantified using a PhosphorImager.

2.8. RNA isolation

For each developmental stage and line studied, two independent RNA purifications were performed using 10–20 seeds. Total cytoplasmic RNA was isolated using the guanidinium isothiocyanate/cesium chloride procedure [33]. The RNA prepared with this protocol was used in the Northern experiments.

Alternatively, RNA was prepared with the SV Total RNA Isolation System (Promega). This method makes it possible to obtain total RNA substantially free of genomic DNA contamination. This is a prerequisite when RT-PCR experiments are to be performed. For every sample studied, PCR amplifications were carried out with non-reverse transcribed RNA; under these conditions no amplified product was ever obtained. Briefly, 70 mg of seeds were homogenized in 250 μl RNA lysis buffer. Then, 500 μl of RNA dilution buffer were added to the suspension. After centrifugation for 10 min at 12 000×*g*, 525 μl of the supernatant were collected and mixed with 200 μl of 95% ethanol. Thereafter, the binding of the RNA onto the spin column, the DNase I treatment and the RNA elution were performed according to the manufacturer's instructions. The corresponding RNA samples were then used in the RT-PCR experiments.

The amount of RNA was determined by spectrophotometry at 260 nm. To check for sample integrity prior to Northern blot and RT-PCR analysis, an aliquot of each RNA sample (2.5 μg) was analyzed on a 1.2% agarose/3% formaldehyde baby gel in 3-[*N*-morpholino]propanesulfonic acid buffer, and the ribosomal RNA bands were stained using 0.05% toluidine blue.

2.9. RT-PCR analysis of Bn-FAE1

Using a StrataScript RT-PCR kit (Stratagene), 2.5 μg of total RNA from each sample were reverse transcribed using random primers. From the 50 μl single-stranded cDNA mixture, a 1 μl aliquot was diluted in the 49 μl PCR reaction mix. After an initial incubation of 10 min at 95°C to activate the enzyme, 35 amplification cycles (30 s at 95°C, 45 s at 60°C and 90 s at 72°C) were performed using a GeneAmp PCR system 9700 (Perkin Elmer, Applied Biosystems) and AmpliTaq Gold DNA polymerase (Perkin Elmer, Applied Biosystems). The primers used for the RT-PCR analysis were (5′-3′) Bn-FAE1 5′: ATC GTA GAC GGT CCA AGT AC, and Bn-FAE1 3′: GAC CCT AAA GCA ATC TGC CA. Using these primers, the *Bn-FAE1* specific fragment was 508 bp long. To discriminate further between the *Bn-FAE1.1* and *Bn-FAE1.2* transcripts, 8 μl of the amplification products were incubated for 2 h at 37°C with 1 μl of *Hin*dIII buffer (REACT 2, Gibco BRL) and 1 μl of *Hin*dIII (10 units/μl, Gibco BRL). The digested products were then analyzed on a 2% agarose gel in TAE buffer. Using this technique, the undigested *Bn-FAE1.1* specific fragment was 508 bp long, whereas the *Bn-FAE1.2* amplification product was cleaved into two fragments of 285 and 223 bp. The *Bn-FAE1* specific fragment obtained by RT-PCR was also subsequently purified from a 2% agarose gel using a QIAEX purification kit (Qia-

gen), and this cDNA was used as a probe for the Northern blot experiments.

2.10. Northern blot analysis

10 μg of each RNA sample were separated in a 1.2% agarose/3% formaldehyde baby gel, transferred to Hybond-N membranes (Amersham) in a solution of 20×SSPE, cross-linked to the membrane by UV irradiation (UV Stratalinker 1800, Stratagene) and prehybridizations and hybridizations were carried out according to the manufacturer's instructions. Labeling of cDNA probes to a specific activity of 5×10^8–2×10^9 cpm/μg was done using a Prime-it II random primer labeling kit (Stratagene). The membranes were exposed in a PhosphorImager cassette, and signals were quantified using ImageQuaNT Software (Molecular Dynamics). To estimate the size of the transcript revealed on the Northern blots, an RNA ladder (Promega) was run in each gel, the corresponding lane was cut after the migration and the bands were stained in agarose gel using 0.05% toluidine blue.

The *Bn-FAE1* fragment was obtained by RT-PCR as described above, and this cDNA was used as probe for the Northern blot experiments. The *A. thaliana* ribosomal RNA cDNA probe was a gift from Dr. B. Ricard.

2.11. Production of antibodies against a recombinant 3-ketoacyl-CoA synthase

A nucleotide sequence of the *FAE1* gene (accession No. U50771) corresponding to the amino acid sequence between V97 and D370 of the encoded 3-ketoacyl-CoA synthase was amplified by PCR with the following oligonucleotides: CEE1 5′ AAA GGT CTC AAT GGT CAT GGA TAT CTT TTA CCA AG 3′ and CEE2 5′ TTT GGA TCC TTA GTC TTT GAA AAG TTT CTT GCC 3′ using a proofreading polymerase. This amplimer was ligated into the pMalC2 expression vector (New England Biolabs). The resulting vector was used to transform the BL21 strain of *Escherichia coli*. A transformant clone was grown in liquid culture to mid-log phase and IPTG was added to a final concentration of 0.5 mM. After 3 h growth at 30°C the cells were sedimented and lysed by hypotonic shock after treatment with lysozyme [34]. A recombinant fusion protein of the predicted size (70 kDa) was affinity purified under non-denaturing conditions from the cytoplasmic fraction. The fusion protein was further purified by SDS–PAGE, collected by electroelution and used to produce a rabbit antiserum. To verify the specificity of the antibody, Western blot experiments with *E. coli* extracts expressing various recombinant FAE1/maltose binding protein fusions were performed and proteins of the expected size were detected (data not shown).

2.12. Immunodetection of Bn-FAE1 proteins

A quantity of 7 μg of microsomal proteins was mixed with buffer A (Tris 25 mM, glycine 192 mM, sodium dodecyl sulfate (SDS) 0.1% (w/v)) to obtain a final volume of 10 μl. The resulting solution was mixed with 10 μl of gel loading buffer (Tris–HCl 62.5 mM, pH 6.8, 2-mercaptoethanol 5% (v/v); glycerol 25% (w/v); SDS 2% (w/v) and bromophenol blue 0.01% (w/v)). After denaturation for 3 min at 100°C, the samples were loaded onto a 12% polyacrylamide gel, and electrophoresis was run in buffer A. To estimate the size of the protein revealed on the Western blots, a protein ladder was run in every gel (Kaleidoscope Prestained Standards, Bio-Rad).

After electrophoresis, proteins were electrotransferred (15 h, 30 V, 4°C) to polyvinylene difluoride membranes (PVDF polyscreen, New England Nuclear) using a Mini Trans-blot Cell (Bio-Rad), and Tris 25 mM, glycine 192 mM buffer, methanol 20% (v/v), pH 8.3. The anti-FAE1 immunoserum was diluted 1/5000, and the secondary antibody (anti-rabbit IgG peroxidase conjugate, Sigma) was diluted 1/5000. Luminescent revelation of the secondary antibodies was done using Chemiluminescence Reagent Plus (NEN) and hyperfilm MP (Amersham).

3. Results

B. napus seeds of representative cultivars used in France (Gaspard, HEAR and ISLR4, LEAR) were sown and grown outdoors in experimental plots in the same field under the same conditions. Seeds were harvested each week during spring 1998 and spring 1999. The first day of pollination was determined

according to the BBCH (Bayer BASF Ciba Hoechst) scale and corresponded to 50% of opened flowers. Although we observed variations in the levels of enzymatic activities and maximal gene expression between seeds harvested in 1998 and 1999, the profiles of the different parameters studied (enzymatic activities and gene expression) during seed development were similar, in the two different experiment sets carried out in triplicate in 1998 and 1999. The results presented in this paper were obtained with seeds collected during spring 1999.

3.1. *Bn-FAE1* gene expression is similar in HEAR and LEAR during seed development

The pattern of the 3-ketoacyl-CoA synthase expression was determined by Northern hybridizations using total RNA isolated from HEAR and LEAR seeds, and a cDNA probe corresponding to a highly homologous region of the *Bn-FAE1.1* and *Bn-FAE1.2* sequences. A typical result obtained for the LEAR line is shown in Fig. 1A. A 1.6 kb transcript corresponding to the theoretical size of *FAE1* mRNA was detected between 4 WAP and 9 WAP. A similar pattern of expression was obtained for the HEAR line. For each genotype, two independent Northern experiments were performed and the steady-state levels for the *FAE1* mRNA were quantified (Fig. 1C). During LEAR development, the relative amount of *FAE1* mRNA was low from 1 WAP to 3 WAP (10% of the maximum), then increased up to 75% of the maximum 5 WAP, and increased slightly up to 9 WAP. Thereafter, *FAE1* mRNA decreased to a low basal level at 10 WAP and remained low in older seeds. The developmental profile obtained for the HEAR genotype was globally similar, with significant amounts of transcripts detected between 5 and 9 WAP and a maximal level of expression at 8 WAP. This difference is thus probably the consequence of varietal differences in growth rate, flowering time, seed filling between the cultivars rather than a different kind of transcriptional regulation. Moreover, we checked that the level of expression was similar in both cultivars by comparing the results obtained with RNA samples from LEAR 9 WAP and HEAR at 8 WAP. To achieve this goal, both samples were analyzed in parallel on the same Northern blot (Fig. 1B). Similar amounts of *FAE1* mRNA were detected in HEAR and LEAR seeds, thereby indicating that the LEAR mutation did not lead to a defect in the quantitative expression of the *FAE1* mRNA.

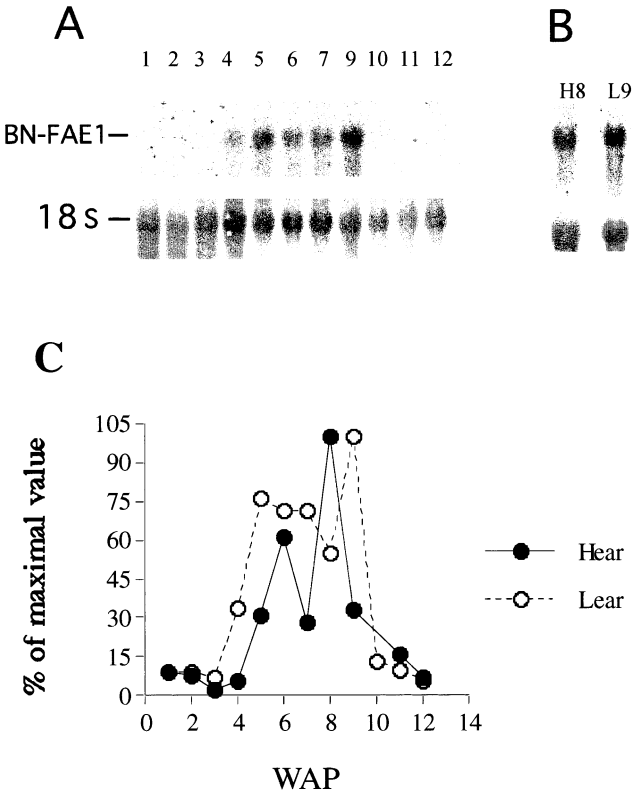


Fig. 1. *Bn-FAE1* mRNA expression in rapeseeds from HEAR and LEAR lines. 10 μg of total RNA from LEAR seeds were applied to an agarose-formaldehyde gel, blotted, and hybridized successively with the *Bn-FAE1* and rRNA cDNA probes. Lanes are marked according to the developmental stage (WAP). Northern blots obtained with LEAR and HEAR seeds from 9 and 8 WAP respectively, and hybridized successively with the *Bn-FAE1* and rRNA cDNA probes. 10 μg of total RNA per lane. For each developmental stage studied, two independent RNA purifications were performed and the corresponding blots were carried out for each cultivar. An example is shown in A. After correction for RNA levels between lanes, using the rRNA results, quantitations were performed using a PhosphorImager with the ImageQuaNT software. Results are presented as the percentage of the maximal level measured during the developmental period studied. Data are mean values for the two experiments.

3.2. *Bn-FAE1.1* was expressed at higher levels than *Bn-FAE1.2* during seed development

Bn-FAE1.1 and *Bn-FAE1.2* genes are very homol-

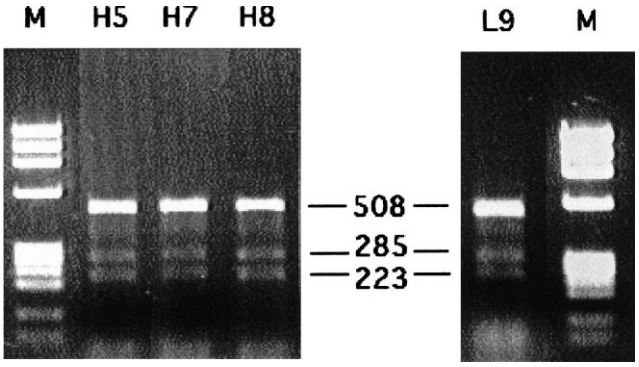


Fig. 2. *Bn-FAE1.1* and *Bn-FAE1.2* expression during HEAR and LEAR seed development. After RT-PCR, the amplified products from HEAR (H) and LEAR (L) seeds were *Hin*dIII digested and analyzed by 2% gel agarose electrophoresis. Lanes are marked according to the developmental stage (WAP) and the genotype. M, size markers (ΦX174 DNA-*Hae*III digested).

ogous, the corresponding encoded proteins sharing 98.2% amino acid identity [16] but the *Bn-FAE1.2* sequence differs by the presence of a *Hin*dIII restriction site at position 1141. To monitor the respective levels of expression of *Bn-FAE1.1* and *Bn-FAE1.2*, we used a RT-PCR/*Hin*dIII digestion strategy. *Bn-FAE1.1* and *Bn-FAE1.2* transcripts were reverse transcribed and amplified (nucleotides 856–1364 of the mRNA sequence) in the same tube. The amplification product corresponding to both transcripts was 508 bp long. The further *Hin*dIII digestion led to two products of 285 bp and 223 bp for the *Bn-FAE1.2* transcript, whereas the *Bn-FAE1.1* PCR product remained undigested. A typical result is shown in Fig. 2. In every sample tested from HEAR and LEAR seeds, we detected a major 508 bp band corresponding to *Bn-FAE1.1* mRNA, and two bands of low intensity corresponding to the digested products of the *Bn-FAE1.2* amplified product. Several control experiments using different amounts of amplimers, increasing amounts of *Hin*dIII, and longer digestion times (up to 12 h) led to the same results (data not shown). Quantifications of the DNA amount per band using fluorescence intensity allowed a direct measurement of the *Bn-FAE1.1*/*Bn-FAE1.2* ratio. In every sample, the *Bn-FAE1.1*/*Bn-FAE1.2* ratio ranged from 4 to 5, indicating that *Bn-FAE1.1* mRNA was the major *Bn-FAE1* isoform expressed during HEAR and LEAR seed development.

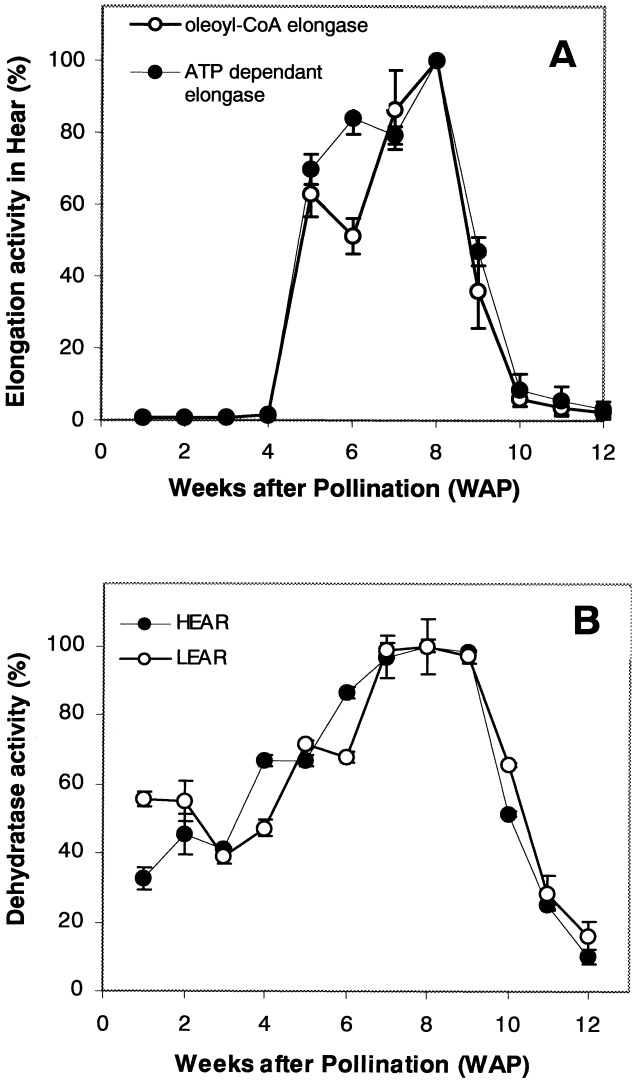
3.3. Elongation activities were present only in HEAR

Acyl-CoA elongase and ATP-dependent elongase activities were determined as reported previously [6]. Working with small amounts of developing seeds led us to redefine the optimal conditions for the elongase activities. The concentrations of oleoyl-CoA, malonyl-CoA and ATP (when present) were respectively 30 μM, 200 μM and 250 μM. Under these conditions and using 20 μg of microsomal proteins, both activities were measured in linear velocity conditions (Fig. 3A). Whatever the development stage, no significant elongation activity was detected in microsomes prepared from LEAR seeds (data not shown), showing that the VLCFMA synthesis was deficient. In contrast with the HEAR cultivar, oleoyl-CoA and ATP-dependent elongation were detected from 1 WAP up to 12 WAP. Both activities, which were weak in the earlier stages, increased from 4 WAP to reach a maximum at 8 WAP and then decreased (Fig. 3A). In view of the work of Norton and Harris [18] which showed that erucic acid increases from 6 to 12 WAP and would be predominant in the triacylglycerols after 8 WAP, our results indicate that the maximum rate of erucic acid synthesis precedes maximum triacylglycerol accumulation. Oleoyl-CoA elongase and ATP-dependent elongase maximal activities were respectively 18.6 and 14.3 nmol/mg/h. In view of these activities it could be estimated that the synthesis of very long chain fatty acids is about 0.1 mg/h/seed. The fatty acid analyses showed (Table 1) that 41–47% of labeling by ATP-dependent elongase was recovered in VLCFMA and 74–78% by oleoyl-CoA elongase. Moreover, in the case of oleoyl-CoA elongation, no changes in the VLCMFA (C20:1, C22:1 and C24:1) labeling distribution was observed, assuming that these fatty acids were synthesized at similar rates at each of the different developmental stages and erucic acid was always the major product.

3.4. 3-Hydroxyacyl-CoA dehydratase activity is present in both LEAR and HEAR

The 3-hydroxyacyl-CoA dehydratase study was carried out using linear velocity conditions (Fig. 3B). In contrast to elongase activities, dehydratase activity was detected from 1 WAP in both cultivars.

Fig. 3. Elongation and dehydratase activities during seed development. Oleoyl-CoA (○) and ATP-dependent elongase (●) activities were measured under the conditions reported in Section 2. 20 μg of protein were incubated for 1 h at 30°C in the presence of 200 μM [2-^{14}C]malonyl-CoA (45 mCi/mmole), 0.5 mM NADH, 0.5 mM NADPH, 1 mM $MgCl_2$, 2 mM DTT, 30 μM C18:1-CoA (oleoyl-CoA elongase) or 0.25 mM ATP (ATP-dependent elongase) in a HEPES 80 mM buffer (pH 7.2). The final volume was 100 μl. Results are the mean values of three independent experiments and are expressed as the percentage of the maximal activity ±S.D. 3-Hydroxyacyl-CoA dehydratase was measured using 3-[1-^{14}C]OH C20:0-CoA (11.4 μM) and 10 μg of microsomal proteins from HEAR (●) and LEAR (○) in a HEPES 80 mM buffer (pH 7.2) containing 1 mM $MgCl_2$, 2 mM DTT and 75 μM Triton X-100. The incubation was run for 15 min at 30°C and the fatty acids were extracted and separated by HPTLC. The radioactivity in the *trans*-2,3 C20:1-CoA was measured as reported in Section 2. Results are the mean values of three independent experiments and are expressed as the percentage of the maximal activity ±S.D.



In HEAR and LEAR lines the synthesis of *trans*-(2,3)-enoyl C20:1-CoA increased from 1 WAP to 7 WAP, was maximal from 7 WAP up to 9 WAP, and then decreased. This result suggests that *Bn-FAE1.1* and *Bn-FAE1.2* mutations present in the LEAR line did not affect the 3-hydroxyacyl-CoA dehydratase, and supports the hypothesis that the 3-ketoacyl-CoA synthase was the only activity missing in LEAR cultivars.

3.5. 3-Ketoacyl-CoA synthase is absent in LEAR seeds

The presence of the 3-ketoacyl-CoA synthase encoded by the gene *Bn-FAE1* was determined using an anti-FAE1 antibody prepared against a recombinant Bn-FAE1.1 fusion protein. A 57 kDa protein corresponding to the molecular mass deduced from the *Bn-FAEI* cDNA sequence was detected in HEAR from 5 to 10 WAP (Fig. 4A). This result shows a good correlation between the immunodetection level and the ATP-dependent and acyl-CoA elongase activity profiles during HEAR seed maturation. In good agreement with the absence of elongation and 3-ketoacyl-CoA activities (Fig. 4B) in LEAR seeds [36], no protein corresponding to 3-ketoacyl-CoA synthase was detected at any developmental stage in these seeds.

4. Discussion

4.1. FAE1 3-ketoacyl-CoA synthase and elongation systems

The LEAR phenotype is due to mutations affecting both E1 and E2 loci controlling erucic acid content in the seed [25]. Fourmann et al. [35] showed that *Bn-FAE1.1* and *Bn-FAE1.2* are linked respectively to E1 and E2 loci. Independent LEAR mutations affected the *Bn-FAE1.1* and *Bn-FAE1.2* genes [35,36] and resulted in a loss of 3-ketoacyl-CoA synthase activity [36]; consequently, it was hypothesized that the 3-ketoacyl-CoA synthases present in HEAR

Table 1
Analysis of the chain length of the fatty acids synthesized by the oleoyl-CoA and ATP-dependent activities

	Fatty acid labeling (%)			
	5 WAP	6 WAP	7 WAP	8 WAP
Oleoyl-CoA elongase				
C18:1	21.3 ± 1.0	21.5 ± 1.6	20.0 ± 0.5	21.1 ± 0.7
C20:1	21.5 ± 0.6	22.2 ± 2.0	21.4 ± 1.4	22.7 ± 1.3
C22:1	30.7 ± 2.0	26.5 ± 1.3	33.4 ± 2.5	33.1 ± 3.0
C24:1	23.9 ± 0.3	25.6 ± 1.3	22.3 ± 0.2	22.8 ± 2.5
VLCFMA	76.1	74.3	77.1	78.6
ATP-dependent elongase				
C14:1	16.5 ± 0.0	15.3 ± 0.7	15.1 ± 0.2	15.0 ± 0.0
C16:1	22.6 ± 0.6	19.1 ± 0.6	18 ± 0.1	18.6 ± 0.5
C18:1	19.9 ± 0.5	20.3 ± 1.8	19.3 ± 0.4	19.2 ± 0.0
C20:1	17.2 ± 0.0	16.2 ± 0.6	17.4 ± 0.2	16.4 ± 0.3
C22:1	12.8 ± 0.1	14.8 ± 0.7	15.5 ± 0.2	15.5 ± 0.1
C24:1	11.1 ± 0.4	13.1 ± 0.2	14.6 ± 0.1	15.2 ± 0.2
VLCFMA	41.2	44.2	47.5	47.2

Fatty acids were analyzed by reversed phase HPTLC and the radioactivity was measured using a PhosphorImager. Results are means ± S.D. from three different experiments.

represented the wild-type functional proteins whereas in LEAR both proteins were affected [35]. Nevertheless, the restoration of VLCMFA synthesis to LEAR has been obtained by the introduction of the jojoba KCS gene (encoding for a 3-ketoacyl-CoA synthase) in LEAR [15]. This result suggests that the presence of only one functional condensing enzyme is sufficient to restore the HEAR phenotype.

Two types of elongases co-exist in rapeseeds: the oleoyl-CoA elongase and the ATP-dependent elon-

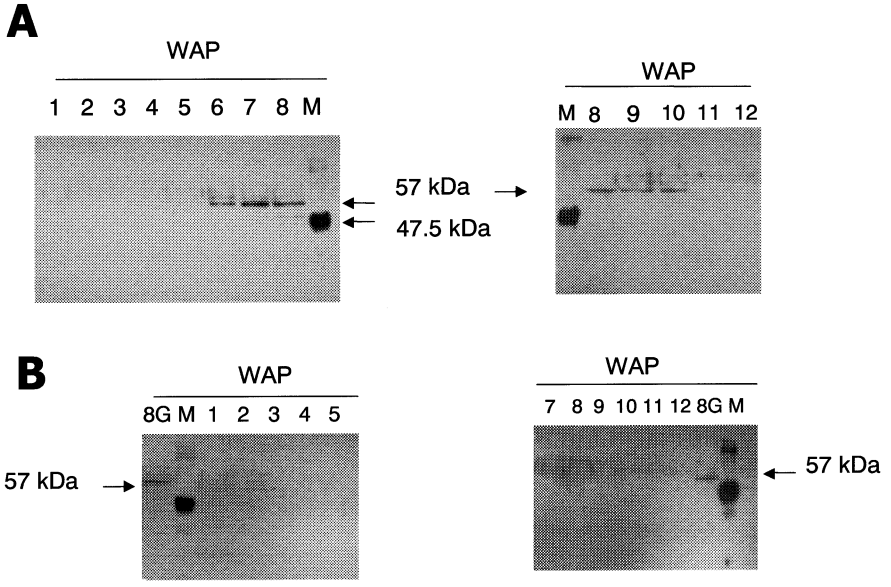


Fig. 4. Immunodetection of the 3-ketoacyl-CoA synthase during HEAR and LEAR seed development. 7 μg of microsomal proteins from HEAR (A) or LEAR (B) seeds were separated by electrophoresis on a 12% polyacrylamide gel. After transfer, the 3-ketoacyl-CoA synthase was detected using the anti-MBP *Bn-FAE1* serum and visualized using chemiluminescence as reported in Section 2. M, molecular markers; 8G, HEAR control (Gaspard 8 WAP).

gase [6]. 'In vitro' the preferred substrate of the acyl-CoA elongase is oleoyl-CoA, whereas ATP-dependent elongase uses an unknown substrate and requires ATP. Our results show that the profile of expression of these two activities is similar during seed maturation in HEAR. Both elongase activities were undetectable before 5 WAP, then both reached a maximum at 8 WAP and then finally decreased between 8 and 12 WAP. In LEAR neither oleoyl-CoA nor ATP-dependent elongation activity was detected during the development of the seed whereas the expression profile of *FAE1* mRNA was the same than in HEAR seeds. Since both oleoyl-CoA and ATP-dependent elongase activities were undetectable whatever the maturation stage of LEAR seeds, our result can be interpreted either as showing that both elongation systems share the same 3-ketoacyl-CoA synthase (condensing enzyme) or that the two elongation complexes use distinct condensing enzymes encoded respectively by *Bn-FAE 1.1* and *Bn-FAE1.2* and that both genes are mutated in LEAR. The first hypothesis is supported by the fact that the complementation of the LEAR can be obtained by the introduction of the KCS gene from jojoba [15], although the presence of the two elongation activities in the transgenic rapeseeds has not been measured. The second hypothesis is supported by the fact that the primer substrate is distinct (i.e. oleoyl-CoA versus an unknown endogenous substrate) for both elongases and by the presence of an ATP binding site only in the protein encoded by *Bn-FAE1.2* [36]. Immunoblots did not provide support for either of these hypotheses since the anti-FAE1 antibody detected only one protein band in HEAR, indicating that it was unable to differentiate between the proteins encoded by genes *Bn-FAE1.1* and *Bn-FAE1.2*, which is not surprising because these two proteins have a very similar amino acid sequence. Additional experiments will be necessary to understand the organization and the role of the different elongation systems in rapeseed.

4.2. Acyl-CoA elongase complex

In contrast to the activity of the 3-ketoacyl-CoA synthase, 3-hydroxyacyl-CoA dehydratase was always functional during seed development in both cultivars. In HEAR seeds, the highest activities of oleoyl-CoA and ATP-dependent elongases were measured from 8 to 9 WAP. The absence of condensing enzyme in LEAR did not modify the activity of the dehydratase gene, and the mutations in *Bn-FAE1.1* and *Bn-FAE1.2* did not affect dehydratase activity. Microsomes isolated from both HEAR and LEAR at each stage of seed development contain an equivalent activity of 3-hydroxyacyl-CoA dehydratase. This result indicates that the regulation of the expression of the dehydratase gene in rapeseed embryos is independent from the expression or activity of the 3-ketoacyl-CoA synthase and also from the intermediates or VLCFMAs. Nevertheless, it has been shown that the high molecular mass complex corresponding to the functional elongation complex was missing in the LEAR seeds [36]. This suggests that the different enzymes of the elongase complex are tightly associated, and that the absence of the 3-ketoacyl-CoA synthase leads to the inability of the different proteins to assemble into a functional multienzymatic complex.

4.3. Expression of Bn-FAE1.1 and Bn-FAE1.2 during rapeseed development

The developmental profile of expression obtained for the *FAEI* mRNA in HEAR is similar to that obtained for the elongation activities, with maximal values measured between 5 WAP and 9 WAP. Moreover, the *FAEI* transcript levels are high only when the 57 kDa FAE1 protein is immunodetected. Our results concerning the respective expression of *Bn-FAE1.1* and *Bn-FAE1.2* suggested: (i) that both isoforms are expressed during rapeseed development; (ii) that they are coordinately regulated because the *Bn-FAE1.1*/*Bn-FAE1.2* ratio remained constant during development; (iii) that *Bn-FAE1.1* is quantitatively the main isoform and represents at least 75% of the total *Bn-FAE1* mRNA. All together, these results strongly suggest that the regulation of elongase activity during rapeseed development occurs mainly at the *FAE1.1* mRNA level. Nevertheless, further work will be required to know whether the variations in transcript levels are due to variations in the corresponding gene transcription level or to variations in mRNA stability. Interestingly, *Bn-FAE1* mRNA regulation appeared to be similar in LEAR and HEAR lines according to the developmental

profiles, the *FAE1* mRNA steady-state levels and the *FAE1.1*/*Bn-FAE1.2* ratios. Thus, the mutations responsible for the LEAR phenotype did not alter the regulation of the expression of the *Bn-FAE1* mRNA isoforms. Nevertheless, the absence of any elongase activity or FAE1 protein in LEAR seeds suggests that the corresponding mutations interfere either with the translation efficiency of the *Bn-FAE1* mRNA and/or the stability of the corresponding protein.

Erucic acid synthesis is governed by alleles at two loci, E1 and E2 [25]. These two loci do not contribute equally to the erucic acid content and it has been reported that the effect of E1 QTL is 2-fold greater than that of E2 QTL [35,37]. The *Bn-FAE1.1* gene encoding for the rapeseed 3-ketoacyl-CoA synthase has been shown to be linked to the E1 locus and *Bn-FAE1.2* is assigned to the E2 locus [16,35]. The fact that *Bn-FAE1.1* is more expressed than *Bn-FAE1.2* is consistent with these previous results; however, since *Bn-FAE1.2* mRNA was detected in all samples, it is difficult to assume that the active 3-ketoacyl-CoA synthase is solely due to the transcription product of the gene *Bn-FAE1.1*. Further work is necessary to understand the role of *Bn-FAE1.1* and *Bn-FAE1.2* in the regulation of erucic acid content in rapeseed.

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