Mutations in the *fatty acid elongation 1* gene are associated with a loss of β-ketoacyl-CoA synthase activity in low erucic acid rapeseed

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Received 17 January 2001; revised 9 February 2001; accepted 9 February 2001

First published online 21 February 2001

Edited by Ulf-Ingo Flügge

Abstract Low erucic acid rapeseed (LEAR) is characterised by a near absence of very long chain fatty acids in the seed oil which has been correlated with a lack of acyl-CoA elongation activity. Here we show that the absence of acyl-CoA and ATP-dependent elongation activities in microsomes isolated from LEAR embryos is associated with an absence of β-ketoacyl-CoA synthase activity encoded by the Bn-*fatty acid elongation 1* (*FAE1*) genes. Size exclusion chromatography of solubilised microsomes revealed the presence of a high molecular mass acyl-CoA elongase complex in high erucic acid rapeseed which was absent in microsomes isolated from LEAR seeds. Although transcripts for the Bn-*FAE1* genes were detected in LEAR embryos, immunoblots using antisera raised against the β-ketoacyl-CoA synthase indicated an absence of this protein. Comparison of the deduced amino acid sequences of immature embryo cDNAs reveals that LEAR alleles of Bn-*FAE1* encode variant β-ketoacyl-CoA synthase proteins.

Key words: Acyl-CoA elongase; β-Ketoacyl-CoA synthase; Erucic acid; *Fatty acid elongation 1*; *Brassica napus*

1. Introduction

Many plant species contain very long chain fatty acids (VLCFAs) as components of their seed oils and of their cuticular waxes. VLCFAs are synthesised by an extraplastidial fatty acid elongation complex (acyl-CoA elongase) using acyl-CoA substrates typically with chain lengths of 16 or 18 carbons from a cytoplasmic pool maintained by de novo lipid biosynthesis in the plastid or by an ATP-dependent elongase using an unknown endogenous substrate. Condensation of two carbon units derived from a malonyl-CoA donor with a long chain acyl-CoA primer occurs via a β-ketoacyl-CoA synthase and the keto group is then removed by a series of three reactions: β-keto reduction to the β-hydroxyacyl-CoA, β-hydroxydehydration to the enoyl-CoA and an enoyl reduction of the double bond (reviewed in [1,2]).

Certain varieties of oilseed rape (high erucic acid rapeseed, HEAR) synthesise a triacylglycerol that contains a high proportion of erucic acid which is used as an industrial feedstock. Concerns over the nutritional safety of erucic acid led to the production of Canola varieties (low erucic acid rapeseed, LEAR) enriched in oleic acid at the expense of VLCFA. LEAR varieties were selected by the introduction of recessive alleles at two loci that control the elongation of fatty acids [3]. Studies in rapeseed have shown that the synthesis of erucic acid occurs via two sequential condensation reactions, controlled by elongase activities present in HEAR embryos but absent from embryos of LEAR varieties [4]. The *FAE1* (*fatty acid elongation 1*) mutants of *Arabidopsis thaliana*, have drastically reduced levels of seed VLCFAs [5,6] and a deficiency of both elongation activities [7], a similar phenotype to LEAR. The cloning of the *A. thaliana FAE1* gene revealed homologies of the encoded protein to condensing enzymes [8]. In rapeseed, the two elongation steps from oleoyl-CoA (C18:1-CoA) to erucic acid are each controlled by alleles at two loci, E1 and E2. The *FAE1* genes encoding rapeseed β-ketoacyl-CoA synthases were shown to be tightly linked to the E1 and E2 loci [9,10].

Despite these recent advances in our knowledge of the biochemistry of the seed elongases and the cloning of the loci controlling the erucic acid content, the nature of the mutations that characterise the agriculturally important LEAR trait remain obscure. Here we compare the component elongase activities in rapeseed varieties that vary in erucic acid content and show that an absence of ketoacyl-CoA synthase is associated with variation in the sequence of the Bn-*FAE1* gene.

2. Materials and methods

2.1. Plant material

Rapeseed lines varying in erucic acid content, B002 (less than 1% erucic acid) and Hokkaido (more than 50% erucic acid), were crossed. 400 near-isogenic high and low erucic acid segregant lines from a backcrossed, B5F2 population (recurrent parent B002), were obtained by allowing flowers of a B5F1 plant to self pollinate. B5F2 seeds were evaluated for fatty acid composition. Segregating lines (B5F3) of HEAR (24) containing greater than 48% erucic acid and LEAR lines (25) containing less than 1% erucic acid were used in this study.

2.2. Isolation of cDNA clones encoding rapeseed β-ketoacyl-CoA synthase

The protocol described in [9] was used to construct and screen a LEAR immature embryo cDNA library in the vector λ ZAPII (Stratagene).

2.3. DNA sequencing

DNA sequences were determined by primer walking with Bn-*FAE1*-

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0014-5793/01/$20.00
PII: S0014-5793(01)02243-8

specific primers: CE51: 5′-CAATCACGGCTTTGCCTCCGGC-3′; CE41: 5′-GCGGCCCCACCAACACGGAACAAGC-3′; CE31: 5′-GCACTACAACCCATGGCCACCAAGG-3′; CE22: 5′-GGAAAGGACTTGTTGCATGTCC-3′; CE23: 5′-CCGATGTTGCTGGTCGAACGG-3′; CE24: 5′-GCTATGAGTTCGCATACATAAACCC-3′ using DyeDeoxy Terminator cycle sequencing (Applied Biosystems) on double stranded DNA templates with an ABI 373A sequencer.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from embryos dissected from the seeds of B5F3 plants isolated at 28 days after pollination, by the protocol described in [11]. RT-PCR was performed using the Pro-Star kit (Stratagene) using 100 ng of DNase-treated RNA as template. High performance liquid chromatography purified primers (Eurogentec, Belgium) were derived from the LEAR and HEAR Bn-*FAE1* cDNA sequences and were designed to detect gene-specific transcripts: CE71: 5′-CGCTATTTTGCTCTCCAACAAGCCTG-3′; CE72: 5′-GCCTCTACATCGATCGGTGCTGGG-3′; CE81: 5′-CGCTATTTTGCTCTCCAACAAGCCTA-3′; CE82: 5′-GCCTCTACATCGATCGGTGCTAGGC-3′.

2.5. Immunodetection of β-ketoacyl-CoA synthase

Total protein was extracted from 0.5 g mid-cotyledonary stage embryos and from leaf tissue in 50 mM NaCl, 0.25% Triton X-100, 2 mM mercaptoethanol, 25 mM Tris–HCl pH 8.0. The homogenate was centrifuged at 13 000×*g* for 10 min at 4°C. One volume of the supernatant was mixed with nine volumes of cold acetone. The protein precipitate was collected by centrifugation, washed in acetone and dissolved in 2% sodium dodecyl sulfate (SDS). Proteins were resolved by SDS–PAGE [12] and immunodetection performed via the enhanced chemiluminescence system (Amersham).

2.6. Overall and intermediate elongation activity measurements

A 15 000×*g* pellet isolated from immature seeds according to [13] was used as the enzyme source. The acyl-CoA elongation was measured by incubating 60 μg protein in 30 μM C18:1-CoA, 0.5 mM NADPH, 0.5 mM NADH, 1 mM $MgCl_2$, 2 mM dithiothreitol (DTT) and 34 μM [2-^{14}C]malonyl-CoA, 0.08 M HEPES buffer pH 7.2 for 1 h. The ATP-dependent elongase was performed using the same conditions except that C18:1-CoA was replaced by 1 mM ATP. The reaction was stopped by addition of 0.1 ml methanolic 5 N KOH (1 $CH_3OH{\cdot}9H_2O$) and the reaction mixture was heated at 80°C for 1 h. The fatty acids were extracted and the content of radioactivity determined.

The 3-ketoacyl-CoA synthase activity was measured under the same conditions reported in [13]. The 3-hydroxyacyl-CoA dehydratase activity was determined by incubating 60 μg of microsomal proteins in the presence of 2 mM DTT, 1 mM $MgCl_2$, 150 μM Triton X-100 and 11 μM [1-^{14}C]3-hydroxy-C20-CoA, 80 mM HEPES buffer (pH 7.2) in a volume of 0.1 ml for 15 min at 30°C. The 3-hydroxy and (*E*)2,3-unsaturated fatty acids were recovered for radioactivity measurements as described in [14]. Control reactions at zero time of incubation were carried out under the same conditions. Activities were determined as the difference between the percentages of [1-^{14}C]3-hydroxy-C20:0-CoA conversion in the assay and the control. Enoyl-CoA reductase was measured under the same conditions as the 3-hydroxydehydratase but in the presence of 0.1 mM NADPH. The activity was determined as the radioactivity recovered in the saturated fatty acid fraction.

Table 1
Total and partial acyl-CoA elongase activities in rapeseed embryo microsomes

	Enzymatic activity (nmol mg^{-1} h^{-1})	
	HEAR	LEAR
Overall elongation:		
Control (without substrate)	0.19 ± 0.02 (4)	0.17 ± 0.02 (4)
C18:1-CoA elongase	5.62 ± 0.44 (4)	0.10 ± 0.02 (4)
ATP-dependent elongase	3.45 ± 0.41 (4)	0.13 ± 0.07 (4)
Intermediate reactions:		
3-Ketoacyl-CoA synthase	2.08 ± 0.04 (6)	0.03 ± 0.04 (6)
3-Hydroxyacyl-CoA dehydratase	50.7 ± 0.9 (4)	4.63 ± 0.2 (4)
(*E*)2,3-Enoyl-CoA reductase	54.7 ± 3.1 (4)	47.5 ± 1.8 (4)

Total and partial acyl-CoA elongation activities in rapeseed microsomal fractions. Elongation was measured as the incorporation of [2-^{14}C]malonyl-CoA into fatty acids. The acyl-CoA elongation was determined in the presence of C18:1-CoA. The ATP-dependent elongase was determined by replacing C18:1-CoA by ATP. The intermediate reactions were determined as described in the text.

2.7. Acyl-CoA elongase solubilization and Superdex 300 chromatography

Acyl-CoA elongase was solubilized from the 15 000×*g* particulate fraction isolated from the seeds of HEAR and LEAR lines by Triton X-100 using a 2.5:1 detergent:protein ratio [13,15]. A total of 15 mg of solubilised protein was loaded onto a 1.6×63 cm Superdex 300 column equilibrated with a 0.08 M HEPES buffer pH 7.0 containing 10 mM 2-mercaptoethanol, 10% ethyleneglycol and 0.02% Triton X-100. The elution was performed using the same buffer at a 0.2 ml min^{-1} rate. Fractions of 1 ml were collected and 3-hydroxyacyl-CoA dehydratase and acyl-CoA elongase activities were determined using 50 μl of each fraction.

3. Results

3.1. Elongation activities in microsomal fractions of HEAR and LEAR

The C18:1-CoA and ATP-dependent elongation were determined using a particulate fraction prepared from immature seeds (Table 1). No VLCFA synthesis was detected when the microsomal fraction prepared from LEAR was examined for overall elongation activities. In contrast, HEAR microsomes synthesised 5.62 nmol mg^{-1} h^{-1} of VLCFA from exogenously added C18:1-CoA and 3.45 nmol mg^{-1} h^{-1} of VLCFA from endogenous substrate in the presence of ATP. The intermediate enzymatic activities involved in the acyl-CoA elongation, the 3-hydroxyacyl-CoA dehydratase and the 2,3-enoyl-CoA reductase activities were similar in both HEAR and LEAR microsomes, whereas the β-ketoacyl-CoA synthase activity was absent in the microsomes prepared from LEAR seeds compared to the activity measured with the HEAR microsomes.

3.2. Expression of FAE1 genes in HEAR and LEAR embryos

To verify that each of the two rapeseed genes [9], designated Bn-*FAE1.1* and Bn-*FAE1.2*, were transcribed, RT-PCR was performed using primers designed to amplify fragments of each gene in a specific manner. A band corresponding to a 405 bp fragment of the Bn-*FAE1.1* transcript was amplified with the primers specific to the Bn-*FAE1.1* gene using RNA isolated from both LEAR (Fig. 1, lane 1) and HEAR (Fig. 1, lane 6) immature embryos. Similarly, a 405 bp fragment was

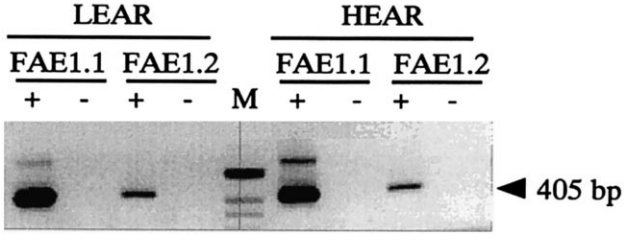

Fig. 1. Expression of Bn-*FAE1* genes in rapeseed embryos. RT-PCR: RNA of immature embryos was used as a template in a RT reaction followed by PCR using either the Bn-*FAE1.1* gene-specific primer pair (lanes 1, 2, 6 and 7) or the Bn-*FAE1.2* gene-specific primer pair (lanes 3, 4, 8 and 9). +/− indicates presence or absence of reverse transcriptase. M: DNA size markers.

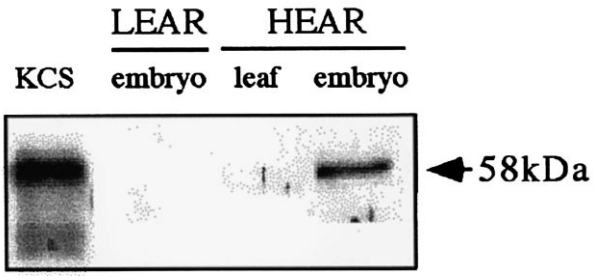

Fig. 2. Immunodetection of β-ketoacyl-CoA synthase. A sample containing 20 μg of immature embryo proteins or a leaf protein extract was resolved by SDS–PAGE, transferred to nitrocellulose and probed using antibody raised against C-terminus of the *Arabidopsis* β-ketoacyl-CoA synthase. KCS: co-migration of ^{35}S methionine-labelled β-ketoacyl-CoA synthase produced by in vitro transcription and translation programmed by a Bn-*FAE1.1* template.

amplified using primers specific to the Bn-*FAE1.2* gene from both LEAR (Fig. 1, lane 3) and HEAR (Fig. 1, lane 8) immature embryos. The absence of amplimers in control reactions lacking reverse transcriptase verified that amplification resulted from RNA templates (Fig. 1, lanes 2, 4, 7 and 9).

3.3. Immunodetection of β-ketoacyl-CoA synthase synthase in HEAR and LEAR embryos

Western blot experiments were performed using antibodies raised against different regions of a recombinant β-ketoacyl-CoA synthase protein. In three independent experiments, an antibody raised against the C-terminal extremity (V_{466}–S_{506}) of the β-ketoacyl-CoA synthase detected a protein migrating with an apparent molecular weight of 58 kDa in HEAR embryos which was not present in LEAR embryos or leaf tissue (Fig. 2). This protein co-migrated on the same gel with a ^{35}S-radiolabelled KCS protein produced by in vitro transcription and translation of the Bn-*FAE1.1*gene. Similar results were obtained with a second antibody raised against a fusion protein containing the central portion (M_{100}–G_{365}) of the β-ketoacyl-CoA synthase (data not shown).

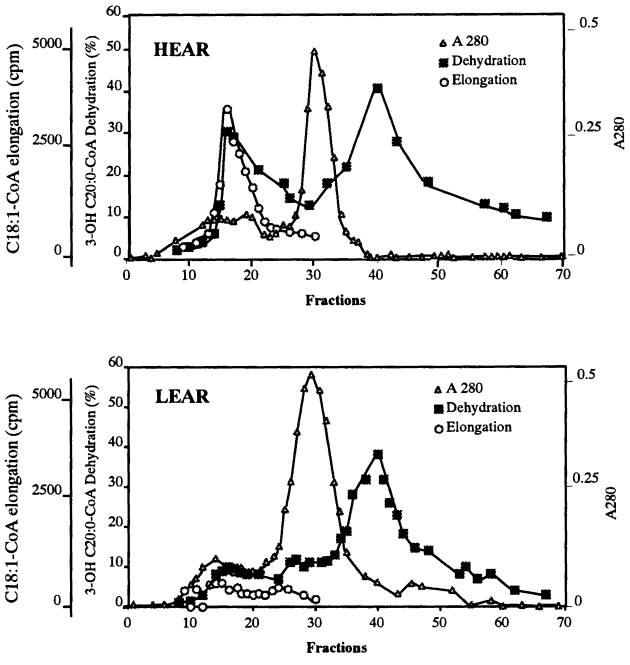

Fig. 3. Size exclusion chromatography of solubilised rapeseed microsomal proteins. A total 15 mg of a solubilised 15 000×*g* particulate fraction isolated from the seed protein was loaded onto a Superdex S-300 column and eluted with a 0.08 M HEPES buffer pH 7.0 containing 10 mM 2-mercaptoethanol, 10% ethyleneglycol and 0.02% Triton X-100. The 3-hydroxyacyl-CoA dehydratase and C18:1-CoA elongase activities were determined in each fraction. Data is typical of three independent experiments.

3.4. Comparison of cDNAs encoding rapeseed β-ketoacyl-CoA synthase

Seven cDNA clones were isolated from the LEAR immature embryo library. The deduced protein sequence of cDNAs L3, L5, L10 and L13 was characterised by the presence of G_{286}, I_{323}, R_{395} and A_{406} in common with the protein encoded by the HEAR cDNA CE7 [9] and the *Brassica rapa* accession number AF054499, whereas the proteins encoded by clones

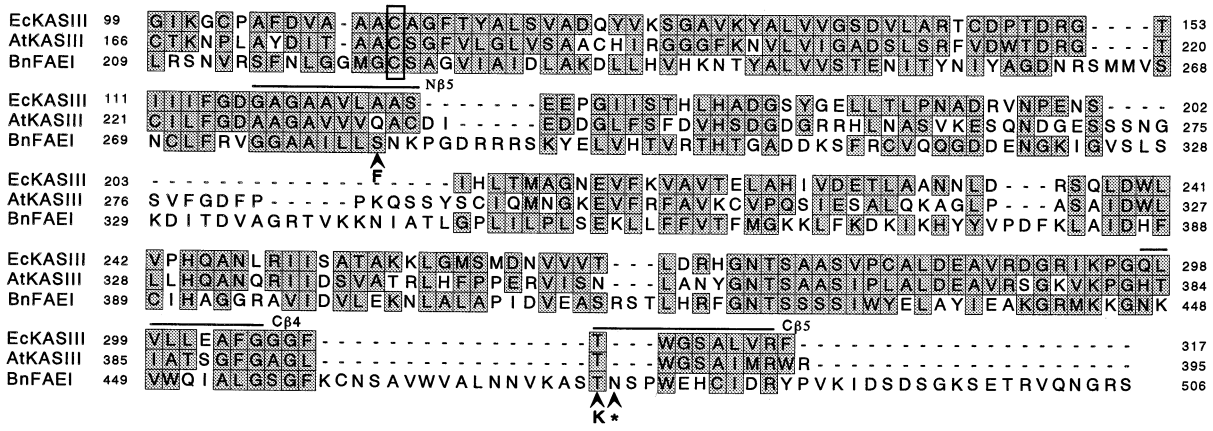

Fig. 4. Comparison of partial amino acid sequences of β-ketoacyl-CoA synthase and β-ketoacyl (acyl carrier protein) synthases. Bn-*FAE1* corresponds to Bn-*FAE1.1* gene product. The single arrow at S_{242} indicates the LEAR allele of Bn-*FAE1.1* and the double arrow indicates the LEAR allele of Bn-*FAE1.2*. The putative active site cysteine is boxed. Shading indicates homologous or identical residues. Secondary structural elements are overlined. AtKASIII accession number P49243; EcKASIII accession number L39891; Bn-*FAE1* accession number AF009563.

L2, L11 and L21 were characterised by R_{286}, T_{323}, K_{395} and G_{406} and thus resembled the protein encoded by the HEAR cDNA CE8 [9], and the *Brassica oleracea* accession number AF054500.

Each member of the two groups of LEAR cDNAs was characterised by additional variation. L3, L5, L10 and L13 were characterised by a non-conservative substitution of phenylalanine residue for a serine at F_{282}. This was the consequence of a single nucleotide substitution of T for C at position 845 in the nucleotide sequence of the open reading frame of Bn-*FAE1.1*. The second group of LEAR cDNAs, L2, L11 and L21, was characterised by a deletion of two nucleotides at A_{1421} and A_{1422}. This resulted in a $T_{472}K$ substitution immediately followed by a stop codon thereby producing a truncated protein.

3.5. Size exclusion chromatography of solubilized rapeseed proteins

Chromatography on a Superdex S-300 column (Fig. 3), of proteins solubilised from HEAR membranes revealed a high molecular weight fraction containing the C18:1-CoA-dependent elongation associated with the presence of the acyl-CoA elongase complex [13]. The ATP-dependent elongase activity was not determined since this activity is extremely sensitive to detergent solubilisation. The dehydratase activity co-eluted with the acyl-CoA elongase complex and as a second low molecular mass peak. Since solubilization by Triton X-100 increased the synthesis of 3-hydroxy fatty acid intermediates [15], it is probable that this second peak corresponds to the activity of free dehydratase subunits and reflects the dissociation of the acyl-CoA elongase complex. In contrast, chromatography of solubilized proteins from a LEAR microsomal fraction revealed an absence of elongation activity and the presence of only one peak of 3-hydroxyacyl-CoA dehydratase corresponding to the low molecular mass form.

4. Discussion

4.1. β-Ketoacyl-CoA synthase activity is absent in LEAR

Fatty acid analysis of seeds of B5F2 segregants (data not shown) confirmed the characteristic of the LEAR trait, the near-total absence of erucic acid and a greatly decreased quantity of eicosanoic acid together with a concomitant increase in C18 lipid species [16]. The mutation(s) that give rise to a LEAR phenotype affect elongation from C18:1 to C20:1 and C20:1 to C22:1 and thus resemble the seed fatty acid elongation (*FAE1*) mutants of *A. thaliana* [5–7]. The accumulation of C18 fatty acids was anticipated since C18 are the precursors of VLCFA.

Two elongation systems co-exist in the particulate, microsomal and oil body fractions of developing rapeseed, an C18:1-CoA-dependent (acyl-CoA elongase) and an ATP-dependent elongase [17,18]. In this context, examination of the β-ketoacyl-CoA synthase sequences for the presence of consensus motifs revealed the presence of a putative ATP/GTP binding site (PROSITE, PDOC00017) at $G_{316-323}T$ in the protein encoded by Bn-*FAE1.2*. Elements of this motif are present in other FAE enzymes and, thus, this motif merits attention in future studies aimed at understanding the physiological significance of ATP-dependent elongation. Each elongation activity was absent in the immature seeds of LEAR, despite measuring acyl-CoA-dependent activities in HEAR that were approximately 2.5-fold higher than those reported by Domergue et al. [18]. These results are consistent with previous studies using rapeseed embryos that have shown that HEAR varieties are able to elongate C18:1-CoA to C20:1 and C22:1 but that C18:1 and C20:1 elongation is absent in LEAR varieties [4]. The absence of acyl-CoA elongation is reflected in the fatty acid composition of LEAR triacylglycerols and suggests that the lesion(s) which results in a LEAR phenotype is present in a factor that is common to each elongation system.

The absence of β-ketoacyl-CoA synthase activity in immature seeds of LEAR is consistent with the restoration of VLCFA biosynthesis to LEAR by transformation with the *FAE1* gene of Jojoba coding for a β-ketoacyl-CoA synthase [19], and confirms the hypothesis of these authors that the mutations that give rise to the LEAR phenotype reside in genes that encode or regulate β-ketoacyl-CoA synthase. We were not able to determine β-ketoacyl-CoA reductase activity since a β-ketoacyl-CoA substrate is not available, however, a mutation in one of the steps subsequent to the condensation reaction could be deleterious because of sequestration of CoA [20].

4.2. Mutations that eliminate β-ketoacyl-CoA synthase activity in LEAR act post-transcriptionally

Gene-specific RT-PCR showed that each gene is transcribed in both HEAR and LEAR. The results obtained from immunodetection experiments using antibodies raised against either the carboxy-terminal extremity or the central portion of the β-ketoacyl-CoA synthase indicated that each protein was absent from immature LEAR seeds. Furthermore, size exclusion chromatography of microsomal proteins solubilised from the immature seeds of LEAR lines revealed the absence of a dehydratase activity, a marker activity for the native elongase complex, associated with a high molecular weight fraction. Taken together, these results suggest that independent mutations that give rise to the LEAR phenotype act post-transcriptionally affecting the quantity or stability of the β-ketoacyl-CoA synthase or affecting its capacity to allow the formation of the acyl-CoA elongase complex.

4.3. Absence of β-ketoacyl-CoA synthase is associated with variation in the sequence of Bn-FAE1 genes

The absence of the elongase complex may be associated with either an absence of the β-ketoacyl-CoA synthase or an inability of the protein to form a functional complex resulting in its degradation. The deduced proteins corresponding to the LEAR *FAE1* alleles are identical in sequence in the regions surrounding the two transmembrane spanning domains located at the N-terminus, the active site cysteine (C_{223}) and a putative leucine zipper (I_{398}, L_{405}, V_{412}, L_{419}) in the corresponding HEAR proteins. The truncated protein corresponding to the Bn-*FAE1.2* LEAR allele lacks 31 residues compared to the corresponding HEAR protein (Fig. 4). The N-terminal half of this sequence is well conserved in *FAE1*-like proteins suggestive of a structural or functional importance. The sequences of the FAE family of proteins are most closely related to β-ketoacyl (acyl carrier protein) synthase III (KASIII), and chalcone synthase condensing enzymes whose structures have been solved [21–24]. Although less homology exists with KASI and KASII, the subunit structure of all of these enzymes comprises an α–β–α–β–α fold, forming the core do-

main from which protrusions extend [25,26]. In KASIII both subunits contribute to the hydrophobic active site cavity. The secondary structural element Cβ5, a component of the core domain is located at the C-terminus. The loss of the analogous β-sheet in the truncated LEAR protein would not allow the formation of the core domain and would potentially destabilise the interaction between the subunits impairing dimerisation and precluding the assembly of a functional enzyme.

The protein encoded by the LEAR allele of Bn-*FAE1.1* contains a $S_{282}F$ substitution. Database searches reveal that this serine is conserved among all FAE1-like proteins and in the rapeseed β-ketoacyl-CoA synthase lies at a hydrophobic to hydrophilic transition between an extended sheet and a random coil structure. The alignment of the FAE proteins to KASIII (Fig. 4) suggest that S_{282} in FAE proteins corresponds to A_{165} in KASIII. If so, then S_{282} would be located close to the end of the secondary structure element Nβ5, located distant from the active site and not on the monomer: monomer interface. Until soluble β-ketoacyl-CoA synthases are available for crystallography, a potential approach for testing the significance of the $S_{282}F$ would be to mutagenise KASIII to produce the deduced analogue, $A_{165}F$, and to test its activity in vitro. This approach has been used to demonstrate that the *A. thaliana fab1* mutation when present in *Escherichia coli* KASI drastically impairs catalytic activity [27]. It is unclear as to the precise effects of the variation in sequence on the protein structure, although the failure to detect the β-ketoacyl-CoA synthase may be the result of a rapid turnover of incorrectly folded proteins.

Acknowledgements: The authors thank Ljerka Kunst for providing antibodies and Martine Devic for critical appraisal of the manuscript and Penny von Wettstein for providing valuable suggestions and insight into the comparative structure of condensing enzymes. This work was supported by the Ministère de l'Education National et de la Recherche Scientifique, Agence de l'Environnement et de l'Matrise de l'Energie, Organisation Nationale Interprofessionelle des Oléagineux, Rustica Prograine Genetique and Seracem.

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