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M. Fourmann · P. Barret · M. Renard
G. Pelletier · R. Delourme · D. Brunel

The two genes homologous to *Arabidopsis FAE1* co-segregate with the two loci governing erucic acid content in *Brassica napus*

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Abstract KCS (β-keto-acyl-CoA synthase) has been proposed as a candidate gene for explaining the erucic acid level in rapeseed. Degenerate PCR primers corresponding to the *FAE1* gene have been designed. Two *B. napus* genes *BN-FAE1.1* and *BN-FAE1.2*, corresponding to the parental species *B. rapa* and *B. oleracea FAE1* genes, were amplified. Polymorphism was revealed for these two genes by acrylamide electrophoresis of the amplification products. These two genes could then be mapped and a co-segregation of these genes with the *E1* and *E2* loci controlling erucic acid content was found. Furthermore, mutations observed for one of these genes could explain part of the low erucic trait of the three LEAR types used in this study.

Key words Fatty acid elongase · Erucic acid · Consensus primers · *Arabidopsis* · *Brassica*

Introduction

Lipids in the seeds of higher plants are mainly composed of C16 and C18 fatty acids. *Brassicacae* species produce Very Long Chain Fatty Acids (VLCFAs, with a number of carbons >18) such as C20:1 (eicosenoic acid) or C22:1 (erucic acid). In its seeds rapeseed (*Brassica napus* L.) accumulates naturally triacylglycerols (TAG) containing erucic acid in the *sn-1* or *sn-3* position of the glycerol backbone to a level of about 50% of the seed oil.

Genetic studies have shown that in rapeseed the erucic acid content depends on the genotype of the embryo. Harvey and Downey (1963) demonstrated that this trait is controlled by two genes, with additive effects, and with no dominance involved. These two genes (*E1* and *E2*) have been mapped in rapeseed using a QTL approach (Ecke et al. 1995; Jourdren et al. 1996a; Thormann et al. 1996).

For nutritional purposes, Low Erucic Acid Rapeseeds (LEAR) were selected by breeders. The decrease from up to 60% of the TAG to less than 2% was achieved by the introduction of recessive alleles (e*1* and *e2*) involved in low biosynthesis of VLCFA (Morice 1974). However, High Erucic Acid Rapeseed (HEAR) cultures are regaining interest for industrial purposes, as erucic acid can be used for making high fluidity lubricants, polymers, paints and inks, cosmetics and pharmaceuticals (Murphy 1996).

The erucic acid synthesised in plant seeds results from a particular elongation pathway located in the cytosol. Mono-unsaturated C18 compounds synthesised in the plastids provide the initial substrates. These acyls are esterified to coenzyme A and further rounds of elongation are catalysed by a protein complex localised in the microsomal fraction: the fatty acid elongase (FAE) complex (Cassagne et al. 1994). Because of the membrane-bound location of this complex, little was known until recently about the enzymes involved in these reactions. This elongation process is presumed to be a four-step process, like that of the plant plastid fatty acids biosynthesis pathway (for a review see Harwood 1996). In the first step, supposed to be the rate-limiting one (Lemieux et al. 1990; Kunst et al. 1992) a β-keto-acyl synthase (KCS) catalyses the condensation of malonyl-CoA on C18:1CoA (and later on C20:1CoA). This component is then reduced to a β-hydroxy-acyl-CoA and dehydrated to form a trans 2-3 enoyl-CoA. The last reduction leads to an acyl-CoA

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M. Fourmann (✉) · G. Pelletier · D. Brunel
1-INRA Station de Génétique et Amélioration des Plantes,
Route de St Cyr, 78026 Versailles cedex, France
Fax:(33) 01 30 83 33 08
E-mail: fourmann@versailles.inra.fr

P. Barret · M. Renard · R. Delourme
2-INRA Station d'Amélioration des Plantes,
B.P. 29, 35650 Le Rheu, France

elongated by two carbons. Biochemical studies showed that different peptides, rather than one single multifunctional peptide, act in this elongation process (Bessoule et al. 1989).

Studies of *Arabidopsis thaliana* mutants deficient in VLCFA showed that the *FAE1* gene product was required in the seeds for the elongations from C18 : 1 to C22 : 1 (Lemieux et al. 1990; Kunst et al. 1992). The *Arabidopsis FAE1* gene has been cloned by transposon tagging (James et al. 1995). It probably codes for the β-keto-acyl-CoA Synthase (KCS) involved in the first key step, because of its sequence similarity with other acyl-CoA condensing enzymes, such as members of the chalcone synthase superfamily. Lassner et al. (1996) purified an element of the FAE complex from jojoba having β-keto-acyl-CoA synthase activity. The corresponding cDNA (based on the predicted peptides) was around 60% identical with the *Arabidopsis* gene. The expression of the jojoba KCS gene in rapeseed restored a HEAR phenotype, as some transformants accumulate mono-unsaturated VLCFA, including erucic acid, in a proportion comparable with HEAR types. Thus, LEAR genotypes could have mutations in the genes coding for the KCS enzyme.

Using an oligonucleotide probe from the *Arabidopsis FAE1* gene, two rapeseed cDNAs coding for KCS enzymes were isolated (Barret et al. 1998). One of them (CE8) mapped at the *E1* locus, confirming the role of KCS in this trait. The hypothesis that HEAR and LEAR genotypes differ in their KCS genes led us to amplify the *FAE1* homologs from *B. napus* and its parental species (*Brassica oleracea* and *Brassica rapa*) using consensus primers. The visible polymorphism between rapeseed genotypes allowed us to map these two *FAE1* genes in doubled-haploid descendants. The co-segregation of these genes with the *E1* and *E2* loci, governing the erucic acid content in rapeseed, was studied.

Materials and methods

Plant material

DNA from four *B. napus* cultivars ('Darmor.*bzh*', 'Yudal', 'Stellar' and 'Drakkar'), *A. thaliana* ('Columbia'), *B. oleracea* (Rapid cycling var. capitata) and *B. rapa* ('R500' accession) was extracted (Doyle and Doyle 1990). 'Darmor. *bzh*' is a dwarf isogenic line resulting from the introduction of the dwarf *Bzh* gene in 'Darmor' (Foisset et al. 1996). *B. oleracea* and *B. rapa* were used as they represent the parental species of *B. napus* (U 1935). Both of them synthesize C22 : 1 to a level of 52 and 55% of their seed oil, respectively.

The four *B. napus* genotypes are either LEAR type ($<1\%$ C22 : 1; 'Darmor.*bzh*', 'Drakkar', and 'Stellar') or HEAR type ($>50\%$ C22 : 1; 'Yudal'). DH progeny (152 lines) were produced from the cross 'Darmor.*bzh*' × 'Yudal' and were used for the construction of a *B. napus* genetic map (Foisset et al. 1996). The progeny were evaluated for erucic acid content and used to map the *E1* and *E2* genes (Jourdren et al. 1996 a). DH progeny (96 lines) were also produced from the cross 'Stellar' × 'Drakkar' and used to map the *L1* and *L2* genes controlling linolenic acid content (Jourdren et al. 1996 b). The genetic map built from the cross 'Darmor.*bzh*' × 'Yudal' was partially transferred to this second cross using RAPD markers (unpublished data).

PCR analysis

Primers were designed with the Oligo 4.0 program (Rychlik 1989) on the *Arabidopsis* and rapeseed *FAE1* sequences in the Genbank database [accession numbers U29142 (James et al. 1995) and U50771 (Barret et al. 1998) respectively]. The chosen pair of consensus primers is located in the second half of the gene at positions 992 and 1515 of the *Arabidopsis* sequence (positions 990 and 1513 of the rapeseed sequence). Primers were degenerate at their 3′ end. Sequences (5′ → 3′) were: upper primer CGGAGCTGACGACAARWCNTT; lower primer TCGATGCAGTGYTSCCANGG.

PCR reactions were performed in a 50-µl final volume containing 30 ng of genomic DNA, 1.5 mM of $MgCl_2$, 0.1 mM of each dNTP, 100 ng of each primer, 1.5 units of *Taq* polymerase (Eurobio) and 1 × *Taq* polymerase buffer (Eurobio). Cycles were 94°C 4 min, (94°C 1 min-53°C 1 min-72°C 2 min) × 30, final extension 72°C 4 min.

Amplification products were resolved on TBE 1 × 5% polyacrylamide non-denaturing gels (Acrylamide/bis-Acrylamide : 29/1) (32-cm plates). Gels were run for 24 h at 220 V in a Hoeffer apparatus (Ref: SE 620, Pharmacia Biotech) regulated at 20°C. Gels were EtBr-stained for 30 min in a 1 mg/l solution, and visualised on a UV table.

Sequencing of PCR products

Some DNA from each band was removed from the gel and re-amplified using the same PCR conditions except for the cycles which were 94°C 4 min, (94°C 1 min-53°C 45 s-72°C 1 min) × 25, final extension 72°C 4 min. Un-incorporated reagents were removed by centrifugation on Microcon columns (Millipore, ref 42413).

Enzymatic hydrolysis of the amplified products from 'Darmor-*bzh*' and 'Stellar' (1 µg) were performed with the restriction enzyme *Hae*III (BRL-30 U) for 2 h at 37°C. Three bands were separated on Nusieve (FMC, ref 50082) agarose gels (0.8%). After elimination of the agarose on microspin columns (Polylabo, ref 8504-00), the obtained fraction was cleaned on Microcon columns.

Sequencing was performed following the Applied Biosystems protocol for Dye-Terminator double-strand sequencing on an ABI373A sequencer.

Sequences were analysed with the GCG Wisconsin package (Version 9.0, Genetics Computer Group (GCG), Madison, Wis.).

Linkage analysis

Segregation of rapeseed *FAE1* genes was studied on the two DH progenies. Linkage analyses were performed using Mapmaker/exp version 3.0b (Lincoln et al. 1992). A maximum recombination fraction of 0.4 and threshold LOD scores of 4.0 and 3.0 were used for the 'Darmor.*bzh*' × 'Yudal' and 'Stellar' × 'Drakkar' progenies, respectively. Centimorgan distances were expressed with the Kosambi function.

Results

FAE1 rapeseed sequences

The separation of the PCR products on long acrylamide gels allowed us to visualise one band for

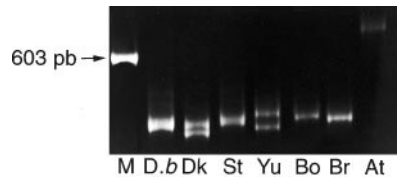

Fig. 1 Electrophoresis profile of the genomic PCR products on a polyacrylamide gel: *M* size marker; *D.b* Darmor.*bzh*; *Dk* Drakkar; *St* Stellar; *Yu* Yudal; *Bo B. oleracea*; *Br B. rapa*; *At A. thaliana*

Arabidopsis, *B. rapa*, *B. oleracea*, *B. napus* cv 'Stellar' and 'Darmor.*bzh*', and two bands for 'Yudal' and 'Drakkar' (Fig. 1). All these bands, corresponding to about 500 nucleotides in length, were sequenced. The *Arabidopsis* sequence obtained was identical over this length to the *Arabidopsis FAE1* gene published by James et al. (1995, Genbank: U29142).

The 'Yudal' and 'Drakkar' nucleotide sequences, and the *B. oleracea* and *B. rapa* representatives, were nearly identical to the rapeseed accession number U50771 (identity: 99.4%) and very similar to the *Arabidopsis FAE1* gene (identity: between 85 and 88%), confirming the amplification in the *Brassicae* of homologs of the *Arabidopsis FAE1* gene. As observed for the intronless *Arabidopsis* and *B. napus* genes in Genbank, we did not find any introns in the amplified part.

Analysis of the *Brassica* sequences obtained permitted us to distinguish two types of gene, depending on their ancestral origin: the BN-FAE1.1 gene ('Drakkar' band 2, 'Yudal' band 1) which corresponds to the unique *B. rapa* gene *BR-FAE1*, and the *BN-FAE1.2* gene ('Drakkar' band 1, 'Yudal' band 2) corresponding to the unique *B. oleracea* gene *BO-FAE1*.

Two PCR products corresponding to the two types of genes should be present in the single 'Stellar' and 'Darmor.*bzh*' band. The analysis of the sequences obtained for the two other rapeseed cultivars and the parental crops showed that a *Hae*III site was present only in the gene of *B. oleracea* origin. We suspected that this would also be the case in the 'Stellar' and 'Darmor.*bzh*' genotypes and submitted their PCR products to *Hae*III hydrolysis. Digestion profiles confirmed the existence of two genes in these cultivars, whose PCR products were co-migrating previously in the acrylamide gels. Sequences of these two nearly identical genes were obtained confirming that each rapeseed cultivar fits the scheme of the addition of the two parental genomes. The *B. napus* sequence in the database (U50771) appears to be of the *B. oleracea* type.

The *B. oleracea* and *B. rapa* nucleotide sequences present nine substitution differences. Eight of these nine substitutions (G1–G8 in Fig. 2) were also found between the two rapeseed genes in all the genotypes studied, leading to a common identity of 98.4% (eight differences in 504 bases, one difference being located in the *Hae*III restriction site).

In the four *B. napus* genotypes studied here, two alleles differing at positions 41 and 93 were found for the gene *BN-FAE1-1* (allele 1 for the high erucic Yudal; allele 2 for the low erucic types), and three allelic forms for the *BN-FAE1-2* gene (allele 1-'Yudal', allele 2-'Stellar'/'Darmor.*bzh*': two-base deletion at position 495; allele 3-'Drakkar': a four-base deletion at position 438) (Fig. 2).

Peptide sequences (Fig. 3) deduced from the coding sequences showed two amino-acid differences (positions 86 and 97) between the *BN-FAE1-1* and *BN-FAE1-2* genes.

Among the *BN-FAE1.1* gene, one change in the amino-acid sequence was shared only by the LEAR types (position 14). Among the *BN-FAE1.2* gene, the deletions in the three LEAR types led to a frameshift and to a stop codon soon after. *B. rapa* and *B. oleracea* also differed from *B. napus* at position 33. In addition *B. oleracea* was different at position 51.

Linkage analysis

The two *FAE1* rapeseed genes were each polymorphic in one of the two crosses: *BN-FAE1.1* in the 'Darmor.*bzh*' × 'Yudal' (DY) cross and *BN-FAE1.2* in the 'Stellar' × 'Drakkar' (SD) cross. *BN-FAE1.1* mapped to the DY9 linkage group (Fig. 4) and was found to be totally linked to the CE8 locus (Barret et al. 1998) and to co-localise with the *E1* QTL (Jourdren et al. 1996a). *BN-FAE1.2* gene mapped to the SD7 linkage group which was co-linear with the DY7 linkage group (Fig. 4). The position of the *BN-FAE1.2* gene corresponded to the location of the *E2* QTL in the DY7 linkage group, as previously determined by Jourdren et al. (1996 a).

Discussion

The consensus primers defined from *Arabidopsis* and rapeseed *FAE1* sequences made it possible to amplify one *FAE1* homolog in each parental species of rapeseed and two *FAE1* homologs in rapeseed itself. Each *BN-FAE1* rapeseed gene could be assigned to the *B. oleracea* or *B. rapa* genome from the sequence alignment.

The use of PCR with consensus primers, coupled with electrophoresis on long polyacrylamide gels, proved to be very efficient for isolating some parts of rapeseed genes without cloning and avoids the less-specific signals that can occur in hybridisation experiments between very related genes. The sequencing of the separated products validates the amplification of the desired gene and provides information on the part of the gene amplified. Electrophoresis of double-stranded DNA on non-denaturing acrylamide gels can

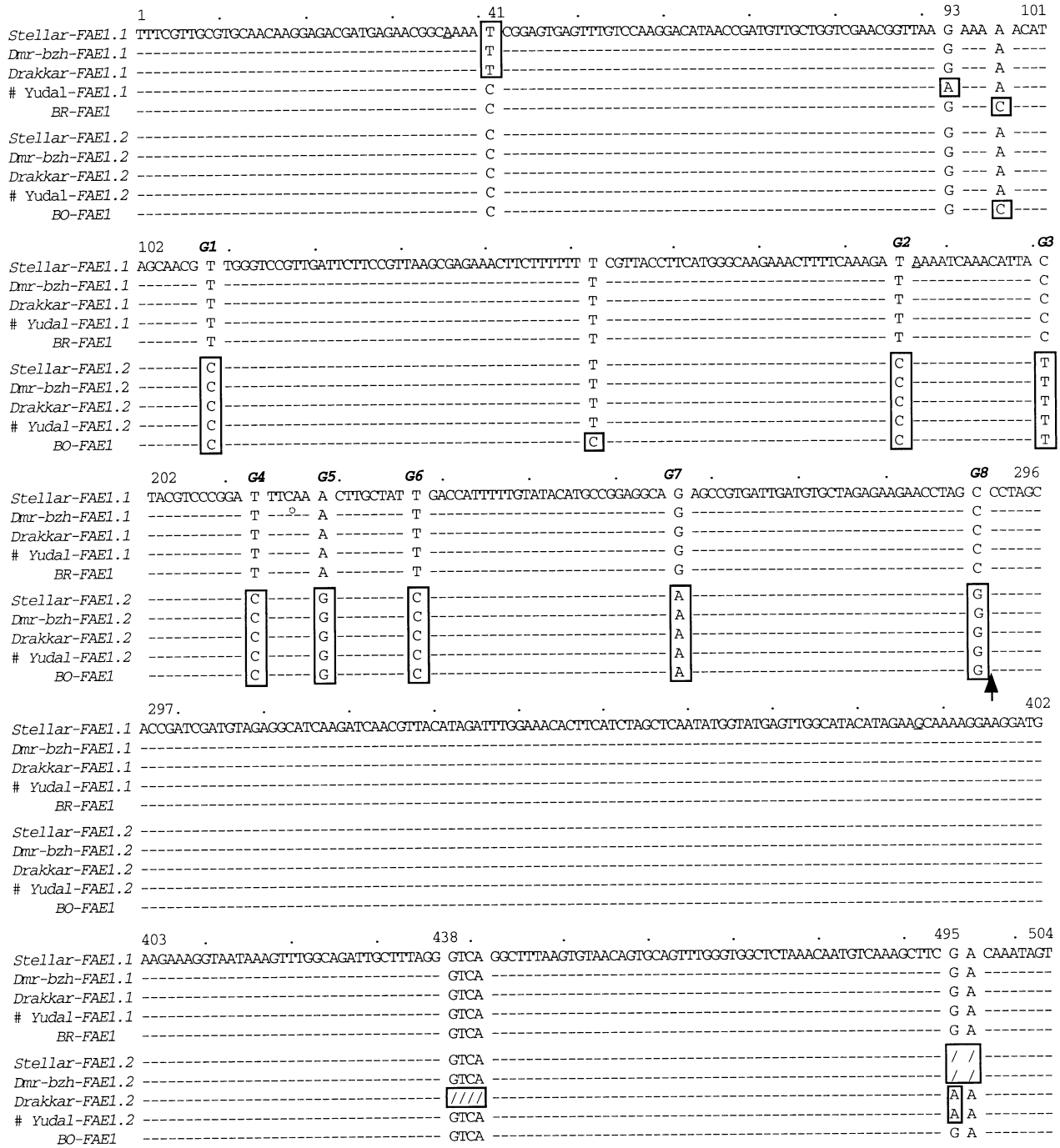

Fig. 2 Nucleotide sequences alignment: -- indicates identity to the first line; / indicates deletion. Nucleotides different from the rapeseed accession U50771 are *underlined*; *BR B. rapa. BO B. oleracea. Dmr* Darmor. *Arrow Hae*III site. # = HEAR genotype

separate DNA fragments differing by size, but also differing in their sequences, due to different conformations of fragments in gels (Kirkpatrick et al. 1993). This was of particular interest in the case of this intronless gene. For example, the visible polymorphism between the 'Yudal' genes reflected only substitutions in the sequence. The observed polymorphisms easily permitted the mapping of these *FAE1* genes on the rapeseed genetic map.

The two genes *BN-FAE1.1* and *BN-FAE1.2* mapped to the DY9 and DY7 linkage groups of the *B. napus* genetic map (Foisset et al. 1996), respectively. This result allowed us to confirm that these two groups are homoeologous, which was previously hypothesised by Barret et al. (1998) since two homoeologous isozyme genes (*Lap-1A* and *Lap-1C*) mapped at

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                1       .  14     .       .       .       .       .       .       . 84
Stellar-FAE1.1  FRCVQQGDDENGK I GVSLSKDITDVAGRTVKK N IATLGPLILPLSEKLLF F VTFMGKKLFKDKIKHYYVPDFKLAIDHFCIHAG
Dmr-bzh-FAE1.1  ------------ I ------------------ N ----------------- F --------------------------------
Drakkar-FAE1.1  ------------ I ------------------ N ----------------- F --------------------------------
# Yudal-FAE1.1  ------------ T ------------------ N ----------------- F --------------------------------
     BR-FAE1    ------------ T ------------------ H ----------------- F --------------------------------

Stellar-FAE1.2  ------------ T ------------------ N ----------------- F --------------------------------
Dmr-bzh-FAE1.2  ------------ T ------------------ N ----------------- F --------------------------------
Drakkar-FAE1.2  ------------ T ------------------ N ----------------- F --------------------------------
# Yudal-FAE1.2  ------------ T ------------------ N ----------------- F --------------------------------
     BO-FAE1    ------------ T ------------------ H ----------------- S --------------------------------
atu29142-FAE1   FRCVQQEDDESGK I GVCLSKDITNVAGTTLTK N IATLGPLILPLSEKFLF F ATFVAKKLLKDKIKHYYVPDFKLAVDHFCIHAG

                85      .  97    .       .       .       .       .       .       .  168
Stellar-FAE1.1  G R AVIDVLEKNL A LAPIDVEASRSTLHRFGNTSSSSIWYELAYIEAKGRMKKGNKVWQIALG SGFKCNSAVWVALNNVKAS TNS
Dmr-bzh-FAE1.1  - R ---------- A ---------------------------------------------------- ------------------ ---
Drakkar-FAE1.1  - R ---------- A ---------------------------------------------------- ------------------ ---
# Yudal-FAE1.1  - R ---------- A ---------------------------------------------------- ------------------ ---
     BR-FAE1    - R ---------- A ---------------------------------------------------- ------------------ ---

Stellar-FAE1.2  - K ---------- G ---------------------------------------------------- ------------------ K*
Dmr-bzh-FAE1.2  - K ---------- G ---------------------------------------------------- ------------------ K*
Drakkar-FAE1.2  - K ---------- G ---------------------------------------------------- ALSVTVQFGWL*
# Yudal-FAE1.2  - K ---------- G ---------------------------------------------------- ------------------ ---
     BO-FAE1    - K ---------- G ---------------------------------------------------- ------------------ ---
atu29142-FAE1   G R AVIDELEKNL G LSPIDVEASRSTLHRFGNTSSSSIWYELAYIEAKGRMKKGNKAWQIALG SGFKCNSAVWVALRNVKAS ANS
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Fig. 3 Peptide sequences alignment: -- indicates identity to the first line; * indicates a stop codon. amino acids different from the rapeseed accession U50771 are underlined; *BR B. rapa. BO B. oleracea. Dmr* Darmor. *Arabidopsis* sequence from Genbank in grey. # = HEAR genotype

approximately the same distance from the *E1* and *E2* genes, respectively.

The complementation experiments of LEAR genotypes with the jojoba KCS gene, as well as the fatty acid content of the *Arabidopsis fae* mutants, indicates the importance of this protein in the erucic acid trait. This information, as well as the digenic determinism of this trait in rapeseed, suggested that it could be controlled by one or two *FAE1* genes. The fact that the *BN-FAE1.1* and *BN-FAE1.2* candidate genes map respectively at the position of the *E1* and *E2* loci controlling the erucic acid content strongly supports this hypothesis, even if one cannot exclude the possibility that another gene tightly linked to *FAE1* is responsible for this variation.

If the two *FAE1* genes in rapeseed control erucic acid synthesis, HEAR genotypes should represent the wild-type functional proteins, and the LEAR types should be affected in their two genes.

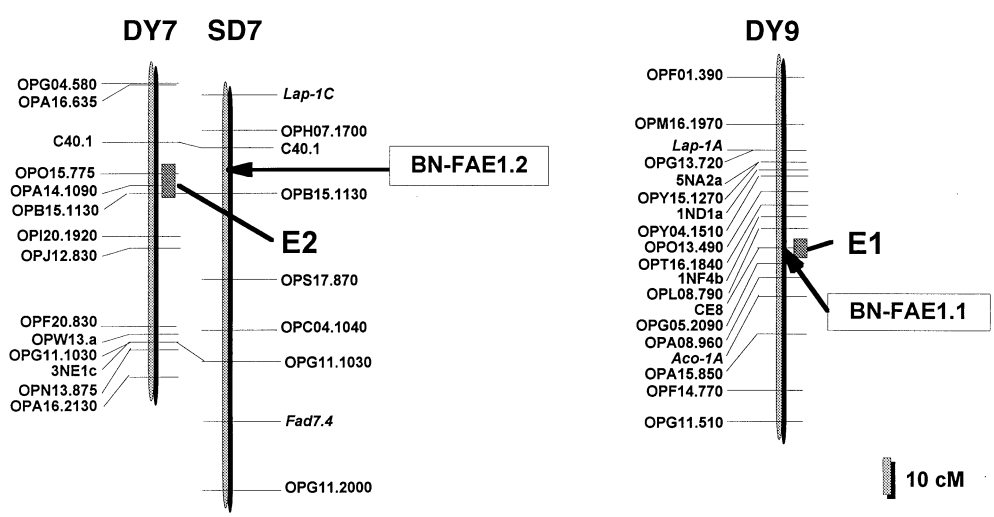

Fig. 4 DY7 and DY9 linkage groups constructed from 'Darmor.*bzh*' × 'Yudal' DH progeny and the SD7 linkage group constructed from 'Stellar' × 'Drakkar' DH progeny. *BN-FAE1.1* and *BN-FAE1.2* are mapped on DY9 and SD7, respectively. The position of the *E1* and *E2* loci is shown with grey boxes indicating the 1-LOD (ten-fold) likelihood confidence intervals as determined by Mapmaker/QTL

In the part of the *BN-FAE1.1* gene that we amplified, no evidence was found in the LEAR cultivars of a dramatic change in this second half of the protein that would not concern the 'Yudal' (HEAR-type) allele. A change at the 14th amino-acid position of our alignment was shared only by the LEAR types (I instead of T), but this amino acid is also present at this position in the *Arabidopsis FAE1* gene and a V is found in the jojoba cDNA, apparently without affecting protein efficiency. As no dramatic change was observed in this part of the gene for the *BN-FAE1.1* alleles, a difference between the high and low erucic acid rapeseeds could be located in parts of the KCS coding sequence not studied here (926 nucleotides upstream and 109 nucleotides downstream). The sequencing of the entire coding sequence for LEAR and HEAR types, as well as the analysis of *BN-FAE1.1* gene expression, would lead to a better understanding of this situation.

The observed deletions in the sequences of the *BN-FAE1.2* alleles of the LEAR genotypes led to frameshifts and premature stop codons, and thus to incomplete proteins. We can hypothesise that these mutations are responsible for the low erucic trait at this locus, although they may represent secondary mutations occurring in an already non-functional gene.

The weight of the *E1* and *E2* genes in the variation of erucic acid content was found to be different in the 'Darmor.*bzh*' × 'Yudal' cross (Jourdren et al. 1996 a), although this did not seem to be the case in another rapeseed cross studied by Thormann et al. (1996). Different alleles at the *E1* and *E2* loci, leading to different levels of erucic acid content in the seeds, have been hypothesised in rapeseed (Jönsson 1977). The characterisation of other LEAR and HEAR alleles at the two rapeseed *FAE1* loci could help to identify them. It might then be possible to determine whether the suspected different alleles would be related to differential expression (differences in the promoter region, post-transcriptional events) or to different proteins being encoded.

The polymorphism that we observed in this part of the gene can already be used to tag each allele of the studied genotypes. The characterisation of other different alleles present in various LEAR and HEAR genotypes is necessary to decide whether the differences identified in this work could be broadly used to differentially tag the high or low erucic acid rapeseeds. If not, the analysis of the whole sequence of the genes could be of value. This would make possible the development of easier tools for use in breeding programs (allele-specific primers; Cleaved Amplified Polymorphic Sequences: CAPS) providing simple molecular-markers for breeders in marker-assisted selection. They would be useful in particular for HEAR breeding where homozygous-high and heterozygous-intermediate classes overlap in the gas chromatography analysis of VLCFA content.

In this study, we chose a candidate gene approach, where a polymorphic tag was obtained for a gene involved in a particular metabolism, in order to validate or invalidate a link with an agronomic trait. The same method was successfully used by Jourdren et al. (1996c) for the candidate gene *FAD3* and the linolenic acid content in rapeseed. This underlines the interest in using sequences from known-function genes for the tagging of agronomic traits under oligogenic control, and appears to be an interesting supplement to the strategy based on saturating the genome with anonymous markers.

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Note added in proof

The nucleotide sequences reported will be registered in Genbank under the accession numbers AF054497 ('Yudal' FAE1-1), AF054498 ('Yudal' FAE1-2), AF054499 (*B. rapa*) and AF054500 (*B. oleracea*).

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