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ORIGINAL PAPER

Zero erucic acid trait of rapeseed (*Brassica napus* L.) results from a deletion of four base pairs in the *fatty acid elongase 1* gene

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Abstract The *fatty acid elongase 1* (*FAE1*) gene is a key gene in the erucic acid biosynthesis in rapeseed. The complete coding sequences of the *FAE1* gene were isolated separately from eight high and zero erucic acid rapeseed cultivars (*Brassica napus* L.). A four base pair deletion between T1366 and G1369 in the *FAE1* gene was found in a number of the cultivars, which leads to a frameshift mutation and a premature stop of the translation after the 466th amino acid residue. This deletion was predominantly found in the C-genome and rarely in the A-genome of *B. napus*. Expression of the gene isoforms with the four base pair deletion in a yeast system generated truncated proteins with no enzymatic activity and could not produce very long chain fatty acids as the control with an intact *FAE1* gene did in yeast cells. In the developing rape seeds the *FAE1* gene isoforms with the four base pair deletion were transcribed normally but failed to translate proteins to form a functional complex. The four base pair deletion proved to be a mutation responsible for the low erucic acid trait in rapeseed and independent from the point mutation reported by Han et al. (Plant Mol Biol 46:229–239, 2001).

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Introduction

Erucic acid is an antinutritional component in rapeseed (Beare-Rogers et al. 1971; Aherne et al. 1976; Badawy et al. 1994; Liu 1985). In 1960s the first variant with low erucic acid content (LEA) was found in a feed rape cultivar called LIHO (Downey and Craig 1964). The first LEA oilseed rape cultivar ORO was bred by using the LEA LIHO as parental material and initiated worldwide endeavors of rapeseed breeding towards LEA quality (Downey and Craig 1964; Harvey and Downey 1963). Since then almost all of the LEA rapeseed cultivars have been developed in China and other countries by traditional crossing method with the LEA gene source from the ORO (Harvey and Downey 1963). No other confirmed mutant for LEA content has been reported in rapeseed (*Brassica napus*). This single genetic source of LEA has aroused great concern about the inbreeding effect and genetic erosion in rapeseed breeding (Harvey and Downey 1963).

Erucic acid is genetically controlled by two additive alleles located separately in A- and C-genome chromosomes in *B. napus* (Jönsson 1977; Anand and Downey 1981, Fourmann et al. 1998). The genotypes $E^AE^AE^CE^C$, $E^Ae^AE^Ce^C$ (or $E^AE^Ae^Ce^C$, $e^Ae^AE^CE^C$) and $e^Ae^Ae^Ce^C$ are responsible for approximately 40, 20 and 0% erucic acid in seeds, respectively (Barret et al. 1998). The *fatty acid elongase 1* (*FAE1*) gene encodes a seed-specific condensing enzyme, β-ketoacyl-CoA synthase (KCS), serving as the rate-limiting enzyme in erucic acid biosynthesis (Lemieus et al. 1990; James et al. 1995; Lassner et al. 1996).

The LEA trait of rapeseed of the ORO origin was attributed to a point mutation of the *FAE1* genes from cytosine to thymine at the base site 845, causing substitution of a single amino acid residue from serine to phenylalanine at position 282 of the coded protein (Han et al. 2001). A reverse mutation

using site directed mutagenesis from the phenylalanine 282 residue to a serine residue in the *FAE1* polypeptide from *B. napus* cv. Westar restored the elongase activity and erucic acid formation (Katavic et al. 2002). Based on the alignment of the coded amino acid sequences, Cys223 was thought to be the putative active site of the KCS (Ghanevati and Jaworski 2001; Ghanevati and Jan 2002), and His391 and Asn424 were also thought to play an important role in the catalysis (Ghanevati and Jaworski 2001; Olsen et al. 1999; Huang et al. 1998; Qiu et al. 1999; Davies et al. 2000; Ferrer et al. 1999; Jez et al. 2000; Todd et al. 1999). However, these amino acids are all present in the HEA and the LEA rapeseed cultivars analyzed so far (Han et al. 2001; Katavic et al. 2002; Roscoe et al. 2001).

Fourmann et al. (1998) observed a two base pair deletion and a four base pair deletion in the fragment of the *FAE1* gene isolated from the LEA rapeseed variety, which were predicted to lead to frameshift mutations and premature termination of translation. They hypothesized that the deletions at the locus were responsible for the LEA trait. However, this speculation was not confirmed, nor could they exclude the possibility that it was a secondary mutation occurring in an already non-functional gene (Fourmann et al. 1998).

In the previous studies, we isolated the complete coding sequences of the *FAE1* genes from a variety of *Brassica* species and studied the polymorphism of single nucleotides (SNPs) of this gene (Wu et al. 2007). Based on the SNPs of the *FAE1* gene we established the methods to distinguish the C-genome *FAE1* gene from the A-genome *FAE1* gene (Wu et al. 2007). In particular we found that the four base-pair deletion between T1366 and G1369 of the *FAE1* gene exists in a number of the LEA Chinese rapeseed cultivars.

In this study we aimed to determine whether four base pair deletion in the *FAE1* gene is really responsible for LEA trait. We also want to know how different it is between the *FAE1* mutations in their functions and impacts.

Materials and methods

Plant materials

One HEA and seven LEA rapeseed cultivars (*B. napus*) were used as experimental materials, as shown in Table 1. The Zhongyou 821 and Zhongshuang series (Zhongshuang 9, 7, 4 and 2) were bred by our institute, and the Huashuang series (Huashuang 2 and 3) were bred by the Huazhong Agricultural University. All these cultivars are maintained in our institute and were provided by the Rapeseed Germplasm Collection of this institute.

Cloning *FAE1* coding regions

Based on the *FAE1* sequences of *B. napus* deposited in Genbank, the primer pairs were designed and synthesized (Sangon, Shanghai, China) as FAE1F (5′ ATCGGATC CATGACGTCCGTTAACGTAAAGCTCCTT 3′) and FAE1R (5′ ATCGAATTCTTAGGACCGACCGTTTTGG ACA 3′).

Genomic DNA was isolated from leaves of *B. napus* according to the protocol described by Saghai-Maroof et al. (1984).

Table 1 Mutation analysis of the *FAE1* gene from *B. napus* cultivars

Source of *FAE1* gene	Erucic acid content	Allele of *FAE1* gene	Type of mutation		
			C845T	Δ1366–1369	Δ1422–1423
Zhongyou 821	High	A	No	No	No
		C	No	No	No
Zhongshuang 2	Low	A	**Yes**	No	No
		C	No	**Yes**	No
Zongshuang 4	Low	A	**Yes**	No	No
		C	No	No	**Yes**
Zhongshuang 6	Low	A	**Yes**	No	No
		C	No	No	**Yes**
Zhuangshuang 7	Low	A	**Yes**	No	No
		C	No	**Yes**	No
Zhongshuang 9	Low	A	**Yes**	**Yes**	No
		C	No	**Yes**	No
Huashuang 2	Low	A	**Yes**	No	No
		C	No	**Yes**	No
Huashuang 3	Low	A	**Yes**	No	No
		C	No	**Yes**	No

The *FAE1* genes were amplified from the genomic DNA with KOD Plus kit (Toyobo, Osaka, Japan) using the primers FAE1F and FAE1R mentioned above. PCR products were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany). The *FAE1* genes from different sources were subcloned into the pZErO-2 vector (Invitrogen, Carlsbad, CA, USA) through *Bam*HI/*Eco*RI restriction enzyme sites and sequenced with the M13 forward primer and M13 reverse primer (Sunbiotech, Beijing, China).

Approximately ten clones bearing PCR products from each cultivar were sequenced. Based on the confirmed sequencing results, several cultivars and *FAE1* genes were selected for the further analyses.

RT-PCR analysis

Total RNA was isolated from leaves, flowers and seeds of Zhongshuang 9 at 30 days after flowering (DAF) using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The polyA RNA was reverse transcribed into cDNA with 100 ng of total RNA as template using the M-MLV RTase cDNA Synthesis Kit (Takara, Shiga, Japan).

From the single-stranded cDNA mixture a 1 μL aliquot was used as the PCR template to amplify the coding region of the *FAE1* using the primers FAE1F and FAE1R. The RT-PCR products were purified and cloned into the vector pZErO-2 and sequenced as described above. The nucleotide sequences of the cDNA were compared with the genomic information data and with sequences in Genbank.

Heterologous expression of the *FAE1* isoforms in yeast

All the constructs containing a *FAE1* gene insert were sequenced with M13 forward and reverse primers by cycle sequencing. The sequenced *FAE1* genes from different species were subcloned from pZErO-2 into the pYES2/NT C vector (Invitrogen, Carlsbad, CA, USA) through *Bam*HI/*Eco*RI sites downstream of the galactose-inducible promoter to generate N-terminus $(His)_6$Gly fused proteins.

The pYES2 constructs harboring the different *FAE1* isoforms were transformed separately into the yeast stain InvSc1 (Invitrogen, Carlsbad, CA, USA) using a lithium acetate procedure according to the manufacturer's instructions. The pYES2/NT C provided in the kit was transformed at the same time as the control. Transformants were selected by growth on synthetic complete medium lacking uracil (SC-ura) supplemented with 2% (w/v) glucose.

Transformed yeast cells were transferred into liquid SC-ura supplemented with 2% (w/v) glucose and grown overnight at 28°C with shaking. The overnight cultures were diluted with the SC-ura supplemented with 2% (w/v) galactose and 1% (w/v) raffinose to give an initial OD_{600} of 0.02. These cultures were grown at 20°C with shaking to an OD_{600} of 1.5.

Each yeast cells leavening was divided into two equal parts for immunoblot and lipids analysis.

Immunoblot analysis of the *FAE1* expressed in yeast cells

Yeast homogenates were prepared by the method of Tillman and Bell (Katavic et al. 2004). The cells were harvested by centrifugation and washed with 10 mL of ice-cold isolation buffer containing 80 mM HEPES–KOH pH 5.2, 5 mM EGTA, 5 mM EDTA, 10 mM KCl, 320 mM sucrose and 2 mM dithiothreitol. Then cells were centrifuged briefly and the pellet resuspended in isolation buffer. Cells were broken during three 90 s pulses with a Mini-Beadbeater (Biospec product, Bartlesville, OK, USA) using 0.5 mm glass beads.

The supernatant was collected after brief centrifugation to remove the unbroken cells and cell fragments. The microsomal membrane pellet was recovered by ultracentrifugation at 100,000*g* for 60 min (Katavic et al. 2002). The pellet was solubilized and purified with His·Bind resin (Novagen, Darmstadt, Germany) as described (Huang et al. 1998).

The purified proteins were separated on a 10% SDS-PAGE gel (Laemmli 1970). For Western blot analysis, proteins were transferred to nitrocellulose membrane by electrophoretic transfer with a mini trans-blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Western blot analysis was performed according to the standard protocols (Sambrook et al. 1989). Protein bands were detected using the anti-HisG antibody (Invitrogen, Carlsbad, CA, USA) followed by alkaline phosphatase-conjugated goat anti-mouse IgG (SABC, Luoyang, China) and color development.

Analysis of yeast lipids

Yeast cell fatty acid methyl esters (FAMEs) were prepared by the method of Katavic et al. (2002, 2004). Cells were harvested from overnight cultures by centrifugation and washed with distilled water. Cell pellets were saponified in methanolic-KOH [10% (w/v) KOH, 5% (v/v) H_2O in methanol] for 2 h at 80°C. After saponification, samples were cooled on ice and then washed with hexane to remove non-saponifiable material. The remaining aqueous phase was then acidified with 6 M HCl. Free fatty acids were extracted in hexane. The solvent was removed under a stream of N_2 and the free fatty acids were transmethylated in 2 mL 1% H_2SO_4 in methanol for 1 h at 60°C. FAMEs were extracted into hexane, the solvent was removed by vacuum and the residue was dissolved in hexane for gas chromatography (GC) analysis.

GC analysis was performed on a gas chromatogram (Agilent 5890N) fitted with a 30 m FFAP column, ID

0.25 mm narrowbore, film thickness 0.5 μm. The GC conditions were as follows: injected volume, 1 μL; injector temperature and flame ionization detector temperature, 260°C; nitrogen used as carrier gas in split mode at a split vent ratio of 50:1; column head pressure was set to 172.7 kPa (25 psi) to make up a total flow of 78.2 mL/min and a column flow of 1.5 mL/min (average velocity 42 cm/s); hydrogen flow 40 mL/min; air flow 400 mL/min; running temperature program, 160°C for 1 min, then increasing at 4°C/min to 240°C and holding at this temperature for 10 min.

Immunoblot analysis of *FAE1* from seeds

His·Bind resin purified *FAE1* from yeast cells bearing the wild type *FAE1* gene was prepared in complete Freund's adjuvant (FCA), and then injected subcutaneously at 20 sites on the thigh of two New Zealand white rabbits. The first booster injection was given with *FAE1* protein prepared in incomplete Freund's adjuvant (FIA) 15 days later, followed by booster injections every 15 days thereafter. Rabbits were bled 45 days after the initial immunization and again every 15 days subsequently. The serum was separated and the titer was measured by ELISA using yeast cell lysis fractions containing *FAE1* as the detection antigen, with serum from non-injected rabbits used as control. High-titer antibodies containing serum were stored at −20°C.

The developing seeds of *B. napus* cv. Zhongyou 821, Zhongshuang 6 and Zhongshang 9 were collected at 30 DAF. The microsome was prepared according to the method described by Puyaubert et al. (2001). Seeds (0.5 g) were homogenized in 10 mL buffer containing 80 mM HEPES pH 7.2, 10 mM *β*-mercaptoethanol, 0.32 mM sucrose and 5% polyvinylpyrrolidone (w/v). The homogenate was filtered and centrifuged at 10,000*g* for 5 min and followed by 100,000*g* 15 min. The pellet was resuspended in 500 μL 80 mM pH 7.2 HEPES buffer containing 10 mM L-mercaptoethanol and centrifuged again for the same condition. The final pellets containing microsome fragments were resuspended in the same buffer and diluted to the same concentration.

The microsomal proteins were separated by SDS-PAGE. The rabbit-anti-FAE1 polyclonal antibody was used to detect the *FAE1* proteins by Western blot as described above.

Results

Gene cloning and characterization

PCR amplifications of genomic DNA from the HEA Zhongyou 821 and seven LEA rapeseed cultivars generated products with a length approximately 1.5 kilo base pairs (kb). Sequencing of these *FAE1* genes demonstrated that each genotype produced two isoforms of the *FAE1* gene according to their sequence characteristics, each corresponding separately to the A-genome or C-genome (Wu et al. 2007).

Alignment of the isoforms with those from Genbank detected 61 SNPs along the coding sequences and 25 mutations in the deduced amino acid sequences. Twenty-five SNPs were identified to be A/C genome specific. Three sites were distinguished in the *FAE1* genes between HEA and LEA cultivars. The first site was a point mutation at base site 845 from cytosine in HEA to thymine in LEA cultivars. The second site was a four base pair deletion TCAG at 1366–1369 and the third site was the two base pair deletion GA at 1422–1423. As shown in Table 1, the A-genome *FAE1* gene all contained the point mutation in all the seven LEA cultivars, and the C-genome *FAE1* contained the four base pair deletion Δ1366–1369 or the two base pair deletion Δ1422–1423 without containing the point mutation in any cultivar (Table 1). Exceptionally, the A-genome *FAE1* in Zhongshuang 9 had both the point mutation and the same four base pair deletion as is normally found in the C-genome. The unique sequences of the *FAE1* isoforms from Zhongshuang 9 were deposited into Genbank with accession numbers AY888037 and AY888044.

To understand the consequence of the four base pair deletion, the gene sequences were translated into the putative amino acid sequences. It was found that a serine (Ser) residue at position 282 changed to a phenylalanine (Phe) with the point mutation (Fig. 1), in agreement with the characteristics reported for HEA and LEA rapeseed cultivars, respectively (Han et al. 2001). The four base pair deletion led to a frameshift mutation and a truncated protein which was 466 amino acid residues in Zhongshuang 9 in contrast to the protein with 506 amino acid residues in HEA rapeseed Zhongyou 821 and LEA rapeseed Zhongshuang 6 (Fig. 1).

Transcriptional analysis of the novel *FAE1* mutations in *B. napus*

Transcriptional analysis of Zhongshuang 9 was carried out to confirm whether the mutated *FAE1* gene transcribes normally. A PCR product approximately 1.5 kb in length was amplified with the reverse transcription product from developing seeds of Zhongshuang 9 as template. No band was visible from its leaves or flowers, or from the control reactions without reverse transcriptase (Fig. 2). These data confirmed that the amplification resulted from the mRNA template.

The RT-PCR products were sequenced and compared with the *FAE1* genes amplified from the genomic DNA.

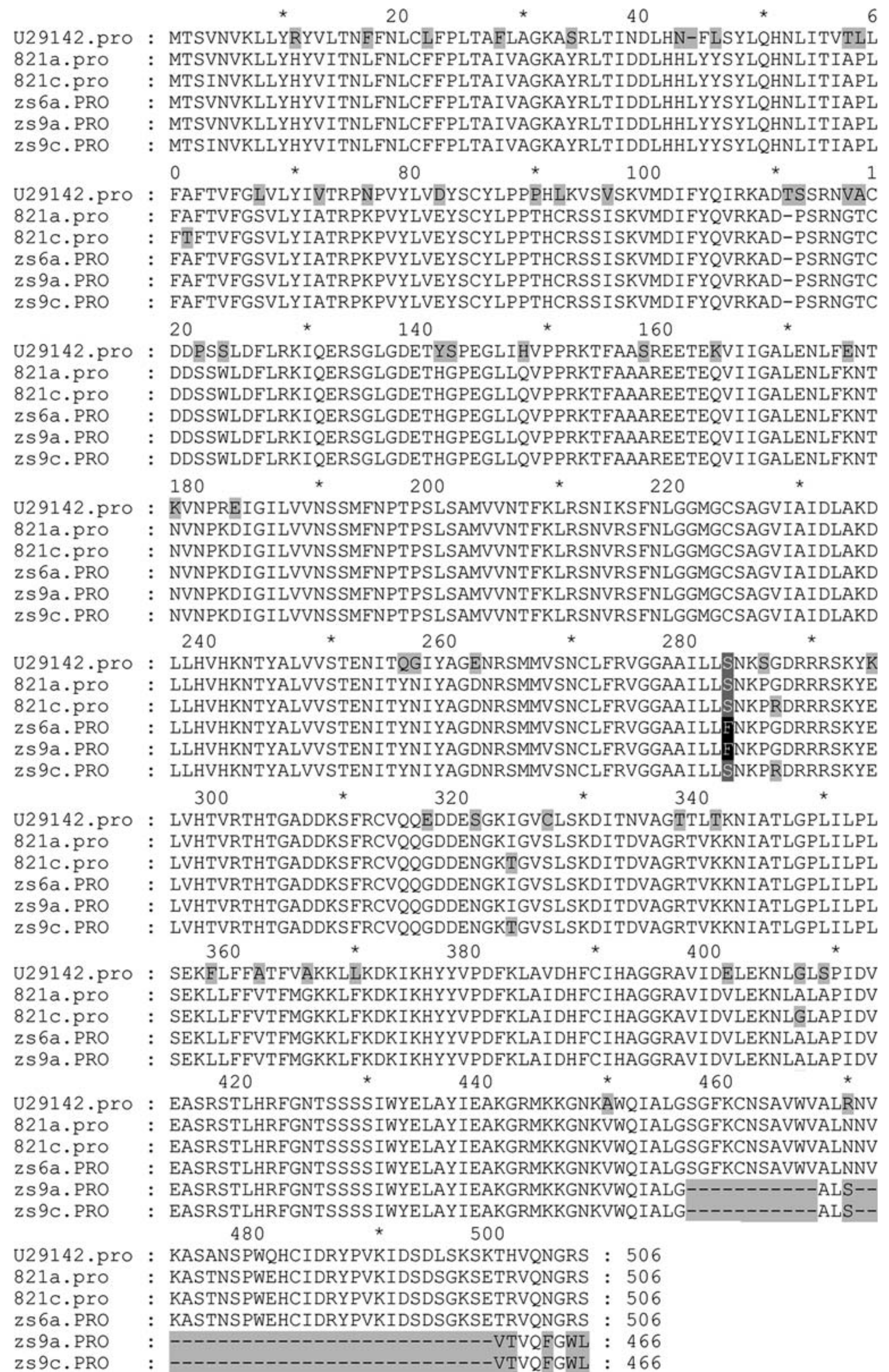

Fig. 1 Deduced amino acid sequence alignment of the *FAE1* gene from different species. The Phe at position 282 is indicated by *black shading* and the Ser at the corresponding position indicated by *gray shading*. U29142: *FAE1* amino acid sequence of *Arabidopsis thaliana* (accession number U29142); 821a: *FAE1* amino acid sequence in A-genome from HEA rapeseed Zhongyou 821 which is identical to AF274750; 821c: *FAE1* amino acid sequence in C-genome from Zhongyou 821; zs6a: *FAE1* amino acid sequence in A-genome from LEA rapeseed Zhongshuang 6; zs9a: *FAE1* amino acid sequence in A-genome from LEA rapeseed Zhongshuang 9 (accession number AY888044); zs9c: *FAE1* amino acid sequence in C-genome from Zhongshuang 9 (accession number AY888037)

The comparison showed that the cDNA sequences of these genes were identical to the corresponding genomic sequence. The characteristic deletion of the four base pairs and the Ser282 in the deduced protein sequence of C-genome *FAE1* gene was evident in the cDNA transcribed from the genomic sequence.

This result demonstrated that the *FAE1* gene with the four base pair deletion could be transcribed normally.

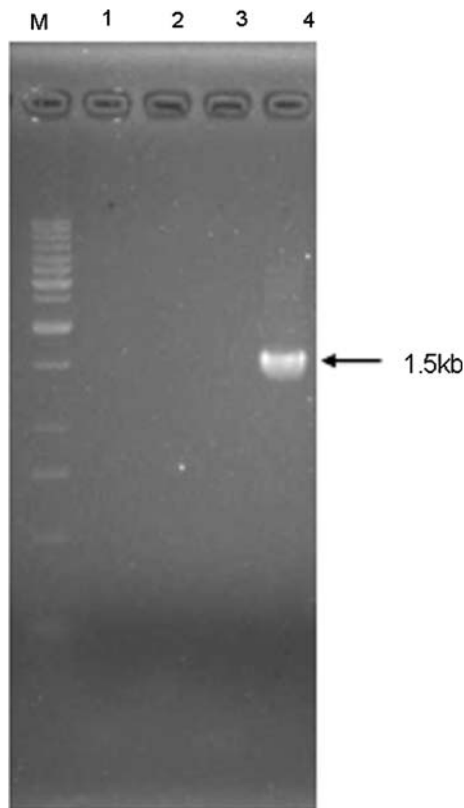

Fig. 2 Expression of the mutated *FAE1* gene in leaves, flowers and embryos of LEA Zhongshuang 9. RT-PCR: RNA from different source was used as template for RT reactions and was followed by PCR using primer pair FAE1F and FAE1R. M: 1 kb DNA Ladder (Fermentas, Vilnius, Lithuania); 1: RNA from seeds without reverse transcriptase as template; 2: RNA from leaves as template; 3: RNA from flowers as template; 3: RNA from seeds as template

Heterologous expression of the *FAE1* mutations in yeast and immunoblot analysis

The N-terminus $(His)_6$Gly fused *FAE1* protein expressed in yeast cells was purified and analyzed by Western blot using the anti-HisG antibody. No protein band was observed for the negative control pYES2/NT C. The colored protein bands could be divided into two groups. The fused *FAE1* from *B. napus* cv. Zhongshuang 9 migrated faster than those from cv. Zhongyou 821 and cv. Zhongshuang 6, indicating that the fused *FAE1* genes from *B. napus* cv. Zhongshuang 9 encoded smaller proteins (Fig. 3).

In addition, the size and density of the bands indicated that different *FAE1* genes expressed at different levels, and the mutants with deletion showed a relatively lower expression.

Analysis of yeast lipids

The fatty acid composition of induced yeast cell lysates was analyzed by GC as shown in Fig. 4. By comparison with

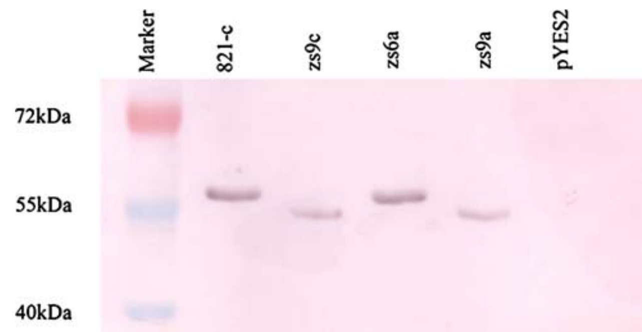

Fig. 3 Immunodetection of His-tag fused *FAE1* protein expressed in yeast cells using antibody raised against His-tag as probe. Marker: PageRuler Prestained Protein Ladder (Fermentas, Vilnius, Lithuania); 821-c: protein from yeast expressing the Zhongyou 821 C-genome *FAE1* gene; Zs6a: protein from yeast expressing the Zhongshuang 6 A-genome *FAE1* gene; Zs9a and zs9c: protein from yeast expressing the A- and C-genome *FAE1* gene from the Zhongshuang 9; pYES2: protein from yeast transformed with empty plasmid pYES2 NT/C

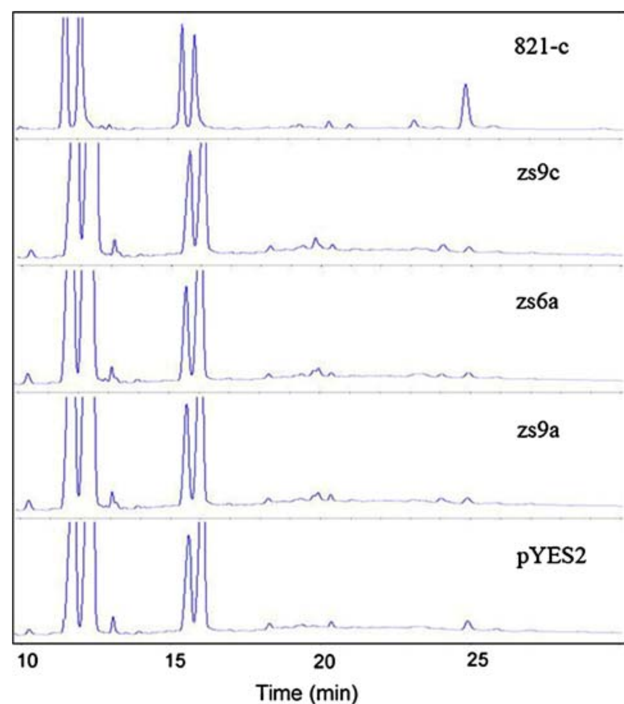

Fig. 4 GC chromatogram of FAMES showing fatty acid profiles from yeast cells transformed with His-tag fused *FAE1* genes. 821-c: FAMES prepared from yeast lysates expressing HEA rapeseed Zhongyou 821 C-genome *FAE1* gene; Zs6a: FAMES from yeast lysates expressing LEA Zhongshuang 6 A-genome *FAE1* gene; Zs9a and zs9c: FAMES from yeast lysates expressing A- and C-genome *FAE1* gene from LEA Zhongshuang 9; pYES2: FAMES prepared from yeast lysates transformed with empty plasmid pYES2 NT/C

the authentic C22:1 isomer, our results showed that only the yeast cells expressing the *FAE1* genes from Zhongyou 821 produced the erucic acid which is not normally found in yeast. The fatty acid composition of yeast cells expressing *FAE1* genes from Zhongshuang 6 and Zhongshuang 9 was identical to the negative control. These data confirmed

that the wild type *FAE1* genes could be functionally expressed in the yeast system and produce an active enzyme, while the *FAE1* genes from Zhongshuang 9 and Zhongshuang 6 both expressed inactive enzymes.

Immunodetection of the FAE1 proteins in HEA and LEA rapeseed embryos

Rabbit anti-*FAE1* serum was collected for the Western blot analysis and used to detect the presence of the *FAE1* proteins.

The result of immunodetection of the *FAE1* protein in HEA and LEA rapeseed embryos showed that a protein of approximately 56 kDa was detected in HEA rapeseed Zhongyou 821 but no protein band with the expected molecular weight was detected in Zhongshuang 6 and Zhongshuang 9 (Fig. 5). This implied that the expression and accumulation of the *FAE1* KCS in seeds of both LEA rapeseed Zhongshuang 6 and Zhongshuang 9 was very low and not distinguishable by Western blot analysis.

Discussion

The LEA trait was attributed to a point mutation of the *FAE1* genes from cytosine to thymine at the base site 845 (Han et al. 2001). This point mutation was also found in all the Chinese cultivars and without exception in the A-genome *FAE1* gene. It is reasonable that the point mutation might be firstly occurred in the A-genome.

The four base pair deletion was mostly found in the C-genome. It was found not only in Zhongshuang 9, but also in many other LEA rapeseed cultivars (Table 1). Although the LEA materials tested in this study are all winter type Chinese cultivars, the same base pair deletion was also reported in the LEA spring rapeseed cultivar Drakkar (Fourmann et al. 1998). These results implied that the four base-pair deletion in the C-genome *FAE1* is not a rare case, and is of independent origin to the point mutation responsible for LEA trait.

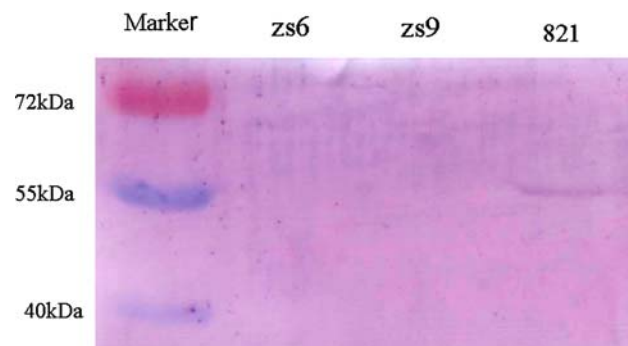

Fig. 5 Immunodetection of the *FAE1* protein in HEA and LEA rapeseed embryos using antibody raised against *FAE1* as a probe. Marker: PageRuler Prestained Protein Ladder (Fermentas, Vilnius, Lithuania); Zs6: protein from embryos of LEA Zhongshuang 6. Zs9: protein from embryos of LEA Zhongshuang 9; 821: protein from embryos of HEA rapeseed Zhongyou 821

It is interesting that Zhongshuang 9 bears the same deletion of four base pairs in both A- and C-genomes. We hypothesized that the four base pair deletion might occur first in the C-genome and shift to the A-genome through homologous exchange in this case. Zhongshuang 9 is one of the most popular LEA rapeseed cultivars in China, which was developed by multiparental crossing from Zhongyou 821, Zhuangshuang 2 and Zhongshuang 4, and by microspore culture. As shown in Table 1, all the parents of Zhongshuang 9 do not contain the four base pair deletion in the A-genome. The homologous exchange may have occurred during microspore culture and haploid meiosis.

It is still unknown when or in which variety the four base pair deletion of the *FAE1* gene first occurred.

Heterologous expression of the *FAE1* mutations in yeast cells and immunoblot analysis confirmed that *FAE1* genes could be expressed and the *FAE1* proteins encoded by the *FAE1* gene with the deletion were relatively smaller in molecule weight in agreement with the result of sequence analysis. Yeast cells which normally do not produce erucic acid formed erucic acid when the functional *FAE1* gene from HEA Zhongyou 821 was introduced. This result was in agreement with the findings of other researchers (Han et al. 2001; Katavic et al. 2002; Ghanevati and Jan 2002). However, yeast cells did not form erucic acid when the *FAE1* gene either from LEA Zhongshuang 9 or from Zhuangshuang 6 was introduced. This result confirmed that the LEA trait is really dependent on *FAE1* gene and the *FAE1* gene with either the point mutation or the four-base pair deletion or both loses its function.

The LEA trait may be derived from the *FAE1* gene's regulatory elements rather than the *FAE1* gene itself. If it is the case, transcription might not occur. RT-PCR analysis in this study showed that all the mutated *FAE1* genes were transcribed in developing seeds and the sequence of the mRNA was consistent with the genomic data. That is to say, the LEA trait of Zhongshuang 9 is related to the mutation in the coding region of the *FAE1* genes rather than the regulatory elements. All these observations suggest that the quantity or stability of the *FAE1* KCS from LEA Zhongshuang 9 was post-transcriptionally affected.

The LEA trait may also be derived from inactivity of the encoded enzyme or decreased expression of the *FAE1* gene. In developing seeds of the plant, absence of the elongase complex may be associated with either an absence of translation of the *FAE1* KCS, or from an inability of the translated protein to form a functional complex. Western blot analysis showed that the *FAE1* KCS protein was detected during seed development only in HEA Zhongyou 821, but

not found in any of the LEA cultivars (Fig. 5). This result appears to contradict to the result from yeast expression, because LEA *FAE1* genes from Zhongshuang 9 were expressed in yeast cells at a relatively high level. It is possible that the regulation of elongase protein may be quite different in yeast and in plants.

In previous reports both the important substrate-specific and the membrane binding domains are located at the N-terminus of the *FAE1* KCS and the activity essential key amino acid residues Cys223, His391 and Asn424 are in the middle of the peptide (Ghanevati and Jaworski 2001; Ghanevati and Jan 2002; Blacklock and Jaworski 2002). Database searches revealed that the C-terminus sequence was conserved among all *FAE1*-like proteins. Engineering the His-tag at the C-terminus of the *FAE1* KCS led to a significant loss of activity of the recombinant protein while the microsomal pellet containing the N-terminus His-tagged *FAE1* KCS retained the same level of condensation activity as the microsomal pellet containing the wild-type protein (Ghanevati and Jan 2002). Based on the analysis above, it was considered that the loss of integrity of the C-terminus might lead to the loss of the activity of the C-genome *FAE1* from Zhongshuang 9.

It is worthwhile to notice that there is another mutation characterized by the two base pair deletion GA at 1422–1423 which was found in the C-genome in this study. This mutation is obviously independent from the four base pair deletion.

In conclusion, the zero erucic acid trait can be derived not only from the point mutation by substitution of a single amino acid residue from serine to phenylalanine at position 282 of the coded *FAE1* protein (Han et al. 2001), but also from a natural deletion of four base pairs between T1366 and G1369 as shown in this study. The mutated genes were transcribed normally and encoded shortened proteins which are inactive and unstable in plants. The two base pair deletion at G1422–A1423 of the *FAE1* gene might be another mutation for LEA trait. Further studies on this mutation are to be undertaken.

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References

Aherne F, Bowland J, Christian R, Hardin R (1976) Performance of myocardial and blood seral changes in pigs fed diets containing high or low erucic acid rapeseed oils. Can J Anim Sci 56:275–284

Anand I, Downey R (1981) A study of erucic acid alleles in digenomic rapeseed (*Brassica napus* L.). Can J Plant Sci 61:199–203

Badawy I, Atta B, Ahmed W (1994) Biochemical and toxicological studies on the effect of high and low erucic acid rapeseed oil on rats. Nahrung 38:402–411

Barret P, Delourme R, Renard M, Domergue F, Lessire R, Delseny M, Roscoe T (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, Nera E, Heggtveit H (1971) Cardiac lipid changes in rats fed oils containing long–chain fatty acids. Can Inst Food Technol 4:120–124

Blacklock B, Jaworski J (2002) Studies into factors contributing to substrate specificity of membrane-bound 3-ketoacyl-CoA synthases. Eur J Biochem 269:4789–4798

Davies C, Heath RJ, White SW, Rock C (2000) The 1.8 A crystal structure and active-site architecture of beta-ketoacyl-acyl carrier protein synthase III (FabH) from *Escherichia coli*. Structure 8:185–195

Downey R, Craig B (1964) Genetic control of fatty acid biosynthesis in rapeseed (*Brassica napus* L.). J Am Oil Chem 41:475–478

Ferrer J, Jez J, Bowman M, Dixon R, Noel J (1999) Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. Nat Struct Biol 6:775–784

Fourmann M, Barret P, Renard M, Pelletier G, Delourme R, Brunel D (1998) The two genes homologous to *Arabidopsis FAE1* co-segregate with the two loci governing erucic acid content in *Brassica napus*. Theor Appl Genet 96:852–858

Ghanevati M, Jaworski J (2001) Active-site residues of a plant membrane-bound fatty acid elongase β-ketoacyl-CoA synthase, FAE1 KCS. Biochim Biophys Acta 1530:77–85

Ghanevati M, Jan G (2002) Engineering and mechanistic studies of the *Arabidopsis FAE1* β-ketoacyl-CoA synthase, FAE1 KCS. Eur J Biochem 269:3531–3539

Han J, Lühs W, Sonntag K, Zähringer U, Borchardt D, Wolter1 F, Heinz1 E, Frentzen1 M (2001) Functional characterization of β-ketoacyl-CoA synthase genes from *Brassica napus* L. Plant Mol Biol 46:229–239

Harvey B, Downey R (1963) The inheritance of erucic acid content in rapeseed (*Brassica napus* L). Can J Plant Sci 44:104–111

Huang W, Jia J, Edwards P, Dehesh1 K, Schneider G, Lindqvist Y (1998) Crystal structure of β-ketoacyl-acyl carrier protein synthase II from *E. coli* reveals the molecular architecture of condensing enzymes. EMBO J 17:1183–1191

James DW, Lim E, Keller J, Plooy I, Ralston E, Dooner H (1995) Directed tagging of the Arabidopsis *FATTY ACID ELONGATION 1* (*FAE1*) gene with the maize transposon activator. Plant Cell 7:301–319

Jez J, Ferrer J, Bowman M, Dixon R, Noel J (2000) Dissection of malonyl-CoA decarboxylation from polyketide formation in the reaction mechanism of a plant polyketide synthase. Biochemistry 39:890–902

Jönsson R (1977) Erucic-acid heredity in rapeseed (*Brassica napus* L. and *Brassica campestris* L.). Hereditas 86:159–170

Katavic V, Barton D, Giblin E, Reed D, Kumar A, Taylor D (2004) Gaining insight into the role of serine 282 in *B. napus FAE1* condensing enzyme. FEBS Lett 562:118–124

Katavic V, Mietkiewska E, Barton D, Giblin E, Reed D, Taylor D (2002) Restoring enzyme activity in nonfunctional low erucic acid *Brassica napus* fatty acid elongase 1 by a single amino acid substitution. Eur J Biochem 269:5625–5631

Laemmli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

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

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

Liu H (1985) Rapeseed genetics and breeding. Shanghai Scientific and Technology Publishing House, Shanghai (in Chinese)

Olsen J, Kadziola A, von Wettstein-Knowles P, Siggaard-Andersen M, Lindquist Y, Larsen S (1999) The X-ray crystal structure of beta-ketoacyl [acyl carrier protein] synthase I. FEBS Lett 460:46–52

Puyaubert J, Garbay B, Costaglioli P, Dieryck W, Roscoe T, Renard M, Cassagne C, Lessire R (2001) Acyl-CoA elongase expression during seed development in *Brassica napus*. Biochim Biophys Acta 1533:141–152

Qiu X, Janson C, Konstantinidis A, Nwagwu S, Silverman C, Smith W, Khandekar S, Lonsdale J, Abdel-Meguid S (1999) Crystal structure of beta-ketoacyl-acyl carrier protein synthase III. A key condensing enzyme in bacterial fatty acid biosynthesis. J Biol Chem 274:36465–36471

Roscoe T, Lessire R, Puyaubert J, Renard M, Delseny M (2001) Mutations in the fatty acid elongation 1 gene are associated with a loss of β-ketoacyl-CoA synthase activity in low erucic acid rapeseed. FEBS Lett 492:107–111

Saghai-Maroof MA, Soliman K, Jorgensen R, Allard R (1984) Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, New York

Todd J, Post-Beittenmiller D, Jaworski J (1999) *KCS1* encodes a fatty acid elongase 3-ketoacyl-CoA synthase affecting wax biosynthesis in *Arabidopsis thaliana*. Plant J 17:119–130

Wu Y, Xiao L, Wu G, Lu C (2007) Cloning of fatty acid elongase1 gene and molecular identification of A and C genome in Brassica species. Sci China Ser C-Life Sci 3:343–349