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Research Paper

Characterization of *FAE1* in the zero erucic acid germplasm of *Brassica rapa* L.

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The modification of erucic acid content in seeds is one of the major goals for quality breeding in oil-yielding *Brassica* species. However, few low erucic acid (LEA) resources are available, and novel LEA genetic resources are being sought. *Fatty acid elongase 1* (*FAE1*) is the key gene that controls erucic acid synthesis. However, the mechanism for erucic acid synthesis in *B. rapa* lacks systematic study. Here, we isolated zero erucic acid lines from 1981 Chinese landraces of *B. rapa* and found that the formation of LEA is not attributable to variations in *FAE1* coding sequences, as reported for *B. napus*, but may be attributable to the decrease in *FAE1* expression. Moreover, the *FAE1* promoter sequences of LEA and high erucic acid materials shared 95% similarity. Twenty-eight bases deletions (containing a 24-base AT-rich region) were identified approximately 1300 bp upstream from the *FAE1* start codon in the LEA accessions. The genotype with the deletions co-segregated with the LEA trait in the segregating population. This study isolated an LEA *B. rapa* resource that can be exploited in *Brassica* cultivation. The promoter variations might modify the expression level of *FAE1*, and the results shed light on novel regulation mechanisms for erucic acid synthesis.

Key Words: erucic acid, *FAE1*, *Brassica rapa*, expression, promoter, deletions.

Introduction

Brassica crops are important in global agriculture and oil production. High concentrations of erucic acid (C22:1) in the seed of oil-yielding *Brassica* species have been reported to be nutritionally undesirable (Badawy *et al.* 1994, Beare-Rogers 1971, Gopalan *et al.* 1974). Therefore, a major objective for breeding is to identify and apply genetic resources to produce low erucic acid (LEA) (<2%) seeds. In the 1960s, the first LEA variant was found in a feed rape cultivar called Liho (Downey and Craig 1964), and the first LEA *B. napus* cultivar, ORO, was bred using Liho (Downey and Craig 1964, Harvey and Downey 1964). Then, the first LEA *B. rapa*, SPAN, was bred (Downey 1964), and the LEA *B. juncea* resource Zem was isolated (Kirk and Oram 1978). Most LEA cultivars of the above species were developed by the introduction of recessive alleles from the donor varieties ORO, SPAN and Zem, or their derivative lines. This single LEA genetic resource has caused great concern about inbreeding effects and genetic erosion (Harvey and Downey 1964). *B. rapa* is the ancestral parent species of *B. napus* and *B. juncea* and has become increasingly attractive to biologists and plant breeders, largely due to its higher diversification and economic importance. Therefore, an exploration of novel zero erucic acid lines of *B. rapa* will expand the genetic resource of LEA genes and promote independent innovation capacity for quality improvement of oilseed via interspecific crosses.

The *fatty acid elongase 1* (*FAE1*) gene encodes the first enzyme (β-ketoacyl-CoA synthase, KCS) in erucic acid biosynthesis and serves as the rate-limiting enzyme for this process in higher plants (James *et al.* 1995). This 1521-bp *FAE1* gene, with no intron, has been isolated from *Arabidopsis thaliana*, *B. napus*, *B. oleracea* and *B. rapa* (Das *et al.* 2002, Fourmann *et al.* 1998, James *et al.* 1995). Many recent reports have addressed the relationship of *FAE1* to erucic acid content. The two *FAE1* genes in *B. napus* (*FAE1.1* and *FAE1.2*) were found to be tightly linked to the E1 and E2 loci controlling erucic acid content (Barret *et al.* 1998, Fourmann *et al.* 1998, Jourdren *et al.* 1996). Subsequently, Roscoe *et al.* (2001) found that an absence of erucic acid (22: 1Δ13) in LEA rapeseed was correlated with a lack of acyl-CoA elongation activity. The LEA trait in *B. napus* can be attributed to the substitution of a single amino acid residue from serine to phenylalanine at position 282 of the encoded protein (Han *et al.* 2001, Katavic *et al.* 2002, 2004). Cys223 was considered to be another putative active site of FAE1 (Ghanevati and Jaworski 2001, 2002), and the conserved Asn424 and His391 residues were confirmed to be important in FAE1-

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KCS activity (Davies *et al.* 2000, Ghanevati and Jaworski 2001, Huang *et al.* 1998, Jez *et al.* 2000). However, all of these amino acids are present in both LEA and high erucic acid (HEA) rapeseed (Han *et al.* 2001, Katavic *et al.* 2002, Roscoe *et al.* 2001). More recently, Wu *et al.* (2008) attributed the LEA trait of rapeseed to a four-nucleotide deletion in the *BnFAE1* gene. Similarly, four substitution-type single-nucleotide polymorphisms (SNPs), one in *FAE1.1* and three in *FAE1.2*, were identified to distinguish low erucic types from high erucic types in *B. juncea* (Gupta *et al.* 2004, Xu *et al.* 2010). These results indicate that the LEA phenotype was due to variations in the *FAE1* coding sequence (CDS) in *B. napus* and *B. juncea*. Despite these recent advances in the biochemistry of seed elongases and cloning of *FAE1*, the nature of the mutations that characterize the agriculturally important LEA trait remains obscure, particularly in *B. rapa*. Sequence alignment of the *FAE1* gene between the HEA and LEA *B. rapa* revealed three SNPs due to transition-type base substitutions at positions 591 (G/A), 735 (C/T), and 968 (C/T) (Wang *et al.* 2010). Among the three variations, only the 968 (C/T) led to an amino acid change, which may have caused the phenotypic difference (Wang *et al.* 2010, Xu *et al.* 2010).

Here, we analyzed the erucic acid content of 1981 *B. rapa* landraces and found that over 90% of the samples had more than 40% erucic acid. An LEA landrace was isolated and *FAE1* was analyzed. We found that the variation in the CDS of *FAE1* did not cause the variable erucic acid content of *B. rapa*, and the LEA *B. rapa* formation may be attributable to the decrease in *FAE1* expression. The 24-base AT-rich region deletion in the *FAE1* promoter of the LEA *B. rapa* may be responsible for altering the expression of *FAE1*. A molecular marker was developed based on the deletions, and the genotype with the deletions co-segregated with the LEA trait in the segregating population. In the study, the formation of LEA in *B. rapa* is explained in a novel way. The discovery of the LEA landrace could be used in LEA breeding.

Materials and Methods

Plant materials

The seeds of most of the *B. rapa* accessions were obtained from the Chinese Crop Germplasms Information System (CGRIS, a germplasm repository for collecting worldwide genetic resources of oilseed crops). In this study, 1981 *B. rapa* landraces from China were used to reveal the erucic acid variation among landraces and to screen for zero erucic acid landraces. Twenty-four inbred *B. rapa* accessions (11 landraces selected from the 1981 landraces and 13 modern elite cultivars including 4 foreign cultivars) were selected to clone the CDS of *FAE1* (**Supplemental Table 1**). The GenBank accession numbers for the nucleotide sequences range from KF999615 to KF999639. All of the self-pollinated seeds were harvested for determination of the erucic acid content.

To establish an F2 population segregating for different alleles at the *FAE1* promoter locus, Sanjiecaizi (an LEA type containing zero erucic acid, *e*/*e*) was crossed with Nanhualinggongdacaizi (an HEA type containing 55% erucic acid, *E*/*E*) (**Supplemental Table 1**). The effect of the deletions on erucic acid formation was examined using 118 F2 seeds.

Fatty-acid analysis

Approximately thirty mature oven-dried seeds were first ground in tubes and then ground to a fine powder using small pestles. After dissolution in 1 mL petroleum ether, each sample was treated using ultrasonic irradiation (using ultrasonic power, i.e., 100 W, at 20°C) for 20 min. After saponification in 3 mL of 0.4 N methanolic-KOH, the samples were sonicated for 20 min and centrifuged, and the supernatant was then collected. The supernatant samples were added to 3 mL of H_2O and vortexed for 15 min. After centrifugation, the 2-μl upper layer was loaded into a gas chromatographic analyzer (GC, Agilent 6890N). The GC conditions were set according to Hu *et al.* (2009). The erucic acid content was determined by measuring the area of the peak.

Fifty seeds from each sample with an erucic acid content less than 20% were analyzed using the half-seed method (one cotyledon was analyzed using the GC, and the other was sown and harvested by self-crossing) to isolate zero erucic acid materials. Fatty-acid analysis of the half-seed method was the same as the method described above except one-tenth of the reagents was used. The half-seed method was also used for erucic acid determination of the F2 seeds (Gupta *et al.* 2004).

Cloning the FAE1 coding and promoter sequences

Based on the *FAE1* sequences from BRAD Bra034635.1 (Wang *et al.* 2011, http://www.brassicadb.org), the forward primer (FAEF1: 5′-ATTCTCCGACACACACACTG-3′) and the reverse primer (FAER1: 5′-AGAGAAACATCGTAGCC ATCA-3′) were designed to isolate the CDS of *FAE1*. To obtain the full length of the upstream sequence of *FAE1*, one pair of specific primers (PFAE40F: 5′-TGCATCCATAGAT ATCCTGT-3′; PPR: 5′-AACGGAAAGAAGCAAAGGT-3′) was designed within the 3′ and 5′ sequences of Bra034634 and *FAE1*. The primers of pM120F (5′-TCGGTAAAAGAA AAATCA-3′) and pM468R (5′-CTCATCTAAACTATATTA AGTG-3′) were designed based on the deletions of the LEA promoter for genotyping the segregating population (**Fig. 1**).

Total DNA was extracted from leaves according to the methods described by Murray and Thompson (1980). Genomic DNA was used as a template for PCR, which was carried out using a KOD-plus kit (Toyobo, Japan) and the primers mentioned above. The reactions were prepared in a total volume of 50 μl containing 1 μl of genome DNA, 1 μl of each 10 μM primer, 5 μl of 10× buffer, 2 μl of Mg^{2+}, 5 μl of dNTPs, 1 μl of KOD-plus and 34 μl of ddH_2O. The PCR was carried out in a PTC-200 Peltier Thermal Cycler (Bio-Rad, USA) using the following program: 5 min of initial

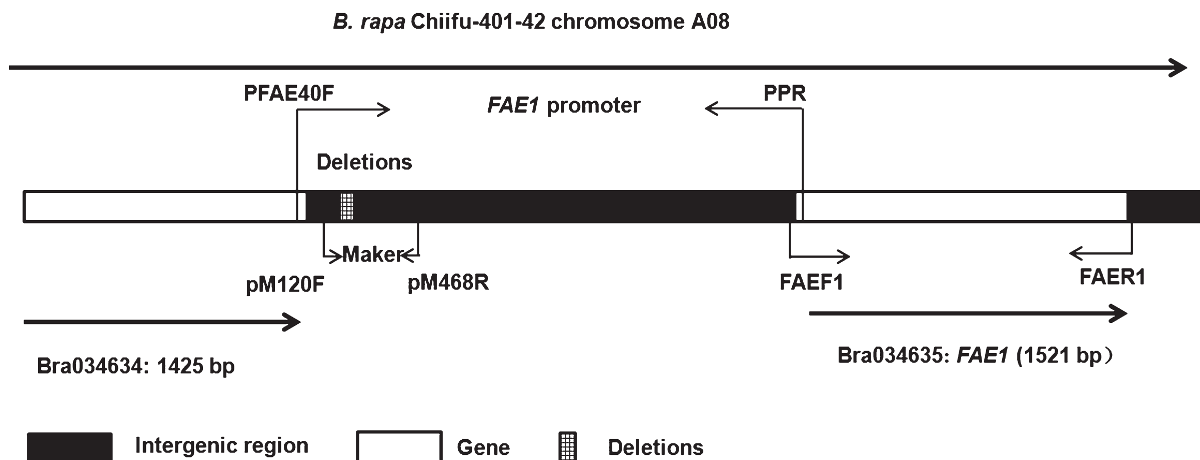

Fig. 1. Schematic representation of the position of *FAE1* and its promoter in the *B. rapa* chromosome.

denaturation at 94°C; 35 cycles of 1 min at 94°C, 1 min at 56°C and 1 min 50 sec at 68°C for *FAE1;* and a final extension of 10 min at 68°C. The annealing temperature was 54°C for *FAE1* promoter amplification.

A nucleotide of adenine was added to the end of the PCR products and they were purified using a PCR purification kit. The *FAE1* genes from different sources were cloned into the pEASY-T1 vector using a pEASY-T1 Cloning Kit (Transgen Biotech, China) and sequenced using the M13 forward and reverse primers (Shanghai Sangon, China). Approximately 5 clones bearing PCR products from each cultivar were sequenced. The sequence alignment was carried out using the Vector NTI suite 9.0 software package.

Determination of FAE1 transcript levels

The transcript levels of *FAE1* in the developing seeds of the inbred LEA Sanjiecaizi, HJa 96368 and HEA Nanhualinggongdacaizi were analyzed using quantitative reverse transcription (qRT-PCR). Total RNA was extracted according to the protocol described for *Arabidopsis* seeds (Vicient and Delseny 1999). RNA pellets were dissolved in DEPC-treated water, quantified by absorbance at 260 nm and checked for quality using agarose gel electrophoresis. Total RNA samples were reverse transcribed to first-strand cDNA using a ReverTra Ace-α-TM qPCR RT kit (Toyobo) and stored at –80°C. The expression of the *FAE1* gene was normalized to *ACTIN* using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The *FAE1* and *ACTIN* primers and the amplification conditions have been described previously (Hu *et al.* 2009).

Real-time PCR reactions were performed in triplicate using the 2× SYBR Green qPCR Master Mixes (Toyobo). The iCycler iQ5 (Bio-Rad) was used for all amplifications. For each gene, triplicate sets of the PCR reaction samples were prepared and run in an 8-tube strip (Bio-Rad). The PCR experiments were repeated for each plate to ensure that similar results were obtained. The PCR protocol consisted of an initial denaturation step at 94°C for 2 min, followed by 40 repeats of denaturation at 94°C for 10 sec and a combined primer annealing/elongation step at 58°C for 20 sec. A melting curve cycle followed the amplification cycle to confirm PCR product specificity: 94°C for 1 min, 58°C for 1 min, and acquisition of a melting curve from 55°C to 95°C with temperature change values of 0.5°C and a dwell time of 30 s.

Statistical analysis

Box plots of erucic acid contents in F2 individuals grouped by *FAE1* genotypes were drawn and the differences in the erucic acid contents among different genotypes were tested using R software Version 3.11 for Windows (http://www.r-project.org/). $P < 0.01$ was considered statistically significant.

A Chi-square test was applied to determine the segregation of the genotypes in the F2 population. The expected ratio of the homozygous (Sanjiecaizi, *e*/*e*) to heterozygous (*E*/*e*) to homozygous (Nanhualinggongdacaizi, *E*/*E*) genotypes was 1 : 2 : 1.

Results

Survey of erucic acid content variation in Chinese landraces of B. rapa

The phenotypic distribution of the erucic acid content in the 1981 *B. rapa* lines is shown in **Table 1**. There were no lines that contained less than 10% erucic acid. There were five classes of phenotypes (10–20%, 20–30%, 30–40%, 40–50% and 50–60%). Among the samples, 92.07% (1824 accessions) had a high erucic acid content, i.e., more than 40%. Of these, 52.70% had an erucic acid content of 40–50%, and 39.37% had an erucic acid content up to 50–60%. From the perspective of geography, we found that the winter *B. rapa* from the Yangtze valley (comprised of Jiangsu, Anhui, Jiangxi, Hunan, Hubei, Sichuan and Yunnan and Xizang provinces) had an erucic acid content of 40–60%, whereas spring *B. rapa* from Qinghai, Gansu and Xinjiang

Table 1. Survey of erucic acid content variation in Chinese landraces of *B. rapa*

Origin	Number of accessions with different erucic acid contents						Total
	0–10%	10–20%	20–30%	30–40%	40–50%	50–60%	
Jiangsu					10	37	47
Zhejiang		2			29	48	79
Anhui		3			70	138	211
Jiangxi		1			117	10	128
Hunan					64	25	89
Hubei				3	95	131	229
Sichuan		4		2	75	84	165
Guizhou		3	7	19	134	149	312
Yunnan					37	84	121
Shaanxi				6	168	10	184
Henan				5	30	9	44
Shanxi		1		1	59	23	84
Guangdong					13	4	17
HongKong					1		1
Taiwan					2	1	3
Fujian					14	10	24
Gansu			2	35	8	1	46
Qinghai			3	40	13	2	58
Xinjiang			4	3	1		8
Xizang		1	2	8	103	14	128
Mongolia		2			1		3
Total	0	17	18	122	1044	780	1981
Proportion	0	0.86%	0.91%	6.16%	52.70%	39.37%	100%

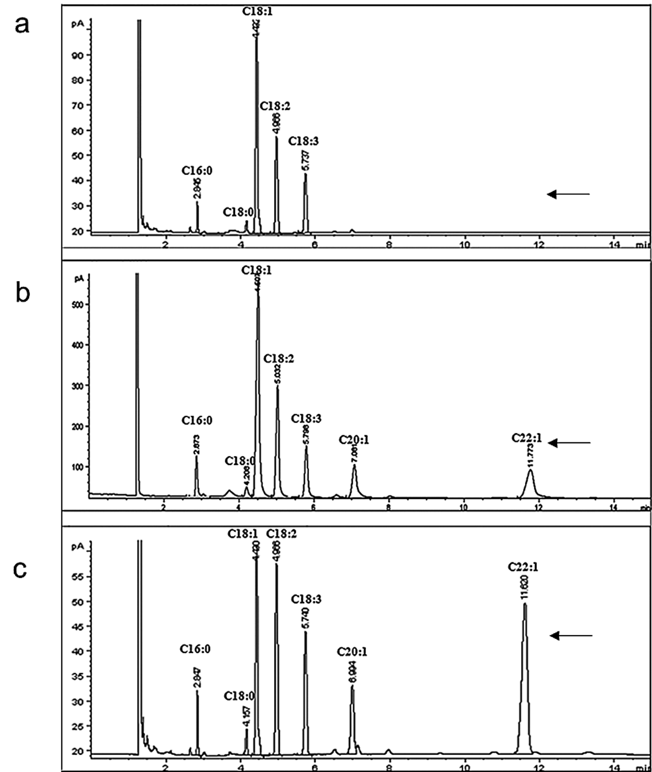

Fig. 2. GC analysis showing fatty acid profiles of different individuals of Sanjiecaizi. a: Sanjiecaizi-1 (erucic acid content: 0); b: Sanjiecaizi-28 (erucic acid content: 13.84%); **c**: Sanjiecaizi-49 (erucic acid content: 33.22%).

had an erucic acid content of 30–40%. In 18 accessions, the erucic acid contents were between 20% and 30%. Only 17 landraces had 10–20% erucic acid; these were distributed across Anhui, Sichuan, Guizhou, and so on. The 17 landraces with 10–20% erucic acid in the seeds were then used to isolate zero-EAC individuals.

Isolation of zero erucic acid B. rapa

Half-seed GC analysis of the 17 landraces with 10–20% erucic acid showed that their erucic acid contents varied from 0% to 36.6% (data not shown). The Sanjiecaizi individuals had the largest variation of erucic acid content (varying from 0 to 34%) among the 17 landraces, and the erucic acid contents of the other 16 landraces varied from 11.0% to 36.6%. Among the fifty seeds of the Sanjiecaizi analyzed, in 26 seeds (52%), the erucic acid contents were zero; in three seeds (6%), between 10% and 20%; in thirteen seeds (26%), between 20% and 30%; and in eight seeds (16%), between 30% and 40% (**Fig. 2**, **Supplemental Table 2**). The Sanjiecaizi individuals with zero erucic acid were planted and self-pollinated, and we ultimately obtained stable zero erucic acid *B. rapa* lines through several self-pollinations.

FAE1 polymorphisms in the 24 B. rapa with different erucic acid contents

The 24 accessions of *B. rapa* whose erucic acid contents were between 0 and 55% (with a normal distribution) were collected for analysis of the CDS of *FAE1*, and the materials contained three zero erucic acid *B. rapa* accessions: HJa96368 (a cultivar from Finland), Sanjiecaizi (a landrace from China), and Qingyou 11 (a cultivar from China). PCR amplification of each of the 24 lines generated a band at 1.5 kb that was cloned into the pEASY-T vectors. The corresponding clones were designated by the name of the lines. Alignment of the *FAE1* CDS revealed 26 SNPs along the CDS, with a similarity of up to 98–100% (**Supplemental Table 1**). The SNPs led to 13 mutations in the deduced amino acid sequences. Most samples had three variable sites (at positions 122, 591 and 735) (**Supplemental Table 1**). However, no sites of the *FAE1* CDS were distinguishable between the HEA and LEA accessions. The results indicate that the variation in the *FAE1* CDS did not contribute to the variable erucic acid phenotype. Moreover, the CDS of the LEA cultivars HJa 96368 from Finland and Qingyou 11 from China shared 100% identity. However, the LEA landrace Sanjiecaizi had three variations (at positions 591, 735 and 968) compared with the CDS of HJa 96368 and Qingyou 11.

FAE1 gene expression in LEA and HEA B. rapa

To confirm whether the *FAE1* gene expression affected the erucic acid content of *B. rapa*, we carried out transcriptional analysis in the developing seeds of inbred landraces LEA Sanjiecaizi, HJa 96368 and HEA Nanhualinggongdacaizi. The mRNA developmental profiles during seed development differed between the HEA and LEA seeds (**Fig. 3**). In the HEA accession, the *FAE1* expression could be detected 10 days after flowering (DAF). The expression decreased at 15–20 DAF and increased significantly at 20–25 DAF. The maximal expression levels were reached at 25

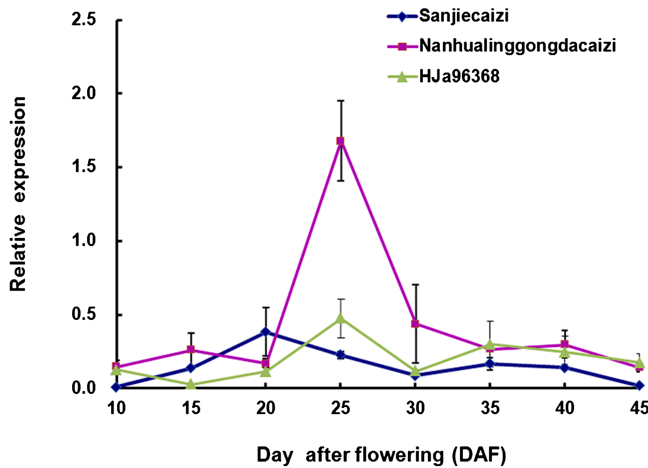

Fig. 3. The expression profile of the *FAE1* gene during seed development in HEA *B. rapa* Nanhualinggongdacaizi and LEA *B. rapa* Sanjiecaizi and HJa 96368.

DAF. The expression decreased sharply at 25–30 DAF and then steadily declined at 30–45 DAF. In contrast, in LEA *B. rapa*, the *FAE1* gene expression remained stable at lower levels compared with HEA *B. rapa* at most developmental stages. The maximal expression level was measured at 20 and 25 DAF in LEA Sanjiecaizi and HJa 96368, respectively. In the Sanjiecaizi individuals, the *FAE1* gene transcripts decreased at 20–30 DAF and increased during 30–35 DAF. After 35 DAF, the transcript levels continued to decrease until no transcription was detected during the final stage of seed development. In HJa 96368, the *FAE1* gene transcripts increased at 15–25 DAF, decreased during 25–30 DAF and increased during 30–35 DAF. After 35 DAF, the transcript levels continued to decrease. A comparison of the peak transcript levels indicated that the *FAE1* gene transcript levels in HEA were 4.4-fold and 3.5-fold those measured in LEA *B. rapa* Sanjiecaizi and HJa 96368, respectively. Generally speaking, the *FAE1* gene transcript was more abundant in the HEA than in the LEA accession during seed development.

FAE1 promoter analysis in LEA and HEA B. rapa

The promoter regions of the *FAE1* gene from the LEA *B. rapa* Sanjiecaizi and Qingyou 11 and HJa 96368, and the HEA Nanhualinggongdacaizi were isolated based on the Chiifu-401-42 sequence (Wang *et al.* 2011) (**Fig. 1**). The alignment results showed that the three LEA promoter sequences shared 95% similarity with the HEA sequences; however, the sequence of the landrace LEA Sanjiecaizi was 100% similar to the LEA cultivar Qingyou 11 and the foreign cultivar HJa 96368. There were 56 SNPs and 37 INDELs between the HEA and LEA promoter sequences. Among the INDELs, a total of 28 bases were deleted at a position approximately 1300 bp from the translation initiation site (ATG) of the LEA allele promoter (**Fig. 4**). The deletions caused the LEA sequence to lose a 24-base AT-rich region. The variations were mainly located in the region shown in **Fig. 4**.

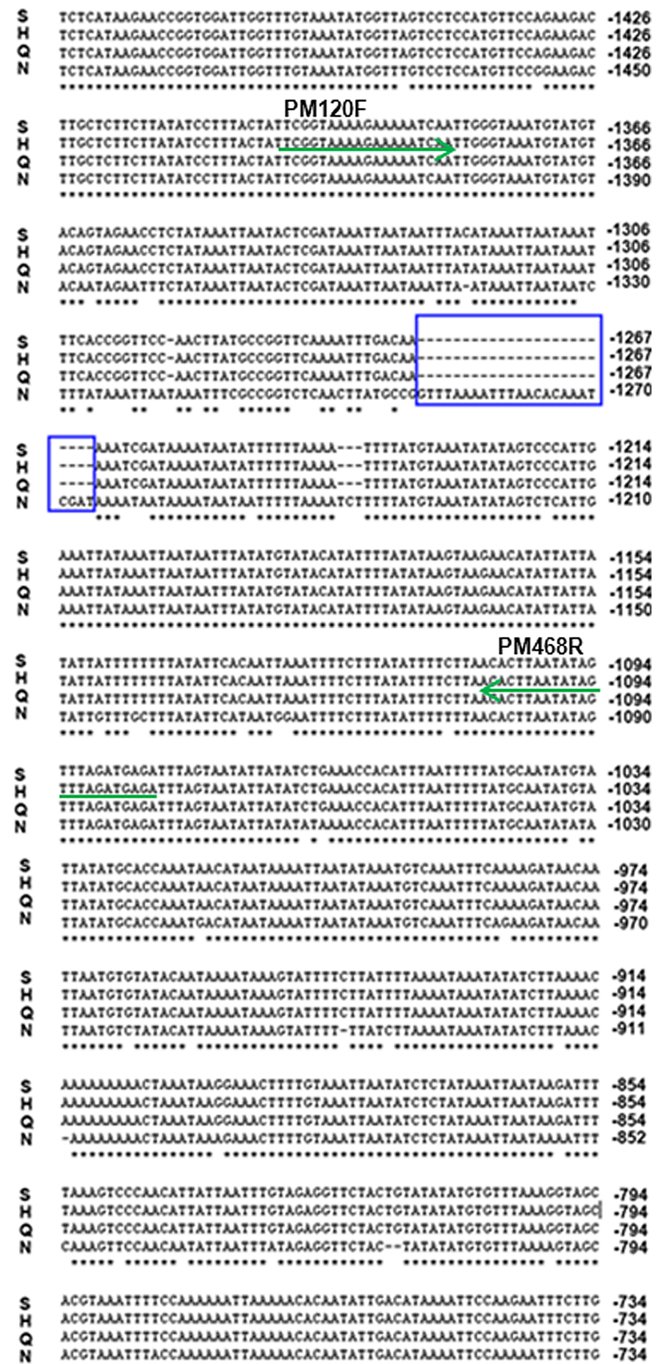

Fig. 4. The sequences alignment results of the HEA and LEA *FAE1* promoters. N: HEA *B. rapa* Nanhualinggongdacaizi (accession number: KF999632). S: LEA *B. rapa* Sanjiecaizi (accession number: KF999615). H: LEA *B. rapa* HJa 96368 (accession number: KF999623). Q: LEA *B. rapa* Qingyou 11 (accession number: KP718763). ▭ The A/T-rich sequence deleted from LEA *FAE1* promoters. The promoter regions do not contain CpG islands. The sequence data presented here have been submitted to GenBank. → The positions of pM120F and pM468R.

Association between the erucic acid content and the 28 bases deletions in the promoter region

The primer pair pM120F/pM468R was specifically

designed for the region carrying the 28 bases deletions in the promoter of *FAE1* to reveal the relationship between the phenotypes and the deletions (as shown in **Fig. 4**). The amplicons could be accurately scored by size difference on 2.5% agarose gels. The primers produced a 317-bp fragment in the zero erucic acid Sanjiecaizi, whereas a 344-bp fragment was produced in the HEA Nanhualinggongdacaizi; 317-bp and 344-bp fragments were produced in the heterozygous F1 individuals (with an erucic acid content of 30.36 ± 5.02%), as predicted (**Fig. 5**a). In 118 F2 plants from the Sanjiecaizi (*e*/*e*) × Nanhualinggongdacaizi (*E*/*E*) cross, 30 plants with amplicons resembling the zero erucic acid parent Sanjiecaizi were LEA lines (0.75% ± 1.32%); 38 plants with amplicons resembling the HEA parent Nanhualinggongdacaizi had an erucic acid content of 43.64 ± 10.60%, and 50 heterozygous individuals had an erucic acid content of 35.12 ± 7.94% (**Fig. 5**a, **5**b). The segregation ratio of the three genotypes in the F2 population agreed perfectly with the expected ratio of 1 : 2 : 1 ($\chi^2 = 3.83$, P = 0.147). The result showed that all LEA plants were *e*/*e* homozygous and the erucic acid content of the *e*/*e* genotype was highly significantly different from that of the *E*/*E* and *E*/*e* genotypes ($p < 0.01$) (**Fig. 5**b). Therefore, the homozygosity with the 28 bases deletions co-segregated with the LEA phenotypes.

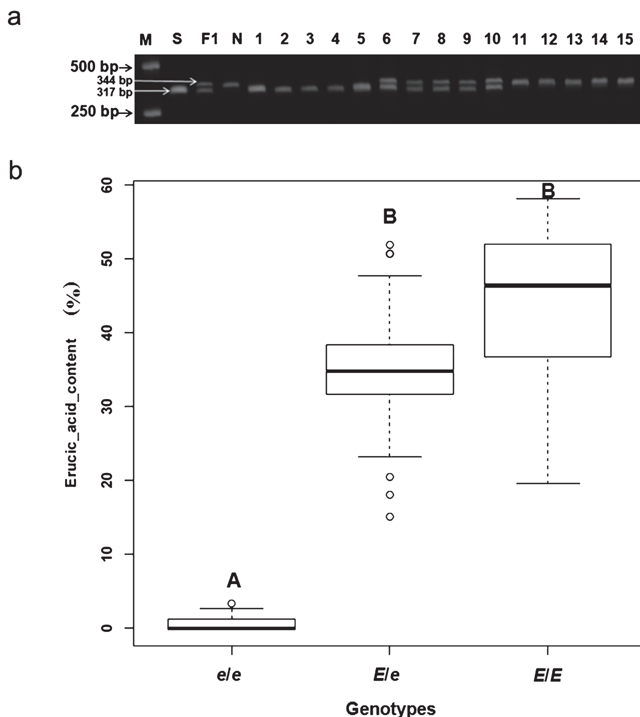

Fig. 5. The amplicons amplified by the primer pair pM120F and pM468R in the F2 population of Sanjiecaizi (*e*/*e*) × Nanhualinggongdacaizi (*E*/*E*). The amplicon contained one insertion in addition to the 28 deletions (as shown in **Fig. 4**). Therefore, the fragments amplified from the *e*/*e* genotype were 27 bases shorter than those from the *E*/*E* genotype, which was confirmed using sequencing. (a) M, DNA ladder; S, Sanjiecaizi (*e*/*e*), zero erucic line, 317 bp; F1 plants with a erucic acid content of 30.36 ± 5.02%, Sanjiecaizi × Nanhualinggongdacaizi, 344 bp and 317 bp; N, Nanhualinggongdacaizi (*E*/*E*), high erucic line, 344 bp; 1 to 5, F2 plants with a low erucic acid content (average 0.75% ± 1.32%); 6 to 10, F2 plants with a erucic acid content of 35.12 ± 7.94%; 11 to 15, F2 plants with a high erucic acid content (43.64% ± 10.60%). (b) Box plot showing the erucic acid content range and median for the three genotypes. *e*/*e*: homozygous genotype resembling the zero erucic acid parent Sanjiecaizi. *E*/*e*: heterozygous genotype containing both alleles of the two parents. *E*/*E*: homozygous genotype resembling HEA Nanhualinggongdacaizi. Note: Capital letters indicate significant differences at the 0.01 level.

Discussion

Because of the effect of erucic acid on human health, there is a maximal allowed level of erucic acid in food oil in most countries (5% in the EU). Downey (1964) bred SPAN, the first LEA *B. rapa* cultivar in the world. The first zero erucic acid *B. rapa* cultivar Torch was registered based on SPAN in 1973. In 1989, Tian bred the first LEA *B. rapa* cultivar named Qingyou 11 in China using the introduced LEA genetic resource from SPAN (Liu 1985). To identify the LEA genetic resources in the landraces, we analyzed the erucic acid contents of 1981 *B. rapa* landraces from China. There were no lines that contained less than 10% erucic acid. At least 92.07% (1824) of the *B. rapa* lines had erucic acid levels up to 40%. Another 17 *B. rapa* landraces had 10–20% erucic acid content, which indicates it may be possible to isolate LEA from these lines. However, there was no LEA resource in the collection. We deduced that highly self-sterile, cross-pollinating plants make the LEA accessions hard to maintain. Therefore, there are very few erucic acid-free winter *B. rapa* cultivars registered. By generation selection, we ultimately isolated the LEA landrace Sanjiecaizi.

The sequence alignment of the *FAE1* CDS indicates that the LEA landrace Sanjiecaizi had three variations compared with the LEA HJa 96368 and Qingyou 11 cultivars. In addition, there were 26 SNPs among the varieties, and these SNPs led to 13 amino acid changes. The 591 (G/A), 735 (C/T) and 968 (C/T) variations that had been reported to be associated with erucic acid content in *B. rapa* (Wang *et al.* 2010) appeared simultaneously in HEA and LEA *B. rapa*. The 845 (C/T) variation detected in *FAE1* of the LEA *B. napus* A genome (Wu *et al.* 2008) did not occur between HEA and LEA *B. rapa*. Thus, it may be concluded that the LEA genetic resources in the A genomes differed between *B. rapa* and *B. napus* (Wang *et al.* 2010). In addition, the other 19 variations at other positions were not correlated with the erucic acid content of *B. rapa* through statistical analysis (data not shown). Based on the analysis above, we demonstrated that these altered DNA and amino acid sequences are not responsible for LEA.

A previous study showed that *FAE1* gene(s) expression was found to be restricted to the embryo and was temporally regulated during seed development. In addition, the highest transcript levels were found at approximately 24–30 DAF, concomitant with the accumulation of 22 : 1 in rapeseed oil

(Han *et al.* 2001). Our results show that the *FAE1* gene was transcribed normally in both HEA and LEA accessions. The peak transcript levels of the LEA Sanjiecaizi, HJa 96368 and the HEA Nanhualinggongdacaizi occurred at 20, 25 and 25 DAF, respectively, which is basically identical to the results of previous reports. However, the transcription level in HEA *B. rapa* cultivar Nanhualinggongdacaizi was much higher than that detected in LEA Sanjiecaizi and HJa 96368. It is clear that the formation of LEA *B. rapa* is due to the decreased expression level of *FAE1*. In *B. napus*, though the *FAE1* expression in the LEA cultivar is much higher than in the HEA cultivar (Hu *et al.* 2009, Wu *et al.* 2008), there is no relationship between the expression and the erucic acid types. The LEA trait in *B. napus* is determined by a four-base pair deletion in the CDS of the *FAE1* gene that leads to a frameshift mutation (Wu *et al.* 2008). Similarly, in *B. juncea*, SNPs in *FAE1.1* and *FAE1.2* were identified for distinguishing the low from the high erucic types (Gupta *et al.* 2004). In *Sinapis alba*, the SNPs in the *FAE1* CDS did not affect enzyme functionality; however, the transposable element insertion of the promoter and epigenetic modification decreased the expression of *FAE1* sharply, which caused LEA formation (Zeng and Cheng 2014). Similarly, we found that LEA *B. rapa* is attributed to the expression level, not the variation of the *FAE1* CDS, and the regulation mechanism for LEA in *B. rapa* is different from that in *B. napus* and *B. juncea*.

To determine why the expression of *FAE1* decreased in LEA *B. rapa*, we analyzed the promoter region of *FAE1*. The alignment of the promoter region of the *FAE1* gene indicated that a total of 28 bases were deleted at a position approximately 1300 bp from the translation initiation site (ATG) of the LEA promoter. The deletions contained a 24-base A/T-rich region. The A/T-rich sequences are able to act as quantitative, non-tissue-specific enhancer elements in higher plants (Chen *et al.* 1988, Sandhu *et al.* 1998). A 33-bp double-stranded oligonucleotide homologous to AT-rich sequences can increase anaerobic stress-induced transcription of the maize *Adhl* promoter (Czarnecka *et al.* 1992). A previous study suggested that a 31-bp A/T-rich sequence and a 26-bp random A/T sequence were able to enhance GUS expression, and the enhancer activity was correlated with the number of copies of the A/T-rich sequence (Sandhu *et al.* 1998). In our study, the deletions in the LEA promoter also occurred at an A/T-rich region. A PCR-based marker was developed based on the deletions and was detected in the F2 population. The segregation ratio of the three genotypes agreed perfectly with the Mendelian model of a single major gene that controls the trait, which was consistent with the report of the segregation of erucic acid phenotypes of the F2 seeds (Dorrell and Downey 1964). Most importantly, the homozygosity with the 28 bases deletions co-segregated with the LEA phenotypes. Therefore, we propose that the divergent promoter may be associated with the differential transcription of *FAE1* in the HEA and LEA accessions. Our next step is to reveal the relationship between the expression and the deletions in the promoters using a transgenic approach in which the only difference between the HEA and LEA lines will be the 28 bases deletions. Other transcription factors may be simultaneously involved in the regulation of *FAE1*.

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