Breeding Science 66: 831–837 (2016)
doi:10.1270/jsbbs.16132

Research Paper

Development and validation of functional CAPS markers for the FAE genes in *Brassica juncea* and their use in marker-assisted selection

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Low erucic acid is a major breeding target to improve the edible oil quality in *Brassica juncea*. The single nucleotide polymorphism (SNP) in *fatty acid elongase 1* (*FAE1.1* and *FAE1.2*) gene was exploited to expedite the breeding program. The paralogs of *FAE1* gene were sequenced from low erucic acid genotype Pusa Mustard 30 and SNPs were identified through homologous alignment with sequence downloaded from NCBI GenBank. Two SNPs in *FAE1.1* at position 591 and 1265 and one in *FAE1.2* at 237 were found polymorphic among low and high erucic acid genotypes. These SNPs either create or change the recognition site of restriction enzymes. Transition of a single nucleotide at position 591 and 1265 in *FAE1.1*, and at position 237 in *FAE1.2*, leads to a change in the recognition site of *Hpy99I*, *BglII* and *MnlI* restriction enzymes, respectively. Two CAPS markers for *FAE1.1* and one for *FAE1.2* were developed to differentiate low and high erucic acid genotypes. The efficiency of these CAPS markers was found 100 per cent when validated in *Brassica juncea*, and *B. nigra* genotypes and used in back-cross breeding. These CAPS markers will facilitate in marker-assisted selection for improvement of oil quality in *Brassica juncea*.

Key Words: erucic acid, *FAE1*, CAPS, SNP, *Brassica juncea*.

Introduction

Improved oil quality coupled with high yield is essential in any oilseed crop. Low cost edible oil from rapeseed mustard (*Brassica spp.*) is preferred by resource poor families in Asia and Africa. Indian mustard (*Brassica juncea*), occupying more than 80 per cent of the total area of rapeseed mustard in India, is predominantly used for oil extraction and consumption in most parts of the country. Despite possessing very low amount of polyunsaturated and saturated fatty acid as compared to other edible oils, the high concentration (35–50%) of erucic acid (C22:1) is a major disadvantage to Indian mustard oil. Presence of high erucic acid in edible oil makes it nutritionally undesirable for human consumption as it causes myocardial infarction and increased blood cholesterol (Mortuza *et al.* 2006, Renard and Mcgregor 1992). On the other hand, the reduction in erucic acid content increases the oleic acid and imparts better ratio of linoleic and linolenic acids in seed oil (Jagannath *et al.* 2011). As per the international norms, erucic acid should be less than 2 per cent of the total fatty acids. It is therefore, imperative to reduce the erucic acid level to less than 2 per cent in seed oil for better human health, especially for the resource poor consumers. The first source identified for Low Erucic Acid (LEA) content in *B. juncea* was 'Zero Erucic Mustard' (Zem) (Kirk and Oram 1978). This genotype and its derivatives were used in breeding programs for development of LEA cultivars through phenotypic selection following tedious biochemical assay. Studies have now confirmed that erucic acid content is controlled by *fatty acid elongase 1* (*FAE1*) gene that encodes the enzyme β-ketoacyl-CoA synthase (KCS) in erucic acid biosynthesis pathway and catalyzes the first four enzymatic reactions in synthesis of very long chain monounsaturated fatty acids (VLCMFAs) (Gupta *et al.* 2004, James *et al.* 1995, Millar and Kunst 1997). The mutation in *FAE1* gene leads to the loss of function in enzymatic activity and reduces the accumulation of VLCMFAs in seeds (Katavic *et al.* 2002, Roscoe *et al.* 2001). In many cultivars of *B. napus*, a frame shift mutation occurred due to four base pair deletion in the *FAE1*, which leads to the premature stop of the translation after the 466th amino acid residue (Wu *et al.* 2008). Similarly, a reverse mutation from the phenylalanine to a serine residue at 282 in *FAE1* gene of LEA cultivar 'ORO' through site directed mutagenesis restored the elongase activity and erucic acid formation

Communicated by Katsunori Hatakeyama
Received August 10, 2016. Accepted October 14, 2016.
First Published Online in J-STAGE on December 7, 2016.
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(Katavic *et al.* 2002). The diploid *Brassica* species (*B. rapa* and *B. nigra*) have one copy of *FAE1* gene while the amphidiploid species (*B. napus* and *B. juncea*) have two copies with additive effect (Gupta *et al.* 2004, Lühs *et al.* 1999, Yan *et al.* 2015). Several reports dealing with the mapping of *FAE1* loci in *B. napus* and *B. rapa* are available, however, the information about the location in *B. juncea* is scanty. The *FAE1* paralogues (designated as *FAE1.1* and *FAE1.2*) are located on chromosome A8 and C3 in *B. napus* (Ecke *et al.* 1995, Qiu *et al.* 2006), whereas, in *B. juncea*, these paralogues were mapped on LG17 and LG3 (Gupta *et al.* 2004). The LG17 is corresponding to chromosome A8 but the location of *FAE1.2* is still unknown. The *FAE1* gene is 1521 bp long without introns and encodes a protein of 507 amino acids (Xu *et al.* 2010). Several SNPs were reported between LEA and high erucic acid (HEA) genotypes in *B. napus* and *B. rapa* (Wang *et al.* 2010, Wu *et al.* 2008, Yan *et al.* 2015). Gupta *et al.* (2004) found substitution-type single-nucleotide polymorphisms (SNPs) in *FAE1.1* and *FAE1.2* to distinguish low erucic from high erucic types in *B. juncea*.

Single nucleotide polymorphisms (SNPs) are the most common type of variation in DNA (Brookes 1999). SNPs which include single base substitution and small insertions/deletions, are the smallest unit of inheritance and can be used as perfect molecular markers when identified within genes underlying observed traits (Lv *et al.* 2013, Uribe *et al.* 2014). SNP variations could involve four different nucleotides at a particular site leading to only two different possible combinations (Cho *et al.* 2015, Yan *et al.* 2015). The abundance, relative stability, ubiquity and interspersed nature of SNPs make them ideal candidate molecular markers for the construction of high-density genetic maps, quantitative trait loci (QTL) fine mapping, marker-assisted breeding and genetic association studies (Rafalski 2002). In addition, SNPs located in known genes provide a fast alternative to analyze the fate of agronomically important alleles in breeding populations, thus providing functional markers (Fusari *et al.* 2008). Though, numerous SNP genotyping assays are available such as Taqman, single-base extension, allele specific primer extension and direct hybridization, but these assays require specialized detection equipments (Lee *et al.* 2004). The PCR and simple gel based markers, thus, would be more advantageous in marker-assisted breeding. Cleaved Amplified Polymorphic Sequence (CAPS) markers where the single nucleotide change disrupts or creates a restriction enzyme recognition site, allow the conversion of single nucleotide polymorphisms into a marker through PCR and restriction endonuclease (Di *et al.* 2015, Michaels and Amasino 1998). Recently, a dCAPS marker was developed and used in marker-assisted selection for low erucic acid in *B. rapa* (Karim *et al.* 2016). Since *Brassica juncea* has two paralogs of this gene, there is a need to develop corresponding CAPS for both of them. Once these markers are developed, the trait can be precisely transferred to any genetic background without the tedious and time taking biochemical assay. Therefore, this study was planned to develop functional CAPS markers based on SNPs present in the candidate gene *FAE1*, validate and evaluate the efficiency of CAPS markers in differentiating LEA and HEA genotypes and utilize them in Marker-Assisted Backcross Breeding (MABB). The outcome from this study, in form of information and material, shall help in improving efficiency of the selection process and development of improved genotypes with LEA.

Materials and Methods

Plant materials

Eighteen genotypes of *B. juncea* (AABB) maintained at Division of Genetics ICAR-IARI and three of diploid *B. nigra* (BB) collected from ICAR-NRCPB, New Delhi were taken for this study. Two back cross populations (BC_1F_1) were developed by crossing LEA varieties Pusa Mustard 24 (PM24) and Pusa Mustard 30 (PM30) with HEA varieties Pusa Vijay and Pusa Bold (**Table 2**).

Molecular analysis

DNA of each individual genotype was extracted from young expanding leaf tissue following the standard cetyltrimethylammonium bromide (CTAB) protocol (Doyle and Doyle 1990). DNA was quantified using nanodrop (NanoDrop 2000, Thermo Scientific, USA) and quality was examined by electrophoresis. The DNA was diluted to a final concentration of 20 ng/µl with HPLC grade water and stored at –20°C for PCR amplifications.

Sequencing of FAE1 paralogs genes and development of CAPS

The full-length *FAE1.1* and *FAE1.2* genes from Pusa Mustard 30, a low erucic acid genotype of *B. juncea*, were amplified using forward and reverse primers 5′ATG ACG TCC ATT AAC GTA AAG CTCC3′ and 5′ATT AGG ACC GAC CGT TTT GGA CA3′ (Gupta *et al.* 2004), cloned and sequenced. Both these sequences were aligned with the sequences of *B. juncea* genotypes present in the NCBI GenBank (KP074955, KP074953, KP074949, KP074963, AF274750, AJ558197, AJ558198, AF274750). SNPs in these amplified DNA fragments were also confirmed with those identified by Gupta *et al.* (2004). The SNPs at position 591 and 1265 in *FAE1.1* were found polymorphic between the LEA and HEA genotypes. Similarly, SNP at position 237 of *FAE1.2* for low erucic acid was found polymorphic between genotypes having high and low erucic acid content. Using these SNPs (**Table 1**), CAPS primers were designed for both the genes. Two independent CAPS markers for *FAE1.1* and one CAPS marker for *FAE1.2* were developed. For detecting CAPS markers, amplification reactions were carried out in a total volume of 15 µl containing 50 ng template DNA, 1X PCR buffer, 0.1 mM of each dNTP, 1U Taq DNA polymerase (Agilent Technologies, USA) and 10 pmol of each forward and reverse primer. Conditions of the PCR amplification were as follows: 94°C for 4 min, then 35

Table 1. Details of the primers and restriction enzymes used in CAPS marker development

Gene	Primer name	Primer sequence	Restriction enzyme	Amplicon size (bp)
FAE1.1	CAPS591	F-TCGTGGCTTGACTTCTTGAG R-GGACCTATTATCACCAGCGTAAA	*Hpy99 I*	432
	CAPS1265	F-ACGTTAGGTCCGTTGATTCTTC R-GGGTATCTGTCGATGCAATGT	*BG1II*	427
FAE1.2	CAPS237	F-TAACCATCGCTCCACTCTTTG R-TCAAGAAGTCAAGCCACGAC	*MnlI*	219

cycles each at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 5 min. The amplified PCR products were digested using suitable restriction endonuclease enzyme in a final volume of 20 µl according to the manufacturer's instructions.

Phenotyping for erucic acid content

Erucic acid content in the seeds of twenty one genotypes and individual plants of BC_1F_1 generation were analyzed by a gas liquid chromatograph (Perkin Elmer Clarus 600) using flame ionization detector (FID). The conditions maintained were column temperature: 150°C–270°C, Injector Temperature: 250°C and Detector temperature: 250°C. GLC was programmed for the temperature at the rate of 10°C per minute increase and finally it was maintained at 270°C. Peaks of the fatty acid methyl esters were identified by comparing their retention time with that of the known standards, run under similar separation conditions (Sujata *et al.* 2008).

Results

Phenotyping of B. juncea genotypes

The erucic acid content in the oil of all *B. juncea* genotypes are given in **Table 2**. Eight single zero Indian mustard genotypes viz., PM24, PM30, PDZ-1, RCL-1, RCL-2, RCL-3, EC 597325 and Heera had erucic acid less than 2 per cent, six (NRCDR-02, DRMRIJ 31, Laxmi, Pusa Vijay, Pusa Bold and RH0749) had high erucic acid content (30–45%) while two genotypes [BioYSR and LS-1 (selection from Laxmi)] had intermediate levels (20–30%) of erucic acid. The yellow seeded, white rust resistant exotic genotypes BEC144 and Donskaja revealed intermediate erucic acid content, while exotic lines EC 597325 had less than 2 per cent erucic acid. All the three *B. nigra* genotypes were found to be high in erucic acid content (**Table 2**).

Two BC_1F_1 populations, developed from the cross [(PM24 × Pusa Vijay) × PM24] and [(PM30 × Pusa Bold) × Pusa Mustard 30], comprising of 136 and 122 individuals respectively, were phenotyped for erucic acid content. The segregation pattern of the erucic acid trait in both the BC_1F_1 populations fit well in 1:2:1 ratio ($\chi^2 = 4.536$ and 0.972, respectively) (**Table 3**), indicating digenic inheritance. The heterozygotes for these genes expressed an intermediate level of erucic acid content, as compared to homozygous individuals, indicating additive gene action. As reported by Gupta *et al.* (2004), *FAE1.1* gene contributes more to the phenotype as compared to *FAE1.2*. Our results confirmed that

Table 2. Genotyping for *FAE1.1* and *FAE1.2* genes and erucic acid content in different genotypes

Species	Genotype	Erucic acid content (%)	Genotype *FAE1.1* (E1)	Genotype *FAE1.2* (E2)
B. juncea (Indian genotypes)	PM24	0.25	e1e1	e2 e2
	PM30	0.21	e1e1	e2 e2
	PDZ-1	0.13	e1e1	e2 e2
	RCL-1	0.25	e1e1	e2 e2
	RCL-2	0.31	e1e1	e2 e2
	RCL-3	0.24	e1e1	e2 e2
	Pusa Vijay	39.12	E1E1	E2 E2
	Pusa Bold	43.1	E1E1	E2 E2
	NRCDR-02	38.26	E1E1	E2 E2
	DRMRIJ 31	35.15	E1E1	E2 E2
	Laxmi	42.5	E1E1	E2 E2
	RH0749	39.74	E1E1	E2 E2
	LS-1	27.42	E1E1	e2 e2
	BioYSR	22.93	E1E1	E2 E2
	Heera	0.13	e1e1	e2 e2
B. juncea (Exotic genotypes)	EC 597325	0.20	e1e1	e2 e2
	Donskaja	14.7	e1e1	E2 E2
	BEC144	23.79	e1e1	E2 E2
B. nigra	NG1	24.3	–	E2E2
	NG2	32.02	–	E2E2
	NG3	30.55	–	E2E2

Table 3. Genotyping and phenotyping of BC_1F_1 populations for erucic acid trait

BC_1F_1 population of cross	Total plants	Erucic acid content (%) <2.0%	2–14%	15–31%	χ^2 value	P value
PM24 × P. Vijay	136	31	78	27	3.176	0.2043
PM 30 × P. Bold	122	29	69	24	2.508	0.2853
Genotype		$e_1e_1e_2e_2$	$E_1e_1e_2e_2/e_1e_1E_2e_2$	$E_1e_1E_2e_2$		

plants heterozygous for *FAE1.1* gene were, in general, having more erucic acid content (12.28 ± 0.45) than the plants which were heterozygous for *FAE1.2* gene (7.67 ± 0.8%).

SNP detection

The PCR amplification of *FAE1* gene produced a single amplicon in both low and high erucic acid genotypes. However, two different sequences were identified on sequencing the clones of PM30, confirming earlier reported SNPs (Gupta *et al.* 2004). The *FAE1.1* and *FAE1.2* gene sequences of PM30 were aligned with the sequence of erucic acid genes from *B. juncea* genotypes downloaded from NCBI GenBank ((KP074955, KP074953, KP074949, KP074963, AF274750, AJ558197, AJ558198, AF274750). Several SNPs were found and majority of them were same as reported by

Gupta *et al.* (2004). Allele-specific primers for these SNPs, as suggested by Liu *et al.* (2012), and promoter based marker developed by Yan *et al.* (2015) in *B. rapa* failed to distinguish the low and high erucic acid containing genotypes.

Development of CAPS markers

The identified SNPs were converted into CAPS markers for their deployment in breeding programs. Out of seven earlier reported SNPs, base substitution from A to G at position 591 in *FAE1.1* gene was found to be polymorphic between LEA and HEA genotypes. This transition creates the restriction site recognized by enzyme *Hpy99I* in HEA genotypes. However, there are two other restriction sites for *Hpy99I* enzyme in *FAE1.1* gene. The primers were designed meticulously to have only one restriction site for *Hpy99I* enzyme in the amplicon. Similarly, at position 1265 in *FAE1.1* gene a transition from C to T leads to change the cleavage site of *BglII* restriction enzyme.

Out of the three earlier reported SNPs in *FAE1.2* gene, the one at position 237, leads to a change in the recognition site of restriction enzyme *MnlI* in LEA genotypes. This restriction enzyme *MnlI* has seven restriction sites in *FAE1.2*. Every precaution was taken in designing the primers that have only one restriction site in the amplicon. The 219 bp amplicon of *FAE1.1* and *FAE1.2* gene was digested with the enzyme *MnlI.* In HEA genotypes, it produced two fragments of size 87 bp and 112 bp, whereas, in LEA genotypes, *FAE1.2* remains undigested and generated three bands (**Fig. 1**).

Validation of CAPS markers

The CAPS markers, developed in this study, were validated in the 21 diverse genotypes possessing variable erucic acid content in their oil (**Table 1**). All genotypes generated fragments of expected size for each CAPS maker. The CAPS markers for *FAE1.1* gene amplified 432 bp and 427 bp fragments for SNP591 (CAPS591) and SNP1265 (CAPS1265) respectively. The primers of CAPS591 and CAPS1265 amplified a single amplicon of same size in both *FAE1.1* and *FAE1.2* genes. However, on digestion with restriction enzyme, only *FAE1.1* amplicon was digested, while *FAE1.2* remains unaffected. On digestion with *Hpy99I* enzyme the amplicon of CAPS591, generated three fragments: one undigested product of *FAE1.2* (432 bp) and two fragments of size 224 and 198 bp of *FAE1.1* gene in HEA genotypes, whereas, there was no digestion in LEA genotypes (**Fig. 1A**). Similarly, on digestion with *BglII* enzyme for CAPS1265 generated three fragments in LEA genotypes: one undigested fragment of *FAE1.2* and two digested products of size 209 bp and 198 bp of *FAE1.1*, whereas, only one fragment was obtained in HEA genotypes (**Fig. 1A**, **B**).

In case of *FAE1.2* gene, the SNP237 converted into CAPS237 marker, amplified single amplicon of 219 bp. On digestion with restriction enzyme *MnlI*, both the alleles of *FAE1.1* and *FAE1.2* were digested in HEA genotypes and

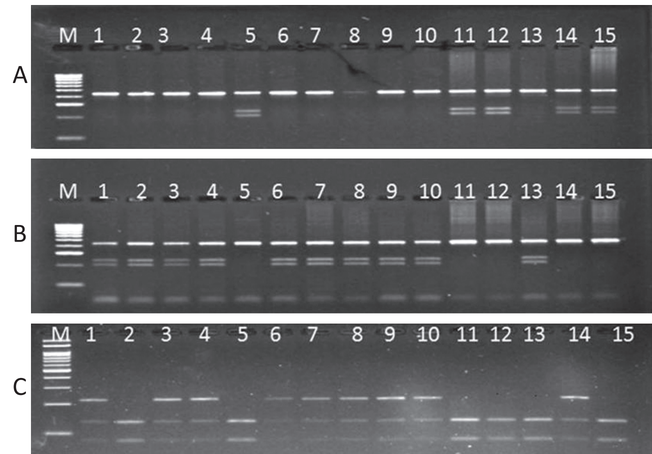

Fig. 1. Detection of SNP591 (A) and SNP 1265 (B) of *FAE1.1* and SNP237 (C) of *FAE1.2* using CAPS markers in genotypes PM24 (1), BEC144 (2), Heera (3), PM30 (4), BioYSR (5), EC 597325 (6), PDZ-1 (7), RCL-1 (8), RCL-2 (9), RCL-3 (10), NRCDR-02 (11), DRMRIJ 31 (12), Donskaja (13), LS-1 (14) and RH0749 (15).

produced 87 and 112 bp fragments, whereas, in LEA genotypes, the *FAE1.1* allele was digested and *FAE1.2* remained undigested. By using CAPS591 and CAPS1265 it is easy to identify homozygous and heterozygous individuals for *FAE1.1* gene in breeding population, whereas CAPS237 behave as a dominant marker for *FAE1.2* gene (**Fig. 1C**).

B. nigra genotypes were also screened with these three CAPS markers. These markers generated the same amplicon sizes as were observed in *B. juncea* genotypes. To detect the SNPs using restriction enzymes, the *Hpy99I* and *BglII* enzymes failed to digest, whereas, *MnlI* completely digested the amplicon. This showed that the *B. nigra* genotypes have only *FAE1.2* gene.

High erucic acid Indian mustard genotypes were having dominant alleles while genotypes having less than 2 per cent erucic acid (LEA) were recessive at both the loci. LS-1 was found to have dominant allele at *FAE1.1* locus and recessive at *FAE1.2* locus possessed intermediate erucic acid content. Exception was a genotype BioYSR, having an intermediate erucic acid content (22.93%), but dominant alleles at both the loci. The European genotypes, on the other hand, were having erucic acid content as per their haplotype.

Use of CAPS markers in MAS

A limited back cross breeding program was initiated to improve the traits of agronomic importance in LEA varieties (PM 24 and PM 30) using popular cultivars Pusa Vijay and Pusa Bold. The BC_1F_1 individuals were genotyped with CAPS markers developed for erucic acid in this study (**Fig. 2**). Around 136 BC_1F_1 individuals from the cross (PM 30 × Pusa Vijay) × PM 30 and 122 from (PM 24 × Pusa Bold) × PM 24 were phenotyped for erucic acid content in seed oil (**Table 3**). Individuals from both the back cross populations were genotyped with above mentioned CAPS

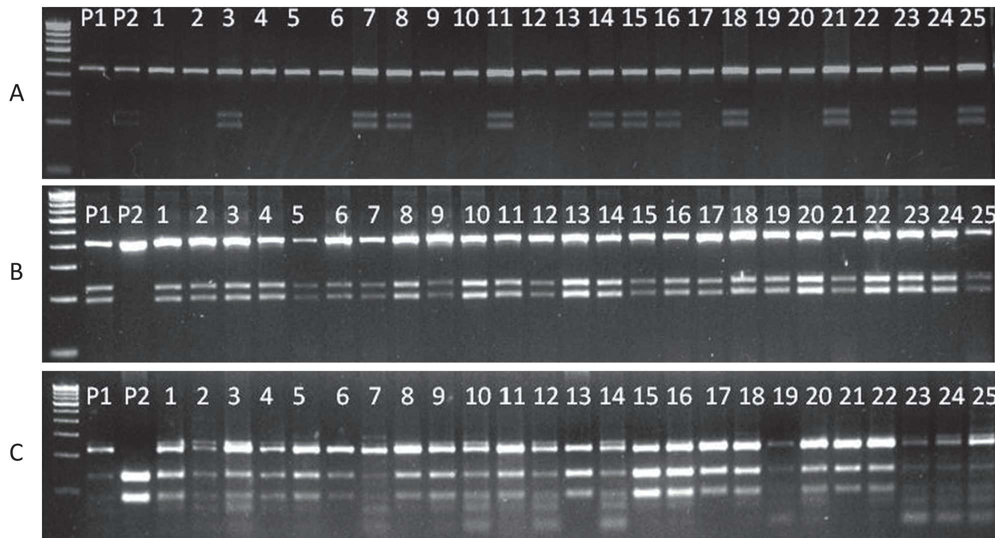

Fig. 2. Amplification patterns of newly developed CAPS markers in BC_1F_1 individuals derived from the cross between Pusa Mustard 30 (P1; low erucic acid, $e_1e_1e_2e_2$) and Pusa Bold (P2; high in erucic acid, $E_1E_1E_2E_2$). A: Plants 3, 7, 8, 11, 14, 15, 16, 18, 21, 23, and 25 have the dominant allele of *FAE1.1* (E_1) as shown by the restriction pattern with the CAPS marker CAPS591. B: The digestion pattern with CAPS1265 revealed the presence of recessive allele (e_1) in all individuals. C: The restriction pattern with the dominant marker CAPS237 for gene *FAE1.2* demonstrated that the individuals were either recessive homozygous (e_2e_2) or heterozygous (E_2e_2).

markers. The individuals having less than 2 per cent erucic acid were homozygous at both the loci while the intermediate types were identified heterozygous at either of the loci.

Discussion

The *FAE1* gene encoded β-ketoacyl-CoA synthase (KCS) enzyme catalyzes the synthesis of very long chain monounsaturated fatty acids (VLCMFA), a major constituent of seed oil (Lühs *et al.* 1999). The mutation in *FAE1* genes results in the loss of function in condensation enzymatic activity during the synthesis of VLCMFA (Katavic *et al.* 2002). It is well documented that erucic acid trait is governed by two independent genes *FAE1.1* and *FAE1.2* in allotetraploids *B. napus* and *B. juncea* (Bhatia and Alok 2014, Gupta *et al.* 2004, Yan *et al.* 2015). Native genotypes of Indian mustard are high (>35–45%) in erucic acid content having both homozygous dominant genes. The exotic LEA genotype, such as EC 597325, has homozygous recessive alleles at both the loci while Donskaja and BEC144 genotypes have recessive alleles at *FAE1.1* and dominant homozygous allele at *FAE1.2* locus. These genotypes thus, possess intermediate erucic acid content in their oil (14.7% and 23.79%, respectively). Kirk and Hurlstone (1983) also reported that the east European group has intermediate erucic content (<25%) due to presence of dominant alleles for high erucic acid level at one locus only. In contrast, the genotype BioYSR had intermediate levels (22.93%) of erucic acid content in seed oil despite having dominant homozygous alleles at both *FAE1* loci. The *B. nigra* genotypes were found to have intermediate to high erucic acid content despite having only one homozygous dominant gene *FAE1.2* (**Table 2**). The variation in erucic acid content among the genotypes indicated the role of other genomic factors in biosynthesis of erucic acid along with dominant allele at either of the loci. Cao *et al.* (2010) detected a QTL in *B. napus* near to *FAE1.1* gene with small effect on erucic acid content. In present study, the segregation pattern confirms digenic and additive inheritance of erucic acid trait in the both BC_1F_1 populations. Several earlier studies have also reported the continuous variation in erucic acid in segregating populations of *B. juncea* (Pandey *et al.* 2013, Singh *et al.* 2015) and *B. napus* (Cao *et al.* 2010, Lühs *et al.* 1999). This phenotypic variation confirms the role of modifiers in biosynthesis of erucic acid. Similar results were found in cereals where the *opaque2* mutant in maize requires modifiers to increase the tryptophan and lysine content beyond a certain limit (Holding *et al.* 2011).

The *FAE1.1* is located on chromosome A8 while the position of *FAE1.2* is still unknown in *B. juncea*. Pradhan *et al.* (2003) and Bhatia and Alok (2014) mapped both these genes on linkage group LG17 and LG3 by using AFLP markers. In *B. napus*, *FAE1* genes are located on chromosome A8 and C3. However, no microsatellite marker has been reported to show linkage with either of the *FAE1* genes in *B. juncea*, which can be used in marker-assisted selection. Yan *et al.* (2015) observed 24 base AT-rich deletion region in the *FAE1* promoter of low erucic acid cultivars of *B. rapa* (A8) and developed a STS marker (pM120F/pM468R). This marker in our study amplified only in exotic *B. juncea* genotypes (data not shown).

The sequence of *FAE1.1* and *FAE1.2* are highly similar and only 32 substitution-type single-nucleotide polymorphisms (SNPs) were observed between these two genes in

B. juncea cultivar Varuna (AJ558197.1 and AJ558198.1), whereas, in *B. napus*, 18 SNPs are found between *FAE1.1* and *FAE1.2* (Wang *et al.* 2010). Gupta *et al.* (2004) detected four SNPs in *FAE1.1* and three in *FAE1.2* alleles. Out of six SNPs found in *B. rapa* (Wang *et al.* 2010), four were also present in *B. juncea* cultivars. Among the three SNPs present in *FAE1.2*, the either of the nucleotide was same in the *FAE1.1*. Only one SNP, present at position 591, could differentiate the *FAE1.1* and *FAE1.2* genes. Several SNP genotyping methods e.g. pyrosequencing (Ronaghi *et al.* 1998), TaqMan assay (Lee *et al.* 1993), targeting induced local lesions in genomes (McCallum *et al.* 2000) have been successfully utilized. All these methods are demanding high skills and specific instruments. Even though, the high throughput genotyping techniques like TaqMan have been used to discriminate the alleles on SNPs in many crops (Shi *et al.* 2015), this marker system will not be able to differentiate the homozygous recessive from heterozygous allele for *FAE1.2*, because either of the SNP of *FAE1.2* is present in *FAE1.1*. On the other hand, the allele specific-PCR (AP-PCR) SNP detection system has been recommended on low cost grounds, even though the allele-specificity is about 30 per cent to highest 81 per cent (Liu *et al.* 2012). On PCR amplification with AP-PCR primer, allele specific PCR fails to differentiate the LEA and HEA cultivars on the basis of these SNPs since amplicon was always observed due to presence of two paralogs of this gene.

In a marker-based breeding program, a simple gel based technique is required to save time and improve the efficiency of selection. The CAPS marker system can potentially utilize single nucleotide polymorphism for development of a PCR-based marker. Four SNPs in *FAE1.1* and three in *FAE1.2* were identified to distinguish low erucic from high erucic types in *B. juncea* (Gupta *et al.* 2004). The SNP at position 591 was found highly conserved in *FAE1.1* among *B. juncea*, *B. napus* and *B. rapa* genotypes differing in erucic acid content. Moreover, at this position there is single nucleotide difference between *FAE1.1* and *FAE1.2* genes in *B. juncea* and *B. napus* (Gupta *et al.* 2004, Wang *et al.* 2010). This single substitution of nucleotide from G to A in low erucic acid genotypes, leads to a change in the cleavage site of restriction enzyme *Hpy99I*. The primers were designed in such a way that only one recognition site was present in the amplicon. Similarly, the SNP arising from change of T to C at locus 1265 generates the recognition site for *BglII* in LEA genotypes. The amplicons generated by CAPS591 and CAPS1265 digested in LEA and HEA genotypes with respective restriction enzymes concluded that all genotypes are homozygous at position 591 and 1265 corresponding *FAE1.1* gene. Among the three SNPs reported in *FAE1.2* gene, one positioned at 237 was present in recognition site of enzyme *MnlI*. The substitution of nucleotide A to C, in LEA genotypes, alters the recognition site of *MnlI* enzyme. Although, this SNP was not observed in *B. rapa* and *B. oleracea* (Wang *et al.* 2010), it proves that the *FAE1.2* is B genome specific. On restriction with *MnlI* enzyme complete digestion of amplicon from *FAE1.1* and *FAE1.2* was observed in wild type, whereas, in heterozygous or recessive homozygous condition at *FAE1.2* locus there would be incomplete digestion. Therefore, CAPS237 could not differentiate the heterozygous and recessive homozygous state at *FAE1.2* locus. The marker helps track the recessive allele in heterozygous and recessive homozygous states in the backcross progenies. Once the nuclear background of the recurrent parent is determined, marker-facilitated progeny evaluation, in BC_1F_2 or successive generations, will help in identification of non-segregating progenies carrying homozygous recessive alleles.

It is evident that the functional CAPS markers developed in this study for *FAE1* loci are highly efficient. The use of these three allele-specific functional markers (CAPS237, CAPS591 and CAPS1265) will thus, reduce time, cost and will improve accuracy in the marker-assisted selection for development of LEA *Brassica* genotypes.

Acknowledgement

The authors thank the Dept. of Biotechnology and Indian council of Agricultural Research, Govt. of India for the financial assistance to carry out this research.

Literature Cited

Bhatia, V. and A. Alok (2014) Molecular marker analysis of the linkage drag around the FAE1 loci of *Brassica juncea* during conventional backcross breeding. J. Crop Sci. Biotechnol. 17: 147–154.

Brookes, A.J. (1999) The essence of SNPs. Gene 234: 177–186.

Cao, Z., F. Tian, N. Wang, C. Jiang, B. Lin, W. Xia, J. Shi, Y. Long, C. Zhang and J. Meng (2010) Analysis of QTLs for erucic acid and oil content in seeds on A8 chromosome and the linkage drag between the alleles for the two traits in *Brassica napus*. J. Genet. Genomics 37: 231–240.

Cho, Y.I., Y.K. Ahn, S. Tripathi, J.H. Kim, H.E. Lee and D.S. Kim (2015) Comparative analysis of disease-linked single nucleotide polymorphic markers from *Brassica rapa* for their applicability to *Brassica oleracea*. PLoS ONE 10: e0120163.

Di, H., X. Liu, Q. Wang, J. Weng, L. Zhang, X. Li and Z. Wang (2015) Development of SNP-based dCAPS markers linked to major head smut resistance quantitative trait locus qHS2.09 in maize. Euphytica 202: 69–79.

Doyle, J.J. and J.L. Doyle (1990) Isolation of plant DNA from fresh tissue. Focus 12: 13–15.

Ecke, W., M. Uzunova and K. Weißleder (1995) Mapping the genome of rapeseed (*Brassica napus* L.). II. Localization of genes controlling erucic acid synthesis and seed oil content. Theor. Appl. Genet. 91: 972–977.

Fusari, C.M., V.V. Lia, H.E. Hopp, R.A. Heinz and N.B. Paniego (2008) Identification of single nucleotide polymorphisms and analysis of linkage disequilibrium in sunflower elite inbred lines using the candidate gene approach. BMC Plant Biol. 8: 7.

Gupta, V., A. Mukhopadhyay, N. Arumugam, Y.S. Sodhi, D. Pental and A.K. Pradhan (2004) Molecular tagging of erucic acid trait in oilseed mustard (*Brassica juncea*) by QTL mapping and single nucleotide polymorphisms in *FAE1* gene. Theor. Appl. Genet. 108: 743–749.

Holding, D.R., B.G. Hunter, J.P. Klingler, S. Wu, X. Guo, B.C. Gibbon, R. Wu, J.M. Schulze, R. Jung and B.A. Larkins (2011) Characterization of opaque2 modifier QTLs and candidate genes in recombinant inbred lines derived from the K0326Y quality protein maize inbred. Theor. Appl. Genet. 122: 783–794.

Jagannath, A., Y.S. Sodhi, V. Gupta, A. Mukhopadhyay, N. Arumugam, I. Singh, S. Rohtagi, P. Burma, A.K. Pradhan and D. Pental (2011) Eliminating expression of erucic acid-encoding loci allows the identification of 'hidden' QTL contributing to oil quality fractions and oil content in *Brassica juncea* (Indian mustard). Theor. Appl. Genet. 122: 1091–1103.

James, D.W., E. Lim, J. Keller, I. Plooy, E. Ralston and H.K. Dooner (1995) Directed tagging of the *Arabidopsis fatty acid elongation 1* (*FAE1*) gene with the maize transposon activator. Plant Cell 7: 309−319.

Karim, M.M., N.N. Tonu, M.S. Hossain, T. Funaki, M.B. Meah, D.M. Hossain, M. Asad ud-doullah, E. Fukai and K. Okazaki (2016) Marker-assisted selection of low erucic acid quantity in short duration *Brassica rapa*. Euphytica 208: 535–544.

Katavic, V., E. Mietkiewska, D.L. Barton, E.M. Giblin, D.W. Reed and D.C. Taylor (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.

Kirk, J.T.O. and R.N. Oram (1978) Mustards as possible oil and protein crops for Australia. J. Aust. Inst. Agric. Sci. 44: 143–156.

Kirk, J.T.O. and C.G. Hurlstone (1983) Variation and inheritance of erucic acid content in *Brassica juncea*. Z Pflanzenzuchtg 90: 331–338.

Lee, L.G., C.R. Connell and W. Bloch (1993) Allelic discrimination by nick-translation PCR with fluorogenic probes. Nucleic Acids Res. 21: 3761–3766.

Lee, S.H., D.R. Walker, P.B. Cregan and H.R. Boerma (2004) Comparison of four flow cytometric SNP detection assays and their use in plant improvement. Theor. Appl. Genet. 110: 167–174.

Liu, J., S. Huang, M. Sun, S. Liu, Y. Liu, W. Wang, X. Zhang, H. Wang and W. Hua (2012) An improved allele-specific PCR primer design method for SNP marker analysis and its application. Plant Methods 8: 34.

Lühs, W.W., A. Voss, F. Seyis and W. Friedt (1999) Molecular genetics of erucic acid content in the genus Brassica. *In*: Proc. 10th Intern. Rapeseed Congr. 26–29.

Lv, H.H., L.M. Yang, J.G. Kang, Q.B. Wang, X.W. Wang, Z.Y. Fang, Y.M. Liu, M. Zhuang, Y.Y. Zhang, Y. Lin *et al.* (2013) Development of InDel markers linked to Fusarium wilt resistance in cabbage. Mol. Breed. 32: 961–967.

McCallum, C.M., L. Comai, E.A. Greene and S. Henikoff (2000) Targeting induced local lesions in genomes (TILLING) for plant functional genomics. Plant Physiol. 123: 439–442.

Michaels, S.D. and R.M. Amasino (1998) A robust method for detecting single nucleotide changes as polymorphic markers by PCR. Plant J. 14: 381–385.

Millar, A.A. and L. Kunst (1997) Very-long-chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. Plant J. 12: 121–131.

Mortuza, M.G., P.C. Dutta and M.L. Das (2006) Erucic acid content in some rapeseed/mustard cultivars developed in Bangladesh. J. Sci. Food Agric. 86: 135–139.

Pandey, S., M. Kabdal and M.K. Tripathi (2013) Study of inheritance of erucic acid in Indian mustard (*Brassica juncea* L. Czern & Coss). Octa Journal of Biosciences 1: 77–84.

Pradhan, A.K., V. Gupta, M. Mukhopadhyay, N. Arumugam, Y.S. Sodhi and D. Pental (2003) A high-density linkage map in *Brassica juncea* (Indian mustard) using AFLP and RFLP markers. Theor. Appl. Genet. 106: 607–614.

Qiu, D., C. Morgan, J. Shi, Y. Long, J. Liu, R.Li, X. Zhuang, Y. Wang, X. Tan, E. Dietrich *et al.* (2006) A comparative linkage map of oilseed rape and its use for QTL analysis of seed oil and erucic acid content. Theor. Appl. Genet. 114: 67–80.

Rafalski, A. (2002) Applications of single nucleotide polymorphisms in crop genetics. Curr. Opin. Plant Biol. 5: 94–100.

Renard, S. and S. Mcgregor (1992) Antithrombogenic effect of erucic acid poor rapeseed oil in the rats. Rev. Fr. Crop Cros. 23: 393–396.

Ronaghi, M., M. Uhlen and P.A. Nyren (1998) Sequencing method based on real-time pyrophosphate. Science 281: 363–365.

Roscoe, T.J., R. Lessire, J. Puyaubert, M. Renard and M. Delseny (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.

Shi, Z., N. Bachleda, A.T. Pham, K. Bilyeu, G. Shannon, H. Niguyen and Z. Li (2015) High-throughput and functional SNP detection assays for oleic and linolenic acids in soybean. Mol. Breed. 35: 176.

Singh, J., D.K. Yadava, S. Vasudev, N. Singh, V. Muthusamy and K.V. Prabhu (2015) Inheritance of low erucic acid in Indian mustard [*Brassica juncea* (L.) Czern. and Coss.]. Indian J. Genet. Plant Breed. 75: 264–266.

Sujata, V., D.K. Yadava, M. Divya, R.S. Tanwar and K.V. Prabhu (2008) A simplified method for preparation of fatty acid methyl esters of *Brassica* oil. Indian J. Genet. Plant Breed. 68: 456–458.

Uribe, P., S. Jansky and D. Halterman (2014) Two CAPS markers predict Verticillium wilt resistance in wild *Solanum* species. Mol. Breed. 33: 465–476.

Wang, N., L. Shi, F. Tian, H. Ning, X. Wu, Y. Long and J. Meng (2010) Assessment of *FAE1* polymorphisms in three *Brassica* species using EcoTILLING and their association with differences in seed erucic acid contents. BMC Plant Biol. 10: 137.

Wu, G., Y. Wu, L. Xiao, X. Li and C. Lu (2008) Zero erucic acid trait of rapeseed (*Brassica napus* L.) results from a deletion of four base pairs in the fatty acid elongase 1 gene. Theor. Appl. Genet. 116: 491–499.

Xu, A., Z. Huang, C. Ma, E. Xiao, X. Zhang, J. Tu and T. Fu (2010) *FAE1* sequence characteristics and its relationship with erucic acid content in *Brassica juncea*. Acta Agron. Sin. 36: 794–800.

Yan, G., D. Li, M. Cai, G. Gao, B. Chen, K. Xu, J. Li, F. Li, N. Wang, J. Qiao *et al.* (2015) Characterization of *FAE1* in the zero erucic acid germplasm of *Brassica rapa* L. Breed. Sci. 65: 257–264.