

Transformation of rapeseed (*Brassica napus* L.) plants with sense and antisense constructs of the fatty acid elongase gene

Alireza Zebarjadi^{1,2}, Mokhtar Jalali Javaran¹, Ghasem Karimzadeh¹, Ahmad Moeini¹, Amir Mousavi², Ali Hatf Salmanian^{*2}

¹Departemant of Plant Breeding, College of Agriculture, Tarbiat Modares University, Tehran, I.R. Iran ²Department of Plant Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, I.R. Iran

Abstract

The biosynthetic pathways of saturated and unsaturated fatty acids consist of many steps controlled by various enzymes. One of the methods for improving oil quality is to change the fatty acid profile through genetic manipulation which requires isolation and characterization of the genes and other cis-acting elements, such as the promoter, involved in fatty acid biosynthesis. β -ketoacyl-CoA synthase (KCS) that is the key enzyme in erucic acid biosynthesis. This enzyme is involved in producing eicosanoic (C20:1) and erucic acids (C22:1) from C18 fatty acids, and is encoded by the fatty acid elongase (FAE) gene. Specific primers were used to amplify the FAE gene and its promoter from genomic DNA by using PCR technique. The putative gene and its promoter were cloned in sense and antisense orientation into the plant expression vector (pBI121). The sense and antisense constructs of the FAE gene were transformed via *Agrobacterium*-mediated transformation into low erucic acid rapeseed (LEAR such as PF) and high erucic acid cultivars (HEAR such as Maplus). The transformed plants were screened on kanamycin-containing media and then analysed by PCR and Southern blotting techniques. Moreover, erucic acid content of the first generation of transgenic (T_0) plants analysed with gas chromatography, showed significant changes in fatty acid composition of transgenic rapeseed plants containing sense and antisense constructs of the FAE gene.

Keywords: *Brassica napus*; Erucic acid; β -ketoacyl-CoA synthase; Gene cloning; Fatty acid elongase gene; Transformation.

INTRODUCTION

Oilseed rape (*Brassica napus* L.) is one of the most important crop for the production of vegetable oils in

the temperate zones of the world (Downey and Robbelen, 1989). The properties of edible vegetable oils are determined to a large extent by the relative content of triacylglycerol fatty acids (Kinney *et al.*, 2002). Fatty acids also determine many of the nutritional and health properties of edible fats and oils. The *Brassica* genus is classified into two basic groups: high erucic acid rapeseed (HEAR) and low erucic acid rapeseed (LEAR). Earlier studies had shown that diets rich in erucic acid are associated with fibriotic myocardium and increased blood cholestrol level, and are therefore, undesirable for human consumption (Gopalan *et al.*, 1974). Oils having high levels of erucic acid have found widespread applications for non-edible purposes such as: manufacture of polymers, cosmetics, lubricants, plasticizers, surfactant detergents and pharmaceuticals (Princen and Rothfus, 1984; Murphy and Sonntag, 1991; Ohlrogge, 1994). Regulation during the development of the seed is necessary to implement strategies that change the erucic acid content. In many higher plants, C16:1 and C18:1 fatty acids are the major components of seed storage triacylglycerols (TAG) but in HEAR, the oil is different since erucic acid (C22:1 Δ 13) represents 45 to 60% of the total fatty acids. Erucic acid biosynthesis is catalysed by the membrane-bound oleoyl-CoA elongase complex through four successive reactions (Bernert and Sprecher, 1977; Fehling and Mukherjee, 1991; Domergue, 2000). The elongation mechanism has now been well characterized due to the isolation of the *FAE1* gene (encoded by 3-ketoacyl-CoA synthase, CE). However, organization of the acyl-CoA elongase in the membrane, the developmental regulation of very-long-chain mono-unsaturated fatty acids (VLCMFA) and triacylglycerol biosynthesis remain unclear (Barret *et al.*, 1998).

*Correspondence to: Ali Hatf Salmanian, Ph.D.
Tel: +98 21 44580365; Fax: +98 21 44580395
E-mail: salman@nrcgeb.ac.ir

The pathway, for the biosynthesis of C18 fatty acid, occurs in the plastid, thereafter the C18 fatty acids are exported out into the cytosol for further modification including the synthesis of VLCFA, such as erucic acid (Millar and Kunst, 1997). The elongation of chain lengths greater than C18 are catalyzed by an enzyme complex known as elongase complex (Harwood, 1988). The key enzyme of the elongase complex, β -ketoacyl-CoA synthase (KCS) was shown to be encoded by the *FAE1* gene and responsible for erucic acid synthesis (Lassner *et al.*, 1996). The gene encoding the enzyme, 3-ketoacyl-CoA synthase, was characterized in *Arabidopsis thaliana* (*FAE1*) and jojoba (James *et al.*, 1995; Lassner *et al.*, 1996). Two homologous sequences have been isolated from embryos of *B. napus* (*Bn-FAE1.1* and *Bn-FAE1.2*). *Bn-FAE1.1* encodes a protein of 506 amino acids and *Bn-FAE1.2*, a protein of 505 amino acids, both of which share 98.2% identity (Barret *et al.*, 1998). Modification of the fatty acid composition to make rapeseed oil more competitive in various segments of the food and industrial oil markets has been an important issue for both plant breeding and molecular genetics in recent years (Friedt and Luhs, 1998; Katavic *et al.*, 2000; Jaworski and Cahoon, 2003).

In this paper, we report the isolation and cloning, in sense and antisense orientation, of the seed specific promoter and *FAE* gene from *B. napus*. Effects of this constructs on the erucic acid content of transgenic rapeseed plants were studied.

MATERIALS AND METHODS

Plant materials: The LEAR (PF) and HEAR (Maplus) cultivars of *Brassica napus* L. were used in this study. These cultivars were grown under similar condition in an experimental field. Genomic DNA was extracted from young leaves by a protocol based on the Cetyl Trimethyl Ammonium Bromide (CTAB) method (Murray and Thompson, 1980).

Plasmids and bacterial strains: The BluescriptII SK+ plasmid and *Escherichia coli* DH5 α were used for cloning and sequencing. The plasmid pBI121 (Novagen) and *A. tumefaciens* LBA4404 were used for plant transformation.

Amplification and cloning of the *FAE* gene and its promoter: The *FAE* promoter and gene were amplified from genomic DNA by PCR method using appropriate primers (primers number 1 and 2 for pro-

motor and primers number 3 and 4 for *FAE1* gene) which were designed on the basis of published data (Accession no. AF275254 for *FAE1* promoter and AF274750 for *FAE1* gene). The PCR reaction for both amplifications was performed in a total 50 μ l final volume, using 2.5 mM of each dNTPs, 10 pmol of each primer, 2.5 mM Mg²⁺ and 2.5 units of *pfu* DNA polymerase. Thermocycler was programmed for one cycle at 94°C for 5 min, followed by thirty cycles at 95°C for 1 min; 63.5°C for 1 min; 72°C for 1.5 min and one cycle at 72°C for 10 min, as a final extension. This programme was used for both amplifications. The PCR products were electrophoresed on 1% (w/v) agarose gel and visualised by ethidium bromide (Eth-Br) staining and UV transilluminator. The resulted bands (promoter and gene) were purified using the PCR clean-up gel extraction protocol (MN company-Germany). The purified *FAE* gene was double digested with *Cfr*9I and *Sac*I enzymes and cloned into the pSK+ vector which was digested with the same enzymes. The promoter region was double digested with *Hind*III and *Cfr*9I enzymes and ligated into the same position in the pSK+ vector. The ligation mixture were used for transformation of *E. coli* competent cells. The presence of inserts in the transformed colonies were screened by selection on MacConkey agar medium containing 100 mg l⁻¹ ampicillin and colony PCR with specific primers. The recombinant plasmids were further analysed by sequencing (in both direction with T3 and T7 standard primers). The sequencing results were compared with other sequences deposited in the Genebank using the BLAST software (Altschul *et al.*, 1990) and ClustalW program (Thompson *et al.*, 1994).

Sense and antisense construction of *FAE1* genes: In the pBI121 binary vector, the CaMV35S promoter was replaced by the *FAE1* promoter through digestion with *Hind*III and *Cfr*9I restriction endonucleases and ligation procedure. The modified pBI121 vector was digested with *Cfr*9I and *Sac*I restriction enzymes to eliminate the β -glucuronidase (*GUS*) gene. The *FAE1* gene which was amplified with primers 2 and 3 (Table 1) was digested with the same enzymes. The sense construct was prepared via ligation of these two components. For antisense construction, the *FAE1* gene was replaced with the same gene which was amplified with primer number 5 as the forward and number 6 as the reverse primer (Table 1). Therefore this PCR fragment has *Sac*I site at the beginning and *Cfr*9I at the end of the gene. The presence and orientation of both constructs in recombinant pBI121 were analysed by PCR and restriction enzyme digestion.

Table 1. The sequence of primers used.

Primer	Sequence (5'→3')	Primer	Sequence (5'→3')
1	CCCAAGCTTTACAACGATACACAAAACCTT	nptF	GTCGCCTAAGGTCACCTATCAGCTAGC
2	GGACCCGGGTGCTCAGTGTGTGTGT	nptR	ATG TTTGAACGATCGGGGATCATG
3	CACCCCGGGATGACGTCGTTAACGT	senF	ATTTACGCTGGTGATAAATAGGTC
4	CATGAGCTCTTAGGACCGACCGTTTTG	senR	CGCGATAATTTATCTAGTTTTGC
5	CACGAGCTCATGACGTCGTTAACGTA	antF	CTACAATGCGTTGGTGGGAAG
6	CATCCCGGGTTAGGACCGACCGTTTTG		

Plant tissue culture, transformation and regeneration: Seeds of *B. napus* were surface sterilized with 1.5% (v/v) sodium hypochlorite and 0.01% (v/v) Triton X-100 by vigorous shaking for 10 min. The seeds were washed 3 times in sterile distilled water and were germinated aseptically on MS medium (Murashige and Skoog, 1962) in glass bottles (15-20 seeds per bottle) at 25°C in a 16 h light/8 h dark photoperiod. Plant transformation and regeneration were performed by procedure which described by Moloney and colleagues (1989). In brief, the 5 days old cotyledons were excised in such a way that they included approximately near 2 mm of petiole at the base. Care was taken to eliminate the apical meristem which sometimes adheres to the petioles. The excised cotyledons were placed on MS medium containing 3% (w/v) sucrose and 0.7% (w/v) agar enriched with 4.5 mg l⁻¹ benzyladenine (BAP) as a cytokinin. Single colonies of the *A. tumefaciens* strain LBA4404 containing the modified binary plasmid pBI121 (sense and antisense constructs) were grown overnight at 28°C in LB medium supplemented with 50 mg l⁻¹ kanamycin. Explants were then inoculated with *A. tumefaciens* for 20-30 seconds and the cultivation was continued on the same medium which solidified with agar (8 g l⁻¹) at 25°C in the dark. After 2 days of co-cultivation, explants were transferred to the same medium containing 15 mg l⁻¹ kanamycin (for selection of transgenic plant cells) and 200 mg l⁻¹ cefotaxime (for elimination of *Agrobacteria*). Subculturing was carried out at 10 days intervals. Transgenic plants were selected on the basis of kanamycin resistance, mature plants were regenerated and cultured in perlite, and were then transferred to soil and grown to maturity.

Analysis of transgenic plants: PCR and digestion analysis of integrated constructs- Total DNA was extracted from leaves of putative transformants according to the Murray and Thompson (1980) protocol. Total DNA from transformed and control plants were analysed for the presence of the sense, antisense constructs and the *nptII* gene by PCR. The primer pairs used for DNA amplification were: nptF and nptR

primers for the *nptII* gene, senF and senR primers for the sense construct and antF and antR primers for the antisense construct (Table 1). For further confirmation, each of two PCR products were digested at the *SacI* site.

Southern blot analysis- Southern hybridization was conducted according to Sambrook and Russell (2001) procedure with some modification. For each plant, 15 µg of genomic DNA was digested with restriction endonuclease *EcoRV* and separated by electrophoresis on a 0.8 % (w/v) agarose gel. The DNA fragments in the gel were then transferred to a nylon membrane. Prehybridization and hybridization were performed using a standard method essentially as described by the protocol. Two probes were prepared by the PCR DIG Probe Synthesis Kit (Roche, Germany). The first probe was obtained from recombinant pBI121 vector with the primers (Table 1) senF and senR for detection of sense construct and another was prepared from the same template with the primers antF and antR for detection of the antisense construct. After hybridization with DIG labeled probes (65°C, overnight), the membranes were washed twice with washing buffer (maleic acid 0.1 M, NaCl 0.15 M, 3%(v/v) tween 20, pH 7.5) and detected by the AP- conjugated anti-digoxigenin antibody and NBT/BCIP color substrate solution by the procedure described by the manufacturer.

Fatty acid analysis- Total fatty acids from the putative transgenic and control plants were converted into methyl esters by incubation at 70°C for 30 min in the presence of 1 ml of absolute methanol containing 10 % (w/v) of boron trifluoride. After addition of 1.5 ml of 2.5% (v/v) aqueous NaCl, the methyl esters were extracted with hexane and analysed by GC.

RESULTS

PCR amplification of genomic DNA from the HEAR variety of *B. napus* with specific primers (1,2 and 3,4

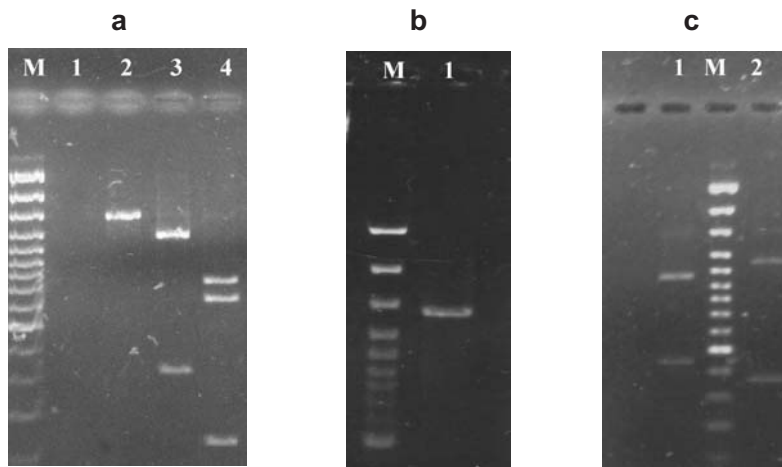


Figure 1. Amplification of the *FAE* gene, its promoter and confirmations of the amplification products by restriction enzyme digestion. **a)** Lane 1: Negative control. Lane 2: Amplification of the *FAE* gene with specific primers (1521 bp). Lane 3: Restriction enzyme analysis of the *FAE* gene by *EcoRV* digestion (331 and 1190 bp). Lane 4: Restriction enzyme analysis of the *FAE* gene by *HindIII* digestion (106, 642 and 773 bp). **b)** Lane 1: Amplification of the *FAE* promoter with specific primers (1432 bp). **c)** Lane 1: Restriction enzyme analysis of the *FAE* promoter by *EcoRI* digestion (462 and 970 bp). Lane 2: Restriction enzyme analysis of the *FAE* promoter by *XbaI* digestion (341 and 1091 bp). Lane M: 100 bp ladder.

in Table 1) generated two PCR products; these two fragments, The *FAE* gene and its promoter, have sizes of approximately 1500 bp and 1400 bp respectively. The *FAE* gene and its promoter were isolated and further analysed by restriction enzyme digestion (Fig. 1a,b,c), and cloned into the pSK+ plasmid separately. The clones were confirmed by PCR, restriction enzyme analysis and sequencing. The authentic PCR fragments were subcloned into a plant binary vector (pBI121) and the resulting clones and orientation of constructs were confirmed by PCR and restriction enzyme digestion (Fig. 2a,b). These constructs were transferred to *A.tumefaciens* LBA4404 by the freeze-

thaw standard method (Höfgen and Willmitzer, 1988). The *Agrobacterium* strains were then used to transform *B. napus* using an *Agrobacterium*-mediated petiole cotyledonary transformation. The best morphogenic response was shown by 5 days old explants. According to our results the optimum medium for shoot regeneration was the MS medium contain 4.5 mg^l⁻¹ of BAP, leading to 85% shoot regeneration after 20 days of culture.

Explants from the two varieties of *B. napus* (PF and Maplus) were co-cultivated with the *Agrobacterium* strain carrying the recombinant binary vector. Transformed shoots were first transferred to shoot elongation medium (MS medium without any hormones) (Fig. 3a,b) and then in MS medium containing with 2 mg^l⁻¹ of indol-butyric acid (IBA) and

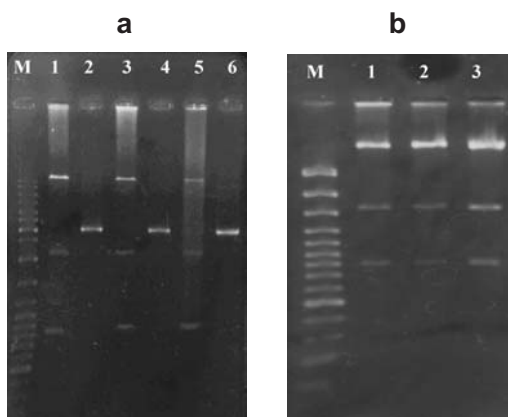


Figure 2. Confirmation of sense and antisense constructs cloned into pBI121 via restriction enzyme analysis. **a)** Lane M: 100 bp plus ladder. Lanes 1, 3 and 5: digestion of the sense construct with *HindIII* (810, 2100 and 12100 bp). Lanes 2, 4 and 6: amplification of the sense construct by specific primers (3000 bp). **b)** Lane M: 100 bp ladder. Lane 1, 2 and 3: digestion of the antisense construct with *HindIII* enzyme (790, 1520 and 12600 bp).

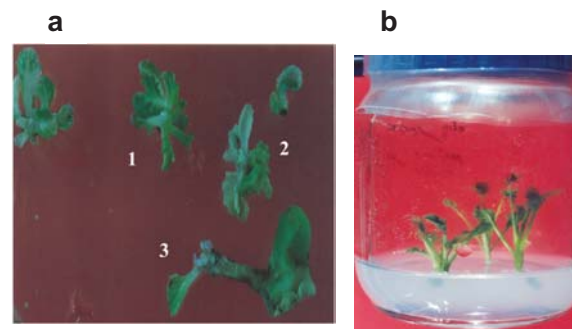


Figure 3. Development and elongation of shoots after transformation. **a)** 1: Regeneration of multiple green shoots from a single explant. 2: Regeneration of a white shoot from a single explant. 3: Unregenerated explant. **b)** Samples of regenerated shoots on shoot elongation medium.



Figure 4. PCR analysis of transgenic plants. Lane 1: negative control (normal plant). Lane 2: positive control (pBI121 plasmid). Lanes 3, 4 and 5: transformed plants (1340 bp). Lane M: 100 bp ladder.

25 mg l^{-1} kanamycin (a lethal concentration of antibiotic for nontransformed shoots). The transgenic plants had a regeneration frequency of approximately about 29% in the medium containing 25 mg l^{-1} of kanamycin. After acclimatization of rooted plantlets to *in vivo* conditions, they were allowed to flower and set seed (data not shown).

Molecular analysis of transgenic plants: Genomic DNA of putative transgenic and non-transgenic plants were analysed for presence of the *nptII* gene by PCR using the *nptF* and *nptR* primers. PCR amplification produced a fragment of 1340 bp in the transgenic plants, but no amplification was observed in the control plants (Fig. 4). PCR analysis for sense and antisense constructs with specific primers (described in materials and methods) generated amplified fragments of 996 bp and 521 bp, respectively (Fig. 5a,b). These two PCR products from the putative transgenic plants were further analysed with restriction enzyme digestion. Digestion of the 996 bp PCR product with the *SacI* enzyme produced 238 bp and 758 bp fragments, while that of the 521 bp PCR product produced 238 bp and 283 bp fragments (Fig. 6a,b).

Southern blot analysis of transformants: Four antisense and four sense (data not shown) transgenic and one non-transgenic lines were analysed by Southern blotting. Extracted genomic DNA samples were digested with *EcoRV* and were hybridized with a Dig labeled 521 bp long probe that consisted of the *FAE* gene and nopalyn synthase (*Nos*) terminator sequences (Fig. 7a). As there is an *EcoRV* site in the recombinant T-DNA construct, the number of hybridization bands indicated the number of integration copies (Fig. 7b). The results of the Southern blotting analysis showed that some of the transformed plants had only a single gene insertion.

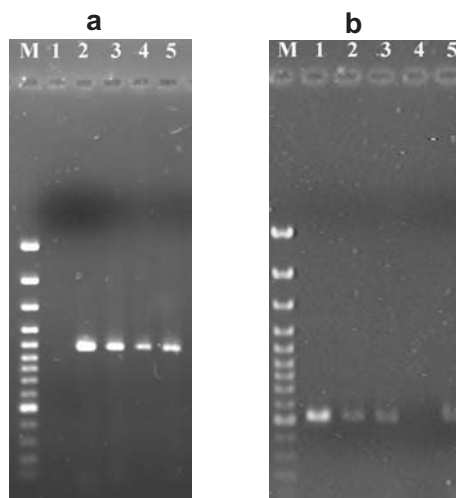


Figure 5. PCR analysis of transgenic plants with integrated sense and antisense constructs. **a)** Lane 1: negative control (normal plant). Lane 2: positive control (PCR product of the recombinant pBI121 plasmid containing the sense construct). Lanes 3, 4 and 5: transformed plants (996 bp). **b)** PCR analysis of the integrated antisense construct by specific primers. Lane 1: positive control (PCR product of the recombinant pBI121 plasmid containing the antisense construct). Lanes 2, 3 and 5: transformed plants (521 bp). Lane 4: negative control. Lane M: 100 bp ladder.

Fatty acid analysis: Seeds of 30 self pollinated kanamycin resistant T_0 plants carrying the *FAE* gene (15 plants contain the antisense construct and 15 plants containing the sense construct) were harvested. Bulk samples of 100 seeds per plant were analysed for fatty

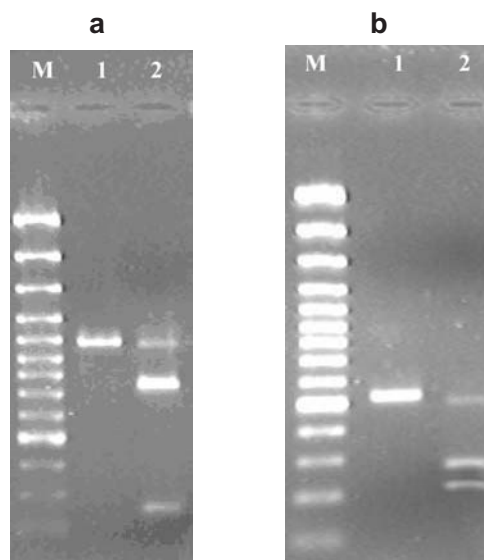


Figure 6. Digestion analysis of integrated constructs with *SacI*. **a)** Digestion analysis of the sense construct. Lane 1: PCR product of the transgenic plant using specific primers for the sense construct (996 bp). Lane 2: The sense fragment digested with *SacI* (758 and 238 bp). **b)** Digestion analysis for the antisense construct. Lane 1: PCR product of transgenic plants using specific primers for the antisense construct (521 bp). Lane 2: Digestion of the antisense fragment with *SacI* (283 and 238 bp). Lane M: 100 bp ladder.

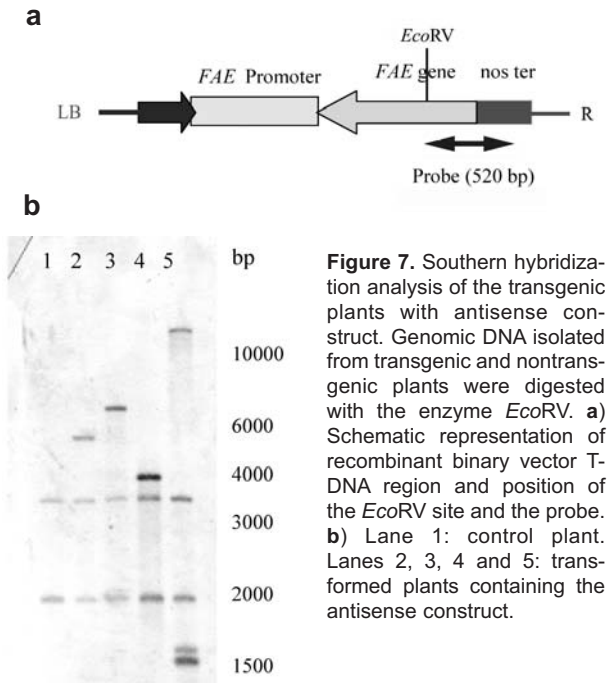


Figure 7. Southern hybridization analysis of the transgenic plants with antisense construct. Genomic DNA isolated from transgenic and nontransgenic plants were digested with the enzyme *EcoRV*. **a)** Schematic representation of recombinant binary vector T-DNA region and position of the *EcoRV* site and the probe. **b)** Lane 1: control plant. Lanes 2, 3, 4 and 5: transformed plants containing the antisense construct.

acid composition by GC (Fig. 8). Five plants bearing the antisense construct had erucic acid levels of approximately 33% (comparing to the HEAR cultivar as control contained 48% erucic acid)(Fig. 8a). Four transformed T_0 plants containing the sense construct showed an increases of over 10% in erucic acid levels (the LEAR cultivar as control contained 1.2% erucic acid) (Fig 8b), while two lines of the LEAR transgenic plants showed a decrease of approximately 0.2% in erucic acid level.

DISCUSSION

The *FAE1* gene has previously been isolated from *A. thaliana* (James *et al.*, 1995), *B. napus* (Han *et al.*, 2001), *B. campestris* (Das *et al.*, 2002) and *B. oleracea* (Barret *et al.*, 1998). The evidence that the *FAE1* gene is responsible for erucic acid synthesis was provided when a β -ketoacyl synthase (coded by *FAE1* gene) isolated from *Simmondsia chinensis* was able to comple-

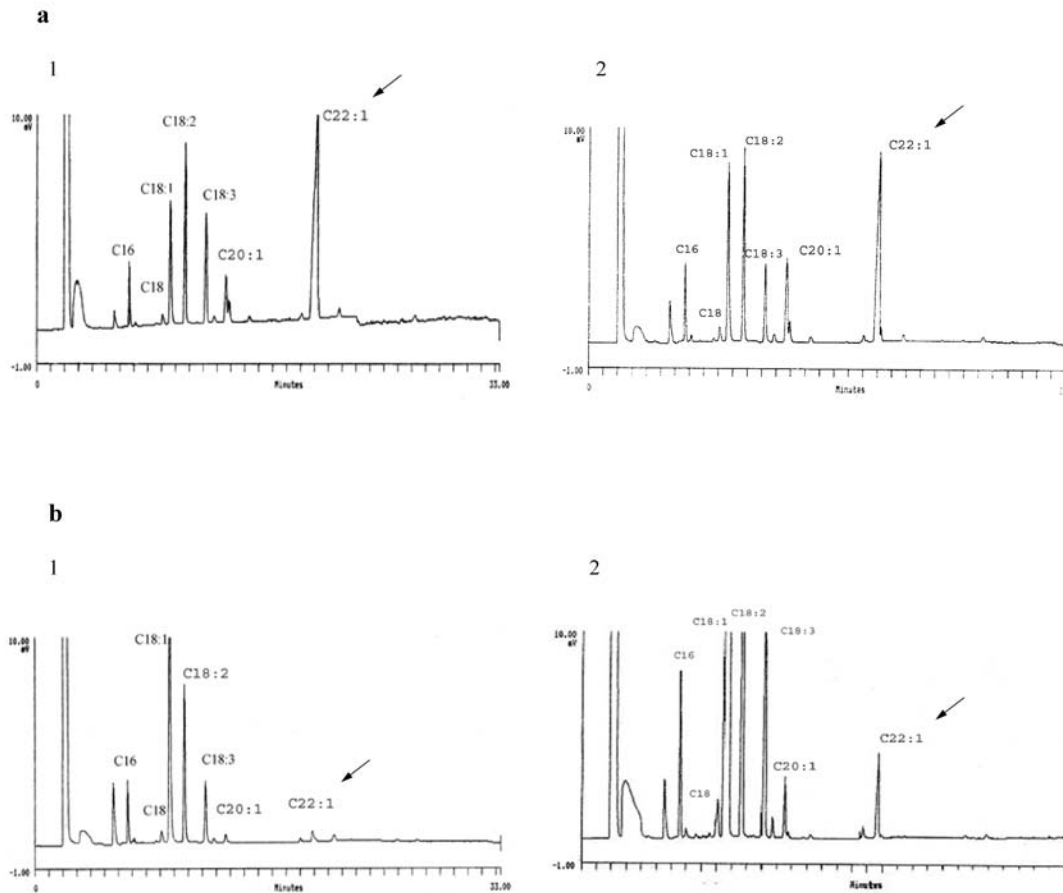


Figure 8. Gas chromatograph analyses of fatty acid contents of seeds from transgenic rapeseed plants. **a)** Fatty acid content of seeds from HEAR cultivar of *B.napus* L., before (1,with 48% erucic acid) and after (2, with 33% erucic acid) transformation with antisense *FAE* gene. **b)** Fatty acid content of seeds from LEAR cultivar of *B.napus* L, before (1, with 1.2% erucic acid) and after (2, with 10% erucic acid) transformation with sense *FAE* gene.

ment the mutation in fatty acid elongation and thus restore the erucic acid level in zero-erucic acid lines of *B. napus* (Lassner *et al.*, 1996). It has been suggested that the variation in erucic acid levels is due to the differences in the *FAE* gene sequences belonging to the low and high erucic acid genotypes (Das *et al.* 2002). Sequence analysis revealed that the *FAE* gene was a 1521 bp fragment that starts with an ATG initiation codon and after alignment has shown a significant sequence similarity to the other β -ketoacyl CoA synthase gene belonging to *B. napus* (AF274750, U 50771), *B. juncea* (Y 11007), *S. chinensis* (U 37088) and *A. thaliana* (U 29142). Analysis of the genomic DNA of *B. napus* *FAE* clones from HEAR cultivars revealed that they have continuous coding regions which are devoid of any interruption by introns. Similar results were also reported earlier (James *et al.*, 1995; Barret *et al.*, 1998; Fourmann *et al.*, 1998; Venkateswari *et al.*, 1999; Han *et al.*, 2001; Das *et al.*, 2002). The promoter sequence contains several A/T-rich elements also present in other seed-specific promoters which have been shown to enhance gene expression (Thomas, 1993; Sandhu *et al.*, 1998). In addition, the analysis of transgenic plants containing the *GUS* gene under the control of the seed-specific promoter from the *B. napus* Askari cultivar revealed that *GUS* activity was only detected in developing seeds of transgenic rapeseed plants, however there was no color development in leaves or other organs such as roots, stems or buds (Han *et al.*, 2001). It is clear that, reduction in erucic acid content of the seeds of oil producing plants has positive nutritional effects, but this reduction can also change fatty acid composition of cell membrane or has other diverse effects on plant growth and development. Therefore, it is necessary and very important to control the erucic acid level only in the seeds in transgenic canola plant.

Finding appropriate plant cells with high capacity for accepting the foreign gene is an important criteria in plant transformation by *Agrobacterium*. In *B. napus* L., different parts of the plant have been used for *A. tumefaciens* transformation but in these experiments the cut surfaces of cotyledonary petioles were used as the target cells. Results showed that this target is a vigorous source of new shoot material and that shoot development occurs very rapidly. The origin of these shoots has been shown by Sharma (1987) to be cells located in the vicinity of the cut end of the petioles. The value of *Agrobacterium*-mediated plant transformation is measured by the number of independent transformed plants expressing the gene of interest, per explant used. This can be the effect of the genotype of the species to be transformed, the virulence of

Agrobacterium used for transformation, the antibiotic used as selectable marker, regeneration capacity of the target cells and the accessibility of the bacterium to the regenerable cells. By this method both lines of *B. napus* (PF and Maplus) were successfully transformed and regenerated. In our hands, this transformation and regeneration method can be act genotype-independently. In the control experiments using non-selective regeneration medium, high shoot formation was observed in all explants. In the medium containing kanamycin (25 mg l⁻¹), regeneration of control explants were almost completely inhibited. In transformation experiments, shoot regeneration on non-selective medium were reduced, presumably due to inoculation with *Agrobacterium*. However on selective medium, many white, non-transformed escape shoots were formed in addition to the green putative transgenic shoots. Putative transformants that rooted on kanamycin-containing medium were confirmed as transgenic by PCR analysis and Southern blotting. Most of the transgenic plants carried one copy of *FAE* gene and only a few carried two or three copies. Two bands (about 2000 and 3500 bp) were observed on all lanes. Presence of these two bands were due to high sequence similarity (approximately 63%) between the probe and endogenous *FAE* gene.

Furthermore, the Southern blotting analyses on transgenic rapeseed plant showed that the level of gene expression is correlate with the number and site of integrations of the expression cassette and this is in agreement with some previously reported results (Kahrizi *et al.*, 2007 and Wang *et al.*, 2003). Similar multiple inserts in the case of Brassicaceae may be an intrinsic feature and it may be dependent on the selection schemes and the levels or types of antibiotics used (Moloney *et al.*, 1989). Our results from GC analysis have shown that introduction of the antisense construct appears to have partially silenced the activity of endogenous *FAE* gene in *B. napus*. Therefore, the use of antisense technology enabled the reduction of the erucic acid level in HEAR cultivars by approximately 15%. In a LEAR variety, the overexpression of the *FAE* gene led to an increase in erucic acid level by approximately 8%. Comparison of these results have shown that in this experiment the ability to suppress the gene activity via the antisense construct of the *FAE* gene was stronger than gene silencing mechanisms by its overexpression. The change in erucic acid level in the seeds of transgenic conola, make changes in the level of other fatty acids as well. In transgenic rapeseed (HEAR cultivar, Maplus) with antisense construct of the *FAE* gene, decrease in the level of erucic acid (C22:1) cause accumulation of upstream fatty acids

such as oleic (C18:1) and linoleic (C18:2) (Fig. 8a). Fatty acid composition in transgenic rapeseed plant (LEAR cultivar, PF) with sense construct of *FAE* gene is different. The GC chromatogram (Fig. 8b) shows that, increase in erucic acid level can cause changes in the level of other fatty acids (C18:1, C18:2, C18:3 and C20:1) as well. These finding can also show the more general effects of *FAE* gene in erucic acid synthesis and other fatty acid composition in seeds of oil producing plants. Moreover, the transgenic rapeseed plants (LEAR and HEAR) could provide new insight into the complex mechanisms of oil accumulation. Our data show that the sense and antisense technology can be effectively used to modify the biosynthetic profile of fatty acids, specially that of erucic acid, through targeting the *FAE* gene into seed of oily plants.

Acknowledgments

This study was supported by the National Institute of Genetic Engineering and Biotechnology (NIGEB) and Tarbiat Modares University. We would like to thank the LEMBKE company, Germany, for kindly providing us with the Maplus cultivar of *B. napus* seeds. Our special thanks to Dr. P. Shariati for critical reading of this manuscript.

References

- Altschul SF, Gish W, Miller W, Myers EW (1990). Basic local alignment search tool. *J Mol Biol.* 215: 403-410.
- Barret P, Delourme R, Rendar M, Domergue F, Lessire L, Delseny M, Roscoe TG (1998). A rapeseed *FAD1* gene linked to the E1 locus associated with variation in the content of erucic acid. *Theor Appl Genet.* 96: 177-186.
- Bernert JT, Sprecher H (1977). An analysis of partial reactions in the overall chain elongation of saturated and unsaturated fatty acids by rat liver microsomes. *J Biol Chem.* 252: 6736-6744.
- Das S, Roscoe TJ, Delseny M, Srivastava PS, Lakshmikumaran M (2002). Cloning and molecular characterization of the Fatty Acid Elongase1 (*FAE1*) gene from high and low erucic acid lines of *Brassica campestris* and *Brassica oleracea*. *Plant Sci.* 162: 245-250.
- Domergue F, Chevalier S, Creach A, Cassagne C, Lessire R (2000). Purification of the acyl-CoA elongase complex from developing rapeseed and characterization of the 3-ketoacyl-CoA synthase and 3-hydroxyacyl-CoA dehydrate. *Lipids* 35: 487-494.
- Downey RK, Robbelen G (1989). *Brassica species In Oil Crops of the World* (G. Robbelen, R. K. Downey and A. Ashri, eds), pp. 339-362. McGraw-Hill, New York.
- Fehling E, Mukherjee KD (1991). Acyl-CoA elongase from a higher plant (*Lunaria annua*): Metabolic intermediates of very long-chain acyl-CoA products and substrate specificity. *Biochem Biophys Acta.* 1126: 88-94.
- Fourmann M, Barret P, Rendar 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.
- Friedt W, Luhs W (1998). Recent developments and perspectives of industrial rapeseed breeding. *Fett/Lipid.* 100: 219-226.
- Gopalan GD, Krisnamurthy D, Shenolikar IS, Krisnamurthy KA (1974). Myocardial changes in monkeys fed on mustard oil. *Nutr Metab.* 16: 352-365.
- Han J, Lush W, Sonntag K, Zahringer U, Borchardt SD, Wolter FP, Heinz E, Frentzen M (2001). Functional characterization of β -ketoacyl-CoA synthase genes from *Brassica napus* L. *Plant Mol Biol.* 46: 229-239.
- Harwood JL (1988). Fatty acid metabolism. *Ann Rev Plant Physiol Plant Mol Biol.* 39: 101-18.
- Höfgen R, Willmitzer L (1988). Storage of competent cells for *Agrobacterium* transformation. *Nucleic Acids Res.* 16: 9877.
- James DW Jr, Lim E, Keller J, Plooy I, Ralston E, Dooner HK (1995). Directed tagging of the *Arabidopsis* fatty acid elongation 1 (*FAE1*) gene with the maize transposon activator. *Plant Cell* 7:309-19.
- Jaworski J, Cahoon EB (2003). Industrial oils from transgenic plants. *Curr Opin Plant Biol.* 6: 178-184.
- Kahrizi D, Salமான AH, Afshari A, Moeini A, Mousavi A (2007). Simultaneous substitution of Gly96 to Ala and Ala183 to Thr in 5-enolpyruvylshikimate-3-phosphate synthase gene of *E. coli* (k12) and transformation of rapeseed (*Brassica napus* L.) in order to make tolerance to glyphosate. *Plant Cell Rep.* 26:95-104.
- Katavic V, Friesen W, Barton DL, Gossen KK, Giblin EM, Lucwic T, An J, Zou J, MacKenzie SL, Keller WA, Males D, Taylor DC (2000). Utility of the *Arabidopsis* *FAE1* and Yeast *SLC1* genes for improvements in erucic acid and oil content in rapeseed. *Biochem Soc Trans.* 28: 935-937.
- Kinney AJ, Cahoon EB, Hitz WD (2002). Manipulating desaturase activities in transgenic crop plants. *Biochem Soc Trans.* 30: 1099-1103.
- Lassner MW, Lardizabal K, Metz JG (1996). A jojoba beta-Ketoacyl-CoA synthase cDNA complements the canola fatty acid elongation mutation in transgenic plants. *Plant Cell.* 8:281-92.
- Millar AA, Kunst L (1997). Very long chain fatty acid biosynthesis is controlled through the expression and specificity of the condensing enzyme. *Plant J.* 12: 121-131.
- Moloney MM, Walker JM, Sharma KK (1989). High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. *Plant Cell Rep.* 8: 238-242.
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissues. *Physiol Plant* 15: 473-497.
- Murphy IS, Sonntag JG (1991). Erucic, behenic: feedstocks of the 21st century. *Theor Appl Genet.* 96:177-186.
- Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 8: 4321-4325.
- Ohlrogge JB (1994). Design of new plant product: engineering of fatty acid metabolism. *Plant Physiol.* 104: 821-826.
- Princen LH, Rothfus JA (1984). Development of new crops for industrial raw materials. *J Am Oil Chem Soc.* 61: 281-289.
- Sambrook J, Russell DW (2001). *Molecular Cloning. A Laboratory manual.* 3rd Edition. Cold Spring Harbor Press. New York.
- Sandhu JS, Webster CI, Gray JC (1998). A/T-rich sequences act as quantitative enhancers of gene expression in transgenic tobacco and potato plants. *Plant Mol Biol.* 37: 885-896.
- Sharma KK (1987). Control of organ differentiation from somatic tissues and pollen embryogenesis in anther culture of *B. juncea*. *Ph.D. Thesis.* Dept of Botany, University of Delhi, India.
- Thomas TL (1993). Gene expression during plant embryogenesis and germination: an overview. *Plant Cell* 5: 1401-1410.

Archive of SID

- Thompson JD, Higgins DG, Gibson TJ (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673-4680.
- Tofer R, Martini N, Schell J (1995). Modification of plant lipid synthesis. *Science* 268: 681-686.
- Venkateswari J, Kanrar S, Kirti PB, Malathi VG, Chopra VL (1999). Molecular cloning and characterization of fatty acid elongation 1 (*BjFAE1*) gene of *Brassica juncea*. *J Plant Biochem Biotech.* 8: 53-55.
- Wang HY, Li YF, Xie LX, Xu P (2003). Expression of bacterial *aroA* mutant, *aroA-M1*, encoding 5-enolpyruvylshikimate 3-phosphate synthase for the production of glyphosate-resistant tobacco plants. *J Plant Res.* 116: 455-460.