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Control of erucic acid biosynthesis in Camelina (*Camelina sativa*) by antisense technology

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ABSTRACT

Oil seeds now make up the world's second-largest food source after cereals. In recent years, the medicinal- oil plant *Camelina sativa* has attracted much attention for its high levels of unsaturated fatty acids and low levels of saturated fatty acids as well as its resistance to abiotic stresses. Improvement of oil quality is considered an important trait in this plant. Erucic acid is one of the fatty acids affecting the quality of camelina oil. Altering the fatty acid composition in camelina oil through genetic manipulation requires the identification, isolation, and cloning of genes involved in fatty acid biosynthesis. The Fatty Acid Elangase 1 (*FAE1*) gene encoded the enzyme β-ketoacyl COA synthase (KCS), which is a key factor in the biosynthesis of erucic acid. In this study, isolation and cloning of the *FAE1* from the *Camelina sativa* were performed to prepare an antisense structure. The fragments were isolated from the DNA source of the genomic Soheil cultivar with an erucic acid content of about 3% (in matured seeds) using PCR. After cloning *FAE1* into the Bluescriprt II SK$^+$ vector and sequencing, these fragments were used for the preparation of antisense structure in the pBI121 plant expression vector. The approved structure was transferred to the camelina plant via the *Agrobacterium*-mediated method. Also, the conditions of tissue culture and gene transfer were optimized. Moreover, the erucic acid content of the immature seeds of T0 transgenic plants was analyzed with gas chromatography (GC). Results showed significant changes in erucic acid levels of two control plants (0.88%), while two lines of the *RFAE1* transgenic plants showed a decrease of approximately 0% in erucic acid level. It can be concluded that the antisense structure can be effective in reducing erucic acid.

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Introduction

The annual increase of 1.5% in the global population has caused so many problems in the world including food supply (1). Camelina (*Camelina sativa* L) is native to northern Europe and South Asia and is known as false flax or Gold of Pleasure. This valuable plant is a member of the *Brassicaceae* family (2). Camelina is mainly a self-pollinated species (3) with a low percentage of cross-pollinated at about 0.01-0.28% (4). The number of camelina chromosomes 2n= 40 has been reported (5).

Possession of several valuable agronomic traits makes camelina a valuable crop. For example, camelina has a relatively short growing season (85-100 days) and contains spring and winter cultivars. In addition, its oil yield (from 400 to 850 kg /ha (per) is typically comparable to *Brassica jansa* and *Brassica rapa* and above that of soybean (6). Camelina also has the capacity to tolerate drought stress, so it is less dependent on irrigation. Due to mentioned proper properties in this plant, improving the quality of the oil and its meal is one of the corrective goals that can be pursued in camelina. The quality of camelina oil is determined by its fatty acid composition. On the other hand, its anti-nutritional factors (especially glucosinolates) and its ratio of fiber and protein also affect its quality. Quality can also be attributed to the proportion of oil and protein in the seed, especially the oil because it is valuable (7).

About 35 percent of the oil is extracted from camelina. It contains a high percentage of unsaturated fatty acids (90%), its omega 3 is about 35 percent and due to its vitamin E and high tocopherol, the oil putrefy late. The fatty acid combination is an important qualitative factor that determines the nutritional or industrial value of oil Camelina seed's oil combination (Palmitic acid, Stearic acid, Oleic acid, Linoleic acid, Linolenic acid, Eicosanoic acid, Ecosadienoic acid, Erucic acid and …) varies depending on cultivar, location, environmental conditions and extraction method.

Erucic acid is a long-chain fatty acid containing a double bond. Camelina oil typically contains 2.8% to 3.4% erucic acid. This fatty acid is found in the *Brassicaceae* family and is harmful to humans. Plant breeders are trying to breed varieties that lack erucic acid. Very long-chain fatty acids are made from oleic acid by the addition of two-carbon units and this process is controlled by the *FAE1* complex. This complex contains the following four different enzymes (8) and the *FAE1* gene plays a key role in this process (9).

1. Compression of malonyl coenzyme A with a long chain of acyl-coenzyme A to form a ß-Ketoacyl-CoA.
2. Revival of ß-Ketoacyl-CoA to ß-hydroxyacyl CoA.
3. Dehydration Converting to Inol CoA.
4. Revival Inol CoA, which forms an elongated acyl coenzyme.

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The elongation complex inserts a ß-ketoacyl group of coenzyme into the acyl chain, which is accomplished by the activity of an enzyme called ß -ketoacyl synthase (KCS), which plays a key role in the biosynthesis of erucic acid (10). Therefore, for the genetic manipulation of the amount of erucic acid present in camelina, it is necessary to identify, clone and transfer the synthesizer gene in this enzyme.

In changing the profile of camelina fatty acids to improve the quality of the oil, it is better to use modern methods such as genetic engineering to change the profile of camelina fatty acids. One of the most important strategies used to change the composition of fatty acids; is gene silencing (10):

This method is based on antisense, in which a double-stranded RNA molecule prevents the expression of a particular gene, which is necessarily homologous to the double-stranded RNA. Inhibition of gene expression is accomplished by mRNA degradation, and for this reason, this process is called a silencing mechanism after gene translation. The antisense result is a reduction in the desired gene product.

In this paper, we report tissue culture, transformation, and adaptation, in sense and antisense orientation, of the seed-specific *FAE1* gene from *C. sativa*, in order to improve the quality of the oil of this plant.

Materials and Methods

Plant materials

The *Camelina sativa* cultivar ''Soheil'' was used for all experiments. This cultivar was prepared by Biston Shafa Company, Iran. The resulting Soheil cultivar is a cross between Blaine Greek and Calena cultivars and is purified by doubled haploid method. The mentioned cultivar has about 3% erucic acid, 35% oil, 90% unsaturated fatty acids, and about 35% omega-3s in matured seed. Genomic DNA was extracted from young leaves by kit (Vivantis).

Plasmids and bacterial strains

The Bluescriprt II SK+ plasmid and *Escherichia coli*-DH5-alpha were used for cloning and sequencing. The plasmid *pBI121* (Novagen) and *A. tumefaciens* LBA4404 were used in order to produce competent cells and plant transformation.

Amplification and cloning of the *FAE1* and *RFAE1* genes

Based on the *FAE1* sequences in Genbank (Accession no.GU929420.1 for *FAE1* gene), the primer pairs were designed and synthesized for *FAE1* (sense sequence) and *RFAE1* (antisense sequence) (Table 1). The *FAE1* primers were used to amplification of *FAE1*gene full length.

The *RFAE1* primers were used for the amplification of antisense of the entire *FAE1* gene or complete antisense (*TRFAE1*). PCR analysis of transgenic plants was carried out with integrated *TRFAE1* construct**.** Detected *TRFAE1* gene was performed by PCR using the TFAE1 F and TFAE1 R primers.

Genomic DNA was isolated from leaves of *C. sativa* with a Vivantis kit (Plant DNA extraction). The *FAE1* genes were amplified from the genomic DNA using the primers mentioned above (Vivantis kit). The purified *FAE1* genes were double digested with *Cfr9I* and *SacI* enzymes and sub-cloned into the PTG19-TPCR cloning vector (Vivantis kit) which were digested with the same enzymes.

Tailed PCR products were ligated into PTG19-T Vector based on the TA-cloning scheme according to the manufacturer's instructions. A 1:3 (vector to insert) molar ratio was used. Ligation reaction set up in 30 µl volume containing 2 µl PTG19-T plasmid, 16 µl of fresh PCR product, 1 µl T4 DNA ligase enzyme, 1 µl 10X buffer and 10 µl nuclease-free distilled water. After a gentle mix and a brief centrifuge, the ligation reaction mixture was incubated overnight at 10ºC. Recombinant vectors were stored at -20°C until transformation. Preparation of competent cells from Escherichia coli strain DH5-α was performed by the calcium chloride method. Then the PCR product was cloned into the PTG19-T vector and transformed into *E. coli* (DH5α strain). The presence of inserts in the transformed colonies was screened by selection on Mac Conkey agar medium containing 100 mgL^{-1} ampicillin and colony PCR with specific primers. The recombinant plasmids were further analyzed by sequencing.

Antisense construction of *FAE1* genes

The modified pBI121 vector was digested with *Cfr*9I and *Sac*I restriction enzymes to eliminate the β-glucuronidase (GUS) gene. In the antisense constructions, the *FAE1* gene was replaced with the same genes. Therefore, this PCR fragment has *SacI* site at the beginning and *Cfr91* at the end of the gene. The presence and orientation of constructs in recombinant pBI121 were analyzed by PCR and restriction enzyme digestion.

Plant tissue culture, transformation and regeneration

Seeds of *C. sativa* were surface sterilized with 70% ethanol for 1 min and 5% (v/v) sodium hypochlorite by vigorous shaking for 10 min. The seeds were washed 3 times in sterile distilled water and germinated aseptically on 1/2 MS medium (11) in glass bottles (30-40 seeds per bottle) at 25°C in a 16 h light/8 h dark photoperiod. Plant

Table 1. The sequence of primers used for the *FAE1* gene and *TRFAE1* construct as a complete antisense of the *FAE1* gene. Where, F: Forward; R: Reverse.

Name	Primer	Sequence (5′→3′)
FAE1	F	CGC GAT AAT TTA TCC TAG TTT GC
	R	CTA CAA TGC GTT GGT GGA AG
RFAE1	F	CAC **GAG CTC**[1] ATG ACG TCC GTT AAC GTA
	R	CAT **CCC GGG**[2] TTA GGA CCG ACC GTT TTG
TFAE1	F	GTC GCC TAA GGT CAC TAT CAG CTA GC
	R	CTC TCA TCG TCT CCT TGT

[1] *SacI* recognition site. [2] *Cfr9I* recognition site.

transformation and regeneration were performed by the procedure described (12). Cotyledons segments of 5, 7, 9 and 14 days old camelina seedlings were used as explants to evaluate the effectiveness of shoot regeneration. The composition of the media only differed by the content of macro salts, types of antibiotics (Table 2), sucrose and phytohormones. In particular, we examined the effect of several concentrations of benzyl amino purine (BAP) and several alternatives of BAP and naphthalene acetic acid (NAA). The regenerative ability of *C. sativa* was tested using 9 media (Table 3).

One of the most common problems during the tissue culture process of camelina is the phenomenon of vitrification. Vitrification is a serious problem since it can affect shoot multiplication and culture vigour and can impede the successful transfer of micro-propagated plants to in vivo conditions (13). In this experiment, the ratio of ammonium to nitrate and agar concentration (Table 4) were studied for the effects of these factors on vitrification.

Care was taken to eliminate the apical meristem which sometimes adheres to the petioles. Single colonies of the *A. tumefaciens* strain LBA4404 containing the modified binary plasmid recombinant pBI121 (antisense constructs) were grown overnight at 28°C in LB medium supplemented with 10 mgL^{-1} kanamycin. Explants were then inoculated with *A. tumefaciens* for 10 min and the cultivation was continued on the same medium which solidified with agar (8 gl-1) at 25°C in the dark. After 72 hours of co-cultivation, explants were transferred to the same medium containing 10 mgL^{-1} kanamycin (for selection of transgenic plant cells) and Cefotaxime and Meropenem (for the elimination of Agrobacteria). Subculturing was carried out at 14 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.

In order to prepare an explant of cotyledon explant, germinated seeds will be used for 5 to 7 days. After removing the terminal buds, the cotyledon leaves are placed on the culture medium so that about 2 mm of the petiole is immersed in the medium (14).

Table 2. Composition types of antibiotic for the in vitro plantlet regeneration of *Camelina sativa* cultivar.

Types of antibiotic	Cefotaxime mg/L	Meropenem mg/L
1	200	50
2	400	50
3	200	-
4	400	-
5	-	100

Fatty acid analysis

In order to measure the percentage of fatty acids in camelina seed oil, gas chromatography (GC) was used. For this purpose, first, the seed oil was extracted. Analysis of fatty acids was performed by Novin-Azma Kimia Analysis Company with test method 2-1-13126.

Results

PCR amplification of genomic DNA from the Sohil variety of *C. sativa* with specific primers (number primers FAE1 F and FAE1 R) generated PCR product, the *FAE1* gene size is 1518 bp (Figure 1a); also PCR amplification with specific primers (primers RFAE1 F and RFAE1 R), generated PCR products, the size *RFAE1* is 1518 bp (Figure 1b).

The purified *RFAE1* gene was cloned into the PTG19-T vector and transformed into *E. coli* (DH5α strain). The presence of insert in the transformed colonies was screened by selection on Mac- Conkey agar medium (Figure

Table 3. Composition of nutrient media for the in vitro plantlet regeneration of *Camelina sativa* cultivar.

Types of nutrient media	MS (NH_4NO_3 / KNO_3)	Sucrose, g/L	BAP mg/L	NAA mg/L
1	N	30	0.22	-
2	1:4	20	0.45	-
3	1:6	30	0.9	-
4	N	20	0.22	0.3
5	1:4	30	0.45	0.4
6	1:6	20	0.9	0.5
7	N	30	-	0.3
8	1:4	20	-	0.4
9	1:6	30	-	0.5

Table 4. Composition of ammonium to nitrate and agar for the in vitro plantlet regeneration of *Camelina sativa* cultivar.

Ratio of ammonium to nitrate and agar	NH_4NO_3 (gr)	KNO_3(gr)	Agar(gr)
1	1/6x (0.275)	6x (11.4)	8.2
2	1/3x (0.55)	3x (5.7)	8
3	1/2x (0.83)	2x (3.8)	7.8

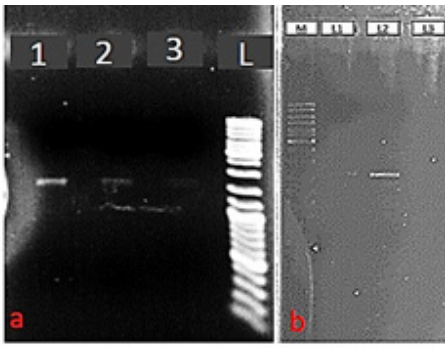

Figure 1. a) The *FAE1* gene *of* the amplification by PCR. L1, L2 and L3: Amplification of the *FAE1* gene with specific primers (1518 bp). **b)** The *RFAE1 of* the amplification products by PCR. L1 and L2: Amplification of the *RFAE1* gene with specific primers (1518 bp). L3: Negative control.

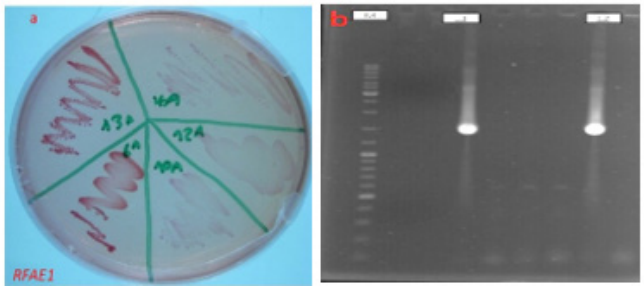

Figure 2. Colonies were screened by selection on Mac Conkey agar medium and Confirmation by PCR of *RFAE1* construct. **a)** Colonies were screened for *RFAE1* construct. **b)** Confirmation by PCR; Lane M: 100 bp plus ladder. L1 and L2: Amplification of the *RFAE1* construct (1518 bp).

2a) and colony PCR with specific primers. (Figure 2b).

The *RFAE1* was isolated and further analyzed by restriction enzyme digestion, and cloned into the pSK+ plasmid separately the clones were confirmed by PCR, restriction enzyme analysis and sequencing. The authentic PCR fragments were sub-cloned into a plant binary vector (recombinant pBI121) and the resulting clones and orientation of construct were confirmed by PCR and restriction enzyme digestion (Figure 3).

These constructs were transferred to *A.tumefaciens* LBA4404 by the freeze-thaw standard method (15) (Figure 4a). The *Agrobacterium* strains were then used to transform *C. sativa* using an *Agrobacterium*-mediated petiole cotyledonary transformation. The best morphogenic response was shown by 7 days old explants. According to our results, the optimum medium for shoot regeneration was the MS medium containing 4.5 mgL^{-1} of BAP, which led to 70% shoot regeneration after 20 days of culture. Explants from a variety of *C. sativa* 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) (Figure 4b) and then in MS medium containing 2 mgL^{-1} of indol-butyric acid (IBA) and 25 mgL^{-1} kanamycin (a lethal concentration of antibiotic for no transformed shoots). The transgenic plants had a regeneration frequency of approximately about 30 % in the medium containing 8 mgL-1 of kanamycin. After acclimatization of rooted plantlets to in vivo conditions, they were allowed to flower and set seeds.

Molecular analysis of transgenic plants

Genomic DNA putative transgenic and non-transgenic plants was analysed for the presence of the *TRFAE1* gene by PCR using the TFAE1 F and TFAE1 R primers. PCR amplification produced a fragment of 750 bp in the transgenic plants, but no amplification was observed in the control plants (Figure 5).

Fatty acid analysis

Flowers self-pollinated kanamycin-resistant T0 plants

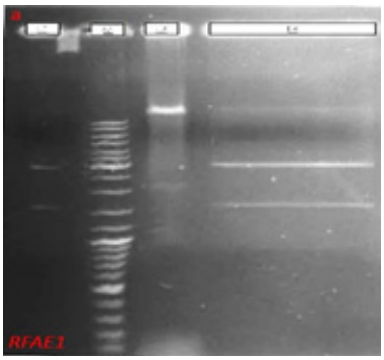

Figure 3. Confirmation of *RFAE1* construct cloned into recombinant pBI121 via restriction enzyme analysis. M: 100 bp plus ladder. L1 and L3 digestion of the *RFAE1* construct with *Cfr91*, *SacI*, and L2 pBI1400 plasmid.

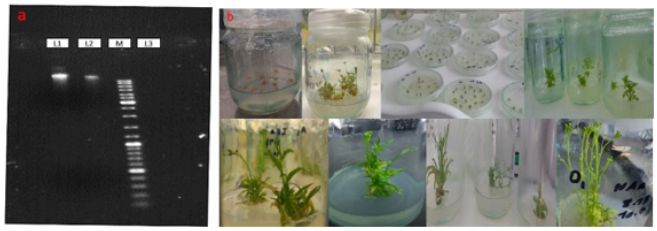

Figure 4. a) Confirmation of construct was transferred to *A.tumefaciens* LBA4404 by PCR, L1 product of the recombinant pBI121 plasmid containing *RFAE1* construct. L3: negative control. **b)** Development and elongation of shoots after transformation.

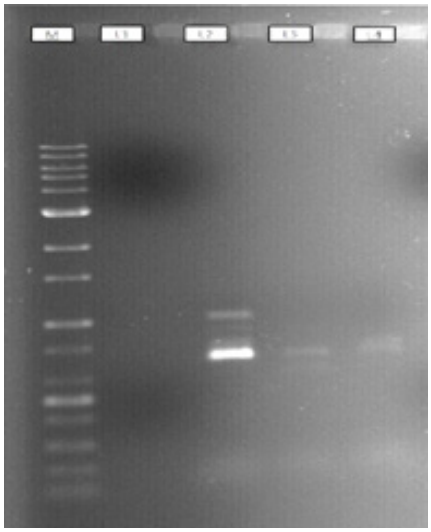

Figure 5. PCR analysis of transgenic plants with integrated *TRFAE1* construct**.** L1 and L5: negative control**.** L2, L3 and L4, PCR amplification produced a fragment of 750 bp in the transgenic plants with *TRFAE1* construct.

carrying the *FAE*1 gene (plants containing the *RFAE1* construct) were harvested and analyzed for fatty acid composition by GC (Figure 6). Two control plants bearing had erucic acid levels of approximately 0.88% (Figure 6a), while two lines of the *RFAE1* transgenic plants showed a decrease of approximately 0% in erucic acid level (Figure 6b).

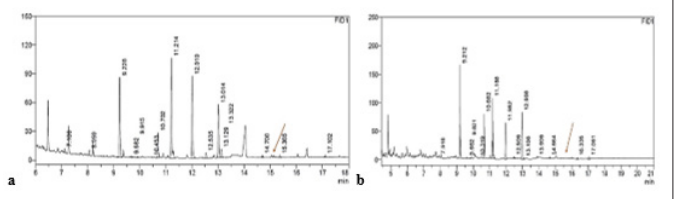

Figure 6. Gas chromatography analyses of fatty acid contents of flowers from transgenic camelina plants. **a)** Fatty acid content of flowers from control cultivar of *C. sativa* L. (0.88% erucic acid). **b)** Fatty acid content of flowers from *RFAE1* transgenic cultivar of *C. sativa* L. (0% erucic acid).

Discussion

In a report conducted by Zabarjedi et al. (2006) (14), sense and antisense structures transferred the *FAE* gene to canola through the *Agrobacterium* method. This transfer was performed in both cultivars with low erucic acid (LEAR such as PF) and with high erucic acid (HEAR such as Maplus). The modified plants were screened in an environment containing kanamycin and they were then analyzed by PCR method and southern blotting.

In addition, the amount of erucic acid in the first generation of the transgenic plant (T0) was analyzed by chromatography, which showed significant changes in the fatty acid composition of the transgenic plants that contain meaningful and meaningless structures of the *FAE* gene.

In another report, a fluorescent protein (DsRed) was used as a visible selective marker in the genetic modification of camelina. In this method, mature transgenic seeds are easily screened from a large number of non-transgenic seeds. By using this method, more than one percent of the transgenic seeds was obtained. Genetic analysis showed that most transgenic plants contain only one transgenic version. The results showed that the genetic engineering method could improve the agronomic properties of camelina, which these properties include the profile of fatty acids present in its seed oil (11).

In a study conducted by Wu et al. (2008) (16), complete coding sequences of the *FAE* gene were isolated from eight rape cultivars with zero and high erucic acids. Four base pair deletions between T1366 and G1369 in the *FAE1* gene, which in some cases led to a frame-shift mutation, and eventually to a premature termination stop of the translation.

This deletion was predominantly found in the C-genome and rarely in the A-genome of *B. napus*. The expression of gene isoforms in the deletion of four base pairs in the yeast system generated truncated proteins with no enzymatic activity and could not produce very long chain fatty acids as the control, with the intact *FAE1* gene did in yeast cells. In the developing rape seeds, the *FAE1* gene isoforms were typically transcribed by removing four base pair but failed to translate proteins into functional complexes. In this report, they succeeded in obtaining low erucic acid in rapeseed by deletion of four pais pairs through mutation.

Kang et al. (2011) (7) identified three *FAD2* genes to produce suitable oils for industrial use in camelina by antisense method. In industrial oils, it is better to increase oleic acid (MUFA) and reduce unsaturated fatty acids (PUFA), linoleic acid and linolenic acid. Although the three *CsFAD2* genes were very similar in sequence, they showed different expression patterns. The expression of *CsFAD2-1* was detected in all the tissues examined, including developing seed, flower, leave, root and stem, but the expression of *CsFAD2-2* and *CsFAD2-3* was mainly seen in seeds.

In 2013, Yemets et al (18) regulated tissue culture, regeneration of seedlings and rooting in Peremozhets and Mirazh camelina cultivars. In this study, they were able to obtain the best effective concentrations of disinfectants and duration of plant material treatment, phytohormone ratio and sucrose concentration in the formation of branching and NAA concentration in seedling rooting. In this study, they were able to obtain the best effective concentrations of disinfectant factors and duration of herbal treatment, phytohormone ratio and sucrose concentration in seedling formation and NAA concentration in seedling rooting. They also produced transgenic plants in camelina using the agrobacterium method and the pGH217 transporter, which had β-glucuronidase (Gus) reporter's gene.

Yan et al. (2015) (19) separated the lines with zero erucic acid *Brassica rapa* and found that the formation of plants with low erucic acid was not related to changes in the *FAE1* coding sequence, 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 promoter variations might modify the expression level of *FAE1*, and the results shed light on novel regulation mechanisms for erucic acid synthesis.

Camelina is an under-exploited crop species but has a great potential for economic importance. In this report, we demonstrate that camelina can be efficiently transformed by a simple method that does not involve lengthy and tedious tissue culture procedures. Recent studies have shown that the plant camelina has special and unique properties, the most important of which are low agriculture expectations and low sensitivity of the plant to pests and diseases. Because the production of camelina in many climates costs less to produce than other oilseeds, it is a better choice than other vegetable oils to be used as biofuels. But erucic acid, a component of the *Brasicaseae* family of fatty acids, is harmful to human nutrition, and (modifiers) reformers are trying to achieve varieties that do not contain erucic acid. Changing the amount of erosive acid in seeds is one of the main goals of improving the quality of the function of the oil of Brassica species (17).

Considering the above-mentioned points about the characteristics of the camelina plant, its requirements and valuable benefits, as well, considering that on the one hand, our country needs more and better quality oil products and on the other hand it is involved in climatic issues and problems, including consecutive droughts, it seems that the cultivation and development of the camelina plant is a big step towards achieving the goals of stable development

in our dear country. The optimal conditions for the establishment of an in vitro culture, in particular, the effective concentrations of the sterilizing agent and the treatment time of *C. sativa* seeds, were established at the beginning of our experiments.

In studies of the regeneration of camelina shoots as the best types of explants, for the initiation of organogenesis in vitro, many species of Cruciferae family cotyledons and hypocotyl explants were used.

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