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Full Length Article

Generation of Transgenic *Camelina sativa* with Modified Seed Fatty Acid Composition

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Abstract

Camelina sativa is an oilseed crop that is currently being developed mainly as an alternative source of bioenergy. To improve its seed fatty acid composition, RNAi was conducted to suppress the expression of fatty acid desaturase 3 (*FAD3*) and fatty acid elongase 1 (*FAE1*) in developing seeds for reduction of undesired polyunsaturated fatty acids (PUFA) and very-long-chain fatty acids (VLCFAs), which are associated with unsatisfactory fuel properties. At the early flowering stage, camelina wild type (cv. Licalla) was transformed by *Agrobacterium tumefaciens* mediated floral-dip vacuum-infiltration method. We successfully obtained six transgenic plants. The expression of *FAD3* and *FAE1* genes were successfully repressed as indicated by quantitative RT-PCR (Q-PCR) analysis. The downregulation of the *FAD3* and *FAE1* genes by RNAi gene silencing resulted in the reduction of linolenic acid (18:3) and eicosenoic acid (20:1) to about 11% and 4% from about 27% and 15%, respectively in the seeds of untransformed plants. In contrast, linoleic acid (18:2) significantly increased from about 25% to 42%, however oleic acid (18:1) was only slightly increased by about 4–5%. Our results demonstrated that seed-specific suppression of key fatty acid modification genes including *FAD3* and *FAE1* effectively changed fatty acid composition in camelina oil. An easy genetic transformation protocol should allow for future metabolic engineering for desirable fatty acid profiles and other biotechnology studies in camelina. © 2019 Friends Science Publishers

Keywords: *Camelina sativa*; *Agrobacterium tumefaciens*; Fatty acid desaturase 3; Fatty acid elongase 1; Transgenic plant

Introduction

The growing popularity of biofuels as an alternative fuel source has been promoted over the past decade by their environmental and economic benefits (Durrett *et al.*, 2008; Lu *et al.*, 2011). Recently, vegetable oils have increasingly been used in biofuels such as straight vegetable oil (SVO) or biodiesel and jet-fuels (Huber *et al.*, 2006; Moser, 2010; Steen *et al.*, 2010). It is desirable to develop oilseed feedstock for biofuel to limit the competition with food oils. A low-cost feedstock is also preferred to maximize revenue since 80–85% of biodiesel production costs are due to feedstock (Soriano and Narani, 2012). *Camelina sativa* (L.) Crantz (also known as false flax or gold-of-pleasure) is a re-emerging cruciferous oilseed crop due to its unique agronomic attributes including relatively lower input compared to other oilseed crops like rapeseed (Putnam *et al.*, 1993). Camelina is widely adapted to many regions of the world and has great potential in food, feed and industrial applications (Berti *et al.*, 2016). It also can be used as a rotation crop to improve cereal-based agricultural systems for diversified crop production (Obour *et al.*, 2018). Most notably, camelina oil has been processed and used as a next-generation jet fuel and has been tested successfully in flights by the U.S. military and by several commercial airliners (Moser, 2010; Shonnard *et al.*, 2010).

The fuel properties of biodiesel are affected by the fatty acid (FA) composition of the feedstock (Knothe, 2008; Moser and Vaughn, 2010). A major limitation of camelina oil is its high concentrations of polyunsaturated fatty acids (*e.g.*, linoleic acid, 18:2, n-6; a-linolenic acid, 18:3, n-3) especially linolenic acid, and the very-long-chain fatty acids (eicosenoic acid, 20:1; erucic acids, 22:1) (Vollmann *et al.*, 2007), which are known to be associated with low oxidative stability, poor cold flow and high melting point, respectively, of the biodiesel (Knothe, 2008). During oil biosynthesis in seed, oleic acid (18:1) is the major de novo fatty acid, which is modified before being incorporated into triacylglycerols primarily through desaturation and elongation pathways (Ohlrogge and Browse, 1995). The fatty acid desaturases *FAD2* and *FAD3* sequentially turn the 18:1 into polyunsaturated 18:2 and 18:3, while the fatty acid elongase *FAE1* promotes the synthesis of very-long-chain fatty acids (Kang *et al.*, 2011). In this study, we suppressed

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the expression of *FAD3* and *FAE1* genes in camelina seeds using a simple and efficient transformation method by a vacuum infiltration of *Agrobacterium*-mediated infection of plants at the early flowering stage. The transgenic seeds contained reduced amounts of polyunsaturated fatty acids and long-chain unsaturated fatty acids.

Materials and Methods

Plant Material and Growth Conditions

Camelina sativa cultivar "Licalla" was used in this study. Seeds were sowed in 14-cm pots (5 seeds/pot) filled with vermiculite, perlite and nutritional soil Mix (2:1:2). Plants were grown in the plant growth chamber at the Tianjin University of Science and Technology. Growth conditions were set at 20/16°C (day/night) and 60% relatively humidity.

Plant Transformation Vector and Agrobacterium Strain

To create an expression RNAi vector G2 (pBinGlyBar1-FAD3-FAE1), a 272-bp *FAD3* fragment which is identical in the three camelina *FAD3* genes (Csa05g033930, Csa07g013360, Csa16g014970) was amplified using primers 5'-CACCACCAGAACCATGGCCA-3' and 5'-CGGAGAGTGGACCGAAGATGAATG-3', and a 252-bp *FAE1* fragment covering all three camelina FAE1 genes (Csa10g007610, Csa12g009060, Csa11g007400) was amplified using primers 5'-GGAATACTTCGTCTAGCTC-3' and 5'-GTCCGACCGTTTTTTGACATGAGTC-3'. The *FAE1* and *FAD3* PCR products were assembled sequentially in an inverted repeat orientation of either sides of a *Flaveria trinervia* pyruvate orthophosphate dikinase (PDK) intron and an Arabidopsis *FAD2* intron, respectively. These RNAi cassettes were assembled in a binary vector pBinGlyBar1 as described previously (Nguyen *et al.*, 2013).

Agrobacterium (*Agrobacterium tumefaciens* strain EHA105) competent cells were prepared by using $CaCl_2$, and the cells were divided into 100 μL per tube, quick frozen in liquid nitrogen and stored at -80°C. Agrobacterium cells were cultured in the YEB medium composed of yeast extract (1.0 g/L), beef extract (5.0 g/L), peptone (5.0 g/L), $MgSO_4$ (0.5 g/L), and sucrose (5.0 g/L) and was adjusted to pH 7.0–7.4. The medium was solidified with 15 g/L agar powder in petri dishes. Infiltration medium was composed of 1/2 MS, 0.05% (v/v) silwet L-77 and 50 g/L sucrose and adjusted to pH 5.7. The plasmid G2 was transformed into agrobacterium using a freeze-thawing method followed by culturing in 800 μL YEB medium to recover at 37°C for 2 h. After centrifugation at 4,500 rpm for 45 s at room temperature, cells were re-suspended in the remaining 100 μL of medium and spread on YEB plates containing 50 mg/L rifampicin and 50 mg/L kanamycin at 28°C. Several single colonies were picked randomly and inoculated into a culture beaker flask containing 25 mL of YEB medium supplemented with 50 mg/L of rifampicin and 50 mg/L of kanamycin. To confirm the presence of transgenes, plasmids were extracted using the GeneJET Plasmid Miniprep Kit from overnight cultured bacteria, and digested with the HindIII restriction enzyme.

Transformation of Plants by *Agrobacterium tumefaciens*-mediated Vacuum Infiltration

To prepare the infiltration medium, a single colony picked from a freshly streaked plate was inoculated into a culture tube containing 5 mL of YEB medium supplemented with 50 mg/L of rifampicin and 50 mg/L of kanamycin. An overnight culture of the 5 mL of bacteria was then transferred into 500 mL of the same medium and grew in a shaking incubator at 28°C for 24–48 h. Bacteria were harvested by centrifugation at 4,000 rpm for 15 min and resuspended in 300 mL of infiltration medium.

Camelina plants at the early flowering stage were placed inside a 310-mm-tall vacuum desiccator connected with a vacuum pump. The inflorescences were submerged into the Agrobacterium solution in a 300-mL beaker prepared as above. The desiccator was vacuumed to a pressure of 85 kPa and held for 5 min. Plants were placed in plastic bags for 24 h, then returned to normal conditions in greenhouse for continued growth.

Screening of Transgenic Plants

Seeds from T0 plants treated with agrobacterium were harvested and sowed in soil. The herbicide phosphinothricin (PPT) mixed with water at a ratio of 1:300 was used to screen transgenic plants by spraying on plants grown to two-leaf stage, with a stem length of approximately 3 cm. The survived T_1 plants were transferred to new pots for continued growth. To determine whether they were true transgenic plants, PCR was performed using the following *bar* gene-specific primers: 5′-GCTGAAGTCCAGCTGCCAGAAAC-3′ (forward) and 5′-GAGACAAGCACGGTCAACTTCC-3′ (reverse). The 20-μL reaction mixture contained 2× PCR Master Mix (10 μL), cDNA template (1 μL), 0.5 μL of each primer (10 μmol/L), and H_2O (8 μL). Cycling parameters began with an initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, followed by extension at 72°C for 2 min and a final extension of 72°C for 10 min. PCR products were analyzed by electrophoresis in 1.0% agarose gels.

Fluorescence Quantitative PCR (Q-PCR) Analysis of Gene Expression

RNA was extracted from developing seeds (16 days after flowering) using a Plant RNA Kit, and cDNA was synthesized using the HiFi-Script cDNA Kit. To monitor gene expression, Q-PCR was performed using the SYBR Green PCR master mix (Invitrogen). The following primers

were used to amplify coding regions of *FAD3*: 5′-ACCAAGGCAACTTCTCATCGT-3′ (forward) and 5′-CTTCGGTCCACTCTCCGTTC-3′ (reverse); *FAE1*: 5′-AGATCGGAGACGGTCCAAGT-3′ (forward) and 5′-CCGCAACAACGGTTATGTCC-3′ (reverse); and Actin7 gene: 5′-TGATGATGCTCCCAGGGC-3′ (forward) and 5′-GTGAGAAGCACAGGATGC-3′ (reverse). The 25 μL reaction mixture contained 2× SYBR Green master Mix (12.5 μL), cDNA template (1 μL), 0.5 μL of each primer (10 μmol/L), and H_2O (10.5 μL). Q-PCR was performed on the ABI step one plus system (Applied Biosystems). Melting curve analysis was done with an initial activation at 95°C for 1 min, then followed by 50 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 15 s.

Fatty Acid Analysis of Seeds

Seed fatty acids were analyzed following previously described methods (Kang *et al*., 2011). To prepare fatty acid methyl esters (FAMEs), 3–5 crushed seeds were put in 1 mL of 2.5% H_2SO_4 (v/v) in methanol and heated at 90°C for 60 min. After samples were cooled down, 200 μL of hexane and 1.5 mL of 0.9% NaCl (w/v) were added. After brief centrifugation, one hundred microliters of the organic phase were transferred to autoinjector vials. One microliter of sample was injected into a Shimadzu 2010 GC fitted with a HP-Innowax column (30 m × 0.25 mm × 0.25 m) (Agilent Technologies). The GC oven temperature was programmed for an initial 180°C for 1.5 min, followed by an increase of 6°C/min to 240°C, and maintained for 3 min.

Statistical Analysis

Student's t-tests were performed within the Microsoft Excel software using default settings for significance in differences.

Results

Transformation of *Agrobacterium tumefaciens* and Camelina Plants

A plasmid named G2 was prepared that includes the RNAi sequences for the camelina *FAD3* and *FAE1* genes driven by the seed specific glycinin promoter and the Basta selection marker (Fig. 1A). The construct was confirmed by restriction enzyme analysis using HindIII. Based on the plasmid map, the enzyme digestion would result in four fragments of 8,889 bp, 3,013 bp, 1,603 bp and 559 bp in size. As expected, electrophoretic analysis of enzyme-digested products (Fig. 1B) revealed four accurate bands. This plasmid was then transferred into the *Agrobacterium tumefaciens* strain. To ensure the presence of G2, plasmid was extracted from agrobacterium and digested with HindIII, which showed the correct bands in the electrophoresis gel (Fig. 1B). The agrobacterium culture

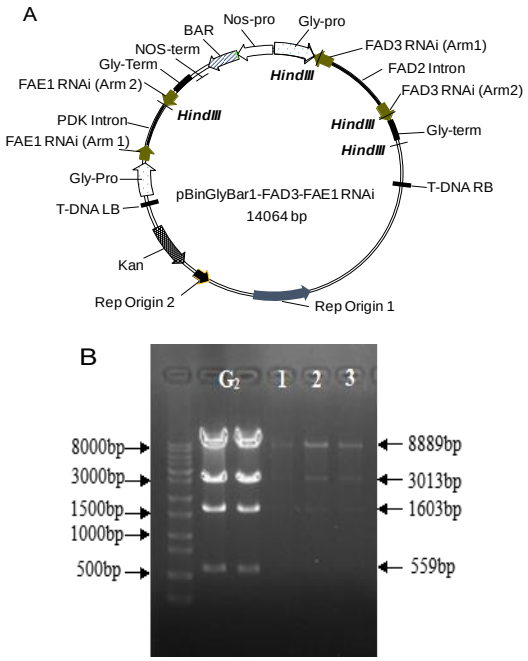

Fig. 1: An RNAi construct of pBinGlyBar1-FAD3-FAE1. A. A map containing key features including the *FAD3*, *FAE1* hairpin structures, Bar selection gene, and HindIII sites. B. Gel electrophoresis of plasmid digested with HindIII. G2: Plasmid extracted from E. coli. 1-3: Plasmids extracted from Agrobacterium

was then used to transform camelina plants when they reached at the early flowering stage with many buds but few open flowers (Fig. 2) following the method (Lu and Kang, 2008).

Screening of Transgenic Plants

Two-week old T1 seedlings were sprayed with the PPT herbicide to screen for putative transgenic plants. As shown in Fig. 2, most of the plants leaves began to curl and dry until the whole plant died after a week. Plants that survived were transferred to new soil for continued growth. We were able to obtain six putative transgenic plants that survived from repeated herbicide spraying treatments.

For confirmation of herbicide resistant plants, PCR results revealed the presence of bands that corresponded to expected *bar* gene fragment (420 bp) (Fig. 3), indicating that the herbicide resistance selection was effective.

Effect of *FAD3* and *FAE1* Suppression by RNAi

Transgenic effects on *FAD3* and *FAE1* gene expression in camelina were determined by Q-PCR experiments. As shown in Fig. 4, the expression of *FAD3* in all of the six transgenic plants was significantly downregulated and the decreased expression of *FAE1* was also observed in six transgenic plants though at different degrees. These results clearly indicated that *FAD3* and *FAE1* genes were successfully repressed by the RNAi transgenes.

To estimate the effect of *FAD3-FAE1*-RNAi on fatty acid metabolism in seed, fatty acid composition was analyzed using single seeds from untransformed and T2 transgenic seeds. In camelina seeds, major fatty acids include saturated palmitic acid (16:0) stearic acid (18:0), monounsaturated oleic acid (18:1) and eicosenoic acid (20:1) and polyunsaturated linoleic acid (18:2) and linolenic acid (18:3). Since T2 seeds are heterozygous for the transgene, many seeds showed the same fatty acid profiles as the non-transformed ones. However, there were several seeds clearly showed different fatty acid composition than the non-transgenic controls. Most significantly, 18:3 and 20:1 in a putative transgenic seed were dramatically reduced compared to the non-transgenic seed (Fig. 5). These results indicated that RNAi suppression of *FAD3* and *FAE1* genes, which are responsible for 18:3 and 20:1 biosynthesis respectively, effectively reduced these fatty acids in transgenic seeds.

All T2 plants were grown and sprayed with the herbicide. Consequently, two homozygous lines were obtained that all plants were resistant to the PPT herbicide. Their seeds were analyzed for fatty acid composition. Linolenic acid (18:3) was reduced to about 11% and eicosenoic acid (20:1) was reduced to about 4% from the levels of 26.9% and 14.9% in untransformed seeds, respectively. While linoleic acid (18:2) was significantly increased due to decreased 18:3, oleic acid (18:1) was only moderately increased by just 4–5% of total fatty acids compared to non-transgenic seeds (Table 1).

Discussion

It is evident from the literature (Lu and Kang, 2008; Nguyen *et al*., 2013; Snapp *et al.*, 2014; Jiang *et al.*, 2017; Chhikara *et al.*, 2018; Ozseyhan *et al.*, 2018; Zhu *et al.*, 2018) that camelina can be transformed by an agrobacterium-mediated floral dipping procedure. This simple method has made camelina a unique platform for translational biology (Collins-Silva *et al.*, 2011; Hines and Travis, 2016). The factors influencing transformation efficiency may include the plant growth conditions, the concentration and virulence of *Agrobacterium tumefaciens* strains. It has been shown in Arabidopsis that female reproductive tissues are primary targets of *Agrobacterium* mediated transformation, thus the critical timing of *Agrobacterium* infection was during roughly 3 days before anthesis when the gynoecium developed as an open, vase-like structure that fused to form closed locules (Desfeux *et al.*, 2000). Camelina is a close relative to *Arabidopsis* (Kagale *et al.*, 2014). Therefore, it is crucial to transform plants at the early flowering stage with fewer open flowers but many buds. In this study, transgenic plants were screened by the herbicide PPT, which was a simple and practicable method since plants that do not contain the bar gene or in which bar gene expression was silenced were killed by herbicides.

Fig. 2: Generation of transgenic Camelina plants. A. Early stage flowers ready for Agrobacterium infection; B. Seedlings to be sprayed with PPT and the herbicide resistant plant; C. A PPT-surviving plants growing in a pot

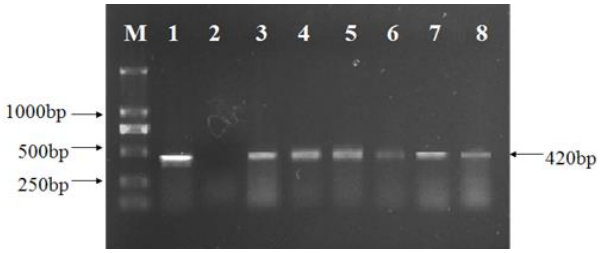

Fig. 3: PCR analysis of transgenic plants. M: DL 2000 DNA marker; 1. Plasmid positive control; 2. Negative control of non-transgenic plants; 3-8: Herbicide-resistant transgenic plant

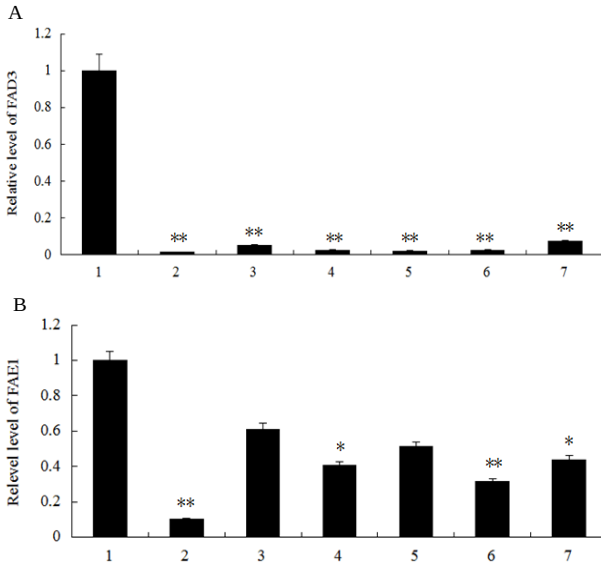

Fig. 4: Expression of *FAD3* (**A**) and *FAE1* (**B**) in untransformed (1) and transformed camelina (2-7), monitored by Q-PCR

Compared to the DsRed maker successfully used previously (Lu and Kang, 2008), this method is more laborious and time consuming. However, herbicide resistance provides an additional marker for transgenic plants screening, which is particularly useful when genes need to be transformed into plants that already contain the DsRed or other selection markers.

Table 1: Fatty acid composition of transgenic camelina seeds compared with Licalla

Line No.		Fatty acids (% mol)						
		16:0	18:0	18:1	18:2	18:3	20:0	20:1
Licalla		10.1	4.3	13.8	25.3	26.9	3.4	14.9
FAD3FAE1 RNAi	L2-3	10.9	3.8	18.6	41.9	11.1	2.7	3.9
	L6-2	10.2	5.8	17.9	44.3	11.7	2.3	4.4

For each line, data represent average of 5 seeds per plant from 3 plants. Only major fatty acids are included

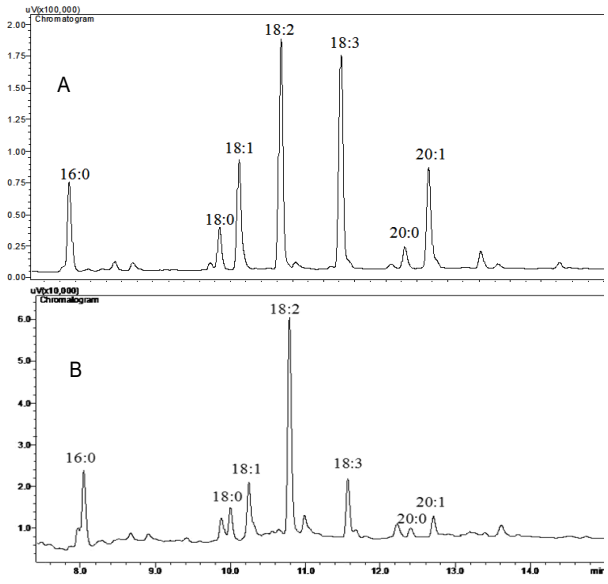

Fig. 5: Comparison of fatty acid composition by gas chromatography. Fatty acid methyl esters were prepared from single mature seeds of untransformed (A) and transformed camelina (B)

Camelina is under intensive development primarily for a biofuel feedstock (Berti *et al*., 2016). To improve camelina oils for biofuel production, it is desirable to reduce its high content of polyunsaturated (especially 18:3) and very-long chain fatty acids. We demonstrated that this could be achieved by simultaneously downregulating the expression of key genes including *FAD3* and *FAE1*. Oleic acid (18:1) is more desirable for both food and industrial applications, therefore it is a primary goal to breed high-oleic oilseed varieties. During oil biosynthesis, 18:1 can be desaturated or elongated to make modified fatty acids such as 18:2, 18:3 and 20:1. It was expected that higher 18:1 would accumulate in our RNAi seeds. However, interestingly reducing 18:3 and 20:1 caused increased 18:2 but failed to increase 18:1 (Fig. 5 and Table 1). This was in contrast to the *fad3*/*fae1* mutant in Arabidopsis in which both 18:1 and 18:2 were enhanced (Smith *et al.*, 2003). A possible explanation could be due to the polyploidy of camelina, which contains three copies of homologous genes of each fatty acid desaturases and elongases (Hutcheon *et al.*, 2010; Kang *et al*., 2011). The strong desaturation activity conferred by three *FAD2* genes in camelina might prevent a high 18:1 accumulation but contribute to high levels of 18:2 in our RNAi seeds. Suppression of *FAD2* is needed to increase the level of oleic acid in camelina (Kang *et al*., 2011).

Camelina is a recently emerged oilseed crop under intensive development for oil production and to diversify modern crop production systems (Berti *et al*., 2016; Obour *et al*., 2018). Biotechnology provides an effective tool to rapidly improve camelina and for translational biology research (Collins-Silva *et al*., 2011; Hines and Travis, 2016). Here we demonstrate that seed fatty acids can be effectively modified by RNAi on key genes involved in fatty acid synthesis through a simple transformation procedure. This will allow for future experiments to improve camelina for many agronomic traits including fatty acid composition in seed oils, and make this crop a sustainable source of bioenergy.

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