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Authors: Mehmet Ozseyhan, Pengcheng Li, GunNam Na, Zhenjing Li, Changlu Wang, and Chaofu Lu

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Improved fatty acid profiles in seeds of *Camelina sativa* by artificial microRNA mediated *FATB* gene suppression

Mehmet E. Ozseyhan^a, Pengcheng Li^b, GunNam Na^a, Zhenjing Li^b, Changlu Wang^b, Chaofu Lu^{a,*}

^a Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717, USA

^b College of Food Engineering and Biotechnology, Tianjin University of Science and Technology, Tianjin 300222, China

A B S T R A C T

The fatty acid profile of plant oils determines their quality and uses. Saturated fatty acids are often not desirable from the standpoints of nutrition and some industrial applications. *Camelina sativa* is a re-emerged oilseed crop, however its oil needs to be improved to meet different application requirements. In this study, saturated fatty acids were greatly reduced by down-regulating genes encoding the fatty acyl-ACP thioesterases (*FATB*). An artificial microRNA (*amiFATB*) was created by replacing a microRNA sequence in the camelina *Csa-miR159a* gene with a *FATB* gene specific sequence. Seed-specific expression of *amiFATB* caused a 45% reduction of palmitic acid (16:0) and a 38% reduction of stearic acid (18:0) compared to wildtype seeds. The total saturated fatty acid content was decreased by 35% from 14.6% to 9.4% of total fatty acids. When *amiFATB* was expressed in a high-oleic acid transgenic line, it caused further increased oleic acid content. This work demonstrates that the *FATB* genes in camelina can be effectively knocked down by an artificial microRNA targeting gene-specific sequences, thus provides an additional tool to improve seed oils for desired properties.

1. Introduction

Vegetable oils are an important source of food and feed, and increasingly industrial feedstocks such as biofuels [1]. To provide desired functionalities and nutritional attributes, genetic engineering is being conducted to improve fatty acid composition by manipulating key genes involved in lipid metabolism. In oilseeds, newly synthesized fatty acids are exported from plastids mainly in the form of the monounsaturated oleic acid (18:1) along with smaller amounts of the saturated 16:0 and 18:0. These fatty acids are released from acyl-acyl carrier protein (acyl-ACP) by two different classes of thioesterases (FAT). The FATA has a higher activity on 18:1 while the FATB prefers saturated substrates [2]. A large proportion of oleic acid is modified before being incorporated into triacylglycerol through either desaturation by the desaturases FAD2 and FAD3 acting on the phosphatidylcholine (PC) substrates to form the polyunsaturated linoleic (18:2) and linolenic (18:3) acids, or elongation to form 20:1 and 22:1 by the fatty acid

elongase FAE1 [3]. Controlling these metabolic steps may effectively improve plant oils for required nutritional or industrial properties [4,5].

Camelina (*Camelina sativa*) is a promising oilseed crop that is under intensive development mainly for bioenergy production. An important limitation for camelina is the suboptimal quality of its oil [6], which contains high contents of undesirable polyunsaturated (50%–60% of the total fatty acid), saturated (~10%), and the very-long chain (~12%) fatty acids [7], which are known to be associated with low oxidative stability, poor cold flow and high melting point, respectively, of the biodiesel [8]. Previously, we have successfully decreased polyunsaturated and very-long-chain fatty acids by suppression or deletion of *FAD2* and *FAE1* genes in camelina using RNA interference (RNAi) or CRISPR/Cas9 technologies [7,9,10]. The objective of this study is to decrease saturated fatty acids and to increase oleic acid. Mutations or knockdown of *FATB* genes have been shown to be associated with low saturates in seed oils such as canola and soybean [11,12]. Here, we used a microRNA mediated approach to downregulate the *FATB* genes in camelina. MicroRNAs are short 20–24 nucleotide non-coding RNA sequences. They control gene expression by binding to specific complementary mRNA targets in the untranslated regions or the coding sequence,

leading to mRNA degradation or translational inhibition [13]. Such post-transcriptional gene silencing mechanisms may be used in plants by antisense expression or hairpin-based RNA interference (RNAi), which produce miRNAs to target transcripts of any genes of interest. It is also possible to modify miRNA sequences and create artificial miRNAs to target the gene transcripts alter gene expression and modify seed oil composition [14]. Here we demonstrated that *FATB* genes in camelina were effectively downregulated by an artificial microRNA159a that led to changed fatty acid composition in seed.

2. Material and methods

2.1. Plant materials

Camelina (*C. sativa*) cv. Suneson, and a previously created high-oleic transgenic plant (HiOle1) [9] were used to transform the *FATB* artificial microRNA gene. Plants were grown in a greenhouse at 22 °C under natural light conditions supplemented with high-pressure sodium lights with a 16 h photoperiod (16 h of light and 8 h of darkness). Young leaves, stems, inflorescence, and developing seeds at 16 days after flowering were harvested, chilled in liquid nitrogen, then stored at −80 °C. Seedlings and roots were harvested from plants grown under sterile conditions on MS medium (PhytoTechnology Laboratories, Shawnee Mission, KS).

2.2. Vector construction and plant transformation

The mature sequence in the camelina microRNA (*Csa-miR159a*) was replaced by a 21mer *FATB* artificial microRNA sequence identified by the WMD3 software (<http://wmd3.weigelworld.org/cgi-bin/webapp.cgi>) [15]. Initially, Arabidopsis *FATB* gene sequence was used to identify the amiFATB candidates that were targeted by *Ath-miR159a* without affecting off-target genes. The amiFATB sequences were then compared with the camelina *FATB* sequences to choose one that matched all three homologous copies. The fragment of the *Csa-miR159a* stem loop with amiFATB sequence was synthesized by GeneWiz (South Plainfield, NJ), and was inserted into a binary vector, pPhas-Gly-DsRed2 [9], between the *EcoRI* and *XhoI* sites so that amiFATB was put under the seed-specific glycine promoter. This construct contains a DsRed selection marker for easy isolation of transgenic seeds by fluorescence [16]. Sequences of miRNA and amiRNAs are listed in Table S1.

2.3. Gene expression analysis by RT-PCR

Total RNA was isolated using Norgen miRNA extraction BioKit (Thorold, ON, Canada), measured by NanoDrop (Nanodrop Technologies, Wilmington, DE, USA). For the qRT-PCR or semi-qRT-PCR, the synthesis of first-strand cDNA was performed using SuperScript III reverse transcriptase (Waltham, MA, USA). The primer pairs used for the PCR are listed in Table S1. For the qRT-PCR of *Csa-miR159a*, cDNAs were synthesized using methods described previously [17]. Amplification and quantification of cDNA was done with the CFX96 Real-time system from Bio-Rad and PerfeCTa SYBR Green FastMix from Quantabio (Beverly, MA, USA). The following standard thermal profile was used for PCR: 95 °C for 5 min, 40 cycles of 95 °C for 5 s, 60 °C for 10 s, 72 °C for 1 s and melting curve analysis.

2.4. Fatty acid analysis

Camelina seeds were crushed and lipids were transmethylated according to procedures described previously [18]. The resulting fatty acid methyl esters (FAMES) were analyzed in a gas chromatograph (GC) (Shimadzu Scientific Instruments, Columbia, MD)

fitted with a 15 m × 0.25 mm column (DB-23; Agilent, Lexington, MA) and a flame ionization detector (FID). The GC was programmed for an initial temperature of 180 °C for 1 min followed by an increase of 10 °C/min to 230 °C and maintained for a further 1 min.

3. Results

3.1. Generation of transgenic plants expressing an artificial microRNA

Camelina has three highly similar *FATB* genes (*Csa17g011970*, *Csa14g009990*, *Csa03g011990*) [19]. To ensure effective knockdown of these genes, a 21mer artificial microRNA sequence was designed that targeted all three *FATB* coding sequences (Table S1). The Arabidopsis *Ath-miR159* has been used previously to successfully induce gene silencing in Arabidopsis [14]. Previously, we detected an *Ath-miR159* homolog named *Csa-miR159* in camelina [20]. To determine whether *Csa-miR159* is actively expressed in camelina, we carried out an RT-PCR experiment using RNA extracted from different tissues including young leaf, stem, root and developing seed (16 days after flowering). As shown in Fig. 1, *Csa-miR159* transcripts were detected in all tissues examined. The highest expression level was detected in developing seeds. We reasoned that *Csa-miR159* was able to express the *FATB* artificial microRNA (amiFATB) in camelina. The amiFATB sequence was inserted into a camelina microRNA gene (*Csa-miR159a*) to replace the native mature miRNA sequence. A construct was made by inserting the modified stem-loop region of the *Csa-miR159a* gene under a seed-specific glycine promoter in the pPhas-Gly-DsRed vector (Fig. S1). The amiFATB construct was transformed into an agrobacterium strain (GV3101) and then into the camelina wildtype Suneson plants. We obtained 16 transgenics seeds selected by using the DsRed fluorescent marker [16].

3.2. Reduction of saturated fatty acids in the *FATB*-knockdown seeds

All transgenic amiFATB plants were grown to the T3 generation, and the homozygosity was determined by all-red seeds conferred by the DsRed marker. To examine the expression levels of *FATB* genes, developing seeds at 16 days after flowering were harvested from Suneson and 4 independent homozygous amiFATB lines. RNA was extracted, and RT-PCR was performed using primers that

Fig. 1. Quantitative RT-PCR results of the *Csa-miR159a* gene in camelina tissues.

Fig. 2. The effect of amiFATB on *FATB* expression and saturated fatty acids accumulation. (A) RT-PCR detection of *FATB* in amiFATB transgenics and Suneson. The actin (*ACT2*) was used as a control gene. (B) The levels of 16:0 and 18:0 in Suneson and amiFATB seeds.

amplified all three homologous *FATB* genes (Table S1). The results showed that *FATB* transcript levels were significantly lower in transgenic lines than in the wildtype (Fig. 2A). This indicated that the amiFATB transgene has effectively knocked down *FATB* genes in camelina developing seed.

To assess the effect of the amiFATB transgene on fatty acid metabolism in camelina, fatty acid composition was determined in mature T3 seeds by gas chromatography. The results showed that levels of saturated fatty acids were decreased in the amiFATB seeds compared to those in the non-transgenic Suneson. Especially, a 45% reduction of 16:0 and a 38% reduction of 18:0 were observed in amiFATB compared to Suneson (Fig. 2B). This result was consistent with the enzyme substrate specificity of FATB on saturated (16:0 and 18:0) acyl-ACPs in plastids [2]. Other saturated fatty acids (20:0 and 22:0) were also slightly decreased, consequently the total saturated fatty acids were decreased from 14.6% in Suneson to 9.4% in amiFATB seeds, about 35% reduction compared with non-transgenic seeds (Table 1).

3.3. The effect of *FATB*-knockdown in a high-oleic line

Corresponding to decreased saturated fatty acids, the levels of oleic acid and other unsaturated fatty acids were slightly increased in the amiFATB seeds (Table 1). Previously, we created transgenic camelina lines that accumulated high levels of oleic acid by antisense or hairpin mediated RNAi knockdown on *FAD2*, *FAD3* and *FAE1* genes [7,9]. To explore whether down-regulating *FATB* expression may further increase the oleic acid content, we transformed the amiFATB construct into a high-oleic line (HiOle1) containing the *FAD2-FAD3-FAE1* RNAi construct and a herbicide resistance marker [9]. Twenty transgenic lines were obtained that contained both herbicide and DsRed selection markers. Consequently, six T3 HOamiFATB lines that showed clearly decreased *FATB* transcripts determined by semi-qRT-PCR were analyzed for their seed fatty acid composition. The results showed that saturated fatty acids (16:0, 18:0 and 20:0) were decreased as expected. The

Fig. 3. A comparison of seed fatty acid profiles between a high-oleic line with its *FATB* knockdowns.

composition of unsaturated fatty acids was also changed. Significantly, the oleic acid was increased from about 62% to over 70% (Fig. 3).

4. Discussion

The fatty acyl-ACP thioester *FATB* is a major determinant for saturated fatty acid accumulation in oilseeds. Low palmitate (16:0) in soybean was associated with the mutation of a *FATB* gene [11], and the Arabidopsis *fatb* knockout mutant accumulated only 50% saturated fatty acids of the wild type level [21]. In this study, we demonstrated that seed-specific expression of an artificial micro-RNA (*amiFATB*) significantly decreased *FATB* gene transcripts in developing seeds and caused about 45% and 38% reduction of 16:0 and 18:0, respectively, in camelina seed oil. The reduction of the saturated fatty acid content in amiFATB was at a similar level found in the Arabidopsis *fatb* mutant [21]. In tissues of the *fatb* plant, the remaining about 50% saturated fatty acids were presumably synthesized by other genes including the plastidial glycerol-3-phosphate:acyl-ACP acyltransferase (*ACT1*). Significantly, plant growth of the *fatb* and *fatb/act1* mutants were severely retarded, indicating that saturated fatty acids are essential for normal plant growth [21]. It is therefore infeasible to generate the *FATB* knockout camelina plants, which may affect plant growth while not be able to further reduce saturated fatty acids in seed. We did not observe abnormal seed germination and plant growth of the amiFATB lines.

A major goal of oilseed breeding is to increase the desired oleic acid content. Our results indicated that *FATB* knockdown not only decreased saturated fatty acids, but also caused changes in unsaturated fatty acids especially enhanced oleic acid accumulation. Simultaneous downregulation of *FATB* and *FAD2* in soybean has been shown to increase oleic acid and decrease palmitic acid [22]. Similarly, expression of *amiFATB* in a *FAD2-FAD3-FAE1* RNAi line also increased oleic acid in camelina. Besides decreased saturates, the increased oleic acid in the HOamiFATB seeds seemed to be also at the expenses of linoleic acid (18:2). It is not clear whether reduced

Table 1
Fatty acid composition in Suneson and amiFATB seeds.

		16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	22:1
Suneson	Ave	6.8	3.9	11.5	18.8	36.4	3.3	13.0	1.7	1.3	0.5	2.8
	SD	0.7	0.8	0.7	2.1	2.6	0.7	1.1	0.2	0.2	0.1	0.4
amiFATB ^a	Ave	3.8	2.4	13.4	18.9	37.5	2.8	13.9	1.8	1.3	0.4	3.5
	SD	0.6	0.5	1.6	1.6	4.0	0.4	0.6	0.1	0.2	0.1	0.5

^a Data represent average and standard deviation from 4 independent lines.

flux of 16:0 and 18:0 into the cytosolic triacylglycerol biosynthesis pathway or the *FATB* gene knockdown affected FAD2 activity in the RNAi line. Nevertheless, our results and previous studies [22] suggest that simultaneous knockdown of genes encoding key fatty acid modification enzymes including *FATB*, *FAD2*, *FAD3* and *FAE1* may enhance accumulation of oleic acid in oilseeds. Further increasing oleic acid in HOamiFATB seeds may be achieved by eliminating the very-long-chain fatty acids (20:0 and 20:1) by deleting *FAE1* genes. It was possible to knockout *FAE1* genes in camelina by the CRISPR-Cas9 technology, which blocked fatty acyl flux into the elongation pathway and resulted in enhanced accumulation of 18:1 and its derived polyunsaturated fatty acids (18:2 and 18:3) [10]. Unlike *FAE1* which can be deleted without causing abnormal plant growth, *FATB* and *FAD2* are essential for normal plant growth [21,23,24]. Our results provide an additional tool to effectively downregulate gene expression by artificial microRNAs. Combining this with other biotechnological tools for gene silencing will allow for the development of high-oleic camelina for improved oil qualities.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.bbr.2018.06.051>.

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