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Mutagenesis of the *FAE1* genes significantly changes fatty acid composition in seeds of *Camelina sativa*

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A B S T R A C T

Camelina sativa is a re-emerging low-input oilseed crop that has great potentials. It is necessary to ameliorate camelina oils for optimized fatty acid composition that can meet different application requirements. Camelina seed contains significant amounts of C20-C24 very long-chain fatty acids (VLCFAs) that may not be desirable. We demonstrated that these VLCFAs can be effectively reduced by deactivating the Fatty Acid Elongase1 (*FAE1*) in camelina. The allohexaploid camelina contains three alleles of *FAE1* genes. Ethyl methanesulfonate (EMS) induced mutation at the *FAE1-B* gene caused over 60% reduction of VLCFAs in seed. Homozygous knockout mutants were successfully created in a single generation by simultaneously targeting three *FAE1* alleles using the CRISPR technology with an egg cell-specific Cas9 expression. VLCFAs were reduced to less than 2% of total fatty acids compared to over 22% in the wild type, and the C18 unsaturated fatty acids were concomitantly increased. The *fae1* mutants were indistinguishable from wild type in seed physiology and plant growth. This study demonstrated that the CRISPR/Cas9 technology can be effectively applied to the polyploid crop camelina to rapidly obtain desired traits such as optimal fatty acid composition in its seed oil. Knocking out *FAE1* also provides a means to increase the levels of oleic acid or α -linolenic acid in camelina oils that are desirable for industrial or food/feed uses.

1. Introduction

Vegetable oils constitute one of the most important agricultural commodities. Vegetable oils are mainly consumed by humans, but considerable amounts are also used in industrial applications for manufacturing numerous products such as lubricants and biofuels (Gunstone, 1998). The final use of oils is dependent on their nutritional, physical and chemical properties. Camelina (*Camelina sativa* (L.) Crtz.), also known as false flax or gold-of-pleasure, is a member of the Brassicaceae family and was an established oilseed crop in parts of Eastern and Central Europe prior to World War II (Putnam et al., 1993). More recently, a strong interest has arisen in camelina as a potential biofuel crop in North America and other parts of the world due to its favorable attributes such as low-input production and tolerance to drought and cold temperatures (Pilgeram et al., 2007). Camelina seed has an oil content of ~35% of dry mass for current varieties. Camelina oils are high (~50%) in polyunsaturated fatty acids (PUFA), especially α -linolenic (18:3), an omega-3 fatty acid, and are thus also attracting attention for use in the production of health-promoting foods and feeds. However, the oil profiles of available camelina varieties (Table 1) are

less than ideal for either food or non-food uses. For example, for use as biofuel and other products such as lubricants, it would be beneficial to increase the oxidative stability by reducing the content of highly unsaturated fatty acids (Jaworski and Cahoon, 2003). In contrast, these PUFAs are highly desirable from a nutritional standpoint. Currently, the main source of omega-3 vegetable oils is from flax. Camelina provides an attractive alternative to this type of oil in the food and feed markets (Pilgeram et al., 2007). Camelina seeds contain significant amounts of C20-24 very long-chain fatty acids (VLCFAs), primarily eicosenoic acid (20:1) and lesser amounts of erucic acid (22:1). These VLCFAs need to be reduced for food/feed uses.

Camelina is an allohexaploid species, which could potentially complicate the process of breeding for desired traits due to gene redundancy and their complementary effects (Kagale et al., 2014; Hutcheon et al., 2010; Kang et al., 2011). Plant biotechnology, especially the recent advent of the highly efficient and facile CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated protein 9) system for precision gene modifications, provides an effective tool (Lowder et al., 2015). Camelina can be easily transformed by agrobacterium using a floral vacuum infiltration method, with

Table 1
Fatty acid composition of a low-VLCFA camelina mutant compared with Suneson.

	16:0	18:0	18:1	18:2	18:3	20:0	20:1	20:2	20:3	22:0	22:1
Suneson	5.8	1.8	17.3	20.7	33.0	1.4	14.4	1.4	1.0	0.4	2.8
LE1914	6.7	3.0	22.4	24.1	35.1	0.8	6.1	0.7	0.5	0.3	0.4

Data show averages of seeds harvested from 6 plants.

transgenic seeds obtained in a relatively short period of time after infiltration (4–6 weeks) (Lu and Kang, 2008). This has made camelina a useful crop platform for biotechnology and translational research (Collins-Silva et al., 2011; Hines and Travis, 2016). Recent attempts of CRISPR/Cas9-induced gene mutagenesis in camelina have been successful (Jiang et al., 2017; Aznar-Moreno and Durrett, 2017; Morineau et al., 2017). Understanding genetic and biochemical mechanisms of oil biosynthesis is necessary to effectively modify plant oils for a wide range of applications (Napier and Graham, 2010).

In oilseeds, fatty acids synthesized from acetyl-CoA in the plastids are exported into the cytosol for triacylglycerol (TAG) assembly in the endoplasmic reticulum (ER) (Browse and Somerville, 1991). The newly made fatty acids, mainly oleic acid (18:1) and a small amount of palmitic acid (16:0) and stearic acid (18:0), are first incorporated into phosphatidylcholine (PC) by the *de novo* pathway (Kennedy, 1961). They may also be incorporated into PC by an acyl exchange mechanism known as “acyl editing” mediated primarily by the lysophospholipid acyl-CoA: acyltransferases (Bates et al., 2009, 2012). The oleic acid is further desaturated to form the polyunsaturated linoleic (18:2) and α -linolenic (18:3) acids by the fatty acid desaturases FAD2 and FAD3 in the endoplasmic reticulum (ER) using the phosphatidylcholine (PC) substrate, or elongated into 20:1 and 22:1 by the fatty acid elongase FAE1 (Li-Beisson et al., 2013). Several other biosynthetic enzymes such as phosphatidylcholine diacylglycerol cholinephosphotransferase (PDCT), lysophosphatidylcholine acyltransferases (LPCAT), and diacylglycerol acyltransferases (DGAT) may also affect the fatty acid composition of TAG by regulating the acyl flux through its precursors including PC and diacylglycerols (Bates et al., 2012; Lu et al., 2009, 2011; Marmon et al., 2017). In this study, we demonstrated that mutations at the *FAE1* genes in camelina significantly changed fatty acid composition by decreasing the VLCFA content. Knocking out all alleles of the *FAE1* genes by the CRISPR-Cas9 technology almost eliminated the accumulation of these fatty acids in camelina seeds.

2. Methods

2.1. Mutant isolation and characterization

Camelina (*Camelina sativa*) cv. Suneson was used in this study. Camelina seeds were treated with ethyl methanesulfonate (EMS) to induce mutations as described previously (Büchsen-Schütz-Nothdurft et al., 1998). The M2 generation of the treatments was grown in the field at the Arthur Post Agronomy Farm at Bozeman, MT. Seeds harvested from individual M2 plants were analyzed for fatty acid composition in a high-throughput fashion as described (Lu et al., 2006). Mutant lines showing altered fatty acid composition were collected and grown for another generation. Lines of homogeneity for the low 20:1 content were determined by analyzing at least 10 plants. These lines were chosen for further investigation and sequencing of the *FAE1* alleles. Genomic DNA was isolated from young camelina leaves using the method as described (Lukowitz et al., 2000).

2.2. CRISPR/Cas9 vector construction

The single guide RNA (sgRNA) targeting all three alleles of *Camelina sativa* Fatty Acid Elongase (*CsFAE1-A*, *Csa11g007400*; *CsFAE1-B*, *Csa10g007610*; and *CsFAE1-C*, *Csa12g009060*) (Hutcheon et al., 2010)

was designed by using gRNA prediction algorithms of Harvard CHOP-CHOP website (<http://chopchop.cbu.uib.no>) (Labun et al., 2016). The gRNA was selected according to the following criteria: (1) The proximity to the 5' end, (2) the coverage of all three *CsFAE1* genes, (3) the higher efficiency score, (4) the absence of predicted possible off-targets, (5) minimum self-complementary regions, (6) higher GC content, preferably, between 40 and 70% (Wang et al., 2014; Tsai et al., 2015). The best gRNA that obeys the criteria above was then tested with Cas-OFFinder web-based software (<http://www.rgenome.net/cas-offinder>) for off-target possibility (Bae et al., 2014). *CsFAE1* sgRNA was synthesized by Eurofins MWG Operon USA (Louisville, KY) as two oligonucleotides: gRNA-F, 5'-ATTGTTGGAGATGGGAATAGAAG-3', and gRNA-R, 5'-AAACCTTCTATTTCCCATCTC CAA-3' (sgRNA is underlined). Two oligos were mixed and incubated in a thermocycler at 95 °C for 5 min and then cooled down to 4 °C for 5 min. The *FAE1* gRNA was then cloned into the binary transformation vector pHEE401E (Xing et al., 2014; Wang et al., 2015) (obtained from Addgene; www.addgene.org/71827) by the Golden Gate cloning method (Engler et al., 2008). Finally, the sgRNA-Cas9 cassette was digested with *HindIII* and *EcoRI*, then the entire sgRNA-Cas9 cassette was ligated into the pBinGlyRed2 vector (Lu and Kang, 2008). In the final construct, sgRNA is driven by Arabidopsis U6-26 promoter (Li et al., 2007), then followed by gRNA scaffold and U6-26 terminator. Maize codon optimized Cas9 (*zCas9*) gene is under the control of the egg-cell specific enhancer (EC1.2en) and promoter (EC1.1pro), and transcription ends with *Pisum sativum* rbcS-9E terminator (Wang et al., 2015) (Fig. 1).

2.3. Camelina transformation

The above construct was transformed into *Agrobacterium* strain GV3101 (pMP90) by electroporation with 0.2 cm cuvettes (Bio-Rad, Hercules, CA, USA) at a field strength of 2.5 kv/cm, restorers of 600 Ω and a capacitance of 25 μ F. Selection of transformed bacteria was carried out on LB plates containing 50 μ g/ml kanamycin and 40 μ g/ml gentamycin. Camelina plants were grown in 6" pots (5 plants/pot) filled with 1:1 mix of MSU soil (equal parts by volume of loam soil: washed concrete sand: Canadian sphagnum peat moss with AquaGro, 2000 G wetting agent blended in at 1 lb/cubic yard of soil, aerated steam pasteurized at 70 °C for 1 h) and Sunshine Mix #1 (Bellevue, WA, USA). Greenhouse conditions were 22/18 $\pm$ 1 °C for day/night temperatures, a relative humidity of 30%, and a 16 h photoperiod of natural lighting supplemented when necessary by season. A well-established camelina transformation protocol developed at our laboratory was followed to generate transgenic plants (Lu and Kang, 2008). Transgenic DsRed-expressing seeds were screened by illuminating mature seeds with green LED lights and viewing through a red filter. Transgenic plants were confirmed by PCR using the U6-specific primers (Suppl. Table 1) and genomic DNA extracted from leaves of 15-day old plants by the CTAB method (Lukowitz et al., 2000).

2.4. Seed and fatty acid analyses

For fatty acid analysis, camelina seeds were crushed and lipids were transmethyated according to procedures described previously (Lukowitz et al., 2000). The resulting fatty acid methyl esters (FAMES) were analyzed in a gas chromatograph (GC) (Shimadzu Scientific Instruments, Columbia, MD) fitted with a 15 m $\times$ 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.

Seed weight was determined by weighing 100 seeds. Seed oil content was determined by a bench-top nuclear magnetic resonance (NMR) seed analyzer (MQC23, Oxford Instruments, Concord, MA, USA) and by GC analysis using heptadecanoic acid (17:0) as internal standard (10 mg/ml), which was added to test tubes prior to FAMES

Fig. 1. Mutagenesis in the *FAE1* alleles in *Camelina sativa*. (A) An EMS-induced mutation is shown on top at nucleotide no. 448 from the start codon ATG in the *FAE1-B* gene. Single-letter amino acids are encoded by *FAE1-B*. The sgRNA sequence targeting all three *FAE1* genes (on the reverse strands) is shown at the bottom. Asterisks indicate identical nucleotides. PAM: protospacer adjacent motif. (B) T-DNA region (between the left/right borders) of the CRISPR/cas9 construct. The *FAE1* sgRNA gene is bracketed by the *Arabidopsis thaliana* U6-26 small nuclear RNA promoter (U6-26 pro) and terminator (U6-26 term). zCas9 (*Zea mays* codon optimized) is driven by an egg cell-specific 1.2 enhancer (EC1.2 en) and an egg cell-specific 1.1 promoter (EC1.1 pro), and the translation stopped by a *Pea sativum* Rubisco small subunit 9E terminator (RbcS-9E term). The selection marker DsRed is driven by the cassava vein mosaic virus promoter (CVMV pro).

derivatization. All seeds used for comparison of oil content and seed traits were harvested from plants grown at the same time in the greenhouse.

3. Results and discussion

3.1. A low-VLCFA camelina line contains a mutation at the *FAE1* locus

In our previous experiments to isolate high-oleic camelina mutants, we screened an M2 population derived from ethyl methanesulfonate (EMS)-treated seeds (cultivar Suneson) and obtained several lines that have altered seed fatty acid profiles (Kang et al., 2011). One mutant line, which we designated as LE1914, showed a moderate increase in oleic acid (18:1). More significantly, the mutant seed contained reduced levels of very long-chain fatty acids comprising primarily of eicosenoic acid (20:1) and erucic acid (22:1). Compared to Suneson, which contained 17.2% of these fatty acids, the level decreased to only 6.5% in LE1914 (Table 1). The accumulation of eicosanoic acid (20:0) and other minor VLCFAs, was also significantly reduced in the mutant.

In oilseeds, VLCFA synthesis is catalyzed by the Fatty Acid Elongase1 (*FAE1*) acting on 18:1-CoA (Coenzyme A) (Millar and Kunst, 1997). *Camelina sativa* is a close relative to *Arabidopsis thaliana* (Beilstein et al., 2006; Al-Shehbaz et al., 2006). Like Camelina, *Arabidopsis* seed contains high amounts of VLCFAs, predominantly 20:1 (about 21% w/w of total fatty acids). The *Arabidopsis* mutant *fae1* contains negligible amount (< 1%) of 20:1 in seed oils (Kunst et al., 1992). It has been shown previously that camelina contains three *FAE1* genes and they share very high sequence similarity between these two species (Hutcheon et al., 2010). We sequenced the homology *CsFAE1* genes, *FAE1-A* (Csa11g007400), *FAE1-B* (Csa10g007610) and *FAE1-C* (Csa12g009060), in camelina wildtype (Suneson) and the LE1914 mutant. The *FAE1-B* locus in the LE1914 had a nucleotide change at #448 (C to T), which would convert the codon CAA for amino acid

Glutamine (Gln) into a stop codon TAA (Fig. 1). This result suggested that a single *FAE1* gene mutation caused over 60% reduction in the 20:1 + 22:1 content compared to the wildtype. There was no mutation detected at the other two *FAE1* genes, which are presumably responsible for the remaining VLCFAs in the mutant seed.

3.2. *Cas9* transgenic plants targeting *FAE1* genes caused decreased accumulation of VLCFAs

Characterization of the LE1914 mutant suggests that three *FAE1* genes may function in an additive fashion in camelina seeds. To better understand *FAE1* enzymes in the polyploidy camelina seeds, we attempted to create *FAE1* mutants by the CRISPR/Cas9 technology targeting all three alleles of the *CsFAE1* genes.

Sequences of the 1518bp-long *FAE1* genes (*CsFAE1-A*, *-B*, *-C*) are highly similar to one other (more than 96% identical) and to their orthologs from *Arabidopsis* (Hutcheon et al., 2010). To ensure that gene mutations would cause the loss of enzyme activities, we designed the single guide RNA (sgRNA) sequence near the 5'-end of the *FAE1* coding region (Fig. 1A). The sgRNA sequence was selected based on the camelina genome sequence to avoid potential off-target sites. Nearby single nucleotide polymorphisms (SNPs) between the three genes were present. These SNPs along with sequence variations within the promoter regions (Suppl. Fig. S1) would help determine whether all genes were efficiently targeted and mutated by the Cas9/gRNA complex.

The sgRNA sequence was synthesized as two complementary oligonucleotides and inserted into pHEE401E transformation vector. The sgRNA-Cas9 cassette was sub-cloned into the pBinGlyRed2 vector to use the DsRed as the selection marker. This construct was transformed into camelina plant (Suneson) using the *Agrobacterium*-mediated vacuum infiltration method. We obtained 14 transgenic seeds showing strong DsRed expression, which were successfully germinated in soil for further analysis.

Fig. 2. Eicosenoic acid (20:1) content in single seeds of wild type (WT) and transgenic camelina. Seeds no. 1–5 (white bars) show wild type. Red (A) and brown (B) seeds harvested from 14 T1 transgenic lines are shown in black bars.

Seeds were harvested from all 14 independent T1 lines that showed DsRed segregation indicating single-transgene inserts (Suppl. Table 2). To evaluate the Cas9-induced mutations on *FAE1* genes, five individual T2 seeds showing DsRed expression from each transgenic line were analyzed for their fatty acid composition by gas chromatography (Lu et al., 2006), and compared with non-transgenic seeds of Suneson that had been grown together with the transgenic plants in the greenhouse. We focused on the content of 20:1 since it's the most abundant VLCFA synthesized by the *FAE1* enzyme in camelina. The wild type seeds accumulated about 14% 20:1 of total fatty acids (Seeds no. 1–5, Fig. 2). Of the 70 red seeds analyzed, 19 appeared to be wild type that had 12–14% of 20:1. The other 51 low-20:1 putative mutant seeds were from every transgenic line, showing a wide range of variation (ranging from 0.6 to 11.4% w/w) (Fig. 2A). This result suggested that *FAE1* genes have possibly been mutated by CRISPR/Cas9 in transgenic plants, and that *FAE1* genes are responsible for most of 20:1 accumulation in camelina seed. The transgenic seeds fell into three major groups according to the level of 20:1 (around 5–11%, 2–5%, and < 2%). These might reflect the number (1, 2 and 3) of *FAE1* genes that were disrupted in their genomes.

Germline mutation was expected since the *Cas9* gene was driven by an egg cell-specific promoter. We speculated that *FAE1* mutations might have occurred in brown (*Cas9*-negative) seeds, as well as in red seeds. We then analyzed five single brown seeds from each of the above 14 transgenic lines. As shown in Fig. 2B, low 20:1 seeds were detected from 7 lines, though at a lower frequency (20/70). Specifically, all seeds from line #3 had less than 1% of 20:1, indicating that this line had already reached homozygosity of the *fae1* knockout mutation. Our results thus demonstrated that homozygous camelina mutants could be obtained in a single generation, as previously reported for *Arabidopsis* using the same egg cell-specific promoter-driven CRISPR/Cas9 system (Wang et al., 2015).

3.3. Mutations at the *FAE1* alleles in the *Cas9* transgenic camelina plants

To assess the efficacy of Cas9-induced mutagenesis at the *FAE1* gene sequences, genomic sequences near the sgRNA sites were examined in transgenic plants. DNA fragments flanking the three *FAE1* genes present

in the A, B and C subgenomes of the allohexaploid camelina were amplified by three pairs of gene-specific primers (Suppl. Fig. S1). PCR products from transgenic plants for all three genes were indistinguishable in size from those amplified from Suneson, suggesting no large insertion/deletion occurred at *FAE1* alleles by the Cas9/sgRNA expression. Sequencing of the three PCR fragments amplified from the wildtype plants resulted in sequences identical to the previously published (Kagale et al., 2014; Hutcheon et al., 2010), indicating that the primers were able to specifically amplify the three pairs of the *FAE1* alleles in the camelina subgenomes.

For each *FAE1* gene, 7 of the 14 T1 plants did not contain any nucleotide changes at the target sites. In the remaining 7 transgenic T1 plants, nucleotide deletions or substitutions were detected at the sgRNA target sites in at least one of the three *FAE1* genes (Suppl. Fig. S2). We planted 10 red seeds from each T1 line to grow into T2 plants. Leaf samples of each plant were collected and stored in -80°C for later analysis by extracting genomic DNA and sequencing. Putative homozygous T2 lines, determined by all red seeds they produced, were obtained. We selected one homozygous plant from each of the T2 lines to examine the Cas9-induced mutations at the *FAE1* genes. These plants were confirmed to have the *Cas9* transgenes by PCR using the U6-specific primers (Suppl. Table 1). Sequencing results indicated that nucleotide deletions at the predicted locations near PAM sites occurred in 13 of the 14 lines for each of the *FAE1* genes. Nucleotide substitutions were also detected in 10 plants for each gene (Suppl. Fig. S3).

The high frequency of mutagenesis in the T2 plants compared to T1 plants was consistent with previous studies (Jiang et al., 2017; Morineau et al., 2017), suggesting continued Cas9/sgRNA actions as transgenic plants were advanced to the next generations. Combining with fatty acid analysis (Fig. 2), our sequencing results also indicated that homozygous Cas9-transgenic camelina plants carrying mutations at all three *FAE1* alleles have already been obtained in the T2 generation. Although the mutations affected all *FAE1* genes in 13 T2 lines, results from Fig. 2 suggested that not all mutations would cause *FAE1* inactivation. Some of the nucleotide deletions or substitutions (Suppl. Fig. S3) that led to deletion or substitution of 1–2 amino acids in the translated *FAE1* proteins may not significantly affect their enzyme activities, therefore near or equal to wild type-level 20:1 contents were

<i>FAE1-A</i> (Csa11g007400)		
WT	CCTCAAACGATCTCTACC <u>ACTTCTATTCCCATCTCCAACACAACCTT</u> A TAAACCGTAATTT	
	Y H F Y S	
Line 3-3-1	CCTCAAACGATCTCTACCAC-----TTCCCATCTCCAACACAACCTTATAACCGTAATTT	-5
Line 3-3-3	CCTCAAACGATCTCTACC C -- T TATTCCCATCTCCAACACAACCTTATAACCGTAATTT	-2
Line 3-3-4	CCTCAAACGATCTCT-CC-CTT C T TATTCCCATCTCCAACACAACCTTATAACCGTAATTT	-2, +2
	S L L Y S	
Line 3-3-14	CCTCAAACGATCTCTACCAC--CTATTCCCATCTCCAACACAACCTTATAACCGTAATTT	-2
<i>FAE1-B</i> (Csa10g007610)		
WT	CAGCAAACGATCTCTACC <u>ACTTCTATTCCCATCTCCAACACAACCTT</u> A TAAACCGTAATTT	
Line 3-3-1	CAGCAAACGATCTCTACC A CTT-TATTCCCATCTCCAACACAACCTTATAACCGTAATTT	-1
Line 3-3-3	CAGCAAACGATCTCT-----CATCTCCAACACAACCTTATAACCGTAATTT	-14
Line 3-3-4	CAGCAAACGATCTCTACC C CT--TATTCCCATCTCCAACACAACCTTATAACCGTAATTT	-2
Line 3-3-14	CAGCAAACGATCTCTA-----CATCTCCAACACAACCTTATAACCGTAATTT	-13
<i>FAE1-C</i> (Csa12g009060)		
WT	CCACAAACGATCTCTACC <u>ACTTCTATTCCCATCTCCAACACAACCTT</u> G TAAACCGTAATTT	
Line 3-3-1	CCACAAACGATCTCTACC A CT-----TCCCATCTCCAACACAACCTTGTAACCGTAATTT	-5
Line 3-3-3	CCACAAACGATCTCTACC A CTT-TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT	-1
Line 3-3-4	CCACAAACGATCTCTACC A CTT-TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT	-1
Line 3-3-14	CCACAAACGATCTCTACC A CT--TATTCCCATCTCCAACACAACCTTGTAACCGTAATTT	-2

Fig. 3. Comparison of sequences surrounding the sgRNA sequence in the camelina *FAE1* genes. In WT sequences, bold letters distinguish nucleotide polymorphism between different *FAE1* alleles. The gRNA sequences are underlined. PAM sites on reverse strands are italicized. In transgenic lines, red letters indicate nucleotide substitution; green letters show nucleotide insertion. Deletions are indicated by dashes and the number of deleted nucleotides, and insertions are indicated by + number. Partial sequences of amino acids of the translated *FAE1-A* proteins are also shown under the nucleotide sequences.

Fig. 4. Comparison of fatty acid composition between the best performing Cas9 transgenic seeds and the non-transgenic wildtype camelina (Suneson). Data are means and standard errors from 5 single seeds harvested from 3 individual T3 plants grown in the greenhouse at the same time.

measured in those lines.

We reasoned that the seeds containing the lowest amounts of VLCFAs may have been caused by knockout mutations at all *FAE1* alleles. To test this possibility, we selected a putative homozygous T2 line (#3-3) that all 5 red seeds analyzed contained low amounts of 20:1. We excised small chips from the cotyledons of 20 red seeds for analyzing fatty acid composition. The seeds contained less than 1% of 20:1 were selected to germinate and gave rise to T3 plants (3-3-1, 3-3-3, 3-3-4 and 3-3-14) for sequence analysis. As expected, all three alleles of *FAE1* were mutated at the sgRNA target sites, primarily by deletions near the PAM sites that would cause translational frameshift and resulted in *FAE1* enzyme inactivation (Fig. 3). An exception was in the *FAE1-A* gene in line 3-3-4, where the deletion was compensated by the insertion of two nucleotides so that the gene might still be translated into an

intact protein. However, the substitution of the amino acids Tyr-His-Phe in the wildtype *FAE1-A* with Ser-Leu-Leu might render the mutant enzyme inactive.

Due to limited number of sequences generated in this study, we did not attempt to assess the overall efficiency or the nature of the mutants (such as insertions, deletions, or nucleotide substitutions), which have been extensively evaluated in previous studies in camelina (Jiang et al., 2017; Morineau et al., 2017). However, our results clearly indicated that the Cas9/sgRNA effectively induced gene mutations at the *FAE1* targets, and the desired fatty acid changes can be obtained from a small number (e.g. 14) of transgenic plants. Homozygous knockout mutants of multiple genes in the allohexaploid camelina can be obtained as early as the T2 generation by using an egg-cell specific promoter for Cas9/sgRNA expression.

3.4. Improving fatty acid composition in camelina seeds using *FAE1* mutagenesis

Analyses of fatty acid composition of the Cas9-transgenic homozygous seeds indicated that knocking out all the *FAE1* alleles significantly reduced the VLCFAs. In one of the best performing lines (#3-3), these C20 fatty acids comprised of less than 1.5% of total fatty acids compared to over 22% in the wildtype seeds (Fig. 4). This result was similar to the *Arabidopsis fae1* mutant (Kunst et al., 1992). The small amounts of VLCFAs in these mutants are possibly due to actions by other factors that are non-allelic to *FAE1* (Kunst et al., 1992). The decrease of VLCFAs caused concomitant increases in other fatty acids (C16 and C18), especially the unsaturated fatty acids including oleic (18:1), linoleic (18:2) and α -linolenic (18:3) acids. This is expected since the elongation and desaturation are the two major pathways for 18:1 modification. Knocking out *FAE1* would block the elongation pathway and direct fatty acyl flux toward desaturation. Our results demonstrate that Cas9/sgRNA targeting the *FAE1* genes can be effectively applied to make changes in camelina seed for favorable fatty acid compositions. For instance, one of the desirable traits in camelina seeds is its high concentration of α -linolenic acid (18:3) for food and feed application. Eliminating VLCFAs increased 18:3 content from 39% in Suneson to 50% in the best Cas9 transgenic seed grown in our greenhouse conditions (Fig. 4).

Shutting off the 18:1 elongation pathway by knocking out *FAE1* may provide a tool to increase the oleic acid content for food and industrial applications. Reduction of 18:1 desaturation may be achieved by decreasing the FAD2 activity in camelina seed by RNA interference (RNAi) (Kang et al., 2011; Nguyen et al., 2013) or CRISPR/Cas9 technologies (Jiang et al., 2017; Morineau et al., 2017). Simultaneously knocking out all three *FAD2* genes in camelina is however not feasible since unsaturated fatty acids are essential for plant growth. The *Arabidopsis fad2* mutant failed to survive at low temperatures (Miquel et al., 1993). Camelina *fad2* knockout mutants created by CRISPR/Cas9 showed stunted growth (Morineau et al., 2017). It may be possible to target two of the *FAD2* genes that are predominately expressed in seeds (Lowder et al., 2015) using gene-specific sgRNAs. By contrast, the camelina *FAE1* knockout mutants did not show noticeable undesirable effects on seed physiology and plant growth. Seed germination and plant growth were comparable to Suneson when growing in normal greenhouse conditions. We analyzed transgenic T3 seeds for seed weight and oil content. These traits were similar to wildtype Suneson (Suppl. Fig. S4), and the mutant seeds were visually indistinguishable from the wild type. It therefore appears that *FAE1* genes, which had been shown to be exclusively expressed in seed in *Arabidopsis* (Rossak et al., 2001) and camelina (Suppl. Table S3) (Kagale et al., 2016), are not essential for plant growth. We will further assess the effects of *FAE1* mutations on plant performance in field conditions using homozygous *fae1* knockout plants that are free of Cas9/sgRNA and DsRed transgenes.

4. Conclusion

The oilseed crop camelina has great potentials in food and non-food applications. It is necessary to improve fatty acid composition in its oils to meet different requirements. In this study, we investigated the usage of the Fatty Acid Elongase1 (*FAE1*) in camelina by traditional mutagenesis and new CRISPR/Cas9 approaches. A significant decrease in very long-chain fatty acids in a *FAE1-B* mutant suggested that the three *FAE1* genes in camelina are responsible for synthesizing these VLCFAs and they may act in an additive fashion. By designing a sgRNA targeting all three *FAE1* genes using the CRISPR/Cas9 technology, we obtained the knock-out mutants that accumulated very small amounts of VLCFAs without causing deleterious effects on seed traits and plant growth. Homozygous mutant plants were successfully obtained in a single generation by using an egg cell-specific expression of the Cas9/sgRNA

transgenes. Our results demonstrated the high efficiency of the CRISPR/Cas9 technology in a hexaploid oilseed crop to simultaneously edit the homologous genes. The *FAE1* knock-out camelina seeds had significantly changed fatty acid profiles especially increased C18 unsaturated fatty acids, thus provide ways to breed high oleic acid or high α -linolenic acid varieties for food or industrial applications.

Contribution

M.O., X.M., C.L. designed experiments; M.O., X.M., J.K. conducted research; M.O. and C.L. wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.plaphy.2017.11.021>.

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