2015

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Kim, Hae Jin; Silva, Jillian E.; Vu, Hieu Sy; Mockaitis, Keithanne; Nam, Jeong-Won; and Cahoon, Edgar B., "Toward production of jet fuel functionality in oilseeds: identification of FatB acyl-acyl carrier protein thioesterases and evaluation of combinatorial expression strategies in *Camelina* seeds" (2015). *Faculty Publications from the Center for Plant Science Innovation*. Paper 103.  
http://digitalcommons.unl.edu/plantscifacpub/103

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Toward production of jet fuel functionality in oilseeds: identification of FatB acyl-acyl carrier protein thioesterases and evaluation of combinatorial expression strategies in Camelina seeds

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Received 23 March 2015; Revised 23 March 2015; Accepted 9 April 2015

Editor: Rowan Sage

Abstract

Seeds of members of the genus Cuphea accumulate medium-chain fatty acids (MCFAs; 8:0–14:0). MCFA- and palmitic acid- (16:0) rich vegetable oils have received attention for jet fuel production, given their similarity in chain length to Jet A fuel hydrocarbons. Studies were conducted to test genes, including those from Cuphea, for their ability to confer jet fuel-type fatty acid accumulation in seed oil of the emerging biofuel crop Camelina sativa. Transcriptomes from Cuphea viscosissima and Cuphea pulcherrima developing seeds that accumulate >90% of C8 and C10 fatty acids revealed three FatB cDNAs (CpuFatB3, CvFatB1, and CpuFatB4) expressed predominantly in seeds and structurally divergent from typical FatB thioesterases that release 16:0 from acyl carrier protein (ACP). Expression of CpuFatB3 and CvFatB1 resulted in Camelina oil with capric acid (10:0), and CpuFatB4 expression conferred myristic acid (14:0) production and increased 16:0. Co-expression of combinations of previously characterized Cuphea and California bay FatBs produced Camelina oils with mixtures of C8–C16 fatty acids, but amounts of each fatty acid were less than obtained by expression of individual FatB cDNAs. Increases in lauric acid (12:0) and 14:0, but not 10:0, in Camelina oil and at the sn-2 position of triacylglycerols resulted from inclusion of a coconut lysophosphatidic acid acyltransferase specialized for MCFAs. RNA interference (RNAi) suppression of Camelina β-ketoacyl-ACP synthase II, however, reduced 12:0 in seeds expressing a 12:0-ACP-specific FatB. Camelina lines presented here provide platforms for additional metabolic engineering targeting fatty acid synthase and specialized acyltransferases for achieving oils with high levels of jet fuel-type fatty acids.

Key words: Camelina, Cuphea, FatB acyl-ACP thioesterase, jet fuel oilseed, medium-chain fatty acid.

Introduction

Medium-chain fatty acids (MCFAs), including caprylic acid (8:0), capric acid (10:0), lauric acid (12:0), and myristic acid (14:0), are important for industrial production of detergents, soaps, cosmetics, surfactants, and lubricants (Knaut and Richtler, 1985; Dyer et al., 2008). These fatty acids, along with palmitic acid (16:0), also have potential for use as
feedstocks for the hydrocarbon component of Jet A and Jet A-1 fuels, which are composed primarily of C8–C16 alkanes and aromatic hydrocarbons (Hemighaus et al., 2006; Kallio et al., 2014). The main commercial sources of plant-derived MCFAs are oils from tropical plants including palm kernel (Elaeis guineensis Jacq.) and coconut (Cocos nucifera L.), which are enriched in lauric acid (46–52 mol% of total fatty acids) and myristic acid (16–19 mol% of total fatty acids). Among the few temperate sources of MCFAs are seeds from members of the Cuphea genus, some of which produce >90% of a single MCF (Graham and Kleiman, 1992; Graham, 1998). Examples of Cuphea species with seeds that accumulate high levels of MCFAs include Cuphea viscosissima, with seeds that contain ~25 mol% 8:0 and ~64 mol% 10:0, and Cuphea pulcherrima, with seeds that contain ~95 mol% 8:0. Cuphea oil showed favourable fuel properties for biodiesel in functionality testing due to its high content of MCFAs (Geller et al., 1999; Knothe et al., 2009; Lovestead et al., 2010; Knothe, 2014). Even though many Cuphea species have been characterized as the potential oil crop for MCFAs, non-desired traits such as indeterminate flowering, seed shattering, seed dormancy, viscid and glandular trichomes in vegetative tissues and flowers, and open pollination have limited attempts to domesticate Cuphea species for agronomic production (Olejniczak, 2011). Therefore, Cuphea species have been considered as valuable genetic resources to isolate genes that encode specialized biosynthetic enzymes for transgenic production of MCFAs in established oilseed crops (Dehesh et al., 1996a, b; Leonard et al., 1997, 1998; Slabaugh et al., 1998; Filichkin et al., 2006).

The synthesis of MCFAs is a variation on typical de novo fatty acid synthesis that occurs in plants that generates primarily C16 and C18 fatty acids. De novo fatty acid biosynthesis occurs in the plastids in plants and is initiated by the condensation of acetyl-coenzyme A (CoA) and malonyl-acetyl carrier protein (ACP) by the β-ketoacyl-ACP synthase III (KASIII) to produce a four-carbon β-ketoacyl-ACP. Fatty acids are elongated by sequential condensation of two carbon units from malonyl-ACP by the co-operation of enzymes of fatty acid synthase (FAS). Following its synthesis, 16:0-ACP can be further elongated to 18:0-ACP by KASII or is hydrolysed by acyl-ACP thioesterase to generate free palmitic acid that is exported from plastids. Hydrolysis of 16:0-ACP is catalysed primarily by FatB thioesterases, whereas hydrolysis of ACP esters of the C18 fatty acids, stearic acid (18:0) and oleic acid (18:1), is catalysed principally by FatA thioesterases. As such, acyl-ACP thioesterases are major determinants of carbon chain lengths of fatty acid (Li-Beisson et al., 2013). Divergent FatB enzymes with substrate specificities for saturated fatty acids with chain lengths less than C16 are responsible for the synthesis of MCFAs in seeds of Cuphea species as well as species such as California bay (Umbellularia californica). These divergent FatBs have been used to confer MCFA production to Brassica napus and Arabidopsis thaliana by transgenic expression (Pollard et al., 1991; Voelker et al., 1992; Jones et al., 1995; Dehesh et al., 1996b; Eccleston et al., 1996; Tjellström et al., 2013). An alternative fate to release of 16:0 from ACP by typical FatB thioesterase is elongation of 16:0-ACP to 18:0-ACP, which is initiated by KASII activity. Mutagenesis or RNA interference (RNAi) suppression of KASII genes has been shown to be an effective way of generating 16:0-rich oils, which could potentially contribute to bio-based jet fuel (Pidkowich et al., 2007).

Jet A fuel-type fatty acids (MCFAs and 16:0) released from the plastid of oilseeds must be esterified onto glycerol backbones in the endoplasmic reticulum (ER) for sequestration in triacylglycerol (TAG). Key to achieving high levels of Jet A fuel-type fatty acid accumulation in TAG is the acyltransferases glycerol-3-phosphate acyltransferase (GPAT), lysophosphatic acid acyltransferase (LPAT), and diacylglycerol acyltransferase (DGAT) that catalyse the sequential addition of CoA esters of MCFAs and 16:0 to the TAG glycerol backbone (Thelen and Ohlrogge, 2002; Cahoon et al., 2007; Dyer et al., 2008; Lu et al., 2011). To obtain levels of MCFA and 16:0 accumulation at levels found in seed TAG of many Cuphea species, these fatty acids must be introduced at all stereospecific positions of TAG (Knutzon et al., 1999; Dehesh, 2001; Roscoe, 2005; Burgal et al., 2008; Snyder et al., 2009). Limiting this in most oilseed crops is the strict substrate specificity of LPAT for unsaturated acyl-CoAs, such as oleoyl (18:1)-CoA (Kim et al., 2005; Nlandu Mputu et al., 2009). As such, a major target for engineering high levels of MCFA and 16:0 accumulation in engineered oilseeds is the identification of LPATs and possibly other acyltransferases with specificity for CoA esters of these saturated fatty acids.

In this report, new divergent FatB genes have been identified from transcriptomes of developing seeds from C. viscosissima and C. pulcherrima. The emerging oilseed crop [Camelina sativa (L.) Crantz] was used to characterize FatB genes, with the goal of generating Jet A and Jet A-1 fuel-type fatty acid compositions in vegetable oils. Camelina is a member of the Brassicaceae family and is currently being developed as a non-food oilseed for biofuel, industrial, and high-value specialty oil traits (Iskandarov et al., 2014). Camelina is especially attractive as a biotechnological crop because it is readily transformed by floral Agrobacterium infiltration (Lu and Kang, 2008; Liu et al., 2012). In addition, Camelina is productive in the climate of the North American Great Plains and Pacific Northwest, characterized by limited rainfall and marginal soil fertility. These properties together with its short 100–120 d life cycle have made Camelina attractive for production in fallow seasons and in double-cropping systems (Iskandarov et al., 2014). Camelina seeds are also oil rich (30–40% of seed weight), but the oil contains high levels of polyunsaturated fatty acids, which reduce the biofuel quality of Camelina oil because of their oxidative instability (Johnson et al., 2011; Campbell et al., 2013; Iskandarov et al., 2014). Genetic improvement of Camelina for biofuel and industrial and specialty oil traits has been facilitated in part by the recent release of a genome sequence and seed transcriptomes that include compilation of oil and seed storage gene databases (Nguyen et al., 2013, 2014; Kagale et al., 2014).

In these studies, the newly identified Cuphea FatB genes as well as previously reported FatB genes from several Cuphea species and California bay were expressed individually and in
combination in Camelina seeds to generate oils with a range of MCFA compositions. Co-expression studies of divergent FatB genes with a specialized LPAT from coconut were also conducted to evaluate the effectiveness of this strategy for increasing MCFA accumulation in Camelina seed TAG. RNAi-mediated down-regulation of Camelina KASII was also tested as a means of increasing accumulation of MCFAs as well as 16:0 in seeds from Camelina lines engineered for overexpression of functionally divergent FatBs.

Materials and methods

Plant material, growth, and transformation conditions

Camelina sativa (variety Suneson) seeds were sown into 81 cm² plastic pots containing Fafard Germination Mix media (Hummert International, Saint Louis, MO, USA). Ambient light was supplemented in greenhouses with a combination of metal halide and high pressure sodium lights with a 14:10 light:dark cycle. Plants were grown in a greenhouse at 24 °C to 26 °C, and night temperatures ranged from 18 °C to 20 °C. When outdoor temperatures were >29 °C, supplemental lights were shut off to reduce the need for extra cooling.

Agrobacterium tumefaciens-mediated transformation was used. Plants were transformed by floral dip/vacuum infiltration, and DsRed (Discosoma sp. red fluorescent protein) was used as a visual selection marker (Lu and Kang, 2008). Segregation patterns of T1 seed were used to determine lines containing a single T-DNA insertion, and homozygous lines were subsequently isolated as the T2 progeny of single-insert plants having 100% of seed showing red fluorescence.

Binary vectors

Previously reported binary vectors were used for expression of CpuFatB3, CpuFatB2, UcFatB1, or ChFatB2 in Camelina seeds (Tjellström et al., 2013). Full-length cDNAs for CpuFatB1 and CpuFatB4 genes from C. pulcherrima developing seed and CvFatB1 and CvFatB3 from C. viscosissima developing seed cDNA were amplified by PCR using the oligonucleotide primers with added EcoRI and XbaI restriction sites. The EcoRI- and XbaI-digested fragments containing the genes were inserted into the corresponding sites of the pBinGlyRed3 binary expression vector which contains the strong seed-specific soybean glucinin-1 promoter and a DsRed marker gene (Zhang et al., 2013). The primer sequences are provided in Supplementary Table S1 available at JXBl online. Primers were designed to amplify the open reading frames (ORFs) of those from developing seeds, and product sequences were confirmed by sequencing.

RNA isolation and cDNA synthesis for RT-PCR

Total RNA was isolated from Cuphea roots, stems, leaves, flowers, and developing seeds using a RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) with methods slightly modified from those described previously (Chang et al., 1993). A pre-heated 10 ml of extraction buffer (2% (w/v) cetyltrimethylammonium bromide (CTAB), 2% (w/v) polyvinylpyrrolidone (PVP), 2M NaCl, 100 mM TRIS-HCl pH 8.0, 25 mM EDTA pH 8.0, and 0.05% (w/v) spermidine) was added to the sample (200–300 mg) ground in liquid nitrogen, mixed, and incubated at 65 °C for 10 min. An equal volume of chloroform was added, mixed, and centrifuged at 15 000 g for 10 min at 4 °C. One-third volume of 8 M LiCl was added to the supernatant and incubated on ice overnight. RNA was collected by centrifugation at 15 000 g for 1 h at 4 °C. The pellet was resuspended in 500 μl of RLT buffer, and DNAse I treatment was carried out according to the manufacturer’s protocol. First-strand cDNA was synthesized from 2 μg of total RNA using a RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific, Waltham, MA, USA) with an oligo(dT) primer.

Fatty acid analysis of seed oils

Fatty acid methyl esters (FAMEs) were generated by grinding 10 mg of dry seeds in 2 ml of 2.5% H2SO4 (v/v) in methanol including 900 μg of tri 17:0-TAG (Nu-Chek Prep, Elysian, MN, USA) in toluene (10 mg ml–1) as an internal standard and heated for 45 min at 90 °C in tightly capped tubes. Following cooling, 1.5 ml of water and 1.5 ml of hexane were added to the tubes and mixed. The organic phase was transferred to autosampler vials and analysed on an Agilent Technologies 7890A gas chromatograph fitted with a 30 m length×0.25 mm inner diameter HP-INNOWax column (Agilent, Santa Clara, CA, USA) using H2 carrier gas. The gas chromatograph was programmed for an initial temperature of 90 °C (1 min hold) followed by an increase of 30 °C min–1 to 235 °C and maintained for a further 5 min. Detection was achieved using flame ionization.

Analysis of the sn-2 position of TAG

Total neutral lipid was extracted from seeds using a modification of the Bligh–Dyer method (Bligh and Dyer, 1959). A 30 mg aliquot of Camelina seeds was ground in 3 ml of methylchloroform (2:1 v/v) with 2.7 mg of C17-TAG as an internal standard. Homogenized samples were incubated for 30 min to 1 h at room temperature with agitation, and lipids were partitioned and extracted as described (Cahoon et al., 2006). Total lipids were re-dissolved in 1 ml of heptane. To purify TAG, a 3 ml Supelco Supel Clean LC-Si SPE column (Sigma-Aldrich, Saint Louis, MO, USA) was used. The column was equilibrated with 5 ml of heptane. The total lipid sample in heptane was added to the column and drained into the column bed. To remove extra wax ester, 1.5 ml of heptane:diethyl ether (95:5, v/v) was added to the column and this fraction was discarded. Then, 5 ml of heptane:diethyl ether (80:20, v/v) was added and this fraction was collected as TAG. Analysis of TAG sn-2 fatty acids was conducted using lipase digestion as described (Cahoon et al., 2006).

Phylogenetic tree of Cuphea FatB amino acid sequence clusters

A phylogenetic tree was generated by MEGA6 software, using the minimum-evolution method with 1000 bootstrap replications (Tamura et al., 2013). Multiple sequence alignments were produced using ClustalW (http://www.genome.jp/tools-bin/clustalw). The MSF-formatted alignment was then analysed using the Genedoc sequence analysis program (Nicholas et al., 1997).

454 transcriptome sequencing and transcriptome assembly

Total RNA was extracted from developing seeds (between 14 d and 18 d after pollination) of C. pulcherrima and C. viscosissima using a method as described (Suzuki et al., 2004) without 8 M LiCl treatment. mRNAs were purified from ~1 mg of total RNA by two passes through oligo(dT)–cellulose columns by use of the Illustra mRNA purification kit (GE Healthcare, Pittsburgh, PA, USA). A sequencing library optimized for Roche/454 GS FLX Titanium sequencing was prepared from oligo(dT)-enriched mRNA according to custom protocols used previously (Nguyen et al., 2013). To reduce the number of high copy transcripts, amplified dsDNA library intermediates were partially normalized using Trimmer Direct (Evrogen) protocols. Emulsion PCR and sequencing were performed according to the manufacturer (Roche/454 Life Sciences). High quality sequence reads were trimmed (https://sourceforge.net/projects/estclean/) and assembled using Newbler v2.0.

Acyl-ACP analysis

Acyl-ACPs were extracted as previously described (Bates et al., 2014). The enriched acyl-ACP samples were treated with Asp-N endoproteinase
Kim et al. (Sigma-Aldrich) at a 1:50 protein ratio, and incubated at 37 °C for 2 h. Methanol was added to a final concentration of 50% after enzyme digestion. Mass spectrometry analyses were conducted using a 4000 QTRAP (Applied Biosystems) LC-MS/MS as described (Bates et al., 2014).

Results

Fatty acid profile of Cuphea seeds and leaves

Seeds of C. pulcherrima and C. viscosissima accumulate 94 mol% of 8:0, and 17 mol% of 8:0 and 70 mol% of 10:0 fatty acids, respectively (Graham and Kleiman, 1992; Phippen et al., 2006). To confirm the fatty acid profile of Cuphea seeds and to compare their profile with other tissues, the fatty acid composition was analysed in mature dried seeds and leaves. Seeds and leaves of C. pulcherrima and C. viscosissima had very different fatty acid compositions (Fig. 1). As previously reported, 8:0 accounted for nearly 95 mol% of fatty acids in C. pulcherrima seeds, while the leaves contained primarily 16:0 and large amounts of unsaturated long fatty acids, including 18:3, which made up 61 mol% of the total fatty acids. Similar results were observed in C. viscosissima (Fig. 1). Nearly 90 mol% of the fatty acids in C. viscosissima seed consisted of 8:0 and 10:0, but leaves were enriched in 18:3 in amounts similar to those in C. pulcherrima leaves. This result confirmed that seeds of C. pulcherrima and C. viscosissima are excellent genetic resources for caprylic acid (8:0) and capric acid (10:0).

Transcriptomic analyses using 454 pyrosequencing was conducted on normalized cDNAs from C. pulcherrima and C. viscosissima developing seeds to identify specialized FatBs and metabolic enzymes (e.g. acyltransferases) associated with MCFA synthesis and accumulation. From the transcriptomic analyses, four FatB cDNAs, designated CpuFatB1, CpuFatB2, CpuFatB3, and CpuFatB4, were identified in C. pulcherrima. Of these, only CpuFatB1 activity was tested previously by expression in Camelina seeds, and shown to be a typical FatB that generated seed oils enriched in palmitic acid (16:0) (Horn et al., 2013). In addition, three FatB cDNAs, designated CvFatB1, CvFatB2, and CvFatB3, were identified in 454 transcriptomic analyses of C. viscosissima genes. These cDNAs were nearly identical to C. viscosissima FatB cDNAs previously deposited in the National Center for Biotechnology Information (NCBI) database. Notably, CvFatB3 identified in the transcriptomic analyses differed from the analogous sequence in NCBI by two nucleotides that resulted in one amino acid difference (A instead of V), and the gain of a stop codon resulting in a polypeptide of 388 amino acids rather than 412 amino acids as found in the existing NCBI accession.

Analysis of the spatial expression of FatB transcripts in C. pulcherrima and C. viscosissima

Real-time PCR (RT-PCR) analysis was carried out with total RNAs isolated from roots, stems, leaves, flowers, and developing seeds of C. pulcherrima and C. viscosissima to examine relative expression levels of FatB transcripts from the 454 transcriptomic analyses. Among the four FatB genes from C. pulcherrima, CpuFatB1 and CpuFatB2 displayed ubiquitous expression in the tested organs, whereas CpuFatB4 was expressed predominantly in developing seeds, and CpuFatB3 was expressed exclusively in developing seeds (Fig. 2A). Among the three FatB genes in C. viscosissima, expression of CvFatB1 and CvFatB3 was detected only in developing seeds, while expression of CvFatB2 was detected in all tested organs (Fig. 2B).

Amino acid sequence comparisons are consistent with structure–function relationships related to substrate specificity among the Cuphea FatBs

Based on sequence homology and substrate chain length specificities, Cuphea FatB proteins have been classified

![Fig. 1. Fatty acid composition of seeds and leaves in Cuphea pulcherrima and Cuphea viscosissima. Fatty acids were extracted from leaves and seeds of C. pulcherrima and C. viscosissima and analysed using gas chromatography. Values are the means ±SD from five biological replicates.](image-url)
into three groups: clade I generally has preferential activity towards 16:0-ACP; clade II generally has preferential activity towards 12:0-ACP and 14:0-ACP or broad-range specificity (12:0-ACP to 16:0-ACP); and clade III generally has preferential activity towards 8:0-ACP and 10:0-ACP (Voelker, 1996; Filichkin et al., 2006; Jing et al., 2011). Alignment of deduced FatBs from six Cuphea species showed that C. pulcherrima and C. viscosissima FatB genes could be grouped as seed-specific CpuFatB3 and CvFatB1 (clade III), CpuFatB4 and CvFatB3 (Clade II), and CpuFatB1, CpuFatB2, and CvFatB2 (clade I) (Fig. 3). The identity of amino acid sequences among Cuphea FatB polypeptides ranged from 70% to 93%. The chloroplast transit peptides were predicted based on homology with known FatBs (Fig. 4).

Expression of Cuphea FatB genes in Camelina seed altered fatty acid profiles

Based on seed-specific expression in Cuphea and their phylogeny, CpuFatB3 and CpuFatB4 from C. pulcherrima, and CvFatB1 and CvFatB3 from C. viscosissima were chosen for evaluation to determine the ability to confer Jet A fuel-type fatty acid (C8–C16) production upon transgenic expression in Camelina. Previously reported medium chain-specific FatBs, CpFatB2 from C. palustris (Dehesh et al., 1996a), UcFatB1 from California bay (Pollard et al., 1991; Voelker et al., 1996), and ChFatB2 from C. hookeri ana (Dehesh et al., 1996b), were also tested (Table 1). CpuFatB2 and CvFatB2 were not chosen for Camelina transformation, due to their ubiquitous expression and classification as ‘typical’ FatBs with activity for 16:0-ACP. The cDNAs of eight FatB genes (CpFatB2, UcFatB1, ChFatB2, CpuFatB1, CpuFatB3, CpuFatB4, CvFatB1, and CvFatB3) were introduced into Camelina with strong seed-specific expression mediated by the soybean glycinin-1 promoter (Sims and Goldberg, 1989). Transgenic seeds were selected by fluorescence from the DsRed marker. Previously, transgenic rapeseed expressing UcFatB1 showed a positive correlation between transgene copy number and lauric acid accumulation (Voelker et al., 1996). However, multiple copy numbers of inserted transgenes have complex segregation patterns and might need many generations to establish homozygosity of a line (Micog et al., 2013). Also, multiple copies of the introduced gene have been implicated in transgene silencing (Tang et al., 2007). To avoid these potential problems, 20–30

Fig. 2. Spatial expression of FatB acyl-ACP thioesterases in C. pulcherrima and C. viscosissima. Total RNA was isolated from individual tissues and converted into cDNAs for RT-PCR analyses for evaluation of FatB gene expression in different tissues of C. pulcherrima and C. viscosissima. Cuphea elf4-a1 and actin genes were used as an internal control for RT-PCR.
and fatty acid profiles resembled those of the wild type. This intermediate profile was likely due to the lower levels of 14:0 accumulation, which is characteristic of transgenic seeds expressing single-copy insertions of FatB. In contrast to its reported properties, CvFatB3 expression in Camelina seeds did not produce any detectable MCFAs, and fatty acid profiles resembled those of the wild type. This might be caused by nucleotide sequence differences at positions 442 and 1166 of the CvFatB3 cDNA as described above (Table 2).

Two and three FatB cDNAs were co-expressed to produce more diverse MCFA compositions in Camelina seeds to mimic more closely the carbon chain lengths of Jet A fuel (from 8- to 16-carbon distribution) and to examine the utility of this approach for enhancing MCFA accumulation in Camelina seeds relative to expression of individual FatBs. Co-expression of CpFatB2 and UcFatB1 resulted in the production of 12:0, 14:0, and 16:0 (Table 2). Co-expression of CpFatB2 with ChFatB2 or three cDNAs (CpFatB2, UcFatB1, and ChFatB2) produced fatty acids ranging from 8:0 to 16:0. Although diverse compositions of MCFAs were observed in seeds of the co-expression lines, the amounts of each MFA were less than those obtained with expression of individual cDNAs (Table 2). For example, 12:0 made up 18 mol% of the total fatty acids in seeds of the co-expression lines, and the amount of 14:0 in seeds of CpFatB2-expressing lines was 24 mol% of total fatty acids (Table 2). In contrast, seeds of CpFatB2 and UcFatB1 co-expression lines accumulated only 7 mol% of 12:0 and 12 mol% of 14:0 (Table 2). Myristic acid (14:0) content was also reduced in seeds of CpFatB2 and ChFatB2 co-expression lines and CpFatB2, UcFatB1, and ChFatB2 co-expression lines by nearly 3-fold relative to seeds expressing only CpFatB2. Similarly, lauric acid (12:0) content was reduced by ~9-fold in seeds of lines co-expressing CpFatB2, UcFatB1, and ChFatB2 compared with seeds expressing UcFatB1 alone (Table 2).
To increase MCFAs levels further in *Camelina* seeds, FatB polypeptides were co-expressed with a chimeric lysophosphatidic acid acyltransferase (*CnLPAT*) that has been shown to insert 12:0 at the sn-2 position of the TAG glycerol backbone (Table 1), leading to 12:0 accumulation in transgenic *B. napus* seeds (Knutson et al., 1999). Co-expression of either *CpFatB2* or *UcFatB1* with *CnLPAT* in *Camelina* resulted in further accumulation of MCFAs in the seed (Table 2). However, co-expression of *CnLPAT* with the C8/C10-specific FatB *ChFatB2* did not increase 8:0 or 10:0 fatty acid levels in *Camelina* seeds relative to expression of *ChFatB2* alone (Table 2). Analysis of sn-2 fatty acids of TAG confirmed the effect of *CnLPAT* to increase MCFAs in TAG. Myristic acid (14:0) and lauric acid (12:0) were detected in the sn-2 position of TAG in the co-expression lines of *CpFatB2* with *CnLPAT* and *UcFatB1* with *CnLPAT*, respectively, while these fatty acids were 5- to 10-fold lower in the absence of *CnLPAT* (Fig. 5A, B). Interestingly, 10:0 was not detected at the sn-2 position of TAG in *Camelina* seeds co-expressing *ChFatB2* and *CnLPAT* (Fig. 5C), indicating that *CnLPAT* has substrate specificity for CoA esters of 12:0 preferentially and 14:0 to a lesser extent, but is not active with 10:0-CoA (Table 2).
Similar to a previous report by Tjellström et al. (2013), a positive correlation between 8:0 and 10:0 levels and 18:1 levels was observed. Seeds expressing CpuFatB3, for example, had a 2-fold increase of 18:1. However, although 12:0, 14:0, and 16:0 increase in seeds expressing UcFatB1, CpFatB2, and CpuFatB1, the level of 18:1 decreased to 8, 6, and 7 mol%, respectively, compared with wild-type seeds that had 11 mol% of 18:1 (Table 2). Furthermore, total fatty acid content in 14:0-accumulating seeds expressing CpuFatB2 was increased, whereas a decrease in total fatty acids was detected in seeds of 10:0-producing FatBs, in comparison with wild-type seeds (Supplementary Fig. S1 at JXB online). Notably, co-expression of MCFA-specific FatB and CnLPAT resulted in an increase of MCFA without significant impact on total seed fatty acid content under greenhouse growth conditions (Fig. 6). Interestingly, lines expressing the C10-producing ChFatB2 thioesterase had lower seed fatty acid content than wild-type controls, but this was restored to wild-type levels with co-expression of CnLPAT (Fig. 6).

Disruption of KASII in Camelina seeds increases palmitic acid content but decreases MCFA content in FatB expression lines

Seed-specific suppression of Camelina KASII was examined as an additional strategy to increase palmitic acid (16:0) and MCFA content to mimic Jet A fuel composition. KASII encodes β-ketoacyl-ACP synthase that catalyses the initial step in the elongation of 16:0-ACP to 18:0-ACP, and KASII suppression has been shown to enhance the 16:0 content of seeds (Pidkowich et al., 2007). Five seed-specific KASII-RNAi lines (Supplementary Fig. S1 at JXB online) were chosen for detailed characterization based on the increased 16:0 content of their seed oil and normal phenotype. Seeds from homozygous T4 lines had 26–28 mol% 16:0, nearly 3-fold higher compared with non-transformed lines (Table 3). KASII-RNAi transgenic Camelina seeds showed no significant changes in total seed oil content relative to the wild type (Table 3). Reduced transcript levels of CsKASII in developing seeds of CsKASII-RNAi lines were not detected in comparison with the wild type (Supplementary Fig. S3). In this regard, complete KASII knock-out is lethal and high levels of palmitic acid (16:0) in KASII-RNAi lines lead to aborted ovules in Arabidopsis (Pidkowich et al., 2007). In the present study, transgenic KASII-RNAi lines with increased 16:0 but normal growth phenotypes were selected. Because increased 16:0 and 16:1 is an obvious phenotype of KASII-RNAi, the altered TAG composition of CsKASII-RNAi provides evidence for the partial suppression of CsKASII activity.

To determine whether KASII suppression increases MCFA levels, the KASII-RNAi construct was transformed into a Camelina line homozygous for the UcFatB1 transgene. The resulting 12:0 content in seeds from these lines ranged from 3 mol% to 7 mol% versus 15 mol% (or 5% versus 10% on a weight percent basis to total oil) in the parent line lacking KASII-RNAI suppression (Table 4; Fig. 7). The mol% of C8–C16 fatty acids in KASII-RNAi lines was not significantly affected by suppression in either the wild type or the UcFatB1 background (Table 4), and ranged from 24.5% to 33.7% of total fatty acid in both backgrounds. These results suggested that efficient downstream acylation of MCFAs into TAG is important for the stable accumulation of MCFAs in the transgenic Camelina seeds. In this regard, with high levels of 16:0 production, it is likely that 16:0-CoA is used in preference to 12:0-CoA for acylation on the glycerol backbone.

### Table 1. Sources of FatB acyl-ACP thioesterase and LPAT for MCFA in Camelina

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>GenBank accession no.</th>
<th>Species</th>
<th>Fatty acid product of heterologous expression</th>
<th>Tissue specificity in source plant</th>
<th>Reported transgenic plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpFatB2</td>
<td>AAC49180</td>
<td>C. palustris</td>
<td>14:0 (E. coli and plant)</td>
<td>seed</td>
<td>Arabidopsis thaliana (Tjellström et al., 2013)</td>
</tr>
<tr>
<td>UcFatB1</td>
<td>Q41635</td>
<td>U. californica</td>
<td>12:0 (E. coli and plant)</td>
<td>seed</td>
<td>Brassica napus (Voelker et al., 1996; Tjellström et al., 2013)</td>
</tr>
<tr>
<td>ChFatB2</td>
<td>AAC49269</td>
<td>C. hookeriana</td>
<td>8:0, 10:0 (E. coli and plant)</td>
<td>seed</td>
<td>Brassica napus (Dehesh et al., 1996b; Tjellström et al., 2013)</td>
</tr>
<tr>
<td>CpuFatB1</td>
<td>AGG79283</td>
<td>C. pulcherrima</td>
<td>16:0 (plant)</td>
<td>NR</td>
<td>Camelina sativa (Horn et al., 2013)</td>
</tr>
<tr>
<td>CpuFatB3</td>
<td>AGG79285</td>
<td>C. pulcherrima</td>
<td>8:0, 10:0 (plant)</td>
<td>NR</td>
<td>Arabidopsis thaliana (Tjellström et al., 2013)</td>
</tr>
<tr>
<td>CpuFatB4</td>
<td>AGG79286</td>
<td>C. pulcherrima</td>
<td>NR</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>CvFatB1</td>
<td>AEM72522</td>
<td>C. viscosissima</td>
<td>8:0, 10:0 (E. coli)</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>CvFatB2</td>
<td>AEM72523</td>
<td>C. viscosissima</td>
<td>14:0, 16:0, 16:1 (E. coli)</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>CvFatB3</td>
<td>AEM72524</td>
<td>C. viscosissima</td>
<td>14:0 (E. coli)</td>
<td>NR</td>
<td>NR</td>
</tr>
<tr>
<td>CnLPAT</td>
<td>XP002313814</td>
<td>Cocos nucifera</td>
<td>12:0 (Plant)</td>
<td>seed</td>
<td>Brassica napus (Knutzon et al., 1999)</td>
</tr>
</tbody>
</table>
Table 2. Fatty acid compositions (mol%) of seed lipids in transgenic Camelina expressing MCFA-specific FatB and in the wild type

The MCFA column is the total amount (mol%) of C8–C14 fatty acids, and the Jet FAs column is the total amount (mol%) of C8–C16 fatty acids with chain lengths found in Jet A and Jet A-1 fuels.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>8:0</th>
<th>10:0</th>
<th>12:0</th>
<th>14:0</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
<th>20:0</th>
<th>20:1</th>
<th>22:1</th>
<th>MCFAs (C8–C14)</th>
<th>Jet FAs (C8–C16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>8.9±0.9</td>
<td>4.0±0.7</td>
<td>10.6±0.7</td>
<td>25.1±1.1</td>
<td>40.5±2.0</td>
<td>1.6±0.0</td>
<td>7.5±0.5</td>
<td>1.8±0.1</td>
<td>0</td>
</tr>
<tr>
<td>CpuFatB1 (T6)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>1.6±0.1</td>
<td>43.5±1.0</td>
<td>5.6±0.2</td>
<td>6.5±0.8</td>
<td>24.2±0.5</td>
<td>11.4±0.9</td>
<td>3.0±0.1</td>
<td>3.3±0.3</td>
<td>1.0±0.0</td>
</tr>
<tr>
<td>CpuFatB3 (T2)</td>
<td>0.5±0.1</td>
<td>1.2±0.2</td>
<td>0.2±0.0</td>
<td>1.0±0.1</td>
<td>8.3±0.3</td>
<td>4.6±0.6</td>
<td>21.8±2.3</td>
<td>18.0±1.2</td>
<td>27.1±2.6</td>
<td>2.7±0.5</td>
<td>12.0±0.8</td>
<td>2.5±0.3</td>
<td>2.9</td>
<td>11.2</td>
</tr>
<tr>
<td>CpuFatB4 (T6)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>7.5±0.9</td>
<td>42.7±0.8</td>
<td>4.8±0.3</td>
<td>7.5±0.3</td>
<td>20.0±0.9</td>
<td>11.6±1.0</td>
<td>2.6±0.3</td>
<td>2.3±0.2</td>
<td>0.8±0.1</td>
</tr>
<tr>
<td>CvFatB1 (T6)</td>
<td>0.4±0.0</td>
<td>8.7±0.6</td>
<td>2.4±0.1</td>
<td>3.2±0.1</td>
<td>16.2±0.4</td>
<td>3.8±0.1</td>
<td>12.2±0.1</td>
<td>17.7±0.1</td>
<td>23.1±0.7</td>
<td>2.9±0.1</td>
<td>7.0±0.4</td>
<td>2.4±0.1</td>
<td>14.7</td>
<td>30.9</td>
</tr>
<tr>
<td>CvFatB3 (T6)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>9.3±0.7</td>
<td>3.9±0.4</td>
<td>13.3±0.2</td>
<td>22.8±1.5</td>
<td>33.1±2.0</td>
<td>2.5±0.2</td>
<td>11.8±1.1</td>
<td>3.3±0.3</td>
<td>0</td>
</tr>
<tr>
<td>CpFatB2 (T2)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>23.8±0.8</td>
<td>18.1±0.9</td>
<td>3.3±0.4</td>
<td>5.6±0.9</td>
<td>26.8±3.1</td>
<td>1.4±0.2</td>
<td>4.2±0.4</td>
<td>1.3±0.2</td>
<td>23.8</td>
</tr>
<tr>
<td>UcFatB1 (T1)</td>
<td>–</td>
<td>–</td>
<td>18.4±0.9</td>
<td>2.7±0.1</td>
<td>7.1±0.2</td>
<td>4.0±0.3</td>
<td>8.1±0.7</td>
<td>29.9±0.6</td>
<td>21.5±1.3</td>
<td>1.8±0.2</td>
<td>5.1±0.3</td>
<td>1.2±0.1</td>
<td>21.1</td>
<td>28.2</td>
</tr>
<tr>
<td>ChFatB2 (T2)</td>
<td>0.7±0.1</td>
<td>10.3±0.4</td>
<td>0.9±0.0</td>
<td>0.7±0.0</td>
<td>9.0±0.3</td>
<td>4.7±0.3</td>
<td>14.8±0.6</td>
<td>30.0±0.3</td>
<td>19.9±0.8</td>
<td>1.9±0.1</td>
<td>5.8±0.1</td>
<td>1.2±0.0</td>
<td>12.6</td>
<td>21.6</td>
</tr>
<tr>
<td>CpuFatB2+UcFatB1 (T6)</td>
<td>–</td>
<td>–</td>
<td>9.4±0.8</td>
<td>16.0±0.3</td>
<td>11.2±0.4</td>
<td>2.2±0.1</td>
<td>6.2±0.1</td>
<td>23.7±1.0</td>
<td>22.1±0.7</td>
<td>1.8±0.1</td>
<td>5.7±0.1</td>
<td>1.7±0.0</td>
<td>25.4</td>
<td>36.5</td>
</tr>
<tr>
<td>CpuFatB2+ChFatB2 (T6)</td>
<td>–</td>
<td>–</td>
<td>8.7±0.5</td>
<td>0.8±0.5</td>
<td>9.3±0.0</td>
<td>12.4±0.2</td>
<td>3.4±0.0</td>
<td>16.2±0.4</td>
<td>16.1±0.5</td>
<td>2.1±0.0</td>
<td>7.4±0.1</td>
<td>1.9±0.0</td>
<td>18.8</td>
<td>31.1</td>
</tr>
<tr>
<td>CpuFatB2+UcFatB1+ChFatB2 (T2)</td>
<td>–</td>
<td>–</td>
<td>8.5±0.9</td>
<td>1.6±0.5</td>
<td>9.6±0.2</td>
<td>12.0±0.2</td>
<td>3.3±0.1</td>
<td>21.4±0.3</td>
<td>16.9±0.7</td>
<td>15.8±0.2</td>
<td>1.9±0.0</td>
<td>7.1±0.2</td>
<td>1.8±0.1</td>
<td>19.7</td>
</tr>
<tr>
<td>CpuFatB2+CnLPAAT (T6)</td>
<td>–</td>
<td>–</td>
<td>36.9±1.9</td>
<td>16.8±0.6</td>
<td>3.0±0.3</td>
<td>3.1±0.3</td>
<td>19.2±0.6</td>
<td>16.0±0.2</td>
<td>19.0±0.2</td>
<td>2.3±0.2</td>
<td>0.9±0.1</td>
<td>36.9</td>
<td>53.7</td>
<td></td>
</tr>
<tr>
<td>UcFatB1+CnLPAAT (T6)</td>
<td>–</td>
<td>–</td>
<td>28.4±1.5</td>
<td>3.8±0.2</td>
<td>5.6±0.4</td>
<td>3.1±0.6</td>
<td>7.4±0.4</td>
<td>13.6±0.3</td>
<td>30.4±0.7</td>
<td>1.2±0.1</td>
<td>4.9±0.3</td>
<td>1.5±0.1</td>
<td>32.2</td>
<td>37.8</td>
</tr>
<tr>
<td>ChFatB2+CnLPAAT (T2)</td>
<td>0.5±0.1</td>
<td>9.7±0.6</td>
<td>0.8±0.0</td>
<td>0.7±0.0</td>
<td>9.3±0.2</td>
<td>5.0±0.4</td>
<td>13.1±0.4</td>
<td>31.3±0.6</td>
<td>20.0±0.6</td>
<td>2.2±0.1</td>
<td>6.0±0.5</td>
<td>1.4±0.2</td>
<td>11.7</td>
<td>21.0</td>
</tr>
</tbody>
</table>

Values are the means±SD of five biological replicates.
The number in parentheses (Tn) indicates the generation of the seeds.
Acyl-ACP pool analysis of developing Cuphea and Camelina seeds

The expression of \( \text{FatB} \) genes from \( \text{Cuphea} \) results in increased MCFA levels in \( \text{Camelina} \) seed oils, but does not approach the levels of MCFA that occur naturally in \( \text{Cuphea} \) seeds. This may be due to the evolution of substrate specificity within the components of the FAS complex in \( \text{Cuphea} \) species. To examine this hypothesis, the acyl-ACP composition of developing seeds from \( \text{C. viscosissima} \) and \( \text{Camelina} \) was analysed by electrospray ionization-tandem mass spectrometry (ESI-MS/MS) (Fig. 8). Acyl-ACP pool sizes were estimated based on comparison of relative peak area percentages per unit of protein analysed. In \( \text{C. viscosissima} \), 8:0-ACP made up 40% of the acyl-ACP pools, followed by 10:0-ACP (24%) and 6:0-ACP (19%). Long-chain (≥16) acyl-ACPs were barely detected and made up <1% of total acyl-ACP in developing seeds of \( \text{C. viscosissima} \). In contrast, acyl-ACP pools of transgenic and wild-type \( \text{Camelina} \) seeds were increased at 10 days after flowering (DAF) (Supplementary Fig. S4 at \( \text{JXB} \) online). However, increased percentages of 8:0, 10:0, and 12:0 ACPs were detected in 15 DAF developing seeds from FatB transgenic lines compared with wild-type seeds.

Discussion

The goal of this study was to generate MCFA-rich seed oils for use as feedstocks for jet fuel applications. To identify \( \text{FatB} \) cDNAs capable of generating novel MCFA-rich oil compositions, 454 transcriptomic studies of developing seeds from two \( \text{Cuphea} \) species was performed. To engineer oilseed with altered fatty acid composition, candidate \( \text{FatB} \) cDNAs were expressed individually or in combination with \( \text{CnLPAT} \) as determined by lipase digestion-based analyses. The data represent averages of four biological replicates ±SD. (A) \( \text{CpFatB2} \) and \( \text{CpFatB2} + \text{CnLPAT} \). (B) \( \text{UcFatB1} \) and \( \text{UcFatB1} + \text{CnLPAT} \). (C) \( \text{ChFatB2} \) and \( \text{ChFatB2} + \text{CnLPAT} \).
**Table 3. Fatty acid composition (mol%) of seed lipids in wild-type Camelina and transgenic Camelina of CsKASII-RNAi**

Values are the means ±SD of five biological replicates.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
<th>20:0</th>
<th>20:1</th>
<th>22:1</th>
<th>Total fatty acid content (µg mg⁻¹ seed weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10.1 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>4.4 ± 0.2</td>
<td>14.2 ± 0.4</td>
<td>34.0 ± 0.3</td>
<td>20.7 ± 1.0</td>
<td>2.8 ± 0.1</td>
<td>10.7 ± 0.3</td>
<td>2.7 ± 0.1</td>
<td>222.4 ± 11.2</td>
</tr>
<tr>
<td>CsKASII-RNAi #1–3 (T₃)</td>
<td>27.9 ± 0.5</td>
<td>4.1 ± 0.0</td>
<td>3.7 ± 0.2</td>
<td>6.7 ± 0.5</td>
<td>19.2 ± 1.2</td>
<td>23.6 ± 1.1</td>
<td>3.6 ± 0.1</td>
<td>8.3 ± 0.1</td>
<td>2.8 ± 0.1</td>
<td>243.4 ± 4.7</td>
</tr>
<tr>
<td>CsKASII-RNAi #2–6 (T₃)</td>
<td>27.9 ± 0.8</td>
<td>4.1 ± 0.2</td>
<td>3.7 ± 0.3</td>
<td>6.5 ± 0.1</td>
<td>17.4 ± 1.6</td>
<td>25.8 ± 0.9</td>
<td>3.4 ± 0.2</td>
<td>8.5 ± 0.2</td>
<td>2.0 ± 0.1</td>
<td>263.0 ± 21.0</td>
</tr>
<tr>
<td>CsKASII-RNAi #5–2 (T₃)</td>
<td>26.3 ± 0.2</td>
<td>3.5 ± 0.1</td>
<td>3.8 ± 0.1</td>
<td>7.1 ± 0.2</td>
<td>20.4 ± 0.9</td>
<td>23.8 ± 0.9</td>
<td>3.6 ± 0.0</td>
<td>8.7 ± 0.2</td>
<td>2.7 ± 0.1</td>
<td>247.6 ± 3.4</td>
</tr>
<tr>
<td>CsKASII-RNAi #6–1 (T₃)</td>
<td>28.2 ± 0.2</td>
<td>4.3 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>6.3 ± 0.1</td>
<td>17.0 ± 0.2</td>
<td>25.4 ± 0.4</td>
<td>3.6 ± 0.2</td>
<td>8.4 ± 0.1</td>
<td>2.9 ± 0.1</td>
<td>263.6 ± 10.4</td>
</tr>
</tbody>
</table>

*₅₃ refers to the seed generation analysed.

**Table 4. Fatty acid composition (mol%) of seed lipids in CsKASII-RNAi Camelina expressing UcFatB1**

Values are the means ±SD of five biological replicates.

The Jet FAs column is the total amount (mol%) of C8–C16 fatty acids with chain lengths found in Jet A and Jet A-1 fuels.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>12:0</th>
<th>14:0</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>18:3</th>
<th>20:0</th>
<th>20:1</th>
<th>22:1</th>
<th>Jet FAs (C8–C16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UcFatB1(T₆)</td>
<td>15.1 ± 2.0</td>
<td>2.7 ± 0.5</td>
<td>6.7 ± 0.5</td>
<td>0.0 ± 0.0</td>
<td>4.2 ± 0.5</td>
<td>10.7 ± 1.5</td>
<td>26.7 ± 1.9</td>
<td>19.3 ± 2.6</td>
<td>3.5 ± 0.2</td>
<td>8.7 ± 0.5</td>
<td>2.5 ± 0.1</td>
<td>24.5</td>
</tr>
<tr>
<td>CsKASII-RNAi (T₆)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>28.3 ± 0.9</td>
<td>3.4 ± 1.9</td>
<td>3.7 ± 0.2</td>
<td>6.7 ± 0.4</td>
<td>18.3 ± 1.8</td>
<td>24.9 ± 1.6</td>
<td>3.5 ± 0.2</td>
<td>8.5 ± 0.2</td>
<td>2.8 ± 0.1</td>
<td>28.3</td>
</tr>
<tr>
<td>CsKASII-RNAi</td>
<td>5.5 ± 1.7</td>
<td>1.6 ± 0.2</td>
<td>24.6 ± 1.6</td>
<td>2.6 ± 0.7</td>
<td>4.0 ± 0.4</td>
<td>6.7 ± 0.4</td>
<td>24.6 ± 1.5</td>
<td>19.5 ± 3.4</td>
<td>3.4 ± 0.2</td>
<td>5.7 ± 0.6</td>
<td>1.9 ± 0.5</td>
<td>31.6</td>
</tr>
<tr>
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<td>5.3 ± 1.2</td>
<td>1.6 ± 0.2</td>
<td>20.2 ± 1.2</td>
<td>2.8 ± 1.3</td>
<td>3.7 ± 0.3</td>
<td>6.8 ± 0.5</td>
<td>21.2 ± 1.9</td>
<td>24.8 ± 2.8</td>
<td>3.9 ± 0.3</td>
<td>7.3 ± 2.7</td>
<td>2.5 ± 0.1</td>
<td>27.1</td>
</tr>
<tr>
<td>CsKASII-RNAi</td>
<td>7.5 ± 2.4</td>
<td>2.0 ± 0.6</td>
<td>24.2 ± 2.3</td>
<td>2.4 ± 0.6</td>
<td>4.1 ± 0.5</td>
<td>6.0 ± 0.2</td>
<td>21.3 ± 3.8</td>
<td>20.1 ± 3.5</td>
<td>4.1 ± 0.4</td>
<td>6.0 ± 0.4</td>
<td>2.3 ± 0.3</td>
<td>33.7</td>
</tr>
<tr>
<td>UcFatB1 #2–1 (T₅)</td>
<td>3.0 ± 1.1</td>
<td>1.3 ± 0.1</td>
<td>22.4 ± 2.2</td>
<td>1.9 ± 0.5</td>
<td>3.6 ± 1.1</td>
<td>7.1 ± 0.5</td>
<td>25.0 ± 3.0</td>
<td>22.7 ± 2.4</td>
<td>3.5 ± 0.5</td>
<td>7.1 ± 1.9</td>
<td>2.4 ± 0.5</td>
<td>26.7</td>
</tr>
<tr>
<td>CsKASII-RNAi</td>
<td>4.4 ± 1.4</td>
<td>1.2 ± 0.3</td>
<td>19.7 ± 2.4</td>
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<td>5.3 ± 0.9</td>
<td>8.9 ± 0.7</td>
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<td>18.6 ± 0.9</td>
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<td>4.8 ± 0.8</td>
<td>8.3 ± 0.8</td>
<td>28.0 ± 2.5</td>
<td>19.2 ± 2.3</td>
<td>4.2 ± 0.6</td>
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<td>4.4 ± 0.7</td>
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<td>18.3 ± 3.9</td>
<td>3.7 ± 0.3</td>
<td>6.5 ± 0.4</td>
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₅₃ and ₅₄ refer to the seed generation analysed.
and CvFatB1, were identified from transcriptomics data that were capable of generating 10:0 in Camelina seeds. Also identified were two FatBs (CpuFatB2 and CvFatB2) ubiquitously expressed in Cuphea, as well as a seed-specific FatB (CpuFatB4) that produced oils enriched in 16:0 and lesser amounts of 14:0 upon expression in Camelina seeds. In addition, co-expression of FatB cDNAs with different substrate specificities generated oils with a range of MCFAs, but the accumulation of each MCLA was less than that obtained by the expression of individual FatBs. Furthermore, co-expression of the MCFA-CoA specific coconut LPAT increased overall levels of 12:0 and 14:0 accumulation in Camelina seeds and the amounts of these fatty acids in the sn-2 position of Camelina seed TAGs. This approach did not increase 10:0 at the sn-2 position of Camelina seed TAGs, suggesting that the coconut LPAT uses 10:0-CoA poorly as a substrate. While suppression of KASII was effective in blocking elongation of 16:0 in Camelina seeds, 12:0 accumulation in Camelina...
seeds from expression of a 12:0-ACP-specific FatB was not enhanced by this approach.

Although C. pulcherrima accumulates almost exclusively 8:0 fatty acids in its seed oil, none of the FatB cDNAs isolated from developing seeds of this plant was capable of generating >1 mol% 8:0 upon expression in developing Camelina seeds. Seed-specific expression of CupFatB4 in Camelina seeds, for example, yielded primarily 16:0 and lower amounts of 14:0, and seed-specific CupFatB3 expression in Camelina seeds produced low levels of 10:0 and lower amounts of 8:0, 12:0, and 14:0. Similar results were previously observed with CcFatB1 from C. calophylla seeds, which accumulate ~60% 12:0 (Filichkin et al., 2006). Expression of this thioesterase in Arabidopsis seeds resulted in the accumulation of 16:0 to ~20 mol% of the total fatty acids, but yielded only ~1.5 mol% of 12:0. However, co-expression of CcFatB1 with a Cuphea wrightii β-keto-acyl-ACP synthase in Arabidopsis seeds shifted fatty acid accumulation to ~13 mol% of 12:0 with only a small elevation in 16:0 content compared with non-transformed plants. These results, along with the present results for C. pulcherrima and C. viscosissima FatBs, indicate that the high content of specific MCFAs in Cuphea seeds is determined not only by FatBs but also by their acyl-ACP substrate pools in these seeds resulting from specialization in FAS. Comparative measurements of acyl-ACP pools in seeds of C. viscosissima developing seeds and those of wild-type and transgenic Camelina seeds engineered for Cuphea FatB expression support this hypothesis (Fig. 8).

The most notable difference between developing Cuphea and Camelina seeds was the near absence of ≥C16 acyl-ACPs in C. viscosissima seed and an enrichment of 8:0- and 10:0-ACP in these seeds, in part consistent with the high levels of 8:0 and 10:0 accumulation in C. viscosissima seeds. Conversely, long-chain acyl-ACPs were more abundant in developing wild-type Camelina seeds. The reduction of ≥C16 acyl-ACPs in developing Camelina seeds expressing CupFatB1 and ChFatB2 relative to developing wild-type Camelina seeds (Fig. 8) may be indicative of an efficient termination of acyl-ACP chain elongation by the activities of these enzymes.

In addition to potential bottlenecks in FAS that limit the production of 8:0 and 10:0 fatty acids in divergent FatB-expressing Camelina seeds, the present results, as well as those of others, show that downstream acylation of MCFAs on the TAG glycerol backbone limits accumulation of these fatty acids in the transgenic Camelina seeds. Unlike other unusual fatty acids, the accumulation of MCFAs is believed to follow the traditional Kennedy pathway, which involves the sequential acylation of the sn-1 of glycerol-3-phosphate (G3P) and the sn-2 of lysophosphatidic acid (LPA) with acyl-CoA substrates to produce phosphatidic acid (PA) by GPAT and LPAT, respectively. Then PA is converted to diacylglycerol (DAG), and finally the sn-3 of DAG is acylated to form TAG. The importance of specialized acyltransferases for MCFA accumulation was most evident with the co-expression of UcFatB1 with CnLPAT, which resulted in a nearly 10 mol% increase in 12:0 accumulation compared with expression of UcFatB1 alone. As expected for a specialized LPAT, this increased accumulation was largely due to enhanced amounts of 12:0 at the sn-2 position of the TAG glycerol backbone (Fig. 5). The coconut CnLPAT, however, showed little ability to introduce 10:0 formed by ChFatB2 into the sn-2 position of the TAG glycerol backbone (Fig. 5C), similar to results previously described in transgenic B. napus (Dehesh, 2001). Given the 8:0- and 10:0-rich fatty acid profiles of C. pulcherrima and C. viscosissima seeds, LPATs and other acyltransferases, particularly DGATs (Bafor and Stymne, 1992; Wiberg et al., 1994, 2000), with activity for 8:0 and 10:0 MCFA substrates are expected to occur. Characterization of candidate acyltransferases from C. pulcherrima and C. viscosissima seed transcriptomes is currently a major focus of research.

Consistent with previously reported studies in Arabidopsis (Tjellström et al., 2013), some transgenic Camelina lines producing 8:0 and 10:0 (e.g. CupFatB3 and CvFatB1) showed increased levels of 18:1 and decreased levels of 18:2, suggesting that the Camelina FAD2 is less active on 18:1 when paired with 8:0 or 10:0 on phosphatidylcholine (PC). However, other transgenic lines (e.g. ChFatB2) showed the opposite phenotype: an increase in 18:2. Therefore, the question of alternative pathways for trafficking MCFA into TAG remains unclear and merits further investigation.

Recently, plastid acyl-ACP synthetase, known as an acyl activating enzyme (AAE), has been proposed to be involved in recycling MCFAs into the plastid for further elongation (Tjellström et al., 2013). Exogenous fatty acids have different metabolic fates depending on their chain length and level of unsaturation. For example, a shorter chain length (≤C10) was used preferentially in plastids for synthesis of plastidal lipid, whereas longer substrates (≥C14) were predominantly utilized in cytosolic lipids (Roughan et al., 1987). This activation of MCFAs to ACP, not only added exogenously but also synthesized in plastids, occurs directly via a plastid acyl-ACP synthetase (Koo et al., 2005; Tjellström et al., 2013). The effect of the limiting acyl-ACP synthetase activity for the accumulation of MCFA was significant on 8:0 which was increased by almost 2-fold in aae 15116 seeds expressing CupFatB3 (Tjellström et al., 2013). It is interesting to note that it was not possible to find homologues of Arabidopsis AAE15 (or AAE16) in the Cuphea transcriptome database used here. This suggests that the presence of acyl-ACP synthetase in Camelina seeds may be an additional bottleneck for MCFA accumulation, particularly 8:0 and 10:0 accumulation.

The main problems of present biodiesel include several technical aspects, such as cold flow properties and oxidative stability, as well as feedstock availability and cost (Knothe, 2014). Due to their unique fatty acid composition, Cuphea seed oils have received attention as a source of biodiesel (Geller et al., 1999; Knothe et al., 2009; Lovestead et al., 2010; Knothe, 2014). The evaluation of fuel properties, such as the cetane number, kinematic viscosity, and oxidative stability, showed that biodiesel derived from Cuphea oil had properties superior to those of other vegetable oils for biodiesel fuels (Knothe et al., 2009). In particular, plant oils rich in 10:0 would be more desirable for biodiesel production than conventional plant oil (Knothe et al., 2009). In addition, Cuphea species represent a deep reservoir of divergent FatBs with a range of substrate specificities for generating oils that mimic the C8–C16 hydrocarbon component of Jet A and Jet A-1 fuels. As shown here, multiple FatBs with differing substrate specificities can be co-expressed to obtain Camelina seed oil with a broad mixture of
fatty acid chain lengths ranging from C8 to C16. However, the expression of multiple FatBs was not as effective at generating amounts of individual MCFAs as obtained by expression of single FatBs. While this may be due to suboptimal acyl-ACP pool compositions to match the substrate preferences of divergent FatBs, it cannot be excluded that mixed FatBs might form heterodimers that are less active than homodimers. It is known that plant FatBs function as dimers (McKeon and Stumpf, 1982; Hellyer et al., 1992). Based on structural similarity to other thioesterases, the active sites of the acyl-ACP thioesterase are predicted to lie at the dimer interface (Dillon and Bateman, 2004), and heterodimerization may affect the active site architecture to reduce activity.

Overall, the present findings highlight the need for a more complete understanding of *Cuphea* FAS to tailor acyl-ACP pools predictably in host oilseeds to match the substrate properties of introduced FatBs and to maximize production of individual or mixtures of MCFAs for uses such as biodiesel and jet fuel production. In addition to *FatB* sequences, the transcriptomic analyses of developing *C. pulcherrima* and *C. viscosissima* seeds uncovered a wealth of FAS genes that will be useful for co-expression in *Camelina* to re-create a *Cuphea*-type FAS that possibly has higher 8:0- and 10:0-ACP substrate pools for divergent FatBs. As indicated by the present inability to increase Jet FA accumulation by suppression of the *Camelina* *KASII*, altering native FAS in the host oilseed is likely to be insufficient on its own for generating a specialized FAS for high levels of Jet FA production.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Total fatty acid content of engineered *Camelina* lines.

Figure S2. 16:0 Fatty acid composition of *CsKASII-RNAi* TAG.

Figure S3. Transcript level of *CsKASII-RNAi* in developing seeds.

Figure S4. Acyl-ACP analysis.

Table S1. List of primers used in this study.

Acknowledgements

The research was funded by the Center for Advanced Biofuel Systems (CABS), an Energy Frontier Research Center funded by the US Department of Energy, Office of Basic Energy Sciences under Award Number DE-SC0001295. We thank Zach Smith and James Ford for technical assistance with sequencing of *Cuphea* seed transcriptomes.

References


Knutzon DS, Hayes TR, Wyrick A, Xiong H, Davies HM, Voelker TA. 1999. Lysophosphatidic acid acyltransferase from coconut endosperm mediates the insertion of laurate at the sn-2 position of triacylglycerols in lauric rapeseed oil and can increase total laurate levels. Plant Physiology 120, 739–746.


Supplementary Table 1. List of primers used in studies.

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<th>Primer name</th>
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Figure S1. Total fatty acid content of engineered Camelina lines.
Total fatty acid content of seeds from FatB transgenic plants were analyzed by GC. The data represents means ± SD of five biological replicates. Airstrikes indicate statistical differences compared to wild type (*P<0.05, **P<0.01).
Figure S2. 16:0 Fatty acid composition of *CsKASII*-RNAi TAG. The data represent averages of five biological replicates ± SD.
Figure S3. Transcript level of CsKASII-RNAi in developing seeds

Total RNA was isolated from 20 day-old developing seeds of Camelina and converted into cDNAs for RT-PCR analyses. Camelina elf4-a1 gene was used as an internal control for RT-PCR.
Figure S4. Acyl-ACPs analysis

Acyl-ACPs in 10 DAF developing seeds from Camelina. The data represents means ± SD of five biological replicates.