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Redirection of metabolic flux for high levels of omega-7 monounsaturated fatty acid accumulation in camelina seeds

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Summary

Seed oils enriched in omega-7 monounsaturated fatty acids, including palmitoleic acid (16:1Δ9) and *cis*-vaccenic acid (18:1Δ11), have nutraceutical and industrial value for polyethylene production and biofuels. Existing oilseed crops accumulate only small amounts (<2%) of these novel fatty acids in their seed oils. We demonstrate a strategy for enhanced production of omega-7 monounsaturated fatty acids in camelina (*Camelina sativa*) and soybean (*Glycine max*) that is dependent on redirection of metabolic flux from the typical Δ9 desaturation of stearoyl (18:0)-acyl carrier protein (ACP) to Δ9 desaturation of palmitoyl (16:0)-acyl carrier protein (ACP) and coenzyme A (CoA). This was achieved by seed-specific co-expression of a mutant Δ9-acyl-ACP and an acyl-CoA desaturase with high specificity for 16:0-ACP and CoA substrates, respectively. This strategy was most effective in camelina where seed oils with ~17% omega-7 monounsaturated fatty acids were obtained. Further increases in omega-7 fatty acid accumulation to 60–65% of the total fatty acids in camelina seeds were achieved by inclusion of seed-specific suppression of 3-keto-acyl-ACP synthase II and the FatB 16:0-ACP thioesterase genes to increase substrate pool sizes of 16:0-ACP for the Δ9-acyl-ACP desaturase and by blocking C18 fatty acid elongation. Seeds from these lines also had total saturated fatty acids reduced to ~5% of the seed oil versus ~12% in seeds of nontransformed plants. Consistent with accumulation of triacylglycerol species with shorter fatty acid chain lengths and increased monounsaturation, seed oils from engineered lines had marked shifts in thermotropic properties that may be of value for biofuel applications.

Introduction

Oils containing omega-7 unsaturated fatty acids (FA), comprising primarily palmitoleic acid (16:1Δ9) and its elongation product *cis*-vaccenic acid (18:1Δ11; Figure S1), are not typically abundant in plant seeds but are enriched in seeds of several nonagronomic plant species, including seeds of cat's claw creeper (*Dolichandra unguis-cati* or *Doxantha unguis-cati)* that accumulate up to 64% 16:1Δ9 and 15% 18:1Δ11 (Chisholm and Hopkins, 1965), seeds of sea buckthorn (*Hippophae rhamnoides*) that accumulate ~32% 16:1Δ9 (Kallio *et al.*, 2002), and seeds of macadamia (*Macadamia sp.*) that accumulate 20% to 30% 16:1Δ9 (Bridge and Hilditch, 1950; Saleeb *et al.*, 1973). These fatty acids have uses for a number of industrial applications, ranging from polymer precursor production and biodiesel formulations, and for nutraceuticals. Biodiesel containing fatty acid methyl esters produced from vegetable oils with high monounsaturated fatty acid content, including those with high omega-7 monounsaturated fatty acid content, has superior functional properties relative to biodiesel from common vegetable oils, such as soybean oil, with high polyunsaturated fatty acid levels (Ciubota-Rosie *et al.*, 2013; Durrett *et al.*, 2008; Knothe *et al.*, 2005). These include enhanced oxidative stability, NO_x emission, and ignition quality conferred by increased fatty acid monounsaturation and reduced polyunsaturation (Durrett *et al.*, 2008). In addition, the melting point of the omega-7 fatty acid palmitoleic acid is ~15 °C lower than that of the omega-9 fatty acid oleic acid, resulting in better cold flow properties for palmitoleic acid–rich oils relative to oleic acid–rich oils. Omega-7 fatty acids also have considerable potential as a feedstock for the production of 1-octene using metathesis chemistry (del Cardayre, 2013; Meier, 2009; Rybak *et al.*, 2008). 1-Octene is used commercially as a monomer in the production of polyethylene, including low linear density polyethylene (LLDPE). 1-Octene is currently derived from petroleum. As such, vegetable oils rich in omega-7 fatty acid acids can serve as renewable sources of this industrially important compound. Vegetable oils enriched in omega-7 fatty acids have also been ascribed a number of beneficial health properties, including promotion of cardiovascular fitness and reduction in LDL cholesterol levels (Curb *et al.*, 2000; Mozaffarian *et al.*, 2010; Yang *et al.*, 2011). Currently, the major nutraceutical source of omega-7 fatty acid oils is sea buckthorn seeds (Kallio *et al.*, 2002).

No commercially significant oilseed crops currently produce high levels of omega-7 fatty acid–rich oils. Common vegetable oils, such as soybean oil, typically contain <2% of omega-7 monounsaturated fatty acids, whereas plants such as cat's claw creeper that accumulate high levels of of omega-7 fatty acids in their seed oils have little agronomic potential. As a result, metabolic engineering of omega-7 fatty acid production in existing oilseeds is the most promising approach for generating

vegetable oils enriched in these fatty acids. The initial challenge for engineering omega-7 fatty acid production is that unsaturated fatty acids in oilseeds are formed principally through a series of desaturation reactions beginning with Δ9 desaturation of stearoyl (18:0)-acyl carrier protein (ACP) by the plastid-localized Δ9-18:0-ACP desaturase. This reaction gives rise to the ω-9 unsaturated oleic acid fatty acid. One approach for generating the omega-7 unsaturated palmitoleic acid is Δ9 desaturation of palmitic acid as an ACP- or coenzyme A (CoA)-linked substrate (Figures 1 and S1). The feasibility of this approach was shown by seed-specific expression in *Arabidopsis* of a mutated Δ9-18:0-ACP desaturase with enhanced substrate specificity for 16:0-ACP (Cahoon and Shanklin, 2000). Using a 3-ketoacyl-ACP synthase II (KASII) mutant with elevated 16:0-ACP substrate pools as a background, *Arabidopsis* seeds were obtained with ~30% omega-7 fatty acids, including 16:1Δ9 and its elongation products 18:1Δ11 and 20:1Δ13 (Cahoon and Shanklin, 2000). Levels of 67% of 16:1Δ9 and 18:1Δ11 were subsequently achieved in *Arabidopsis* seeds by inclusion of the seed-specific expression of fungal 16:0-/18:0-CoA desaturases (targeted to the cytosol) and RNAi silencing of *FAE1*, encoding the 3-ketoacyl-CoA synthase responsible for the elongation of C18 and C20 fatty acids (Nguyen *et al.*, 2010).

In this report, we have explored the efficacy of this strategy primarily in camelina (*Camelina sativa*), an emerging Brassicaceae oilseed crop that is particularly suitable for the complex metabolic engineering required for the introduction of the omega-7 fatty acid biosynthetic pathway. Camelina is amenable to *Agrobacterium*-based transformation using a floral vacuum infiltration method (Lu and Kang, 2008). We have previously developed an extensive metabolic engineering tool box of seed-specific promoters and selection markers to facilitate these studies (Horn *et al.*, 2013; Nguyen *et al.*, 2013). Here, we have systematically examined the use of a six transgene strategy incorporating the previously described method from *Arabidopsis* (Cahoon and Shanklin, 2000; Nguyen *et al.*, 2010) that also combined RNAi silencing of *FatB*, encoding the 16:0-ACP thioesterase. Through this approach, camelina oil was generated with ~66% omega-7 fatty acids as well as an unexpected two- to threefold reduction in total saturated fatty acid content relative to conventional camelina oil. These compositional changes were found to significantly impact of the thermotropic properties of camelina oil. We also report the evaluation of a partial metabolic engineering strategy to confer omega-7 fatty acid accumulation in soybean.

Results

Metabolic engineering for enhanced accumulation of omega-7 unsaturated fatty acids in camelina seeds

Experiments were undertaken to increase the omega-7 unsaturated fatty acid content of camelina seeds by systematically testing transgenes and transgene combinations predicted to enhance flux towards the synthesis of palmitoleic (16:1Δ9 or omega-7) and *cis*-vaccenic acids (18:1Δ11 or omega-7), which account for <2% of the fatty acids of wild-type camelina seeds. For these experiments, seeds were evaluated from 5 to 10 independent T_1 lines for each construct. The initial goal was to remodel plastidic pathways by shifting monounsaturated fatty acid synthesis to the Δ9 or omega-7 desaturation of palmitoyl (16:0)-ACP rather than the Δ9 desaturation of stearoyl (18:0)-ACP that typically generates ~90% of the fatty acids released from camelina seed plastids (Figure 1). To enhance substrate

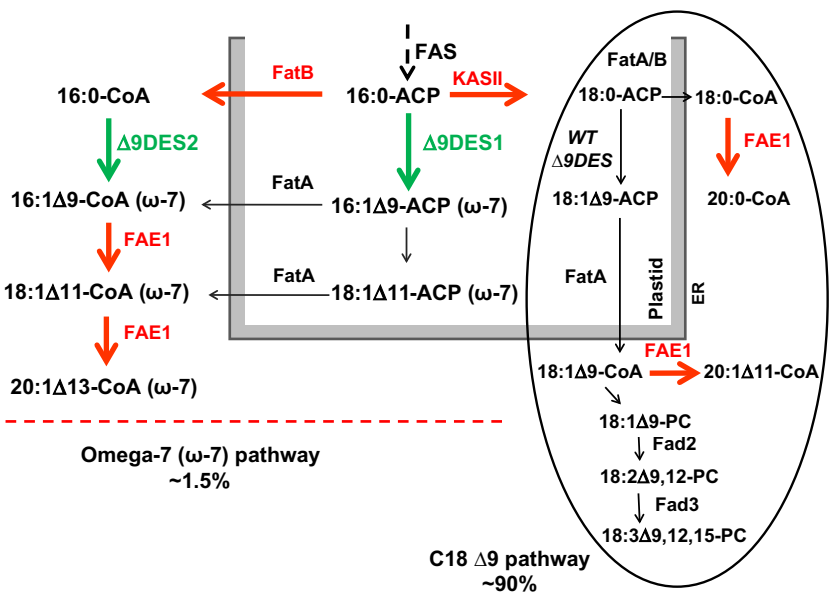

Figure 1 Schematic of the metabolic engineering strategy for enhancement of omega-7 monounsaturated fatty acid biosynthesis. Arrows shown in green indicate up-regulated metabolic steps and red arrows indicate down-regulated metabolic steps. The metabolic engineering strategy involves introduction and up-regulation of a plastid-localized mutant Δ9 acyl-ACP desaturase (Δ*9DES1*; Com25) with enhanced 16:0-ACP activity and an ER-localized Δ9 16:0-CoA-specific desaturase (Δ*9DES2*). RNAi suppression of genes for the plastid-localized 3-ketoacyl-ACP synthase II (*KASII*) and the FatB 16:0-ACP thioesterase (*FatB*) increases pool sizes of 16:0-ACP for the Δ9-16:0-ACP desaturase (Δ*9DES1*). RNAi suppression of the *FATTY ACID ELONGATION 1* (*FAE1*) gene reduces elongation of primarily C18 fatty acids to C20 moieties. As shown, the omega-7 desaturation pathway yields <1.5% omega-7 unsaturated fatty acids in nonengineered camelina seeds, whereas the typical C18 Δ9 pathway typically yields ~90% of the fatty acids in nonengineered camelina seeds.

pool sizes of 16:0-ACP for Δ9 desaturation, β-ketoacyl-ACP synthase II (KASII) genes were suppressed by RNAi ('HP'; Figure 2a). The expected outcome was reduced 16:0- to 18:0-ACP elongation. Consistent with this, seed-specific expression of a camelina KASII RNAi (KASII-HP) transgene yielded an increase in palmitic acid content from 7% in wild-type seeds to 31% in DsRed-positive seeds from T_1 seeds of 10 independent lines (Table 1). This was also accompanied by an increase in total omega-7 unsaturated fatty acids, principally as 16:1Δ9, from 1.4% in wild-type seeds to 9% in the KASII-HP seeds (Table 1 and Figure 3a,b). The mutated Δ9-acyl-ACP desaturase Com25 was then expressed alone or in combination with the *C. elegans* Fat5 ER Δ9-16:0-CoA desaturase (Fahy *et al.*, 2013; Watts and Browse, 2000) in camelina seeds. The result was an increase in omega-7 unsaturated fatty acid content in DsRed-positive seeds from T_1 plants of independent events expressing Com25 to 17% of the total fatty acids, including 2% 16:1Δ9, 8% 18:1Δ11 and 7% 20:1Δ13 (Table 1). The combination of seed-specific expression of Com25 and Fat5 resulted in additional increases in omega-7 unsaturated fatty acids to 23% of the total fatty acids, including 2.7% 16:1Δ9, 11% 18:1Δ11 and 9% 20:1Δ13 (Table 1 and Figure 3c). Of note, these lines were not advanced beyond the T_1 generation and genetic complexity of transgenes was not assessed. As such, it cannot be excluded that multiple transgene insertions may affect the total amount of omega-7 fatty acids accumulated in the engineered seeds.

Combining Com25 and Fat5 expression with KASII-HP increased omega-7 unsaturated fatty acids in seeds from homozygous T_3 lines to as high as 43% of the total fatty acids, including 16.5% 16:1Δ9, 11% 18:1Δ11 and 13% 20:1Δ13 (Table 1 and Figure 3d). To reduce the production of C20 omega-7 unsaturated fatty acids, seed-specific silencing of FAE1 (FAE-HP) was introduced with Com25, Fat5 and KASII-HP. The result was a small increase in total omega-7 unsaturated fatty acids in camelina seeds from homozygous T_3 lines to as high as 44% of the total fatty acids, but a large increase in 16:1Δ9 and 18:1Δ11 to 24% and 18% of the total fatty acids and a reduction in 20:1Δ13 to <1% of the total fatty acids (Table 1, Figure 3e).

To further increase total omega-7 unsaturated fatty acids, a second copy of the Com25 transgene was introduced along with RNAi suppression of the FatB 16:0-ACP thioesterase (FatB-HP; Figure 2b) into the Com25, Fat5, KASII-HP, FAE1-HP line. The total omega-7 unsaturated fatty acid content of the resulting engineered seeds from homozygous T_3 lines was as high as 65% of the total fatty acids, including ~3% of 16:2 and 16:3 derived from 16:1Δ9 in the highest omega-7 producing line (Table 1, Figure 3f, Figure S2). The primary omega-7 monounsaturated fatty acids of the engineered seeds were 16:1Δ9 (32% of total fatty acids) and 18:1Δ11 (30% of the total fatty acids) (Table 1 and Figure 4a). Of note, although the glycinin-1 seed-specific promoter was used in five of the transgenes, the stability of the

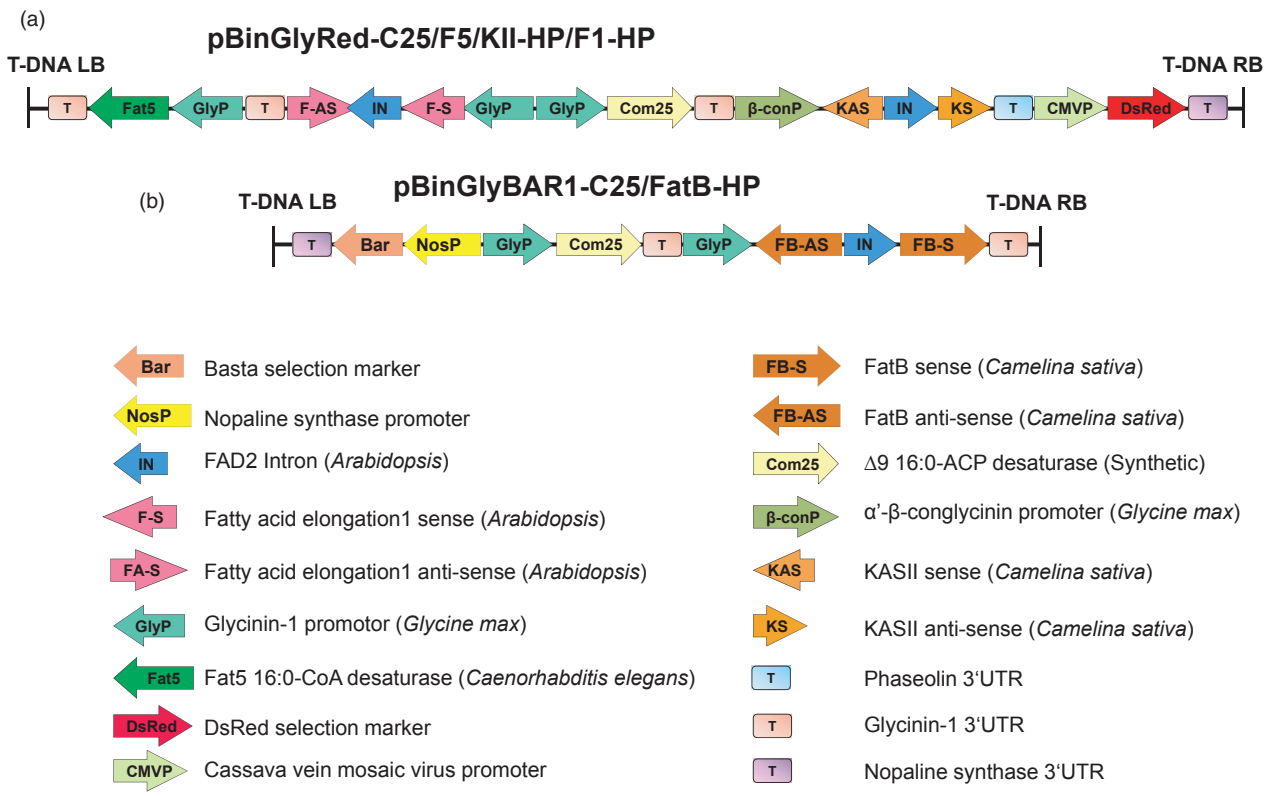

Figure 2 T-DNAs used for generating high omega-7 unsaturated fatty acids in camelina seeds. As shown in (a), pBinGlyRed-C25/F5/KII-HP/F1-HP contains seed-specific cassettes for expression of the Δ9-16:0-ACP desaturase (Com25) and the Δ9-16:0-CoA desaturase genes (Fat5) and RNAi suppression of genes for 3-ketoacyl-ACP synthase (KASII) and fatty acid-CoA elongase (FAE1) with sense and antisense hairpin arms for the target genes. Selection is accomplished with a DsRed fluorescence marker. As shown in (b), a second T-DNA pBinGlyBAR1-C25/FatB-HP was used for retransformation of camelina that contains seed-specific cassettes for the expression of Com25 and for RNAi suppression of the FatB 16:0-ACP thioesterase. Selection is achieved by use of a Basta resistance cassette. Constitutive and seed-specific promoters, 3'UTR sequences, and arrangements of cassettes are also shown.

Table 1 Total fatty acid composition of seeds from nontransformed and engineered lines of camelina

	Fatty acid composition (wt% of total fatty acids)														
Line	16:0	16:1Δ9	16:2	16:3	18:0	18:1Δ9	18:1Δ11	18:2	18:3	20:0	20:1Δ11	20:1Δ13	22:1	Others	Total omega-7*
Non transformed	6.9 ± 0.7	0.2 ± 0.4	ND	ND	3.5 ± 0.2	13.2 ± 2.5	0.7 ± 0.2	20.8 ± 0.2	28.9 ± 3.9	2.4 ± 0.2	12.3 ± 0.9	0.5 ± 0.3	1.9 ± 0.3	1.7 ± 0.8	1.4 ± 0.5
KASII-HP†	30.5 ± 1.2	3.9 ± 0.3	0.7 ± 0.1	ND	3.8 ± 0.4	3.3 ± 0.5	1.9 ± 0.2	16.6 ± 3.8	20.4 ± 2.9	5.2 ± 0.5	5.7 ± 0.8	2.5 ± 0.3	3.8 ± 0.3	1.8 ± 0.8	9.0 ± 0.7
Com25†	4.7 ± 0.9	2.1 ± 0.4	0.2 ± 0.1	0.1 ± 0.1	4.3 ± 0.1	12.0 ± 1.5	7.8 ± 0.7	22.1 ± 3.5	20.6 ± 4.8	3.6 ± 1.0	8.7 ± 0.6	7.1 ± 0.7	2.7 ± 0.3	4.1 ± 1.0	17.3 ± 1.1
Com25/Fat5†	3.6 ± 0.9	2.7 ± 0.5	0.3 ± 0.2	0.1 ± 0.1	3.2 ± 0.4	9.6 ± 1.6	10.9 ± 1.3	20.1 ± 2.1	22.5 ± 2.8	3.1 ± 0.6	8.4 ± 1.0	9.3 ± 1.4	2.9 ± 0.3	3.5 ± 0.6	23.3 ± 1.0
Com25/Fat5/KASII-HP‡	12.8 ± 1.1	16.5 ± 0.9	1.2 ± 0.2	0.7 ± 0.1	2.5 ± 0.1	3.7 ± 0.3	11.4 ± 0.8	9.4 ± 1.1	18.2 ± 0.7	2.3 ± 0.2	3.8 ± 0.5	12.8 ± 2.3	1.8 ± 0.3	2.9 ± 1.2	42.6 ± 1.2
Com25/Fat5/KASII-HP/FAE1-HP‡	7.4 ± 0.8	24.2 ± 0.2	1.3 ± 0.2	0.6 ± 0.7	1.2 ± 0.2	8.3 ± 0.6	17.9 ± 2.2	15.5 ± 1.3	18.0 ± 1.6	0.5 ± 0.2	2.8 ± 0.4	0.3 ± 0.1	0.5 ± 0.1	1.5 ± 0.5	44.3 ± 1.1
Com25/FatB-HP Com25/Fat5/KASII-HP/FAE1-HP‡	4.4 ± 0.5	32.2 ± 0.2	1.5 ± 0.4	1.4 ± 0.2	0.3 ± 0.1	2.1 ± 1.0	29.5 ± 0.7	7.0 ± 0.3	18.0 ± 0.5	0.3 ± 0.1	1.7 ± 0.3	1.1 ± 0.3	0.3 ± 0.2	0.2 ± 0.2	65.7 ± 1.7

ND, not detected.

*Includes content of 16:1Δ9, 18:1Δ11, and 20:11Δ13 as well as 16:2, 16:3, which are derived from the omega-7 fatty acid 16:1Δ9.

†The values shown are the average ±SD from measurements of fatty acids from single DsRed-positive seeds from 10 to 15 T_1 lines.

‡The values shown are the average ±SD from analyses of 20 seeds (T_3 generation) from 3 to 5 independent measurements.

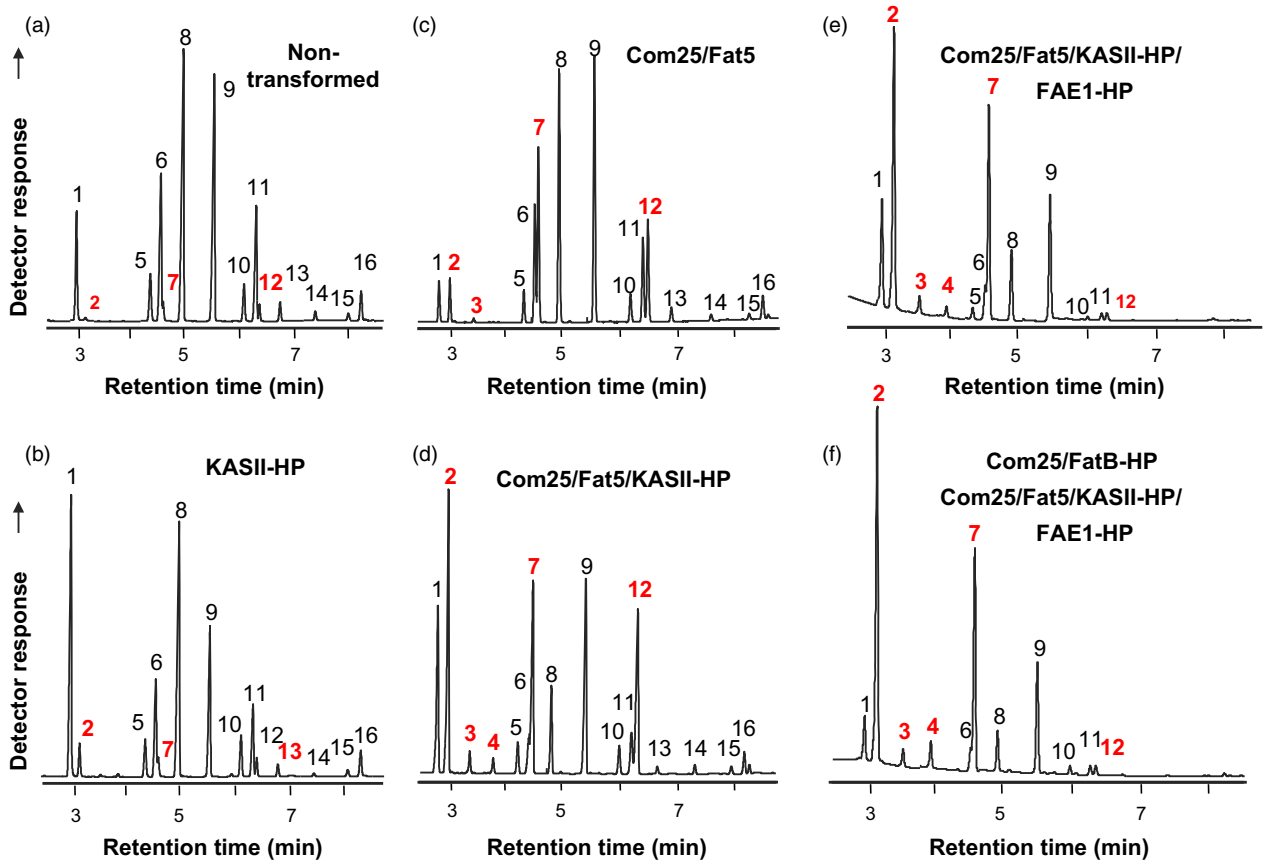

Figure 3 Representative gas chromatograms showing separation and relative quantification of fatty acid methyl esters generated from camelina seeds of nontransformed plants (a) and plants engineered for seed-specific expression of genes for Δ9-16:0-ACP desaturase (Com25) and/or Δ9-16:0-CoA desaturase (Fat5) and/or RNAi suppression (indicated by 'HP') of genes for 3-ketoacyl-ACP synthase II (KASII), fatty acid elongation-1 (FAE1), and/or FatB 16:0-ACP thioesterase. The gas chromatograms in B-F are from seeds expressing a KASII-HP RNAi cassette (b), Com25 and Fat5 (c), Com25, Fat5, KASII-HP RNAi cassette (d), Com25, Fat5, KASII-HP and FAE1-HP RNAi cassettes (e), and Com25 and FatB-HP RNAi cassette + Com25, Fat5, and KASII-HP and FAE1-HP RNAi cassettes (f). Fatty acid methyl ester peaks are as follows: 16:0 (1), 16:1Δ9 (2), 16:2 (3), 16:3 (4), 18:0 (5), 18:1Δ9 (6), 18:1Δ11 (7), 18:2Δ9,12,15 (8), 18:3Δ9,12,15 (9), 20:0 (10), 20:1Δ11 (11), 20:1Δ13 (12), 20:2Δ11,14 (13), 20:3 (14), 22:0 (15), and 22:1Δ13(16). Omega-7 monounsaturated fatty acids or omega-7 pathway-derived unsaturated fatty acids are shown with red labels.

omega-7 trait was observed over at least four generations. Double-bond positions of these fatty acids were confirmed to be in the omega-7 position by gas chromatography/mass spectrometry analysis of thiomethyl derivatives of fatty acid methyl esters (Figure S3). Another notable effect of these engineering efforts was a substantial decrease in the total saturated fatty acids. In this regard, the saturated fatty acid content of wild-type camelina seeds is ~12% of total fatty acids, whereas seeds engineered with the six transgene combination had ~5% saturated fatty acids, including reduction of palmitic acid content from 7% in nontransformed seeds to 4.4% in the engineered seeds and reduction of stearic acid from 3.5% in nontransformed seeds to 0.3% in the engineered seeds (Table 1 and Figure 4b).

Metabolic engineering for enhanced accumulation of omega-7 unsaturated fatty acids in soybean seeds

We also explored whether this engineering strategy can be used for enhanced omega-7 unsaturated fatty acid production in soybean seeds. The more labour- and skill-intensive transformation protocol for soybean limited the numbers of constructs that could be evaluated relative to camelina. In these experiments, the same transgene cassettes as used for camelina transformation were tested for seed-specific co-expression of Com25 and Fat5 and Com25 and Fat5 combined with KASII-HP in soybean. Nine to twelve independent transgenic events were generated for each construct. The top-performing line for Com25 and Fat5 expression resulted in the accumulation of omega-7 unsaturated fatty acids to ~4.5% of the total fatty acids, and the top-performing line for Com25 and Fat5 expression with KASII-HP resulted in the accumulation of omega-7 fatty acids to ~16.5% of the total fatty acids (Table 2, Figure S2). In both cases, the major omega-7 fatty acid that accumulated was 18:1Δ11. Omega-7 unsaturated fatty acids were not detected in soybean seeds from nontransformed plants. Amounts of omega-7 monounsaturated fatty acids accumulated with both constructs were considerably lower than that achieved in camelina seeds with the same transgene cassettes.

Omega-7 unsaturated fatty acids are not excluded from phosphatidylcholine

The relative amounts of omega-7 unsaturated fatty acids were examined in triacylglycerol (TAG) and phosphatidylcholine (PC) of nontransformed camelina seeds and seeds engineered for

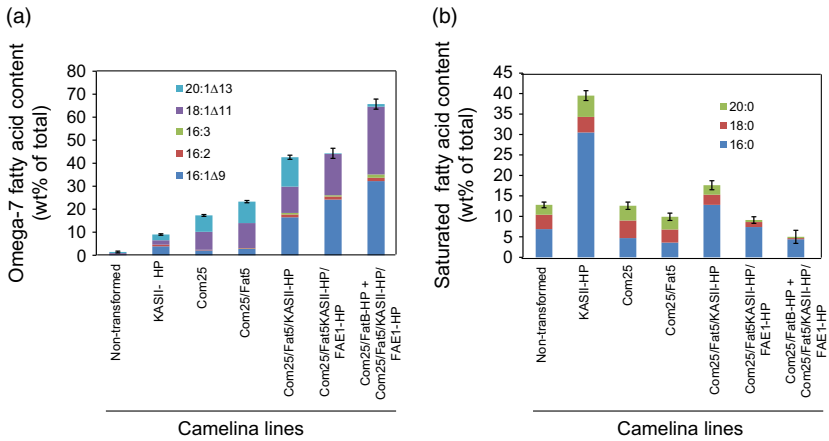

Figure 4 Comparison of amounts of omega-7 unsaturated fatty acids (a) and saturated fatty acids (b) in seeds of nontransformed plants or plants engineered for expression of 3-ketoacyl-ACP synthase II RNAi suppression cassette (KASII-HP); Δ9-16:0-ACP desaturase (Com25); Com25/Δ9-16:0-CoA desaturase (Fat5); Com25/Fat5/KASII-HP; Com25/Fat5/KASII-HP/fatty acid elongation1 RNAi suppression cassette (FAE1-HP); and Com25/FatB RNAi suppression cassette (FatB-HP) + Com25/Fat5/KASII-HP/FAE1-HP. Values shown are the averages of total omega-7 unsaturated fatty acids (a) or total saturated fatty acids (b) in 10 to 15 single seeds from T_1 lines of highest omega-7 unsaturated fatty acid accumulators ± SD for KASII-HP, Com25, and Com25/Fat5 constructs or 20 seeds from nontransformed lines or T_3 homozygous plants of highest omega-7 unsaturated fatty acid accumulating lines ± SD (n = 3–5) for Com25/Fat5/KASII-HP, Com25/Fat5/KASII-HP/FAE1-HP, Com25/FatB-HP + Com25/Fat5/KASII-HP/FAE1-HP constructs. Included in the total amounts of omega-7 unsaturated fatty acids are amounts of 16:2 and 16:3, which are derived from the omega-7 unsaturated fatty acid 16:1Δ9.

highest omega-7 unsaturated fatty acid production (from plants transformed with six transgenes). In seed lipid extracts from high omega-7 unsaturated fatty acid–producing seeds, the content of 16:1Δ9 and 18:1Δ11 was remarkably similar in the PC and TAG fractions (Table 3). In each of these fractions, the 16:1Δ9 content was ~32% of the total fatty acids, and the 18:1Δ11 content was ~29–34% of the total fatty acids. By comparison, the omega-7 unsaturated fatty acid content of TAG and PC from nontransformed camelina seeds was ≤1.1 of the fatty acids of these lipids (Table 3). These results suggest that camelina seeds do not exclude omega-7 fatty acids from membrane lipids.

Omega-7 unsaturated fatty acid accumulation is accompanied by large changes in TAG molecular species

Intact, ammoniated (M + NH_4) TAG molecular species were profiled by ESI-MS/MS by direct infusion of neutral lipid extracts from nontransformed camelina seeds and seeds engineered for high omega-7 unsaturated fatty acid production (~65% of the total fatty acids). Neutral loss scans were performed to validate the fatty acid components of the intact TAG molecular species. Product ion spectra of individual peaks were also obtained to further confirm fatty acid compositions of individual peaks and the number of different TAG species having the identical mass present within a given peak. While the positional distribution of the fatty acids can be suggested by the relative abundance of the two DAG fragments produced, positional distribution cannot be firmly established by this method. Wild-type camelina oil is rich in TAGs containing 52, 54 and 56 carbons, with 16:0/18:2/18:3 (870.8 *m/z*), a mixture of tri-18:2 and 18:1/18:2/18:3 (896.8 *m/z*), and 20:1/18:2/18:3 (924.8 *m/z*) representing the most abundant ammoniated (M + NH_4) TAG species (Figure 5a). In contrast, high omega-7 camelina oil contains TAG species with lower total carbon numbers (48, 50 and 52 carbons). Neutral loss scanning and product ion spectra of individual peaks confirm the presence of 16:1 and 18:1 in the most abundant TAG molecular species (Figure 5b; Figures S4 and S5). For example, the product ion spectrum of the prominent peak at 818.8 *m/z* confirms that this peak is composed entirely of 16:1/16:1/16:1 TAG. Similarly, the peak at 846.8 *m/z* is 16:1/16:1/18:1 (or 16:1/18:1/16:1) TAG. While the mass spectrometer cannot distinguish between oleic acid (18:1Δ9) and cis-vaccenic acid (18:1Δ11) isomers, which have identical masses, it is likely that the 18:1-containing TAG species reflect the high levels of 18:1Δ11 produced by the engineered seeds. Other TAG species that are unique to high omega-7 transgenic lines and not found in wild-type camelina oil include 866.8 *m/z* (16:1/18:3/18:3), *m/z* 868.8 (16:1/18:2/18:3), and high amounts of 16:1/18:1/18:3 (870.8 *m/z*) and 16:1/18:1/18:1 (874.8 *m/z*).

Seed weight, oil content and germination are unaffected by omega-7 unsaturated fatty acid accumulation

The effect of high levels of omega-7 unsaturated fatty acids on seed total oil content, seed weight and seed germination was also examined (Table 4). Under greenhouse conditions, the oil content of seeds from nonengineered plants and seeds from high omega-7 unsaturated fatty acid lines was ~32% of seed weight. Similarly, the germination rate of both sets of seeds was ~97% (Table 4). These results suggest that accumulation of omega-7 unsaturated fatty acids to levels of 65% of the total fatty acids does not negatively affect seed oil content or germination.

Omega-7 unsaturated fatty acid accumulation affects the thermotropic properties of camelina oil

Differential scanning calorimetry of the extracted oils revealed multiple transitions during cooling and heating (Figure 6). During cooling, the onset of crystallization occurred at

Table 2 Comparison of the fatty acid composition of soybean seeds engineered for omega-7 fatty acid production

	Fatty acid composition (wt% of total fatty acids)													
Line	16:0	16:1Δ9	16:2	16:3	18:0	18:1Δ9	18:1Δ11	18:2	18:3	20:1Δ11	20:1Δ13	22:1	Others	Total omega-7
Non transformed	12.6 ± 0.8	ND	ND	ND	3.3 ± 0.2	11.8 ± 1.0	ND	56.0 ± 1.4	13.6 ± 0.9	ND	ND	ND	2.7 ± 0.3	ND
Com25/Fat5	11.7 ± 1.1	0.4 ± 0.1	ND	ND	3.9 ± 0.9	11.8 ± 1.6	4.2 ± 1.6	51.3 ± 2.4	13.3 ± 0.8	ND	ND	ND	3.4 ± 0.3	4.5 ± 1.6
Com25/Fat5 KASII-HP	15.2 ± 1.3	2.4 ± 1.8	ND	ND	4.3 ± 0.6	5.8 ± 1.9	14.1 ± 3.5	41.0 ± 2.6	13.5 ± 1.5	ND	ND	ND	3.7 ± 0.4	16.5 ± 4.1

The values shown are the average ±SD for measurements of fatty acids from 5 to 8 single seed of nontransformed and transgenic (T_1 plants) soybean lines.
ND, Not detected.

Table 3 Fatty acid comparison of triacylglycerol (TAG) and phosphatidylcholine (PC) of nontransformed and high omega-7 fatty acid camelina seeds

		Fatty acid composition (wt% of total fatty acids)													
Line	Lipid	16:0	16:1Δ9	16:2	16:3	18:0	18:1Δ9	18:1Δ11	18:2	18:3	20:0	20:1Δ11	20:1Δ13	22:1	Others	Total omega-7*
Nontransformed	TAG	6.5 ± 0.1	0.2 ± 0.1	ND	ND	3.7 ± 0.2	13.1 ± 0.3	1.0 ± 0.1	20.2 ± 0.5	32.5 ± 1.0	2.6 ± 0.1	11.5 ± 0.1	0.8 ± 0.1	3.3 ± 0.1	4.5 ± 0.2	1.1 ± 0.1
	PC	12.4 ± 0.7	ND	ND	ND	4.7 ± 0.3	24.9 ± 0.6	ND	31.4 ± 1.1	17.2 ± 1.2	ND	5.6 ± 0.4	ND	3.6 ± 0.3	0.2 ± 0.1	ND
Com25/FatB-HP	TAG	4.1 ± 0.1	32.2 ± 0.8	1.5 ± 0.2	1.5 ± 0.2	0.3 ± 0.1	3.7 ± 0.8	29.1 ± 2.8	6.1 ± 0.4	18.2 ± 0.2	1.3 ± 0.1	1.5 ± 0.1	1.3 ± 0.2	0.2 ± 0.1	1.1 ± 0.4	65.9 ± 2.3
Com25/Fat5 KASII-HP/FAE1-HP	PC	3.3 ± 0.2	32.3 ± 0.7	ND	ND	0.1 ± 0.1	2.0 ± 0.3	33.9 ± 3.0	11.5 ± 0.7	15.7 ± 0.5	1.2 ± 0.1	ND	ND	ND	0.1 ± 0.1	66.2 ± 2.6

The values shown are the average ±SD from three independent analyses of lipid extracts from seeds of nontransformed and the highest producing ommega-7 unsaturated fatty acid camelina line (homozygous seeds from T_3 plants).
ND, Not detected.
*Includes content of 16:1Δ9, 18:1Δ11, and 20:11Δ13 as well as 16:2, 16:3, which are derived from the omega-7 fatty acid 16:1Δ9.

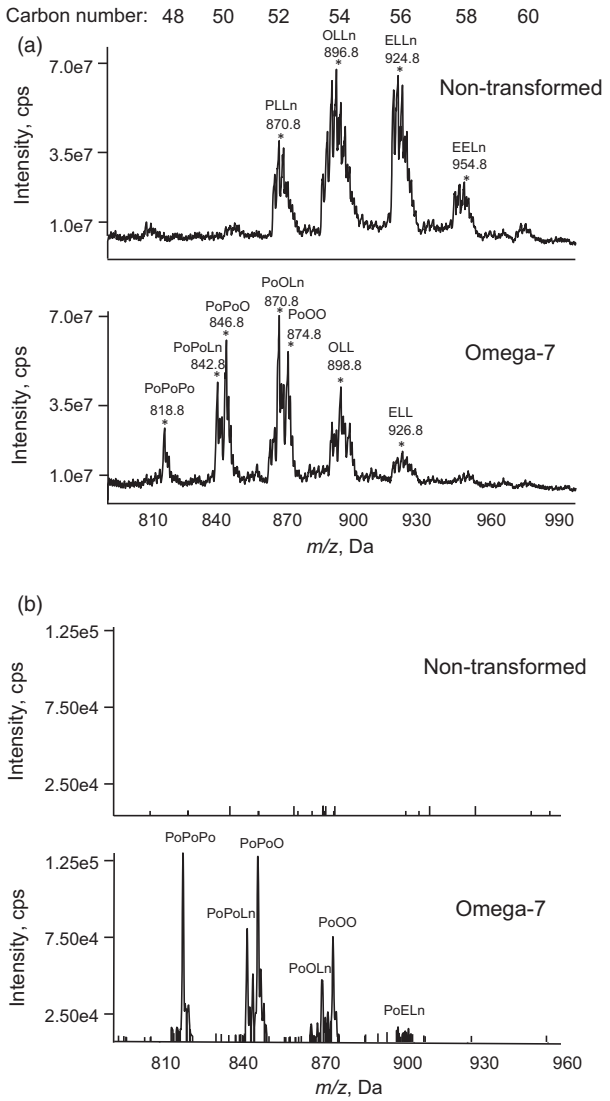

Figure 5 TAG profiling of oil extracts from seeds from nontransformed and high omega-7 lines by ESI-MS/MS. Q1 scan (a) comparing intact, ammoniated ($M + NH_4$) TAG species present in wild-type and high omega-7 camelina oil. Signal intensity is expressed in counts per second (cps). Major TAG species are indicated with an asterisk (*). Peaks to the right of the asterisk represent TAG species with the same carbon number but fewer double bonds (*m/z* increasing by 2 mass units per double bond lost), and peaks to the left of the asterisk represent TAG species with the same carbon number but with more double bonds (*m/z* decreasing by 2 mass units for each additional double bond). (b) Neutral loss scans for loss of 16:1 fatty acid (*m/z* 271) show that novel 16:1-containing TAG species PoPoPo (*m/z* = 818.8), PoPoLn (*m/z* = 842.8), PoPoO (*m/z* = 846.8), PoOLn (*m/z* = 870.8), PoOO (*m/z* = 874.8) are not found in seed oil from nontransformed lines but are abundant in oil from high omega-7 lines. The novel 16:1 containing TAGs were confirmed by product ion scans that show the presence of novel DAGs, PoPo (*m/z* = 547.8) and PoLn (*m/z* = 571.8) (Figure S4 and Figure S5). P = 16:0, Po = 16:1, O = 18:1, L = 18:2, Ln = 18:3, E = 20:1.

−6.0 °C for oil from the nontransformed seeds, while crystallization did not begin until −30.2 °C for oil from the transgenic line enriched in omega-7 fatty acids (Table 4 and Figure 6a). For both oils, a major crystallization exotherm was detected at

Table 4 Seed weight, oil content and germination rate of seeds from nontransformed plants and seeds from highest omega-7 unsaturated fatty acid accumulating line (transformed with Com25/FatB-HP + Com25/Fat5/KASII-HP/FAE1-HP constructs; 'High Omega-7'). T_3 seeds from a homozygous engineered line were used for measurement of seed weight, seed oil content and germination rate

Line	Seed weight (mg)*	Oil content (%)†	Germination (%)‡
Nontransformed	0.95 ± 0.05	31.9 ± 2.7	96.3 ± 1.5
High omega-7	0.95 ± 0.04	32.0 ± 0.7	95.7 ± 2.5

*Values are the average seed weight ±SD from measurements of 80 seeds from a nontransformed and the highest omega-7 unsaturated fatty acid-producing line (n = 3).

†Values are the average oil content ±SD from analyses of three independent measurements.

‡Values shown are the average germination rate ± SD from three soil-containing trays, each sowed with 50 seeds, from the highest omega-7 unsaturated fatty acid-producing line and nontransformed plants.

lower temperatures; the midpoint T_m of this transition was −62.5 °C for the nontransformed and −55.5 °C for the high omega-7 oil.

During heating of the oils, complex melting and recrystallization behaviour was detected (Figure 6b). Oil from the nontransformed seeds began to melt at −54.4 °C; the small endotherm was followed by an exothermic recrystallization event that preceded the largest endotherm, which had a midpoint T_m at −11.2 °C. A small shoulder was apparent following the main endotherm, and melting was completed at 3.1 °C. Oil from the high omega-7 line did not begin to melt until −35.4 °C and was characterized by two endotherms, with T_m's at −25.5 °C and −15.3 °C. In some samples, melting was complete at −5.7 °C, while in other samples, a small shoulder appeared on the endotherm and the melt was not complete until 10 °C. This shoulder may indicate the presence of some impurity in the extracted oil or some heterogeneity among the samples.

In addition to the lowering of T_{on} for crystallization of oil from the high omega-7 line, freezing was completed over a temperature range that was almost 20 °C less than for the oil from nontransformed seeds (Table 5). Similarly, melting transitions during heating occurred over a wider temperature for the oils from nontransformed seeds than for the omega-7 enriched oils (Table 5). In both samples, polymorphism in the TAG crystallization and melting processes also contributes to the complexity of the thermal signals. TAGs typically show multiple endothermic events during heating, as less stable crystalline forms melt, reorient themselves, recrystallize and then melt again, eventually completely liquefying (Tan and Che Man, 2002). Such polymorphism may explain the multiple events seen in heating scans of oils from nontransformed and high omega-7 camelina (Figure 6b).

Discussion

The results presented here demonstrate the ability to engineer the production of omega-7 unsaturated fatty acids to ~60–65% of the total fatty acids of seeds of camelina, compared with ~2% of the fatty acids of seeds from nontransformed plants. This large shift in fatty acid unsaturation from the typical C18 Δ9 unsatu-

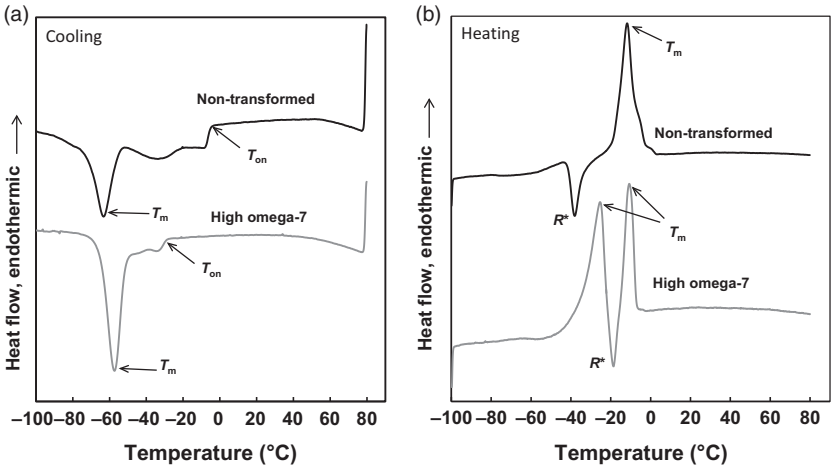

Figure 6 Differential scanning calorimetry (DSC) of TAG from nontransformed and high omega-7 camelina seeds. Representative cooling (a) and heating (b) scans show complex crystallization and melting behaviours of the lipids. Cooling scans (a) reveal a lower crystallization onset temperature, T_{on}, for TAG enriched in omega-7 unsaturated fatty acids compared to TAGs from nontransformed seeds. Subsequent heating (b) shows initial melting endotherms, followed by recrystallization (R*) and subsequent melting for oils from both lines. T_m indicates the midpoint transition temperature for crystallization exotherms (a) and melting endotherms (b).

Table 5 Thermal properties of TAGs extracted from seeds of nontransformed and transgenic camelina

Line	Crystallization T_{on} (°C)	Crystallization T_m (°C)	Melting onset T (°C)	Melting 1st T_m (°C)	Recrystallization nadir T (°C)	Melting 2nd T_m (°C)	Melting offset T (°C)	Melting range (°C)
Nontransformed	−6.5 ± 1.1	−62.5 ± 2.0	−56.1 ± 2.7	−45.8 ± 2.3	−39.7 ± 4.3	−12.5 ± 1.9	3.1 ± 1.2	59.2 ± 2.0
High omega-7	−30.2* ± 4.0	−55.5 ± 2.5	−35.4* ± 1.2	−25.5* ± 0.6	−17.7* ± 1.8	−10.3 ± 0.5	3.6 ± 7.8	39.0* ± 6.7

Values represent average (±SD) acquired from scans of three (Nontransformed) or four (High omega-7) independent TAG extractions. Values for high Omega-7 TAGs designated by * differ ($P < 0.01$) from those from TAG of nontransformed camelina.

rated fatty acid pathway did not result in significant reductions in total seed fatty acid content, seed weight or seed germination under greenhouse conditions. Although no empirical differences in growth of the high omega-7 lines relative to nontransformed plants, field studies are required to conclusively establish that this trait has no agronomic yield penalty.

Key to this engineering strategy is the use of a mutant Δ9-acyl-ACP desaturase with activity enhanced for 16:0-ACP, rather than 18:0-ACP and an ER Δ9-16:0-CoA desaturase. The seed-specific expression of these enzymes combined with KASII suppression to enhance 16:0-ACP substrate pools resulted in seeds with ~44% omega-7 unsaturated fatty acids. The inclusion of a second Δ9-16:0-ACP desaturase transgene and RNAi suppression of FatB increased the total amount of omega-7 unsaturated fatty acids to ~60–65% of the total fatty acids. Although it is not possible to dissect the relative contributions of these additional transgenes, we hypothesize that RNAi suppression of the FatB thioesterase (FatB-HP) provided further increases in 16:0-ACP pool sizes for the Δ9-16:0-ACP desaturase.

An unexpected outcome of the metabolic engineering strategy was a large reduction in total saturated fatty acids in our final transgene combination that included FatB-HP along with expression of 16:0-ACP and –CoA desaturases. In seeds from these lines, saturated fatty acids accounted for ~5% of the total fatty acids compared with ~12% in seeds from nontransformed seeds. Strikingly, not only 16:0 levels were reduced in seeds from these lines, but also 18:0 accounted for ~0.3% of the total fatty acids in transgenic seeds compared with 3.5% in nontransformed seeds. Notably, these saturated fatty acid levels in engineered seeds were lower than those recently reported in *Arabidopsis* seeds modified for expression of the Fat5 Δ9-16:0-CoA desaturase (Fahy *et al.*, 2013).

Another interesting observation from these studies was the near identical content of omega-7 unsaturated fatty acids in TAG and PC of engineered seeds. This observation indicates that camelina seeds do not exclude omega-7 fatty acids from PC, despite the fact that these fatty acids are likely not synthesized on this lipid, in contrast to C18 polyunsaturated fatty acids. Given that germination of engineered seeds is apparently not negatively affected, it is likely that seeds are able to tolerate high levels of omega-7 unsaturated fatty acids in membrane lipids.

The availability of our engineered camelina lines provides an opportunity to evaluate high omega-7 oils in industrial applications, such as 1-octene production, and in nutraceuticals. As a prelude to more extensive functional evaluation of high omega-7 unsaturated camelina oil, we conducted thermal analysis of seed oil from our top engineered line. As indicated by ESI-MS/MS analyses, TAG species from the engineered lines have distinct fatty acid compositions relative to TAG from seeds of nontransformed lines, including reductions in species enriched in C18 polyunsaturated fatty acids and a corresponding increase in species with monounsaturated fatty acids and C16 fatty acids. These differences in TAG composition are reflected in distinct thermotropic properties of the high omega-7 unsaturated camelina oil compared with oil from nontransgenic seeds. The high omega-7 unsaturated fatty acid oil has ~18.5% more C16 fatty acids and ~10% less C18 fatty acids relative to oil from nonengineered seeds (Table 1). This overall decrease in fatty acid chain length should contribute to lower melting points among the TAGs of the high omega-7 line (Knothe and Dunn, 2009). This effect of decreased chain length counters the slight increase in melting points one might expect from the shift from the di-unsaturated 18:2 fatty acids to the monounsaturated omega-7 fatty acids. In a recent study of the melting points of several TAG

species, Knothe and Dunn (2009) reported that TAG of 16:1Δ9 show two endothermic peaks during melting with T_m values of around −28 and −22 °C. By comparison, the T_m of TAG containing 18:1Δ11 was 1.0 °C and T_m for TAGs of 18:2Δ912 was −12.7 °C (Knothe and Dunn, 2009). Thus, the melting points reported here are consistent with TAG containing mixtures of these fatty acids. It is expected that these alterations in thermotropic properties of camelina oil may have important implications for the use of this oilseed in biodiesel applications. In addition, the broader temperature transition range for the oil from nontransformed seeds may indicate more varied TAG molecular species composition, which would cause less cooperativity during freezing and melting. It may also be indicative of the decreased saturated fatty acid content in the transgenic line, as oils with greater saturated fatty acid content melt over a broader temperature range than oils with more unsaturated fatty acids (Tan and Che Man, 2000).

The results reported here are among the highest levels of novel fatty acids generated in an engineered oilseed crop. We did observe that seed-specific expression of the Δ9 16:0-ACP and CoA desaturases with KASII suppression in soybean yielded less than half the omega-7 unsaturated fatty acids compared to camelina engineered with the same transgene combination. Although we do not know the basis for this difference, this observation indicates that our engineering strategy may need additional optimization for transfer to other oilseeds. Future studies for the optimization of this trait may include introduction of additional transgenes for key enzymes, including the Com25 or FAT5 desaturases, and more detailed quantification of target protein levels to determine whether maximal metabolic capacity for omega-7 fatty acid production has been achieved in our current top-performing lines. In addition, we hypothesize that additional increases in omega-7 unsaturated fatty acids can be achieved by seed-specific suppression of the native camelina Δ9-18:0-ACP desaturase to more completely shift flux to Δ9-16:0-ACP desaturation, although the impact of this modification on agronomic fitness, including seed germination, is difficult to predict.

Experimental procedures

Vector construction

Clones encoding KASII were identified from the published camelina seed transcriptome (Nguyen *et al.*, 2013). To construct the camelina KASII sense arm, a 162 base pair region of camelina KASII was amplified with the oligonucleotide primers: 5′-GGG*CTGCAG*AAACAGCAACTATCTACGCA-3′ and 5′- GGG*CTCGAGGCGGCCG*CATGTAATTTATCTGTT-3′ (added restriction sites are italicized). The *Pst*I/*Xho*I restriction enzyme fragment was used to replace the *KASII* sense arm in a modified version of a previously described plasmid utilizing a *FAD2* intron in the previously described vector pGEMT-KASIIHP-FAD2HP (Okuley *et al.*, 1994; Pidkowich *et al.*, 2007). The KASII antisense sequence used was PCR amplified from camelina developing seed cDNA using oligonucleotide primers with flanking *Eco*RI/*Not*I and *Nhe*I restriction sites: 5′- GG*GCTAGC*AGCAACTATCT ACGCA-3′ and 5′- GGG*GAATTCGCGGCCG*CATGTAATTTATCT GTT-3′. The *Eco*RI/*Nhe*I restriction enzyme fragment was used to replace the *Arabidopsis* KASII antisense arm in pGEMT-KASIIHP-FAD2HP. The KASII sense, antisense and *FAD2* intron were assembled and cloned into plasmid pBCon123-Hyg (Zhang *et al.*, 2013) under the control of the soybean α′subunit of β-conglycinin promoter and phaseolin 3′UTR. A cassette including the soybean glycinin-1 promoter and 3′UTR, KASII sense, KASII antisense and FAD2 intron was excised using *Hind*III and cloned into the binary vector pBinGlyRed2 (Nguyen *et al.*, 2013) to generate pBinGlyRed-CsKasII-HP.

A codon-optimized Fat5 gene from *C. elegans* (Fahy *et al.*, 2013; Watts and Browse, 2000) was synthesized (GenScript USA Inc., Piscataway, NJ) with the addition of flanking *Not*I restriction sites. The *Not*I fragment was cloned into the vector pKMS3 (Nguyen *et al.*, 2013) to place Fat5 under the control of the soybean glycinin-1 promoter and 3′UTR to create pKMS3-Fat5S. A cassette comprising the glycinin-1 promoter and 3′UTR flanking Fat5 gene was transferred into to pBinGlyRed-CsKasII to make pBinGlyRed-CsKasII-HP/Fat5. A codon-optimized Com25 open reading frame was synthesized (GenScript USA Inc.) to include flanking *EcoR*I and *Xho*I at the 5′ and 3′ ends, respectively. Com25 was digested with *EcoR*I and *Xho*I and cloned into the corresponding sites of pBinGlyRed-CsKasII-HP/Fat5 under control of the soybean glycinin-1 promoter to generate pBinGlyRed-CsKasII-HP/Fat5/Com25.

A previously described *FAE1* RNAi suppression cassette regulated by a glycinin-1 promoter (Nguyen *et al.*, 2013) was ligated as an *Asc*I restriction enzyme fragment and cloned into the *Mlu*I restriction site of pBinGlyRed-CsKasII-HP/Fat5/com25 to obtain the final vector pBinGlyRed-CsKasII-HP/Fat5/Com25/FAE1-HP (short name is pBinGlyRed/C25/F5/K2/F1, Figure 2, top).

A Com25 fragment flanked by *EcoR*I/*Xho*I restriction sites was cloned into the corresponding sites of pBinGlyRed2 (Nguyen *et al.*, 2013) to obtain the vector pBinGlyRed-Com25. For Fat5, the cassette containing the glycinin-1 promoter, Fat5 open reading frame and the glycinin-1 3′UTR was restricted from vector pKMS3-Fat5S using *Asc*I and cloned into the corresponding site in pBinGlyRed-Com25 to create pBinGlyRed-Com25/Fat5.

A camelina FatB RNAi suppression cassette was constructed similarly to the KASII hairpin structure above with two pairs of primers 5′-GGG*CTGCAG*AAAC AATGTAAAGTACATTGGGT-3′and 5′- GGG*CTCGAGGCGGCCGC*ACTCCACTCTGTTCTTCCTC -3′; 5′- GGG*CTAGC A*ATGTAAAGTACATTGGGT -3′ and 5′-GGG*GAATTCGCGGCCGC*ACTCCACTCTGTTCTTCCTC -3′. The FatB sequence was obtained from the published camelina seed transcriptome (Nguyen *et al.*, 2013), and cDNA generated from developing camelina seeds was used for the PCR template. Com25 open reading frame-containing cassette was cloned in to vector pBinGlyBar1 (Nguyen *et al.*, 2013) to obtain pBinGlyBar1/Com25/FatB-HP (Figure 2, bottom). Both transgenes in this vector were placed under the control of the seed-specific soybean glycinin-1 promoter.

Camelina transformation

Vectors pBinGlyRed-CsKasII-HP, pBinGlyRed-Com25, pBinGlyRed-Com25/Fat5, pBinGlyRed-CsKasII-HP/Fat5, pBinGlyRed-CsKasII-HP/Fat5/Com25, pBinGlyRed-CsKasII-HP/Fat5/Com25/FAE1-HP were transformed into *Camelina sativa* cv. Sunesson using the previously described floral vacuum infiltration method (Lu and Kang, 2008) and transformed seeds were selected by DsRed fluorescence as previously described (Nguyen *et al.*, 2013). The highest omega-7 unsaturated fatty acid–producing line transgenic line pBinGlyRed-CsKasII-HP/Fat5/Com25/FAE1-HP (homozygous T_3 lines) was retransformed with pBinGlyBar1/Com25/FatB-HP (Figure S1), and transformed plants were selected by Basta (glufosinate) resistance as described previously (Nguyen *et al.*, 2013). Camelina plants were grown under greenhouse conditions with 14 h day length (24–26 °C) and 8 h dark

(18–20 °C) with natural and supplemental lighting at 400–500 μmoles/m^2/s as described previously (Nguyen *et al.*, 2013).

Preparation of soybean transformation construct and transformation

Vector pBinGlyBar1-Com25/Fat5 is similar with vector pBinGly-Red-Com25/Fat5 but clone in to pBinBar1 backbone (Nguyen *et al.*, 2013). Com25 fragment was cloned into pBinGlyBar1 vector at *EcoR*I/*Xho*I, and Fat5 cassette including soybean glycinin-1 promoter and 3′UTR from pKMS3-Fat5S was cloned at *Asc*I.

The soybean KASII RNAi cassette was assembled similar camelina KASII RNAi. The KASII sense was amplified with the oligonucleotide primers: 5′- GGG*CTGCAG*AAACAAC AGAT-TTCAGAGCAC-3′ and 5′- GGG*CTCGAGGCGGCCG*CCTATC AACATTCTGAT-3′. The soybean KASII antisense was amplified with oligonucleotide primers 5′- GG*GCTAGC*AGCAACAGA TTTCAGAGCAC-3′ and 5′- GGG*GAATTCGCGGCCG*CCTATCAA CATTCTGAT-3′ The soybean KASII RNAi cassette was added to pBinGlyBar1-Com25/Fat5 at *Mlu*I to create pBinGlyBar1-Com25/Fat5/KASII.

The soybean cultivar Thorne was transformed through an *Agrobacterium*-mediated transformation system as described (Zhang *et al.*, 1999). The final two binary vectors pBinGlyBar1-C25F5 and pBinGlyBar1-C25F5K2 carrying a Basta resistance gene as a selection marker and targeted genes (Com25, Fat5, KASII hairpin) were delivered into the *A. tumefaciens* strain NTL4 by tri-parental mating and were used for transformation into soybean (Zhang *et al.*, 1999). Primary transformants were screened by the leaf paint assay for herbicide tolerance (Zhang *et al.*, 1999). Transgenic soybean plants were grown in a greenhouse with a 14-h photoperiod and 28 °C/26 °C day/night temperature.

Gas chromatographic analysis of fatty acid compositions

Fatty acid methyl esters (FAMEs) were prepared from camelina seeds by transesterification with trimethylsulfonium hydroxide (Butte, 1983) or with 2.5% (v/v) sulphuric acid/methanol as previously described (Nguyen *et al.*, 2013). Fatty acid methyl esters were analysed by gas chromatography with flame ionization detection as previously described (Nguyen *et al.*, 2013). Double bond positions in omega-7 monounsaturated fatty acid 16:1Δ9, 18:1Δ11 and 20:1Δ13 (Figure S1) from engineered were determined GC-MS analysis of dimethyl disulphide adducts prepared from fatty acid methyl esters as described (Yamamoto *et al.*, 1991).

Analysis of triacylglycerol and phosphatidylcholine fatty acid compositions of camelina seeds

Lipids were extracted from camelina seeds (25–30 mg) by grinding to a fine powder in 3 mL methanol/chloroform (2 : 1 v/v). Samples were incubated for 30–60 min at room temperature with agitation, and then, 1 mL chloroform and 1.8 mL water were added (Bligh and Dyer, 1959). The tubes were shaken well, and aqueous and organic phases were separated by centrifugation. The organic phase was transferred into a new tube, and the solvent evaporated under N_2. Lipids were redissolved in 1 mL heptane and applied to an equilibrated 3 mL Supelclean LC-Si SPE column (Supelco, Saint Louis, MO). The TAG fraction was eluted with 4 mL of 80 : 20 (v/v) heptane/ethyl ether. The column was then washed with 4 mL acetone. Total phospholipids were subsequently eluted with 5 mL of methanol:chloroform:water (100 : 50 : 40 v/v/v), and 1.7 mL of chloroform and 1.7 mL of water were added to the eluent. After shaking and centrifugation, the total phospholipids in the organic layer were recovered. The phospholipid fraction was then resolved on silica TLC in chloroform/methanol/water/ammonium hydroxide (65/35/3/2.5 v/v/v/v), and the PC band was scraped from plate and directly transesterified in 2.5% sulphuric acid/methanol (v/v) as described previously (Nguyen *et al.*, 2013). An aliquot of the TAG fraction was also transesterified in 2.5% sulphuric acid/methanol (v/v) and 250 μL of toluene (Nguyen *et al.*, 2013). Following heating at 95 °C for 45 min, fatty acid methyl esters were recovered and analysed by gas chromatography-flame ionization detection as described (Cahoon *et al.*, 2006).

ESI-MS/MS analysis of TAG molecular species

Mass spectrometry analyses were conducted using an Applied Biosystems (Foster City, CA) 4000 QTRAP linear ion trap quadrupole mass spectrometer to characterize TAG molecular species. The total neutral lipid extract for ESI-MS/MS analysis was prepared as described for seed oil content measurement below but without added internal standard and diluted 1:5000 in water/isopropyl alcohol/methanol (55 : 35 : 10 v/v/v) containing 25 mM ammonium formate and 4 μL/L formic acid and directly infused into the mass spectrometer at a rate of 20 μL per minute. A Q1 scan (positive mode) was performed over a mass range of 450–1200 m/z with a cycle time of 2 s. Data were collected for 150 cycles. Instrument settings were as follows: Source temperature 300 °C, ESI needle voltage 5.5 kV (positive mode), desolvation potential (DP) 90, entrance potential (EP) 10, Curtain gas (CUR) 10, and gas 1 (GS1) 50 arbitrary units, gas 2 (GS2) 40 arbitrary units. Product ion spectra were generated for selected TAG molecular species using the instrument settings and flow rates described above, and applying collision energy of 54 and a collision exit potential of 11. Scans were taken over a mass range of 200–1000 *m/z* with a cycle time of 2 s, and data files generated with 50–150 scans. Neutral loss spectra showing the loss of a specific fatty acid from TAG species were generated by direct infusion using instrument settings described above and a source temperature of 400 °C. Scans were taken over a mass range of 500–1475 *m/z* with a cycle time of 3 s. Data was collected for five cycles. Neutral loss of the following fatty acids was monitored: C8:0 (161.0 *m/z*), C10:0 (189.1 *m/z*), C12:0 (217.1 *m/z*), C14:0 (245.1 *m/z*), C16:0 (273.1 *m/z*), C16:1 (271.1 *m/z*), C18:0 (301.1 *m/z*), C18:1 (299.1 *m/z*), C18:2 (297.1 *m/z*), C18:3 (295.1 *m/z*), C20:0 (329.1 *m/z*), C20:1 (327.1 *m/z*), C22:0 (357.1 *m/z*), C22:1 (355.2 *m/z*), C24:0 (385.2 *m/z*), C24:1 (383.2 *m/z*), C26:0 (413.2 *m/z*) and C26:1 (411.2 *m/z*).

Seed oil content measurement

Total lipids were extracted from 30 mg of transgenic camelina seeds as described above with the inclusion of triheptadecanoin (NuChek Prep, Elysian, MN) internal standard, and total fatty acids were measured by transesterification and subsequent analysis by gas chromatography/flame ionization detection as previously described (Nguyen *et al.*, 2013). Fatty acids were quantified relative to the gas chromatographic response of heptadecanoyl methyl ester from the internal standard.

Seed germination measurement

Three soil-containing trays, each sowed with 50 seeds, for the high omega-7 unsaturated line and a nontransformed line were

maintained under greenhouse conditions. After 4 days, germinated seeds from each line were counted.

Differential scanning calorimetry of camelina seed oil

Camelina seeds (500 mg) were ground to a fine powder with glass stirring rod in 2 mL of heptane and then incubated at 40 °C for 1 h. The oil extract in heptane was washed with 1 mL of water and transferred to a 100 × 30 mm class screw cap tube. The oil fraction was dried under nitrogen and heated to 55 °C in a dry-block heater to assure that it was liquid prior to transfer via syringe into preweighed aluminium volatile sample pans (20 μL volume). The pans were hermetically sealed and weighed to obtain the sample weights, which ranged from 2 to 8 mg. Calorimetry was carried out using a DSC-7 (Perkin Elmer, Norwalk, CT) with liquid N_2 cooling and a helium purge gas. The method used was modified from AOCS standard protocol Cj 1-94, which recommends holding samples at 80 °C for 10 min, scanning at 10 °C/min to −40 °C, holding for 30 min at −40 °C, then scanning at 5 °C/min to 80 °C (AOCS, 2004; Ribiero *et al.*, 2009). In our experiments, samples were cooled to −80 or −100 °C to enable detection of the crystallization exotherms. The calorimeter was calibrated to the onset melting temperatures of indium (T_m = 156.6 °C) and heptane (T_m = −90.56 °C), heated at 5 °C/min. Indium was used to calibrate the melting enthalpy (ΔH = 28.45 J/g) at 5 °C/min. Indium was used to calibrate the melting enthalpy (ΔH = 28.45 J/g) at 5 °C/min. Because the cooling and heating rates differed, and the DSC was calibrated to the heating rate, the melting and crystallization onset temperature of indium was measured to enable comparison of temperatures obtained from cooling and heating curves. The crystallization onset of indium was measured at 154.3 °C while cooling at 10 °C. Data obtained from the cooling and heating scans were analysed using the software provided by Perkin-Elmer for the Model 1020 Controller. T_m represents the temperature of the peak maximum or minimum for endotherms and exotherms, respectively. T_{on} represents the onset temperature for crystallization during cooling (Tan and Che Man, 2000).

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Structures of omega-7 fatty acids.
Figure S2 Scatter plots of omega-7 fatty acid content in transgenic single seed from highest producing line of camelina or soybean.
Figure S3 Mass spectra of thiomethyl adducts of fatty acid methyl esters of omega-7 monounsaturated fatty acids 16:1Δ9, 18:1Δ11, and 20:1Δ13 from camelina seeds of a high omega-7 camelina line.
Figure S4 Product ion scans of novel 16:1-containing TAG found in high omega-7 camelina oil.
Figure S5 DAG fragments resulting from product ion scans of m/z 846.8 (a, b) and *m/z* 870.8 to confirm the fatty acid composition of the parent TAG species as PPLn in seed oil from nontransformed and PoPoO in high omega-7 fatty acid camelina oil.