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The coexpression of two desaturases provides an optimized reduction of saturates in camelina oil

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Summary

Reducing the saturate content of vegetable oils is key to increasing their utility and adoption as a feedstock for the production of biofuels. Expression of either the FAT5 16 : 0-CoA desaturase from *Caenorhabditis elegans*, or an engineered cyanobacterial 16 : 0/18 : 0-glycerolipid desaturase, DES9*, in seeds of Arabidopsis (*Arabidopsis thaliana*) substantially lowered oil saturates. However, because pathway fluxes and regulation of oil synthesis are known to differ across species, translating this transgene technology from the model plant to crop species requires additional investigation. In the work reported here, we found that high expression of FAT5 in seeds of camelina (*Camelina sativa*) provided only a moderate decrease in saturates, from 12.9% of total oil fatty acids in untransformed controls to 8.6%. Expression of DES9* reduced saturates to 4.6%, but compromised seed physiology and oil content. However, the coexpression of the two desaturases together cooperatively reduced saturates to only 4.0%, less than one-third of the level in the parental line, without compromising oil yield or seedling germination and establishment. Our successful lowering of oil saturates in camelina identifies strategies that can now be integrated with genetic engineering approaches that reduce polyunsaturates to provide optimized oil composition for biofuels in camelina and other oil seed crops.

Introduction

Vegetable oils are used for both foods and as an industrial feedstock, especially in the production of renewable biofuels (Haslam *et al.*, 2016; Vanhercke *et al.*, 2013). The physical properties and economic value of the oil is a consequence of the fatty acid composition of the triacylglycerol (TAG) which is the major component of the oil and a carbon and energy store in oilseeds, providing for germination and seedling establishment (Maraschin *et al.*, 2019; Theodoulou and Eastmond, 2012). Genetic engineering approaches have allowed extensive alteration and optimization of seed-oil composition for both food uses (Msanne *et al.*, 2020; Wallis *et al.*, 2022) and biofuel applications (Nguyen *et al.*, 2013; Shonnard *et al.*, 2010).

Camelina (*Camelina sativa*) is an ancient crop species that is regaining prominence as a biotechnology crop and an economical source of oil for biofuel and for food. From the bronze age until the mid-20th century camelina was grown throughout Europe and Asia as a source of vegetable oil (Nguyen *et al.*, 2013). After the Second World War, camelina was supplanted as a major oil seed crop by canola (*Brassica napus*) and by soybean (*Glycine max*), but recently camelina has staged a resurgence driven by an interest in renewable biofuels. Its favourable agronomic qualities include the ability to grow on marginal land, enabling biofuel production without cannibalizing arable land. Camelina is also amenable to genetic transformation (Lu and Kang, 2008; Shonnard *et al.*, 2010) and camelina's similarities to the model oil seed plant Arabidopsis (*Arabidopsis thaliana*) provide a strong research foundation for understanding oil synthesis and lipid modification in camelina as well as in other Brassica species (Bai *et al.*, 2019; Collins-Silva *et al.*, 2011; Gomez-Cano *et al.*, 2022; Hutcheon *et al.*, 2010). The advantages of camelina as a crop plant, coupled with the tools available from Arabidopsis research make it a candidate for crop improvement and modification of its qualities for both fuel and food. However, when translating discoveries from Arabidopsis to even closely related species, it is important to recognize that differences in pathway fluxes and metabolic regulation may exist (Bai *et al.*, 2020; Gomez-Cano *et al.*, 2022; Haslam *et al.*, 2016). For this reason, strategies for genetic engineering of oil composition, developed in Arabidopsis need to be retested and refined in the target crop species.

Camelina oil has been successfully used to produce both biodiesel and jet fuel (Fröhlich and Rice, 2005; Shonnard *et al.*, 2010). The preferred oil composition for biodiesel is high in monounsaturates, oleate (18 : 1) and palmitoleate (16 : 1), and low both in polyunsaturates, linoleate (18 : 2) and linolenate (18 : 3), and in saturates, palmitate (16 : 0), stearate (18 : 0) and arachidate (20 : 0). Camelina oil contains about 8% 16 : 0, 3% 18 : 0 and 2% 20 : 0, and these saturated fatty acids detrimentally increase fuel viscosity and form gels at low temperature that compromise engine performance (Damanik *et al.*, 2018; Knothe and Razon, 2017; Sia *et al.*, 2020). Lowering the total saturates from 13% to <5% would provide for a substantial improvement in the low-temperature performance of biodiesel produced from the oil. Since highly unsaturated fatty acids are oxidatively unstable, reducing levels of both polyunsaturated (52%) and fully saturated fatty acids in favour of increased

levels of monounsaturated fatty acids is an important avenue to improve the utility and value of camelia oil (Jiang *et al.*, 2017; Lee *et al.*, 2021).

In plants a multiprotein Type II fatty acid synthase in the plastids produces 16 : 0-ACP (acyl carrier protein). Some of the 16 : 0 is released from the synthase by a FatB acyl-ACP thioesterase (Jones *et al.*, 1995), but most is subject to an additional cycle of the synthase to produce 18 : 0-ACP, which is efficiently desaturated by the stromal 18 : 0-ACP desaturase (Lindqvist *et al.*, 1996). As a result, 18 : 1 is the major fatty acid exported from the plastid to the cytoplasmic acyl-CoA pool, with smaller amounts of 16 : 0 and 18 : 0 (Ohlrogge and Browse, 1995; Wallis *et al.*, 2022). Some 16 : 0-CoA is used for the synthesis of sphingolipids, which have essential roles in the cell (Chen *et al.*, 2006). In camelina seeds, the 18 : 1 may be elongated to 20- and 22-carbon monounsaturates (Kunst *et al.*, 1992), but most is incorporated into phosphatidylcholine of the endoplasmic reticulum where it is available for the synthesis of 18 : 2 and 18 : 3 polyunsaturates (Okuley *et al.*, 1994). The 16 : 0 and 18 : 0 may be elongated to 20 : 0, but they are not desaturated, so these fatty acids make up the saturate portion of the TAG storage lipid. As a result of these metabolic conversions, camelina oil typically contains 13% saturates, 35% monounsaturates and 52% polyunsaturates. Substantial reduction of polyunsaturates in favour of monounsaturates has been achieved in camelina by lowering the expression of the FAD2 18 : 1-phosphatidylcholine desaturase, which is the gateway enzyme for the synthesis of polyunsaturates (Jiang *et al.*, 2017; Lee *et al.*, 2021; Morineau *et al.*, 2017). However, less attention has been paid to reducing the saturate content of camelina oil.

Because 16 : 0-ACP produced in the plastid is the primary source of oil saturates, transgenic expression of engineered and heterologous, plastid-localized 16 : 0-ACP desaturases has been employed in attempts to lower oil saturates in both Arabidopsis and camelina. Although these experiments increased monounsaturates they did not significantly reduce the proportion of saturates (Bondaruk *et al.*, 2007; Nguyen *et al.*, 2010, 2015). This indicates that attempts to use desaturases to lower saturates must focus on 16 : 0- and 18 : 0-containing intermediates of TAG synthesis in the cytoplasm and endoplasmic reticulum. In our experiments with Arabidopsis, we have successfully used a 16 : 0-CoA desaturase from *Caenorhabditis elegans*, FAT5 (Watts and Browse, 2000), to lower 16 : 0 in the seed oil by 70% (Fahy *et al.*, 2013). Alternatively, we have used directed evolution approaches to modify a 16 : 0/18 : 0-glycerolipid desaturase from the cyanobacterium, *Synechococcus elongatus* (Ishizaki-Nishizawa *et al.*, 1996) for activity in eukaryotes. This enzyme, DES9*, also greatly reduces oil saturates when expressed in Arabidopsis seeds (Bai *et al.*, 2016). Camelina is an excellent candidate to produce new crop lines based on these results. Camelina seeds contain approximately 13% saturates, offering an opportunity to test the ability of the desaturases to lower saturates in a widely used biotechnology crop species.

In the work reported here, we found that even very high expression of FAT5 in developing camelina seeds only provided for the modest lowering of oil saturate content, while expression of the DES9* enzyme led to a large decrease in saturates, but compromised seed oil content, germination and seedling establishment. Surprisingly however, coexpression of both FAT5 and DES9* together resulted in transgenic lines that had ~70% reductions in oil saturates while being comparable to parental, untransformed camelina in oil content, germination and seedling establishment. Our findings emphasize the importance of empirical testing and combining approaches to oilseed engineering, and they point to a path for reducing saturates in the oils of other crop plants.

Results

Seed-specific expression of the FAT5 acyl-CoA desaturase in camelina

We chose to first test expression of the *C. elegans* FAT5 in camelina seeds because our use of this gene in Arabidopsis resulted in a 65% reduction in 16 : 0 in the seed oil in several of the transgenic lines (Fahy *et al.*, 2013). Camelina plants were transformed using a floral-dip protocol (Lu and Kang, 2008) with a construct in which FAT5 expression is under control of the strong, seed-specific glycinin promoter and uses the Ds-Red marker to allow visual selection of transgenic seeds (Shockey *et al.*, 2015). We selected and planted 23 red T1 seeds and grew the resulting plants to maturity. We selected samples of red T2 seeds from these individual transformants and analysed them for fatty acid composition of the oil by gas chromatography (GC) as described by Lemieux *et al.* (1990). Compared with untransformed controls (0.3% 16 : 1), 21 transgenic lines had increases in 16 : 1, the product of the FAT5 desaturase. Most lines also had lower 16 : 0 than the controls, with several lines having <5% 16 : 0 (Figure 1). Some, but not all, of these lines had an increase of 5%–20% in total 16-carbon fatty acids (16 : 0 + 16 : 1).

Because the T2 seeds are segregating for the transgene, we calculated the segregation ratios for eight promising lines (Table S1). Three of the lines (3, 11 and 18) that had approximately 75% red seeds (indicating that the T1 parents were hemizygous for a single site of transgene insertion) were selected. We grew ten T2 individuals of these lines and identified a homozygous subline for each of them. All three lines germinated and grew well and produced seeds with 3.8%–4.5% 16 : 0. One of these, line FAT5-11 was investigated in detail.

We prepared RNA samples from developing seeds of homozygous plants of line 11 and measured expression of the FAT5 transgene by quantitative PCR (qPCR). The results show very high expression in excess of 80 times the expression of the endogenous camelina actin gene (*CmACT8*) used as control (Figure 2). Despite this very high expression and the accumulation of 4.9% 16 : 1, the seed oil of homozygous line 11 plants still contained 4.0% 16 : 0, a 48% reduction compared with the untransformed parental control (Figure S1). The FAT5-11 seed also had no significant reduction in either of the other saturates, 18 : 0 and 22 : 0 (Figure S1). The major polar lipid in the seed tissues, phosphatidylcholine, also showed a modest reduction in 16 : 0, to 10.8% compared with 15.4% in the untransformed control (Table S3). These results were disappointing compared with the effect of FAT5 in Arabidopsis where a 65% reduction in 16 : 0 was accompanied by smaller reductions in both 18 : 0 (7%) and 20 : 0 (20%) (Fahy *et al.*, 2013). Our initial characterization indicated that seeds of line FAT5-11 have oil content (280 ± 5 mg/g seed wt.), indistinguishable from untransformed controls (283 ± 5 mg/g seed wt.), and, in subsequent generations, line 11 plants grew well and produced abundant seed. These plant phenotypes were stable to at least the T5 generation and the line is characterized in more detail below.

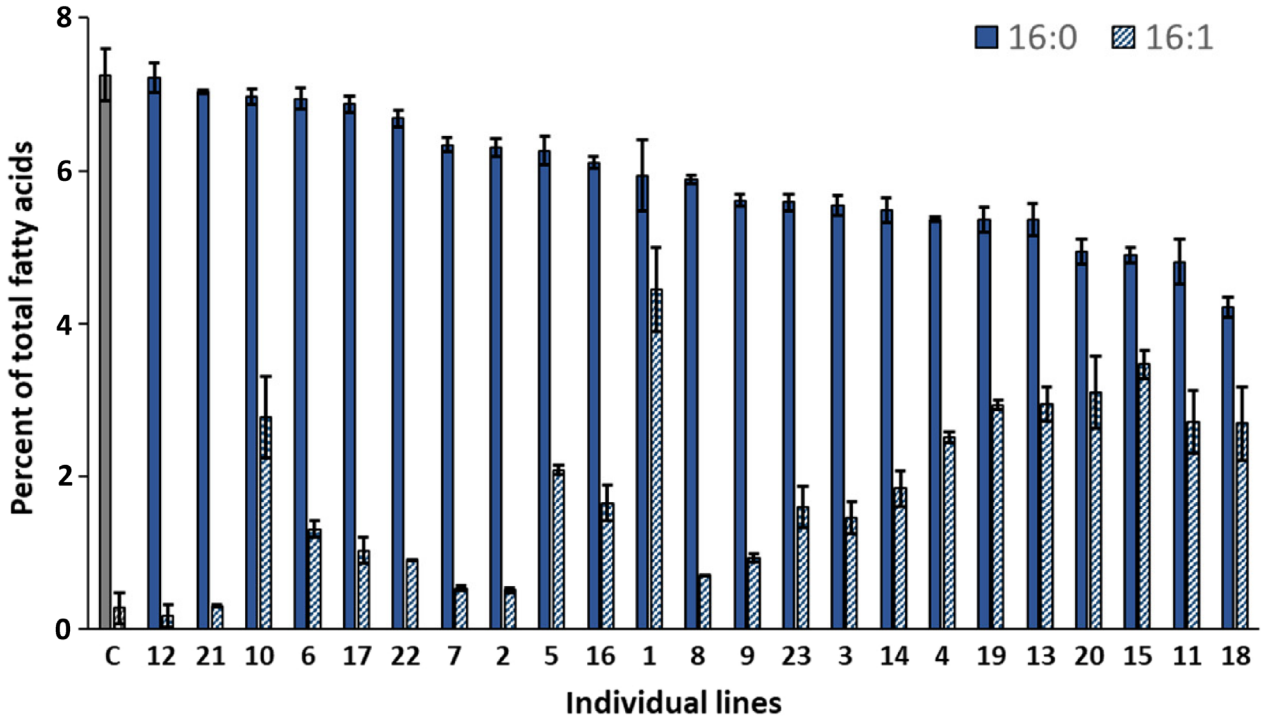

Figure 1 Proportions of 16 : 0 and 16 : 1 fatty acids in T2 seeds expressing the FAT5 desaturase. C, untransformed control. Data are mean ± SEM for three samples of transgenic, red seeds from each independent transformation event.

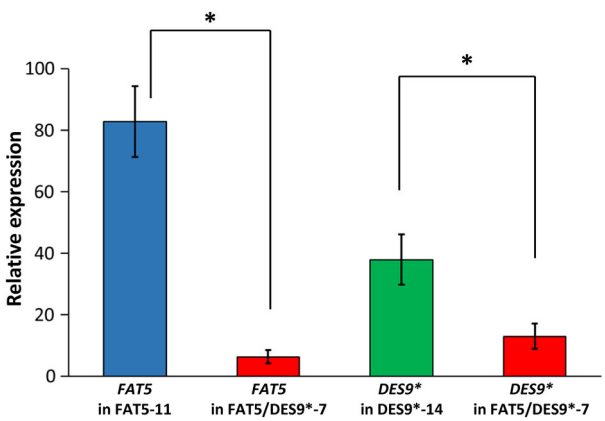

Figure 2 Expression of FAT5 and DES9* transgenes. Quantitative PCR analysis of transcript abundance in developing seeds of lines FAT5-11, DES9*-14 and FAT5/DES9*-7. Data are mean ± SEM for at least 10 biological replicates. * indicates significance ($P < 0.05$) by Student's *t*-test.

Seed-specific expression of the DES9* glycerolipid desaturase in camelina

In previous work, we transformed Arabidopsis with five improved DES9 enzymes that we generated by directed evolution of the 16 : 0/18 : 0-glycerolipid desaturase from the cyanobacterium, *S. elongatus* (Ishizaki-Nishizawa *et al.*, 1996) in the yeast ole1 mutant (Bai *et al.*, 2016). The best of these, here termed DES9*, contained three amino-acid changes (E69R/I129A/G214R) relative to the parental protein, and when expressed in Arabidopsis seed lowered 16 : 0 to only 3.6% of total seed fatty acids compared with 8.4% in the untransformed control. Unfortunately, this and other lines expressing improved DES9 enzymes were all compromised in seed morphology, oil content (reduced by 33% from control), and germination (42% compared to 92% for control). A summary of our data on these lines is included in the Supporting Information (Figure S2 and Table S2).

In light of these unsatisfactory features of transgenic Arabidopsis lines expressing DES9*, we were initially reluctant to test this enzyme in camelina. However, since FAT5 provided for a smaller reduction in 16 : 0 and total saturates in camelina than we measured for Arabidopsis, we decided to investigate whether the consequences of DES9* expression in camelina seeds might be different.

We expressed DES9* under the control of the phaseolin seed-specific promoter with a DsRed selection marker (Shockey *et al.*, 2015). Figure 3 shows the proportions of 16 : 0 and 16 : 1 in samples of red seeds harvested from 14 individual T1 plants, compared to the proportions in brown, untransformed seeds harvested from the same plants. As with the FAT5 transgenics, some of the lines had increases in 16-carbon fatty acids. In the plants on which red seeds contained less than 4% 16 : 0 (T1 plants 1, 2, 6, 8, 9, 10, 11, 12 and 14), the red seeds appeared rather small compared to the brown, untransformed segregants, suggesting that oil content was reduced, as it is in lines of Arabidopsis expressing DES9* (Table S2). This was true both for plants with a single site of transgene insertion (75% red T2 seeds) and plants with multiple insertions (>75% red T2 seeds). One single-insert plant, #14, produced red seeds with only 1.8 ± 0.2% 16 : 0 compared with 8.3 ± 0.3% in the control, but seed size appeared less affected than in other lines. We therefore planted a sample of red T2 seeds and grew plants to maturity. One homozygous plant (all red seeds) that contained an average of 5.0 ± 0.2% total saturates was selected as the parent for the homozygous line DES9*-14 that we characterized in detail. Although seeds of DES9*-14 appeared normal, they

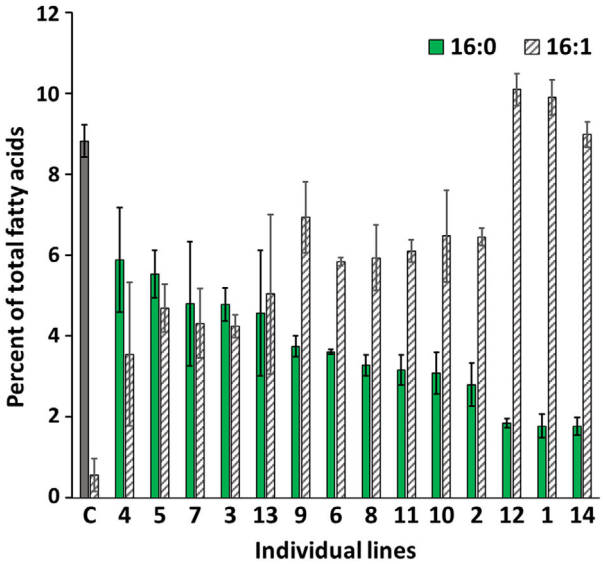

Figure 3 Proportions of 16 : 0 and 16 : 1 fatty acids in T2 seeds expressing the DES9* desaturase. C, untransformed control. Data are mean ± SEM for three samples of transgenic, red seeds from each independent transformation event.

germinated poorly, as did seed from other camelina DES9* lines with <4% 16 : 0. We selected robust line 14 plants that produced abundant seed through to the T4 generation before using them in the comparison studies described below.

FAT5–DES9* expressing camelina

In contrast to our success in lowering 16 : 0 in Arabidopsis seeds by expression of the *C. elegans* FAT5 desaturase (Fahy *et al.*, 2013), the best FAT5 transgenic line that we obtained in camelina has much less of a reduction in seed 16 : 0, despite having very high expression of the transgene (Figures 2 and S1). When we instead used the DES9* desaturase produced by directed evolution, we produced comparable, strong decreases in seed 16 : 0 in both Arabidopsis and camelina, but in both species seed physiology appears to be compromised and germination is poor.

In plants, the exogenous FAT5 desaturase likely acts on 16 : 0-CoA in the cytoplasm and endoplasmic reticulum after 16 : 0 export from the plastid. The DES9* desaturase that we generated by directed evolution in yeast is derived from a cyanobacterial 16 : 0/18 : 0-glycerolipid desaturase (Bai *et al.*, 2016; Ishizaki-Nishizawa *et al.*, 1996); based on our experiments in yeast, we predict that it acts on glycerolipids of the endoplasmic reticulum, including phosphatidylcholine. Because the two desaturase enzymes have different substrates, we decided to investigate the possibility that expressing them together in camelina seeds would provide for a large reduction in oil saturates while ameliorating the highly compromised phenotype that results when DES9* is expressed by itself.

For seed-specific co-expression of the two desaturases, the phaseolin promoter, fused to the Gateway cassette and terminator, was introduced into our Glycinin:FAT5 vector, and then the coding sequence for DES9* was introduced into the new site by an LR Clonase reaction forming a double desaturase vector with DsRed selection. After transformation of camelina, and identification of T1 red seeds, seven lines were grown to maturity. Red seed from all these lines contained <4% 16 : 0, compared to 9.3% in untransformed brown segregants (Figure 4), and some lines again showed modest increases in total 16-carbon fatty acids. The ratio of red to brown seeds produced by T1 transformant #7 (29/8) indicated that the double-desaturase construct had inserted at single site in the genome, so we grew T2 plants and identified a homozygous plant that was propagated to the T3 generation before being investigated in detail. Three additional single-insert lines have similarly low 16 : 0 with seed oil contents and seedling establishment rates similar to the FAT5/DES9*-7 line (Table S4).

Using RNA from developing seeds of the FAT5/DES9*-7 line for qPCR, we found that transcripts of both the FAT5 and DES9* desaturases are expressed, but FAT5 is expressed at less than one tenth of the level in seeds of FAT5-11, while DES9* is expressed at 35% of the level measured in DES9*-14 seeds (Figure 2). Despite the relatively lower expression of both enzymes our initial analysis of homozygous seed of line FAT5/DES9*-7 indicated 58% reduction in 16 : 0 and other saturates, to 6.8 ± 0.2% compared to 16.0 ± 0.3% in the untransformed controls (Figure S3). This line was characterized further in comparison with the lines expressing each individual desaturase.

Comparing single and combined expression of the desaturases

The three transgenic lines that we selected from our transformation experiments, FAT5-11, DES9*-14 and FAT5/DES9*-7, are similar to the other promising lines identified in each transgenic population with respect to decreased content of 16 : 0 and other saturates, oil content, and seed characteristics. Each line maintained their character through subsequent generations. To make a detailed comparison of the three lines and untransformed controls, we began by growing advanced generation plants together, identifying similarly robust individuals of each line and collecting seed from four of these per line to use for subsequent experiments.

In addition to lowering saturates content of the oil, it is critical that lines expressing the desaturases are able to germinate as well as the parental, untransformed camelina seeds and produce

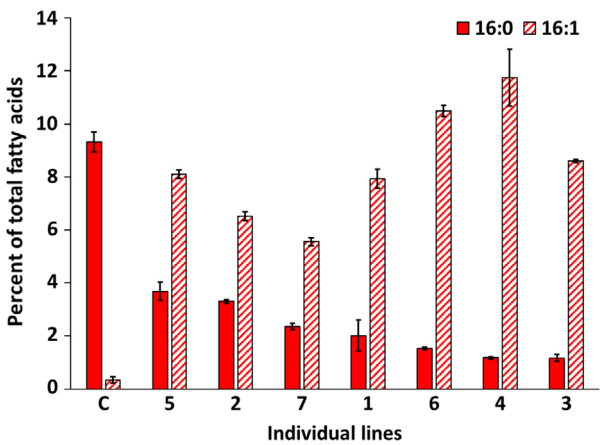

Figure 4 Proportions of 16 : 0 and 16 : 1 fatty acids in T2 seeds coexpressing both the FAT5 and DES9* desaturases. C, untransformed control. Data are mean ± SEM for three samples of transgenic, red seeds from each independent transformation event.

equally robust plants. To make quantitative comparison among our three transgenic lines and the untransformed control, we sowed 72 seeds of each line into potting mix in nine pots. The pots were placed in a randomized pattern in a large growth chamber. Germination (appearance of cotyledons) and establishment (appearance of first true leaves) were assessed separately for each pot over 10 days, then seedlings were harvested and weighed. Two days after sowing greater than 95% of FAT5, FAT5/DES9* and control seeds had germinated. By contrast, fewer than 40% of the DES9* seeds had germinated. Germination of the DES9* seeds continued to be poor so that only 75 ± 6% of these seeds were germinated by day 6 (Figure 5a). Beginning 7d after sowing we scored seedlings for the presence of the first true leaves. On day 7 almost all the FAT5, FAT5/DES9* and control plants had produced true leaves, but even 10 d after sowing only about half of the germinated DES9* plants had produced true leaves (Figure 5b). We interpret this poor germination and establishment of DES9* seedlings as being a consequence of compromised seed physiology resulting from seed-specific expression of the DES9* desaturase. To quantify the effect, we harvested and weighed the 10-day-old plants. Measurements of the fresh weights of plants from each line indicated that while the FAT5, FAT5/DES9*, and control plants were similar with an average of 73 ± 4 mg/plant, the average weight of surviving DES9* plants was 32% lower at 50 ± 2 mg/plant (Figure 5c).

Although we chose the DES9*-14 line because its seed appeared relatively normal, it is possible that poor germination and establishment of seedlings of this line resulted from low-oil content of the seed. To investigate this possibility, and to extend our comparison of all our lines, we measured the oil content and fatty acid composition of mature seeds of each line. The oil content of DES9* seeds is significantly lower than in the untransformed control (Figure 6) by 20%. However, this is unlikely to be the sole cause of the very low establishment in the transgenics, so there may be other negative effects of DES9* expression on the seed physiology of line 14 and other camelina and Arabidopsis (Figure S2b,c) DES9* lines. Neither FAT5-11 nor FAT5/DES9*-7 seeds had significantly lower oil than the untransformed control.

All three of the desaturase transgenic lines had reductions in each of the saturated fatty acids (16 : 0, 18 : 0 and 20 : 0) relative to the untransformed control, and all had increases in 16 : 1 (Figure 7a). The two 16-carbon fatty acids accounted for 7.8% of total fatty acids in the untransformed control, compared with 8.2% in FAT5-11, 10.7% in DES9*-14, and 9.0% in FAT5/DES9*-7. For each of the three saturated fatty acids, proportions in seed of FAT5/DES9*-7 plants were as low or lower than those measured in seeds of DES9*-14. A clearer measure of the effects of desaturase expression in each line is obtained by summing the proportions of saturates, monounsaturates and polyunsaturates in the seed samples (Figure 7b). These results show that decreases in saturates in the transgenics are largely balanced by concomitant increases in monounsaturates, with only minor changes in polyunsaturates. Compared with the saturated proportion (12.9 ± 0.3%) measured in the untransformed control, FAT5-11 has a 34% reduction (to 8.6 ± 0.3%), DES9*-14 has a 56% reduction (to 4.6 ± 0.3%), while coexpression of both the desaturases in FAT5/DES9*-7 reduced saturates by 69% to only 4.0 ± 0.2% of the total seed fatty acids (Figure 7b), although this value is not significantly lower than in DES9*-14 ($P = 0.051$).

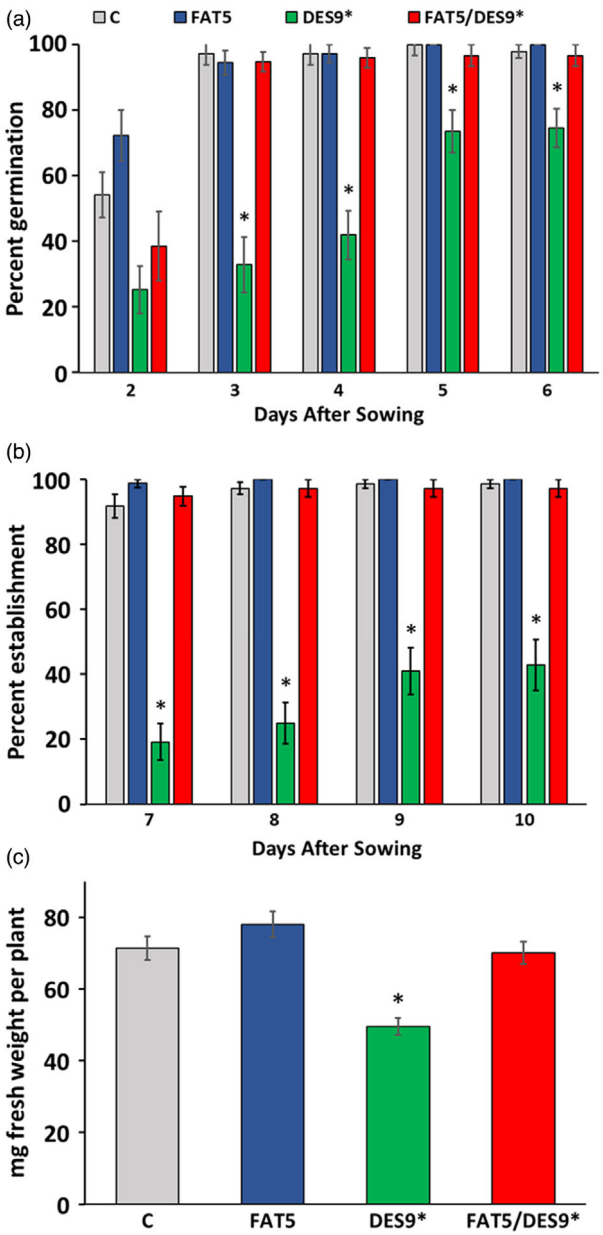

Figure 5 Expression of DES9* alone compromises germination and seedling establishment. Seedlings of DES9*-14 showed reduced germination (a) and seedling establishment (b) compared with untransformed controls and had lower fresh weights after 10-day growth (c). Seedlings of the FAT5 and FAT5/DES9* lines did not differ from control seedlings. Data are mean ± SEM for $n = 9$ (a and b) or $n = 72$ (c). Analysis by one-way ANOVA and *post hoc* Tukey test, * denote significance ($P < 0.05$).

Discussion

When vegetable oils are processed to biofuels the saturates they contain can result in the formation of gels at low temperatures (typically at temperatures below 0°C) that clog filters and cause problems with starting and smooth running of diesel engines (Knothe and Razon, 2017; Sia *et al.*, 2020). Polyunsaturates present a risk of oxidation resulting in poor-storage characteristics, poor-engine performance and formation of varnish coatings on engine parts (Knothe and Razon, 2017). These disadvantages

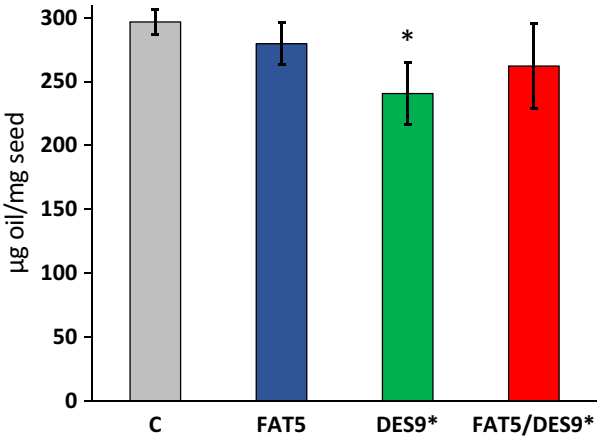

Figure 6 Oil content is modestly reduced in seeds expressing DES9*. Expression of DES9* alone in line DES9*-14 reduced oil content by 20% relative to the untransformed control. Oil contents of FAT5 and FAT5/DES9* seeds did not differ from control seedlings. Data are mean ± SEM, $n = 5$. Analysis by one-way ANOVA and post hoc Tukey test, * denotes significance ($P < 0.05$).

of oil-derived biofuels mean that biodiesel is often limited to a minor component in blends with petroleum based diesel. Because of these problems, the optimum composition of oils used for biofuels would be high in monounsaturates such as oleate and low in both saturates and polyunsaturates – ideally with both saturates and polyunsaturates each below 5% of total fatty acids. Interestingly, this is also the composition that is recognized as the best for food oils (Wallis *et al.*, 2022). In camelina (Jiang *et al.*, 2017; Lee *et al.*, 2021; Morineau *et al.*, 2017) as well as in other oilseeds (Bai *et al.*, 2019; Heppard *et al.*, 1996) very substantial reductions in polyunsaturates have been achieved by blocking expression of the FAD2 18 : 1-phosphatidylcholine desaturase, which is the gateway enzyme of polyunsaturate synthesis (Okuley *et al.*, 1994; Wallis *et al.*, 2022). In the experiments described here, we explore strategies for reducing saturates by converting them to monounsaturates using transgene expression of the FAT5 16 : 0-CoA desaturase from *C. elegans* and a DES9* 16 : 0/18 : 0-glycerolipid desaturase produced by directed evolution of a *S. elongatus* enzyme.

In previous experiments with Arabidopsis, we found that seed-specific expression of FAT5 reduced the proportion of 16 : 0 in the seeds of this species by 65%, along with smaller reductions in 18 : 0 and 20 : 0, with minimal impact on oil content and seed physiology (Fahy *et al.*, 2013). However, when we transformed FAT5 into camelina, 16 : 0 in the seeds was reduced by only 47%, from 7.6 ± 0.2% in the untransformed control to 4.4 ± 0.1% in line FAT5-11 (Figure 7a). This was despite the very strong expression of FAT5 transcript that we measured in developing seeds of FAT5-11 (Figure 2). The reason(s) for this smaller decrease in 16 : 0 proportion in camelina are not evident, but the result reflects other findings that indicate the care with which results in Arabidopsis must be reinvestigated when applying genetic engineering to crop species (Bai *et al.*, 2020; Haslam *et al.*, 2016). Our detailed analysis of FAT5-11 indicates that these plants are indistinguishable from untransformed camelina with respect to seed oil content, germination and seedling establishment (Figures 5 and 6).

The DES9* 16 : 0/18 : 0-glycerolipid desaturase was selected by directed evolution to have high activity in eukaryotic cells (Bai *et al.*, 2016) and in Arabidopsis it provided a reduction in 16 : 0 to 3.6% of total fatty acids in seeds. However, Arabidopsis seeds expressing DES9* had a 33% lower oil content than untransformed controls. Germination and seedling establishment was also compromised (Figure S2, Table S2). Despite these disappointing results, we chose to test seed-specific expression of DES9* in camelina. Similar to our findings in Arabidopsis, camelina T1 plants segregating for the seed-specific DES9* expression construct produced red T2 seeds with 16 : 0 ranging from 5.9% to 1.8%, compared with 8.8% in the untransformed control (Figure 3). However, in most of the lines with <4% 16 : 0 the red seeds appeared smaller than the segregating brown seeds, which lack the DES9* transgene, and they germinated poorly. We chose to generate a homozygous DES9* line from red seeds of plant 14, which had 1.8% 16 : 0 and seed size resembling untransformed segregants, although the seeds did

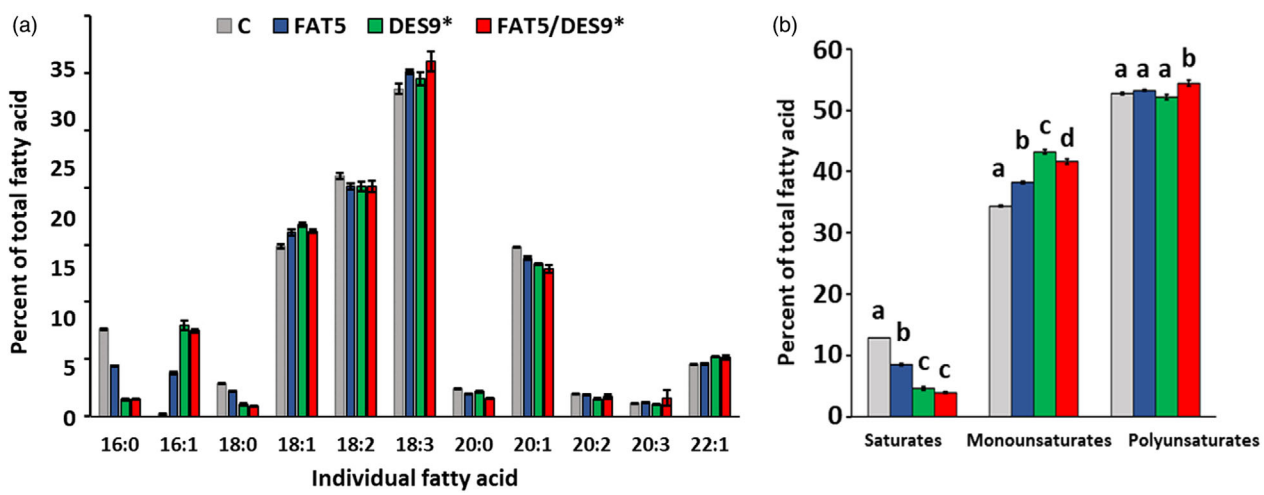

Figure 7 Comparison of the reduction in saturates of camelina seed oil provided by expression of the FAT5 and DES9* desaturases. Fatty acid profiles (a) and summed saturates, monounsaturates and polyunsaturates (b) of total seed oil in lines expressing FAT5 or DES9* alone or coexpressing these two desaturases. Data are mean ± SEM, $n = 5$. In (b), analysis by one-way ANOVA and *post hoc* Tukey test, letters denote significance ($P < 0.05$).

have a lower rate of germination. In our comparison experiments, DES9*-14 seeds did have oil content that was significantly lower than the untransformed control by 20% (234 ± 22 vs. 297 ± 10 mg/plant; Figure 6). Furthermore, this line exhibited greatly reduced germination and establishment (Figure 5), reflecting the same compromised seed characteristics as we found in Arabidopsis seeds expressing DES9* (Figure S2, Table S2).

Our decision to investigate coexpression of both the FAT5 16 : 0-CoA and the DES9* 16 : 0/18 : 0-glycerolipid desaturases was remarkably successful. Our screen identified seven lines with <4% 16 : 0 (Figure 4, Table S4). We chose the FAT5/DES9*-7 line to characterize in detail and compare with lines expressing the two desaturases separately. Despite the reduced expression of both desaturases in FAT5/DES9*-7 relative to the DES9*-14 and FAT5-11 plants (Figure 2), the seeds contain only 1.5% ± 0.1% 16 : 0, the same low level measured in DES9*-14 and very much less than the 7.6 ± 0.1% 16 : 0 in untransformed controls (Figure 7a). The total saturates in seed oil of the double transgenics was only 4.0 ± 0.1%, considerably less than the 12.9 ± 0.2% measured in untransformed controls. Seed oil of DES9* plants contained 4.6 ± 0.3% saturates, and this is not significantly different from the level in the FAT5/DES9* seeds ($P = 0.051$). Importantly however the seedlings of FAT5/DES9* germinated and established as well as the controls (Figure 5, Table S4), indicating that the compromised seed phenotypes found in both camelina and Arabidopsis DES9* transgenics are ameliorated when this desaturase is coexpressed with the FAT5 enzyme.

Relative to the compromised seed characteristics in lines expressing the DES9* desaturases alone, it is likely that the improvements in seed physiology and oil content obtained through coexpression of the DES9* 16 : 0/18 : 0-glycerolipid desaturases alongside the FAT5 16 : 0-CoA desaturase result from the differences in substrate for the two enzymes. For example, moderate expression of the FAT5 desaturases may ensure that sufficient 16 : 0-CoA remains available for sphingolipid synthesis, protein acylation and other important cell functions (Bonaventure *et al*., 2003; Chen *et al*., 2006). Evidently the modest expression of DES9* together with FAT5 is sufficient to achieve very low levels of saturates in the seed oil without triggering the detrimental phenotypes seen in both camelina and Arabidopsis lines expressing DES9* alone. Our successful lowering of oil saturates in camelina define strategies that can now be integrated with genetic engineering approaches that reduce polyunsaturates (Jiang *et al*., 2017; Lee *et al*., 2021; Morineau *et al*., 2017) to provide optimized oil composition for biofuels in camelina and other oil seed crops.

TAG synthesis and oil accumulation in seeds occurs through a network of interrelated pathways (Wallis *et al*., 2022). Recent investigations have established that fluxes and regulation of these pathways can vary considerably across higher plant species (Bates and Browse, 2012), and even between closely related species (Bai *et al*., 2020; Haslam *et al*., 2016). In this context, our results underscore the critical importance of reexamining discoveries made by genetic manipulation in the model oilseed Arabidopsis when translating knowledge to improve and optimize crop species.

Methods

Plants and growth conditions

Camelina seeds of the Suneson cultivar were used for all experiments. Seeds were stored in glass vials with silica gel at −20 °C. Plants were grown in a climate-controlled chamber at 22 °C under continuous illumination of 150 μE/m^2/s, except as otherwise indicated. In tests of germination and seedling establishment, seven seeds were planted per pot, and a total of nine pots per line were used. We scored germination as emergence of the cotyledons from the potting mix, and establishment as the presence of two true leaves.

Cloning and vector construction

Plant transformation vectors containing DES9* were created using an entry clone from Bai *et al*. (2016). The plant transformation vector pOEA2 GW DsRed was recombined with pENTR_DES9* using LR Clonase II (Lu *et al*., 2006). Following identification of successful transformants plasmids were prepared and transformed into *Agrobacterium* strain GV3101 for transformation into Camelina.

Vector pGlycinin DsRed was made Gateway compatible by PCR amplification of the Gateway cassette with appropriate restriction sites added to the primers (EcoRI, XhoI) and followed by ligation to generate pDsRed Glycinin GW. FAT5 was introduced into this vector by an LR clonase reaction from the entry clone used in Fahy *et al*. (2013), resulting in pGlyFAT5 DsRed. To express a second gene in this vector we amplified the phaseolin promoter, Gateway cassette and terminator from pOEA2 with added MluI restriction sites and ligated it into pGly_FAT5_DsRed digested with AscI sites, creating pGlyFAT5_Phase_GW DsRed. DES9* was cloned into pENTR d-topo (Invitrogen) using the manufacturer's instructions. DES9* was introduced into pGlyFAT5_Phase GW DsRed by LR clonase creating pGlyFAT5_PhaseDES9* DsRed. These vectors were transformed into *Agrobacterium* strain GV3101 and grown under selection for both the kanamycin resistant pGlyFAT5/PhaseDES9* DsRed and gentamicin for the helper plasmid.

Plant transformation

Agrobacterium GV3101 cultures (5 mL) were started from single colonies and grown with agitation at 28 °C overnight. Cultures were checked for the presence of the plant transformation vector by colony PCR. In brief, 30 μL of culture was boiled in a PCR tube and centrifuged briefly. Then, a 1 μL aliquot was used as template for colony PCR using ExTAQ (Takara) following the manufacturer's instructions. The rest of each culture was then used to inoculate 500 mL of 2xYT medium with the same kanamycin/gentamicin selection. The cultures were grown for 48 h at 28 °C. Cells were harvested by centrifugation at 4300 ***g*** for 10 min and resuspend in 200 mL infiltration media (50 g/L sucrose, ½ MS salts, 0.5% Silwet L77; Lu *et al*., 2006). Flowering camelina plants were placed in a large polycarbonate desiccator and flowers were impressed in the infiltration medium. A vacuum (25 inches Hg) was applied for between 5 and 8 min, with the vacuum being broken as quickly as possible. Following infiltration plants were stored horizontally in loosely closed black plastic bags overnight. The following day plants returned to their normal growth conditions.

Measurements of transgene expression

Developing siliques 10–20 days after flowering were harvested directly into liquid nitrogen. Then seeds were extracted from frozen siliques and stored at −80 °C. Approximately 50 mg of seed tissue was homogenized with a plastic pestle in a 1.5 mL microcentrifuge tube, and RNA extracted with the QIAGEN Plant RNeasy kit. After homogenization and addition of RLT buffer, the sample was mixed with one volume of chloroform to remove

lipids. After RNA extraction and quantification cDNA was generated with superscript RT III following the manufacturer's directions using 200 ng of RNA. Quantitative RT–PCR was performed with PerfeCTa SYBR Green 2× master mix (Quantabio), using 1 : 5 diluted cDNA with primers for Actin (*CsACT2*) as standard and either *FAT5* or *DES9** (Table S5).

Fatty acid methyl esters generation and analysis

Harvested seeds were placed into 8 mL glass screw capped tubes, with 1 mL of 5% sulfuric acid in methanol and 17 : 0 fatty acid as internal standard. Capped tubes were heated to 85°C in a water bath for 1.5 h, then cooled to room temperature. After addition of 1 mL water, FAMES were extracted with 200 μL hexane with centrifugation at 1000 ***g***, to break any emulsion formed. An aliquot of the hexane was transferred into a GC vial with 300 μL glass insert. Samples were loaded on to an Agilent 6890 GC with autosampler and 1 μL samples were injected onto a Supelcowax 10 (0.5 mm 15 m) column. The program ran with the following conditions, initial temperature 190 °C, 8 °C/min ramp, 230 °C, 1-min hold. Peak areas were converted to μg FAME using the internal standard peak.

Statistical analyses

Statistical analysis using ANOVA and Tukey honest difference test was performed in R Studio (Version 1.2.5001). Student's *T*-test was performed in Microsoft Excel.

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Conflict of interest

The authors declare no conflicts of interest.

Author contributions

All the authors conceived of and designed the original research plans. JDB carried out all the experiments and statistical analysis. JDB wrote the initial text, which was revised with input from all the authors.

Data availability statement

Experimental data to this article can be found online at: https://doi.org/10.7273/000003790.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Fatty acid composition of homozygous seeds of FAT5-11. C, untransformed control. Data are mean ± SEM, $n = 6$.

Figure S2 Characterization of Arabidopsis seed expressing DES9*.

Figure S3 Fatty acid composition of homozygous seeds of FAT5/DES9*-7. C, untransformed control. Data are mean ± SEM, $n = 3$.

Table S1 Segregation of T2 seeds in select FAT5 lines.

Table S2 Oil content and germination of Arabidopsis seed expressing DES9* compared with untransformed Col-0. Data are mean ± SEM ($n = 5$).

Table S3 Fatty acid compositions of phosphatidylcholine from the seed of lines expressing the FAT5 and DES9* desaturases.

Table S4 Characterization of additional camelina lines coexpressing both the FAT5 and DES9* desaturases.

Table S5 Primers used for quantitative PCR.