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Towards the synthetic design of camelina oil enriched in tailored acetyl-triacylglycerols with medium-chain fatty acids

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Abstract

The ability to manipulate expression of key biosynthetic enzymes has allowed the development of genetically modified plants that synthesise unusual lipids that are useful for biofuel and industrial applications. By taking advantage of the unique activities of enzymes from different species, tailored lipids with a targeted structure can be conceived. In this study we demonstrate the successful implementation of such an approach by metabolically engineering the oilseed crop Camelina sativa to produce 3-acetyl-1,2-diacyl-sn-glycerols (acetyl-TAGs) with medium-chain fatty acids (MCFAs). Different transgenic camelina lines that had been genetically modified to produce MCFAs through the expression of MCFA-specific thioesterases and acyltransferases were retransformed with the Euonymus alatus gene for diacylglycerol acetyltransferase (EaDAcT) that synthesises acetyl-TAGs. Concomitant RNAi suppression of acyl-CoA:diacylglycerol acyltransferase increased the levels of acetyl-TAG, with up to 77 mole percent in the best lines. However, the total oil content was reduced. Analysis of the composition of the acetyl-TAG molecular species using electrospray ionisation mass spectrometry demonstrated the successful synthesis of acetyl-TAG containing MCFAs. Field growth of high-yielding plants generated enough oil for quantification of viscosity. As part of an ongoing design–test–learn cycle, these results, which include not only the synthesis of ‘designer’ lipids but also their functional analysis, will lead to the future production of such molecules tailored for specific applications.

Keywords: Acetyl-TAG, biofuels, bioproducts, Camelina sativa, medium chain fatty acid, synthetic biology, vegetable oil.

Introduction

Seed oils are comprised mostly of triacylglycerols (TAGs), energy-dense molecules that consist of three fatty acids esterified to a glycerol backbone (Fig. 1A). The fatty acid composition of the TAG molecular species determines the physical properties of a particular vegetable oil. TAGs from commercially grown oilseed crops typically contain mainly five fatty acids: palmitic, stearic, oleic, linoleic, and linolenic. In contrast, throughout the plant kingdom, a wide variety of fatty acids with different chain lengths and functional groups exist (Badami and Patil, 1980). The presence of these unusual fatty acids often imparts the seed oil with useful functionalities. Among them, medium-chain fatty acids (MCFAs) with chain lengths of 8–14
production in transgenic plants through the expression of the encoding genes. For example, the *E. alatus* diacylglycerol acetyltransferase (EaDaCT) is necessary and sufficient for the synthesis of the acetyl-TAGs that accumulate in the seed endosperm and embryo of that species (Durrett et al., 2010). The seed-specific expression of EaDaCT resulted in acetyl-TAG levels ranging from 47–64 mole percent (mol%) in transgenic *Camelina sativa* lines. Further, EaDaCT expression combined with the suppression of the enzymes for lcTAG synthesis, e.g. diacylglycerol acyltransferase (DGAT1) and phospholipid diacylglycerol acyltransferase (PDAT1), resulted in acetyl-TAG levels as high as 85 mol% in the best transgenic lines (Liu et al., 2015a, 2015b).

Similarly, MCFAs have been synthesised in a variety of transgenic oilseed crops, through the expression of specialised FatB acyl-ACP thioesterases that cause the release of nascent fatty acids before additional cycles of fatty acid synthesis can extend their carbon chain lengths to C16 or longer (Pollard et al., 1991). In a number of different studies, the seed-specific expression of such specialised thioesterases from plants such as the California bay laurel or different *Cuphea* species resulted in the accumulation of MCFAs in transgenic canola (*Brassica napus*) seed (Dehesh et al., 1996; Voelker et al., 1996). Additional MCFA-specific FatB thioesterases were identified in *C. viscosissima* and *C. palustris* (Tjellström et al., 2013; Kim et al., 2015b). Subsequent expression of individual or different combinations of these specialised FatB thioesterases in camelina resulted in a range of fatty acid combinations of 8:0 to 16:0 in the transgenic seed oil (Kim et al., 2015b; where, for example, 8:0 refers to a fatty acid with 8 carbon atoms and 0 double bonds). However, accumulation of MCFAs in transgenic seeds expressing these thioesterases was much lower compared to the levels present in the species that naturally synthesise these unusual fatty acids. The co-expression of a MCFA-specific lysophosphatic acid acyltransferase (LPAAT) from coconut was successful in increasing laurate accumulation at the sn-2 position and thus the overall levels of 12:0 (Knutzon et al., 1999).

Similarly, expression of other MCFA-specific LPAATs from *C. viscosissima* and *C. pulcherrima* caused the accumulation of MCFAs at the sn-2 position in transgenic camelina expressing various *Cuphea* FatB thioesterases (Kim et al., 2015a).

The oilseed crop *Camelina sativa* has emerged as a useful platform for the synthesis of unusual lipids through biotechnology approaches. Importantly, a modified floral dip transformation method (Lu and Kang, 2008) enables the relatively easy development of different transgenic lines, allowing optimisation of transgene combinations. Multiple selectable markers permit the stacking of different transgenic traits through crossing or retransformation (Shockey et al., 2015). In addition, field growth of transgenic camelina plants has allowed the large-scale production and subsequent functional testing of different lipids resulting from metabolic engineering, including acetyl-TAGs (Liu et al., 2015a, 2015b), omega-7 fatty acids (Nguyen et al., 2015), and the nutritionally important omega-3 very long-chain polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Ruiz-Lopez et al., 2014; Betancor et al., 2015, 2016).

Here, we describe a synthetic-biology approach to metabolically engineer camelina to produce tailored lipid molecules...
with a designed structure. By combining the expression of enzymes resulting in the production of MCFAs with those resulting in high levels of acetyl-TAGs, we demonstrate the synthesis of MCFA-containing acetyl-TAGs that have not been found in nature (Fig. 1). Further, some of the transgenic camelina lines were grown in the field to produce these oils for functional testing of physical properties and they provided information to guide subsequent modifications of the lipid structure. Such iterative approaches will lead to targeted production of lipid molecules designed for specific applications.

Materials and methods

Plant transformation

Plasmids expressing EadA-T alone or in combination with DGAT1 RNAi and/or PDAT1 RNAi (Liu et al., 2015a) were modified by replacement of the DsRed selectable marker with the BAR gene that provides resistance to glufosinate. The gene, along with its nopaline synthase promoter, was amplified from the plasmid pBinGlyBar1 (Nguyen et al., 2013) using primers with the sequences 5’-GCAGACCTGATCGTCCGTGATGCCGACGAGCCGA3’ and 5’-AGCGATCGGCACGCTGCCGCAAGCACTCAGGGC - 3’. The amplified product was digested with SacI and PstI and ligated into the corresponding sites of the binary vectors containing DsRed, a process that simultaneously removed the DsRed gene and promoter cassette. The resulting binary vectors (see Supplementary Fig. S1 at JXB online) were introduced into Agrobacterium tumefaciens GV3101 and transformed into the resulting binary vectors (Fig. 2). Transformation with DsRed expressing Camelina oil enriched in acetyl-triacylglycerols with medium-chain fatty acids | 4397

Results and discussion

Synthesis of acetyl-TAGs in MCFA-producing camelina lines

To produce acetyl-TAGs containing MCFAs in planta, we introduced EadA-T into four different camelina backgrounds, ChFatB2, CnFatB2+ChFatB2, CnFatB1+CnLPAAAT and CnFatB2+CnFatB1 (Kim et al., 2015b) using a floral vacuum-infiltration method (Lu and Kang, 2008). T1 plants were selected by spraying young seedlings four times with 0.01% (w/v) glufosinate. Lines containing one transgenic locus were identified based on a 3:1 segregation ratio for glufosinate resistance in the T2 generation. Independent lines with high levels of acetyl-TAGs were further propagated in the greenhouse to generate T3 seed. Large amounts of T3 seed for oil property analysis were harvested from plants grown at the Montana State University A. H. Post agricultural research farm near Bozeman, MT, under guidelines of the Animal and Plant Health Inspection Service, United States Department of Agriculture (USDA APHIS) permit no. BRS 16-032-106r.

Lipid analysis

Seed lipids were extracted using a modified hexane-isopropanol extraction method (Li et al., 2006) with tripentadecanoin (Nu Check Prep, Waterville, MN) added as an internal standard. Acetyl-TAGs and lcTAGs were quantified by separating those fractions using TLC, followed by transmethylation and gas chromatography as described previously (Liu et al., 2015a). For electrospray ionisation mass spectrometry (ESI-MS) analysis, neutral lipids were isolated by elution from total seed lipid extracts using ESI-MS. The kinematic viscosity of the purified acetyl-TAGs and lcTAGs was measured using a calibrated Ubbelohde viscometer (Cannon Instruments, State College, PA) at 40 °C according to the ASTM D445 method (ASTM International, 2017).

Positional analysis of TAGs

The sn-2 fatty acid composition of acetyl-TAGs and lcTAGs in T3 seed oil was determined by using the lipase from Thermomyces lanuginosus (Sigma-Aldrich), which specifically cleaves the sn-1 and -3 ester bonds of TAGs to generate 2-monooacylglycerols (2-MAGs). Samples of TLC-purified acetyl-TAGs or lcTAGs (1.5 mg) were dissolved in 1.0 ml of diethyl ether, after which 5000 U of lipase in 1 ml of 50 mM Tris buffer (pH 7.2) was added. The reaction was incubated at 37 °C for 30 min with constant shaking. Lipids were removed by extracting twice with 3 ml of diethyl ether and separated on boric acid-impregnated silica gel TLC plates using a chloroform:acetone solvent system (80:10, v/v). Next, 10 μg of tripentadecanoin was added to the bands corresponding to MAGs which were then scraped and the lipids extracted using 2 ml of toluene. After transmethylation using a base-catalysed reaction, the resulting fatty acid methyl esters were quantified using gas chromatography.
MCFA-producing lines expressing EaDAcT contained novel scans to identify acetyl-TAG molecular species. The spectra of acetyl-TAG composition of homozygous T3 seeds from independent lines enhances acetyl-TAG accumulation. Scatter plots of the distribution of acetyl-TAGs in Fig. 2. The horizontal lines represent the mean values for each sample group. (This figure is available in colour at JXB online.)

We analysed the composition of neutral lipids extracted from the seeds of homozygous T3 plants using ESI-MS neutral loss scans to identify acetyl–TAG molecular species. The spectra of acetyl-TAGs were obtained by using a stereospecific lipase. Consistent with previous results (Kim et al., 2015a), acetyl-TAGs possessed lower amounts of very long-chain fatty acids compared to lcTAGs (Fig. 4). However, in both genotypes, acetyl–TAGs possessed lower amounts of MCFA compared to the lcTAGs produced in the same lines. For example, in the CpFatB2 + ChFatB2 background, 10:0 levels were about fourfold lower in acetyl-TAGs compared to lcTAGs. Likewise, in the UcFatB1 + CnLPAAT lines, 12:0 levels in acetyl-TAGs were on average half those in lcTAGs (Fig. 4).

**MCFAs are inefficiently incorporated at the sn-2 position of acetyl-TAGs**

As the sn-3 position of acetyl-TAG is occupied by an acetate group, the incorporation of MCFAs at the other two positions is particularly important to achieve high levels of these fatty acids in acetyl-TAGs. The regio-specific incorporation of MCFAs into lcTAGs and acetyl-TAGs in high acetyl-TAG-producing transgenic lines was therefore quantified through the use of a stereospecific lipase. Consistent with previous results (Kim et al., 2015b), lcTAGs in lines expressing CnLPAAT contained ~20 mol% laurate at the sn-2 position whereas plants lacking this MCFA-specific LPAAT possessed minimal levels of MCFAs at this position (Fig. 5). Likewise, only acetyl-TAGs from lines expressing CnLPAAT possessed MCFAs at the sn-2 position. However, the levels of laurate at the sn-2 position of acetyl-TAGs were only 5 mol%, considerably lower than those in lcTAGs produced in the same lines (Fig. 5). Taken together, these results emphasise the importance of a MCFA-specific LPAAT to increase accumulation of these unusual fatty acids at the sn-2 position of storage lipids in engineered oilseeds (Knutzon et al., 1999; Kim et al., 2015a, 2015b). Further, the much lower incorporation of MCFAs in the sn-2 position of acetyl-TAGs relative to lcTAGs also suggests discrimination of EaDAcT relative to endogenous DGAT1 activity for MCFA-containing DAG substrates. This observation was consistent with earlier work where we demonstrated in vitro that EaDAcT preferentially acetylates DAG molecules containing unsaturated DAGs (Bansal and Durrett, 2016a).

**EaDAcT can synthesise MCFA acetyl-TAGs in camelina**

We analysed the composition of neutral lipids extracted from the seeds of homozygous T3 plants using ESI-MS neutral loss scans to identify acetyl–TAG molecular species. The spectra of MCFA–producing lines expressing EaDAcT contained novel lower molecular-mass acetyl–TAG peaks compared to a WT background line expressing EaDAcT (Fig. 3A). The mass of these peaks corresponded to acetyl-TAG molecules containing MCFA. For example, the ammonium adduct of acetyl-dilaurin (indicated by 26:0 in Fig. 3A) causes the novel peak with m/z of 516.5. The presence of MCFA-containing lines in these lower molecular-mass acetyl-TAGs was further confirmed using ESI-tandem MS (ESI-MS²), which produced daughter fragments indicative of MCFA-containing lines (Fig. 3B). Together, these results demonstrated that EaDAcT was able to acetylate MCFA diacylglycerols (DAGs) such as 1,2-dilaurin-sn-3-glycerol and 1,2-dimyristoyl-sn-3-glycerol to generate acetyl-dilaurin and acetyl-dimyristin in camelina seed. Such activities have been shown in vitro in our previous work (Bansal and Durrett, 2016a).

We also quantified the fatty acid composition of the acetyl-TAG and lcTAG fractions of high acetyl-TAG-producing lines in the CpFatB2 + ChFatB2 and the UcFatB1 + CnLPAAT backgrounds. The distribution of these fatty acids typically found in wild-type camelina was generally consistent with previous observations (Liu et al., 2015a): acetyl-TAGs were enriched in more unsaturated fatty acids such as 18:3 and possessed lower amounts of very long-chain fatty acids compared to lcTAGs (Fig. 4). However, in both genotypes, acetyl–TAGs possessed lower amounts of MCFA compared to the lcTAGs produced in the same lines. For example, in the CpFatB2 + ChFatB2 background, 10:0 levels were about fourfold lower in acetyl-TAGs compared to lcTAGs. Likewise, in the UcFatB1 + CnLPAAT lines, 12:0 levels in acetyl-TAGs were on average half those in lcTAGs (Fig. 4).

![Fig. 2. Expression of EaDAcT combined with down-regulation of DGAT1 enhances acetyl-TAG accumulation. Scatter plots of the distribution of acetyl-TAG composition of homozygous T3 seeds from independent camelina lines expressing CnLPAAT and transformed with EaDAcT alone or in combination with RNAi constructs targeting camelina DGAT1 and PDAT1 homeologues. Horizontal lines represent the mean values for each sample group. (This figure is available in colour at JXB online.)](image-url)
Transgenic lines possess a lower seed oil content

When determined gravimetrically, the values for seed oil content of the MCFA-producing lines were lower than those of wild-type camelina. For CpFatB2+ChFatB2 this reduction was quite large (73% of WT) whereas only a minimal reduction was observed with UcFatB1+CnLPAAT (Fig. 6). Similar reductions have been noted for other MCFA-producing camelina lines (Iskandarov et al., 2017). The oil content of acetyl-TAG-producing lines derived from these two backgrounds was further reduced by 10–26%, depending on the specific line (Fig. 6). Previous work has shown that acetyl-TAG-producing lines in a WT background also possess slightly lower oil content (Liu et al., 2015a). Interestingly, expression of a MCFA-specific DGAT1 has been shown to rescue the reduced oil content of camelina lines producing MCFAs (Iskandarov et al., 2017). Here, the development of EaDAcT variants with improved specificity for MCFA-containing DAG might be helpful in overcoming the reduced oil content of these lines. Indeed, there are multiple reports of specialised transferases being able to reverse the reduced oil content caused by the production of unusual fatty acids in transgenic seeds (van Erp et al., 2011; Hu et al., 2012; Li et al., 2012). An alternative strategy could be to overexpress the regulatory factor WRINKLED1 (WRI1), which rescues the reduced oil accumulation in Arabidopsis plants producing hydroxy fatty acids (Adhikari et al., 2016).

Viscosity of MCFA acetyl-TAGs

The viscosity of vegetable oils is an important parameter for their use as fuel, biodegradable lubricant, and other industrial applications. Previously, we demonstrated that acetyl-TAGs
possess a lower kinematic viscosity compared to the lcTAGs found in most vegetable oils (Durrett et al., 2010; Liu et al., 2015a, 2015b). Similarly, other studies have shown that TAGs with MCFAs also possess lower viscosities compared to TAGs with longer chains (Valeri and Meirelles, 1997; Geller and Goodrum, 2000). To determine whether acetyl-TAGs enriched with MCFAs would possess lower viscosity than acetyl-TAGs with regular chain lengths, T4 seeds from two high acetyl-TAG-yielding lines from different MCFA background lines were bulked in the greenhouse and the field to yield enough oil for viscosity testing. The oil was subsequently fractionated using large-scale column chromatography to obtain pure acetyl-TAGs and lcTAGs.

The kinematic viscosities (measured at 40 °C according to ASTM D455) of acetyl-TAGs and lcTAGs from wild-type camelina plants were consistent with previous reports (Liu et al., 2015a, 2015b), with acetyl-TAGs showing a ~30% reduction compared to lcTAGs (Table 1). lcTAGs from UcFatB1+CnLPAAT possessed a slightly lower viscosity of 29.4 mm² s⁻¹ than lcTAGs from wild-type plants (30.7 mm² s⁻¹). However, the viscosity of acetyl-TAGs synthesised in the UcFatB1+CnLPAAT background was essentially the same as that from a wild-type background, with both being 21.9 mm² s⁻¹. Interestingly, lcTAGs from CpFatB2+ChFatB2 possessed a higher viscosity of 34.5 mm² s⁻¹ compared to wild-type lcTAGs. Likewise, the kinematic viscosity of acetyl-TAGs from this background was 23.8 mm² s⁻¹, higher than that of acetyl-TAGs synthesised in a wild-type background (Table 1).

The unchanged or elevated viscosities of acetyl-TAGs containing MCFAs from UcFatB1+CnLPAAT or CpFatB2+ChFatB2 backgrounds, respectively, probably reflects the poor incorporation of MCFAs into these molecules. Initial attempts to enrich the acetyl-TAG fraction for molecules containing MCFAs were unsuccessful. Another, non-exclusive possibility,
MCFA-specific LPAAT enzymes (Kim et al., 2015a) offers the possibility to improve MCFA incorporation into the sn-2 position of DAGs and subsequently acetyl-TAGs. However, strategies beyond increasing MCFA content in DAGs will be required given the apparent discrimination of EaDAcT against saturated DAG molecular species (Fig. 4; Bansal and Durrett, 2016a). It might be possible to use directed molecular evolution approaches, similar to those used to identify high-activity DGAT1 enzymes (Siloto et al., 2009), to select for EaDAcT variants with improved specificity for MCFA-containing DAG molecular species. Alternatively, producing unsaturated MCFA to enable better utilisation by EaDAcT would also result in an increased production of lower molecular-mass TAG species. This latter strategy also has the advantage in that the desired product would be more unsaturated, likely improving the viscosity and cold-temperature properties. Thus, iterative design–test–learn cycles that involve not only increasing the synthesis of tailored lipid molecules in seed oils, but also testing their physical and chemical properties, will lead to the production of lipid molecules designed for specific applications.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. Constructs used to express EaDAcT and suppress camelina acyltransferases.

Fig. S2. Expression of EaDAcT combined with down-regulation of DGAT1 enhances acetyl-TAG accumulation.

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Fig. 6. MCFA acetyl-TAG lines possess a lower oil content. Mean oil content of T₄ seed of independent homozygous lines in the CfpFatB2+ChFatB2 and UcFatB1+CnLPAAT backgrounds expressing EaDAcT and DGAT1-RNAi. Data are means (±SD) of two independent gravimetric experiments. (This figure is available in colour at JXB online.)

Table 1. Kinematic viscosity (at 40 °C) of purified acetyl-TAGs and lcTAGs

| Background                  | TAGs       | Kinematic viscosity (mm² s⁻¹) |
|-----------------------------|------------|-------------------------------|
| Wild-type                   | lcTAGs     | 30.7 ± 0.02                   |
|                             | acetyl-TAGs| 21.9 ± 0.4                    |
| CfpFatB2+ChFatB2            | lcTAGs     | 34.5 ± 0.5                    |
|                             | acetyl-TAGs| 23.8 ± 0.03                   |
| UcFatB1+CnLPAAT             | lcTAGs     | 29.4 ± 0.1                    |
|                             | acetyl-TAGs| 21.9 ± 0.06                   |

* Data represent means (±SD) for five replicate assays
* From seed also expressing EaDAcT + DGAT1-RNAi
* From seed also expressing EaDAcT + DGAT1-RNAi + PDAT1-RNAi

particularly for the TAGs from the CfpFatB2+ChFatB2 background, is that the reduced polyunsaturated fatty acid (PUFA) levels in the acetyl-TAG and lcTAG fractions also contribute to the increase in viscosity. Previous work has shown that double bonds greatly reduce the kinematic viscosity of lcTAGs (Knothe and Steidley, 2005). Likewise, acetyl-TAGs produced in a camelina high-oleic background possess a higher viscosity than acetyl-TAGs containing more PUFAs from a wild-type background (Liu et al., 2015b).

Conclusions

By taking advantage of the combinatorial nature of synthetic biology we have successfully generated tailored lipid molecules not found in nature. However, additional metabolic engineering is needed to improve the incorporation of MCFA into acetyl-TAGs to further alter the physical properties of these molecules. The recent availability of alternative MCFA-specific LPAAT enzymes (Kim et al., 2015a) offers the possibility to improve MCFA incorporation into the sn-2 position of DAGs and subsequently acetyl-TAGs. However, strategies beyond increasing MCFA content in DAGs will be required given the apparent discrimination of EaDAcT against
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