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Engineering the stereoisomeric structure of seed oil to mimic human milk fat

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Introduction

Infant formula is a manufactured food designed to substitute for human breast milk. Around half the calories in human milk are provided by fat (triacylglycerol; TAG) and in infant formula this fat is mainly sourced from plants (1). Although blended vegetable fats can replicate the fatty acyl composition of human milk fat, HMFS are also available, providing a complete fat phase with comparable nutritional properties to >70% in our final iteration. This level of C16:0 enrichment is comparable to human milk fat. We achieved this by relocating the C16:0-specific chloroplast isomerase of the enzyme lysophosphatidic acid acyltransferase (LPAAT) to the endoplasmic reticulum so that it functions within the cytosolic glycerolipid biosynthetic pathway to esterify C16:0 to the middle position. We then suppressed endogenous LPAAT activity to relieve competition and knocked out phosphatidylincholine:diacylglycerol cholinephosphotransferase activity to promote the flux of newly-made diacylglycerol directly into TAG. Applying this technology to oilseed crops might provide a new source of HMFS for infant formula.

Significance

In human milk fat, saturated fatty acids are esterified to the middle position on the glycerol backbone giving the triacylglycerol with more than 70% of the saturated fatty acids to the outer positions. Here we have engineered the metabolism of an oilseed plant so that it accumulates triacylglycerol with more than 70% of the saturated fatty acid palmitate in the middle position, thereby mimicking human milk fat stereoisomeric structure. Applying this technology to oilseed crops (or oleaginous microorganisms) might provide a new source of human milk fat substitute for infant nutrition.
However, plants already possess an LPAT with the appropriate specificity for fatty acyl-CoA expressed from cyanobacteria (11), mammals (12) and algae (13,14). To overcome this limitation, we decided to express an LPAT with specificity for C16:0 against C16:0-CoA as a substrate and this may be achieved by retargeting chloroplast LPAT1 to the ER (20), knock down of LPAT2 and (3) knock out of PDCT. C18:x, long-chain mono- or polyunsaturated fatty acyl groups (black bars); C16:0 and C18:x groups (hatched bars); CoA, Coenzyme A; ACP, acyl carrier protein; G3P, glycerol-3-phosphate; 1-LPA, sn-1 lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; 1-LPC, sn-1 lysophosphatidylcholine; FFA, free fatty acid; LPAT, 1-LPA acyltransferase; PDCT, PCDAG cholinephosphotransferase. 

Results and Discussion

LPAT1 can be redirected to the ER by removing its chloroplast targeting signal

In plant cells, triacylglycerol (TAG) is formed by a cytosolic glycerolipid biosynthetic pathway situated on the endoplasmic reticulum (ER) and the enzyme responsible for acylation of the sn-2 position is lysophosphatidic acid acyltransferase (LPAT) (9) (Fig. 1). ER-resident isoforms of LPAT are commonly found in the endoplasmic reticulum (ER) and the enzyme responsible for acylation of the sn-2 position is lysophosphatidic acid acyltransferase (LPAT) (9,10). To overcome this limitation, we decided to express an LPAT with specificity for C16:0 against C16:0-CoA as a substrate and this may be achieved by retargeting chloroplast LPAT1 to the ER (20), knock down of LPAT2 and (3) knock out of PDCT. C18:x, long-chain mono- or polyunsaturated fatty acyl groups (black bars); C16:0 and C18:x groups (hatched bars); CoA, Coenzyme A; ACP, acyl carrier protein; G3P, glycerol-3-phosphate; 1-LPA, sn-1 lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; 1-LPC, sn-1 lysophosphatidylcholine; FFA, free fatty acid; LPAT, 1-LPA acyltransferase; PDCT, PCDAG cholinephosphotransferase.

from this position in virtually all cases (4,5,8). Even in palm oil that contains ~48% C16:0 in total, only 9% of this occupies the sn-2 position (5). Here we describe a method for modifying TAG biosynthesis, in the model oilseed Arabidopsis thaliana, that results in a stereoisomeric redistribution of acyl groups such that the amount of C16:0 at the sn-2 position increases more than 20-fold to over 70% of the total; a level of enrichment that is comparable to HMF. Applying this technology to oilseed crops might provide a new source of HMFS for infant formula.

Results and Discussion

LPAT1 can be redirected to the ER by removing its chloroplast targeting signal

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selectivity, that resides in the chloroplast (15,16) (Fig. 1). This LPAT uses a C16:0-acyl carrier protein (ACP) substrate but will also accept C16:0-CoA in vitro (17,18). We therefore decided to test whether chloroplast LPAT could be relocated to the ER (Fig. 1). Chloroplast LPAT is an integral membrane protein that is nuclear encoded and contains an N-terminal chloroplast targeting signal (CTS) (19). CTS deletion has previously been used to alter protein localisation (20). Using transient expression in Nicotiana benthamiana leaves, we found that when 101 amino acid residues containing the CTS are deleted from Chloroplast LPAT1 (17) (SI Appendix, Fig. S1) and replaced with a red fluorescent protein (RFP) marker the RFP -LPAT1 protein localises to the ER (Fig. 2A). 

ΔCTS-LPAT1 expression drives C16:0 incorporation into the sn-2 position of TAG

Truncated versions of LPAT1 that lack the CTS are known to be active when expressed in Escherichia coli (18,19). To determine whether ΔCTS-LPAT1 functions in plants and can enable C16:0 to be incorporated into the sn-2 position of TAG, we expressed this truncated protein under the control of the seed-specific soybean

Fig. 1. A simplified diagram illustrating the cytosolic and chloroplastic pathways for de novo glycerolipid biosynthesis in Arabidopsis. Three modifications enabled palmitoyl (C16:0) groups (white bars) to be incorporated into the sn-2 position of TAG in developing seeds: (1) Retargeting of LPAT1 to the ER, (2) knock down of LPAT2 and (3) knock out of PDCT. C18:x, long-chain mono- or polyunsaturated fatty acyl groups (black bars); C16:0 and C18:x groups (hatched bars); CoA, Coenzyme A; ACP, acyl carrier protein; G3P, glycerol-3-phosphate; 1-LPA, sn-1 lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; PC, phosphatidylcholine; 1-LPC, sn-1 lysophosphatidylcholine; FFA, free fatty acid; LPAT, 1-LPA acyltransferase; PDCT, PCDAG cholinephosphotransferase.
Disruption of ER-resident LPAT2 increases C16:0 incorporation into the sn-2 position of TAG. (A) Diagram of LPAT2 locus showing positions of T-DNA insertions in mutant alleles. Effect of lpat2 mutant backgrounds on (B) the percentage of C16:0 esterified to the sn-2 position of TAG, versus sn-1+3, and (C) LPAT2 transcript abundance in seeds expressing ∆CTS-LPAT1. WT = wild type; L11 = homozygous ProGLY:∆CTS-LPAT1 line. Values are the mean ±SE of measurements made on separate batches of dry seeds in B and developing siliques in C from three plants of each genotype (n = 3). LPA T2 expression was normalised to the geometric mean of three reference genes and expressed relative to WT. a, b & c denote values significantly (P < 0.05) different from L11 (ANOVA + Tukey HSD test).

Fig. 4. Bypassing flux through PC increases C16:0 incorporation into the sn-2 position of TAG. (A) Effect of pdct mutant background on percentage of C16:0 esterified to the sn-2 position of TAG in ProGLY:∆CTS-LPAT1 and ProGLY:∆CTS-LPAT1 lpat2-3 seeds. WT = wild type; L11 = homozygous ProGLY:∆CTS-LPAT1 line. (B) Seed weight and (C) percentage oil content of WT and ProGLY:∆CTS-LPAT1 lpat2-3 pdct (All). Values are the mean ±SE of measurements on separate seed batches from between three and six plants in A and five plants in B and C of each genotype (n = 3 to 6). a and b denote values significantly (P < 0.05) different from L11 and pdct, respectively (ANOVA + Tukey HSD test) and c from WT (two-tailed Student’s t test).

ProGLY:∆CTS-LPAT1

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(T1) using a DsRed fluorescent marker system (21) and analysed the total fatty acyl composition of T2 seed batches. We found that several lines exhibited an increase in total C16:0 content, which suggested that the transgene was promoting C16:0 incorporation into TAG (SI Appendix, Table S1). We selected three independent single copy T2 lines (L30, L6 and L11) with high C16:0 content and obtained homozygous T3 seed. When we purified TAG from these homozygous seed batches and determined its stereochemistry using lipase digestion (22), we found that the percentage of C16:0 at the sn-2 position (versus sn-1/3 positions), had increased more than 16-fold, from only 2% in wild type to values ranging between 32 and 39% in the three independent ProGLY:∆CTS-LPAT1 lines (Fig. 2B and SI Appendix, Table S2). ∆CTS-LPAT1 expression was therefore sufficient to allow incorporation of C16:0 into the sn-2 position of TAG, but not to achieve positive enrichment at this position verses the sn-1/3 positions, which can already incorporate a low proportion of C16:0 (9) (Fig. 1).
plants of each genotype (n = 3). In the mean cotyledons and developing true leaves. (0) and 4. WT = wild type; All = its competes with contributions to T AG biosynthesis in Arabidopsis seeds (10) and that qRT-PCR analysis showed that LPAT2 expression is reduced by ~83% in developing lpat2-3 silique, but only by ~24% in lpat2-2. (Fig. 3B). These data support the hypothesis that LPAT2 contributes to TAG biosynthesis in Arabidopsis seeds (10) and that it competes with ACTS-LPAT1. The level of C16:0 enrichment at sn-2 also appears to respond to the strength of LPAT2 repression and achieving a greater reduction than ~83% might therefore lead to even stronger enrichment.

**Disruption of PDCT also enhances C16:0 incorporation into the sn-2 position of TAG**

In developing Arabidopsis seeds >90% of the glycerol backbone in TAG is derived from the membrane lipid phosphatidylcholine (PC), owing to rapid diacylglycerol (DAG)-PC interconversion (26), catalysed mainly by the plant-specific head group exchange enzyme PC:DAG cholinephosphotransferase (PDCT) (27,28) (Fig. 1). Although LPAT is responsible for the initial acylation of glycerolipids at sn-2, once these acyl groups are in PC they may be removed and replaced by acyl editing activities (26,29,30) (Fig. 1). To determine whether bypassing glycerolipid flux through PC (Fig. 1) might increase ACTS-LPAT1-dependent incorporation of C16:0 into the sn-2 position of TAG, we crossed ProGLY:ACTS-LPAT1 L11 into the pdct (reduced olate desaturase) mutant (27). When we purified TAG from ProGLY:ACTS-LPAT1 pdct seed batches and performed positional analysis, we found that the percentage of C16:0 at sn-2 had increased from ~30% in the parental ProGLY:ACTS-LPAT1 line to ~50% in the pdct background (Fig. 1). The data suggest that a more direct flux of newly made DAG into TAG (28) (Fig. 1) favours C16:0 incorporation and/or retention at the sn-2 position. In WT seeds it is conceivable that C16:0 entering the sn-2 position of PC might either be edited from it by the action of lysophosphatidylcholine acyltransferase (LP-CAT) or a phospholipase A2 (28). Interestingly, Lager et al. (29) have provided in vitro evidence that the reverse activities of Arabidopsis LPCAT1 and LPCAT2 can selectively remove certain fatty acyl groups from PC, but C16:0 was not tested. Although rapid DAG-PC interconversion occurs in Arabidopsis seeds (26), it is noteworthy that considerable interspecific variation has been reported in this flux (31) and so the effect of PDCT disruption on C16:0 enrichment at the sn-2 of TAG may differ between oilseeds.

**Disruption of LPAT2 and PDCT has an additive effect on incorporation of C16:0 at sn-2**

To determine whether the combination of reducing LPAT competition and bypassing flux through PC would have an additive effect on ACTS-LPAT1-dependent incorporation of C16:0 into the sn-2 position of TAG (Fig. 1), we crossed ProGLY:ACTS-LPAT1 lpat2-3 with ProGLY:ACTS-LPAT1 pdct. When we purified TAG from homozygous seed batches and performed positional analysis, we found that the percentage of C16:0 at sn-2 had increased from ~56% in ProGLY:ACTS-LPAT1 pdct to ~71% in ProGLY:ACTS-LPAT1 lpat2-3 pdct (Fig. 4A and SI Appendix, Table S4). The combination of just three modifications to the TAG biosynthetic pathway in Arabidopsis (i.e. ACTS-LPAT1 expression, plus LPAT2 and PDCT suppression) is therefore sufficient to replicate the level of C16:0 enrichment at the sn-2 position (versus sn-1+3) that is found in HMF1 (1,2,3). Analysis of TAG composition in ProGLY:ACTS-LPAT1 lpat2-3 pdct (All) seeds using high resolution / accurate mass (HR/AM) lipidomics (32) also confirmed the presence of C16:0 groups at the sn-2 position, since tripalmitin was 27-fold more abundant than in WT (SI Appendix, Fig. S24). By contrast, no dipalmitoyl PC was detected in ProGLY:ACTS-LPAT1 lpat2-3 pdct seeds and molecular species of PC containing one C16:0 group were not increased (SI Appendix, Fig. S2B). These data suggest that an asymmetrical distribution of saturated and unsaturated fatty acyl groups in PC is maintained in Arabidopsis (27,28) (Fig. 1). Although rapid DAG-PC interconversion occurs in Arabidopsis seeds (26), it is noteworthy that considerable interspecific variation has been reported in this flux (31) and so the effect of PDCT disruption on C16:0 enrichment at the sn-2 of TAG may differ between oilseeds.

**Redistribution of C16:0 reduces seed oil content, but not germination or establishment**

Many studies have shown that modifying fatty acyl composition can reduce TAG accumulation in oilseeds and in some cases...
can also impair seed germination and seedling establishment (35,36). Our primary objective in this study was not to alter fatty acyl composition per se, but to change the stereoisomeric structure of TAG. In this manner, any physiological impact of C16:0 enrichment at the sn-2 position of TAG, we compared seed batches from wild type and ProGLY:CTS-LPAT1 lpat2-3 pdct plants that had been grown together under standard laboratory conditions. We found no significant difference (P > 0.05) in seed weight between the two genotypes (Fig. 4B). However, the fatty acid content of ProGLY:CTS-LPAT1 lpat2-3 pdct seeds was significantly (P < 0.05) lower than that of wild type, when expressed as a percentage of seed weight (Fig. 4C). These data suggest that the modifications leading to incorporation of C16:0 into the sn-2 position, reduce TAG biosynthetic flux. This finding is consistent with previous studies in which seed TAG composition has been modified either using genetic engineering or mutant breeding methods (35,36). In warm conditions (20°C), ProGLY:CTS-LPAT1 lpat2-3 pdct seed germination, scored as radicle emergence (Fig. S4) and seedling establishment, scored as cotyledon expansion (Fig. 5B) was not significantly impaired, relative to wild type (SI Appendix, Fig. S3). Finally, although ProGLY:CTS-LPAT1 lpat2-3 pdct seeds following germination in warm conditions (Fig. SD), and this contrasts with some studies where seeds have been modified to incorporate uncommon fatty acyl groups into TAG (35). In cool conditions (10°C), ProGLY:CTS-LPAT1 lpat2-3 pdct seed germination and seedling establishment also appeared not to be significantly impaired, relative to wild type (Supp. Appendix, Table S5). Even an oilseed crop with more modest C16:0 enrichment at the sn-2 position that we have achieved here may still be desirable since clinical trials have reported benefits with as little as 43% of C16:0 at the sn-2 position (1,3,6) and product surveys have found that this level of enrichment is common in infant formulas that are supplemented with HMFS (31).

Materials and Methods

Detailed descriptions of plant material and growth conditions, cloning and Agrobacterium mediated transformation, microscopy, mutant genotyping, lipid analysis, qRT-PCR analysis of gene expression, germination and seedling establishment assays and statistical analysis are provided in SI Appendix, SI Materials and Methods. Primers used are listed in SI Appendix, Table S5.

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Conclusions

In this study we show that the TAG biosynthetic pathway in plants can be engineered so that the stereoisomeric structure of seed storage oil is altered to mimic that of HMFS, with >70% of C16:0 at the sn-2 position. Owing to its potential benefits for infant nutrition (1,3,6), but it has not been feasible to engineer vegetable fats where C16:0 is virtually excluded from the sn-2 position (45,59). Many infant formulas contain HMFS that are made by restructuring vegetable fats using enzyme-based catalysis, but they are relatively costly to produce; particularly for the manufacture of true mimetics with >70% of C16:0 at the sn-2 position (1,7). Translation of our technology from the model species Arabidopsis to an oilseed crop might conceivably provide a cheaper and more sustainable source of HMFS for infant formula, but further research would be required to test this supposition. If HMFS could be obtained directly from a vegetable source this would abrogate the need for enzyme-based catalysis. The infant formula market is currently estimated to use nearly half a million metric tons of vegetable-derived fat per year. Several oilseed crops may be considered as possible hosts for HMFS production, and it is noteworthy that conventional sunflower and genetically modified oilseed rape varieties have already been developed lipids from et al., 387-399 (2011).


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