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Reducing Isozyme Competition Increases Target Fatty Acid Accumulation in Seed Triacylglycerols of Transgenic Arabidopsis

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Reducing isozyme competition between host and transgenic acyltransferases increases the accumulation of ricinoleic and \( \alpha \)-eleostearic acid in seed triacylglycerol of Arabidopsis.
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Abstract

One goal of green chemistry is the production of industrially useful fatty acids (FAs) in crop plants. We focus on hydroxy fatty acids (HFA) and conjugated polyenoic fatty acids (α-eleostearic acid, ESA), using Arabidopsis (Arabidopsis thaliana) as a model. These FAs are found naturally in seed oils of castor (Ricinus communis) and tung (Vernicia fordii), respectively, and are used for the production of lubricants, nylon and paints. Transgenic oils typically contain less target FA than are produced in the source species. We hypothesized that competition between endogenous and transgenic isozymes for substrates limits accumulation of novel FAs in Arabidopsis seeds. This hypothesis was tested by introducing a mutation in AtDGAT1 in a line expressing castor FA hydroxylase (RcFAH12) and acyl-CoA: diacylglycerol acyltransferase2 (RcDGAT2) in its seeds. This led to a 17% increase in the proportion of HFA in seed oil. Expression of castor phospholipid:diacylglycerol acyltransferase1A (PDAT1A) in this line increased the proportion of HFA by an additional 12%. To determine if our observations are more widely applicable, we investigated if isozyme competition influenced production of ESA. Expression of tung FA conjugase, FADX, in Arabidopsis produced ~7.5% ESA in seed lipids. Coexpression of VfDGAT2 increased ESA levels to ~11%. Overexpression of VfDGAT2, combined with suppression of AtDGAT1 increased ESA accumulation to 14-15%. Our results indicate that isozyme competition is a limiting factor in the engineering of unusual FAs in heterologous plant systems, and that reduction of competition through mutation and RNA suppression, may be a useful component of seed metabolic engineering strategies.
Introduction

Production of vegetable oils, in the form of triacylglycerols (TAG), is of great importance for human nutrition and as a source of chemicals for industry. The vegetable oils produced in our food crops are composed of five major fatty acids (FAs) (Bates et al., 2013). Besides these common FAs, numerous uncommon FAs are produced in nature, such as hydroxy, conjugated, epoxy, and short-chain FAs that are, or could be used for industrial purposes (Badami and Patil, 1980). However, the species producing these uncommon FAs are often not suitable for large scale industrialized agriculture (Voelker and Kinney, 2001; Dyer et al., 2008). In order to solve this problem, attempts have been made to produce these uncommon FAs in seeds of crop plants. This has been a long standing goal in the field of lipid research and seemed initially quite straightforward (Voelker and Kinney, 2001; Napier, 2007; Napier and Graham, 2010; Carlsson et al., 2011; Bates and Browse, 2012; Bates et al., 2013; Vanhercke et al., 2013). The approach taken was to identify the enzyme responsible for synthesis of the desired FA, and express the corresponding gene in seeds of high yielding crop plants. Unfortunately, in general only low levels of the desired FAs were produced by comparison to levels in the native plant (Broun and Somerville, 1997; Cahoon et al., 2006). One reason for this discrepancy is that enzymes of TAG synthesis often lack proper substrate specificity and selectivity, leading to poor utilization of substrates containing these unusual FAs (Knutzon et al., 1999; Burgal et al., 2008; Li et al., 2010; Kim et al., 2011; van Erp et al., 2011).

In this paper we focus on the engineering of hydroxy FA (HFA; including ricinoleic acid) and \( \alpha \)-eleostearic acid (ESA), in heterologous plant systems. HFA are used in many industrial applications, including the production of nylon, plastics and lubricants, and they are produced at high levels in the seeds of \textit{Ricinus communis} (castor). However, castor is not suitable for industrialized agriculture and produces the toxic protein ricin, as well as other proteins that can cause allergenic reactions in humans. Currently most of the cultivation of castor for the production of HFA occurs in China, India and Brazil. ESA is used in industrial applications such as inks, coatings and resins and is produced in the seeds of tung tree (\textit{Vernicia fordii} Hemsl., formerly \textit{Aleurites fordii} Hemsl.) (Sonntag, 1979). Tung tree also has problematic agronomic characteristics and can only be grown in limited areas of the United States which are prone to damage from hurricanes. In order to create cheap and reliable sources of these FAs, their synthesis has been studied and attempts have been made to produce them in heterologous plant systems.
The gene responsible for the synthesis of HFA in castor is the *Ricinus communis* FATTY ACID HYDROXYLASE12 (*RcFAH12*). This enzyme hydroxylates the Δ12 position of oleic acid esterified to the sn-2 position of phosphatidylcholine (PC) and is a homologue of FATTY ACID DESATURASE2 (FAD2) (Vandeloo et al., 1995). Tung tree Fatty Acid Conjugase/Desaturase, FADX (Dyer et al., 2002), is responsible for the synthesis of ESA and also is a FAD2 homologue. It converts PC-bound linoleoyl groups to eleostearate (18:3Δ9cis,11trans,13trans) (Dyer et al., 2002). After synthesis on PC, these FAs can be incorporated into TAG by several different metabolic routes and enzymes (Fig. 1). Only the routes and enzymes relevant to this paper will be discussed here, for a more exhaustive description see van Erp et al. (2011). The modified FAs can be hydrolyzed from the sn-2 position of PC by phospholipid:diacylglycerol acyltransferase (PDAT), which then esterifies these FAs to the sn-3 position of diacylglycerol (DAG) in order to generate TAG. The lysophosphatidylcholine (LPC) generated by this reaction can be used by acyl-CoA:lysophosphatidylcholine acyltransferase (LPCAT) to regenerate PC. PC can be converted into DAG by phospholipase C (PLC) or PC:DAG cholinephosphotransferase (PDCT). PLC is involved in the removal of the choline headgroup of PC, to generate DAG with the same FA composition as PC. PDCT exchanges the choline headgroup between PC and DAG. The DAG formed by these enzymes can subsequently be used by PDAT or acyl-CoA:DAG acyltransferase (DGAT) to generate TAG. A reverse activity of LPCAT can hydrolyze the modified FAs from the sn-2 position of PC to generate acyl-CoA and LPC. These acyl-CoAs can subsequently be used by the acyltransferase enzymes of the Kennedy pathway (GPAT, acyl-CoA: glycerol-3-phosphate acyltransferase; LPAT, acyl-CoA:lysophosphatidic acid acyltransferase; and DGAT) to generate TAG containing modified FAs.

Expression of a *RcFAH12* cDNA in Arabidopsis under control of several seed-specific promoters led to accumulation of HFA to only 17% of total seed FAs (Broun and Somerville, 1997; Smith et al., 2000; Smith et al., 2003; Lu et al., 2006), compared to ~90% in native castor seed oil (Badami and Patil, 1980). Engineering attempts to overcome this problem have focused on coexpression of castor acyltransferases such as *RcDGAT2* (Burgal et al., 2008), *RcPDAT1A* (Kim et al., 2011; van Erp et al., 2011), or the castor electron transfer system in Arabidopsis seeds expressing *RcFAH12* (Wayne et al., 2013). Coexpression of *RcDGAT2* and *RcPDAT1A* led to significant increases in HFA levels from 17% to 26-28% of total seed FAs, while coexpression of the electron transfer system did not result in an increase. Production of ESA in Arabidopsis has met similar challenges.
Expression of the FADX genes from either tung tree or bitter gourd (*Momordica charantia*, another species containing high levels of ESA in seeds) in a *fad3 fae1* Arabidopsis line resulted in 7-13% ESA (Cahoon et al., 2006). Two types of DGAT from tung tree showed significantly different affinities towards ESA-containing substrates when expressed in yeast fed tung oil fatty acids (Shockey et al., 2006), but the effects of these and other tung TAG metabolic enzymes *in planta* have not previously been reported. The experiments reported here represent a valuable opportunity to investigate possible commonalities between metabolic engineering strategies for production of various types of value-added oils.

Subsequent research into the underlying causes of suboptimal production of HFA revealed that RcFAH12 expression in Arabidopsis seeds caused metabolic perturbations, leading to the poor accumulation of HFA in TAG and to a reduction in total FA content of seeds (Dauk et al., 2007; Bates and Browse, 2011; van Erp et al., 2011). Overexpression of castor PDAT and DGAT restored FA content in RcFAH12 transgenic seeds to nearly wild type levels while simultaneously increasing HFA content of the seeds (van Erp et al., 2011). Bates et al. (2014) elucidated the mechanism behind this decrease in FA content of seeds expressing RcFAH12, by showing that RcFAH12 expression resulted in feedback inhibition of FA synthesis. These results indicate that efficient transfer of HFA from their site of synthesis on PC into TAG is essential for the engineering of high levels of HFA in heterologous plant systems.

In this paper we investigated whether it is possible to improve the flux of HFA out of PC and into TAG, by reducing substrate competition between endogenous Arabidopsis isoymes and the transgenic counterparts from castor and tung. We focused on substrate competition between AtDGAT1 and/or AtPDAT1 and either RcDGAT2 and RcPDAT1A from castor, or VfDGAT2 from tung. To increase synthesis of HFA, we attempted to replace *AtDGAT1* and *AtPDAT1* with *RcDGAT2* and *RcPDAT1A*, by introducing a null mutation in *AtDGAT1*, and by suppression of expression of *AtPDAT1* with artificial microRNAs. Introduction of an *Atdgat1* null mutation in a plant line expressing *RcFAH12* and *RcDGAT2* resulted in a significant increase in HFA levels. Subsequent overexpression of *RcPDAT1A* resulted in a further increase in HFA levels. In the case of ESA, an established homozygous *FADX* line (producing ~8% ESA in total seed lipids) was re-transformed with either *VfDGAT2* alone or with *VfDGAT2* together with a seed-specific *AtDGAT1* RNAi construct. Analysis of seed lipids from homozygous double-transgenic versus parental single-transgenic *FADX* lines showed higher levels of ESA when tung DGAT2 was present, which were enhanced further when the *AtDGAT1* RNAi was present. The similarities between these two
data sets suggest that endogenous competition is a limitation common to many such engineering strategies.

RESULTS

Eliminating *AtDGAT1* Increases HFA Accumulation in a FAH12 RcDGAT2 Transgenic Line

AtDGAT1 and AtPDAT1 catalyze the final step in the synthesis of TAG in Arabidopsis seeds (Zhang et al., 2009). AtDGAT1 and AtPDAT1 preferentially incorporate common FAs into TAG, while RcDGAT2 and RcPDAT1A prefer HFA (Burgal et al., 2008; van Erp et al., 2011). We hypothesize that substrate competition for common FAs versus unusual FAs occurs between AtPDAT1/AtDGAT1 and the transgenic acyltransferases and that this competition limits the accumulation of unusual FAs in our FAH12 RcDGAT2 transgenic lines.

To test this proposal, the Arabidopsis *dgat1-2* mutant (*tag1-2*; (Routaboul et al., 1999)) was crossed with the CL7 RcDGAT2 line 544 #5 (Burgal et al., 2008) followed by selfing of F1 plants. The CL7 line expresses RcFAH12 in seeds of the *fatty acid elongase1* (*fae1*) mutant (Kunst et al., 1992; Lu et al., 2006). Segregation of the four loci (*FAH12, RcDGAT2, fae1* and *dgat1*) in an F2 population of 460 plants was monitored by PCR assays and gas chromatography of seed fatty acid compositions. An F2 plant determined to be heterozygous for *dgat1* and homozygous for *fae1, RcFAH12*, and *RcDGAT2* was grown to maturity. A population of F3 progeny of this plant (n = 60) were grown and sequencing of *DGAT1* was used to identify nine wild-type and nine *dgat1-2* segregants. Analysis of seed samples from these plants shows a 17% increase in the proportion of HFA from an average of 24.7 ± 0.61% in the *DGAT1* wild-type plants to an average of 29.0 ± 0.9% in the *dgat1* CL7 RcDGAT2 line (Fig. 2) and the amount of HFA per seed increased by 23% (Fig. 3). Introducing the *dgat1* mutation also leads to a reduction in 18:1 and increases in 18:2 and 18:3 (Fig. 2). These changes are likely the result of the known increases in *FAD2, FAD3* and *PDAT1* expression that occur in *dgat1*-mutant seed (Xu et al., 2012). Mutations at the *dgat1* locus cause a 45% reduction in seed FA content (Katavic et al., 1995; Routaboul et al., 1999; Zou et al., 1999). However, introducing a *dgat1* mutation in the CL7 RcDGAT2 background did not lead to any further reduction in total FA/seed or percent oil relative to CL7 RcDGAT2 controls (Fig. 3 A&D), indicating that the castor DGAT2 can maintain rates of oil synthesis in a *dgat1 PDAT1* genetic background. Nevertheless, relative to the *fae1* parental line, total FA/seed,
seed weight and percent oil are reduced by 14%, 10% and 8%, respectively (Fig. 3). Results from previous studies have indicated that *fue1* is comparable to wild-type in all these parameters (Kunst et al., 1992; van Erp et al., 2011).

**Expression of RcPDAT1A in the dgat1 CL7 RcDGAT2 Background Further Increases HFA Levels**

Expression of RcPDAT1A in the *dgat1* CL7 RcDGAT2 background could shift the balance in substrate competition between castor and Arabidopsis enzymes further in favor of incorporation of HFA into TAG. To test this hypothesis, *RcPDAT1A* was transformed in the *dgat1* CL7 RcDGAT2 background using DsRed as a selection marker (Stuitje et al., 2003). Thirty-nine lines with independent transgene insertion sites were generated. From these, four lines with high levels of HFA and segregating 1:3 for brown versus red seeds, indicating a single functional insertion allele, were selected. In order to determine if the increase in HFA levels is due to the presence of *RcPDAT1A*, a T2 population of 40 plants segregating for *RcPDAT1A* was planted. The four tested lines gave similar results and Fig. 4 shows data for one of these lines. HFA levels in T3 seeds increased from an average of 28.0 ± 0.72% in the *dgat1* CL7 RcDGAT2 plants to an average of 31.4 ± 1.12% in the *dgat1* CL7 RcDGAT2 *RcPDAT1A* plants. There were no significant changes in the amount of total FA per seed (Fig. 3A), but there was a 23% increase in the amount of HFA per seed (Fig. 3B) in the *dgat1* CL7 RcDGAT2 *RcPDAT1A* plants compared to the *dgat1* CL7 RcDGAT2 segregants. These increases are somewhat larger than observed previously (2) when *RcPDAT1A* was expressed in the CL7 RcDGAT2 background (26.7% HFA). These improvements likely arise from the introduction of the *dgat1* mutation, which shifts the balance in substrate competition further in favor of incorporation of HFA.

**Reduced Expression of AtPDAT1 in the dgat1 CL7 RcDGAT2 RcPDAT1A Background Does Not Increase HFA Levels Further**

In the *dgat1* CL7 RcDGAT2 RcPDAT1A line, substrate competition between AtPDAT1 and RcPDAT1A / RcDGAT2 can still occur. The *dgat1-1 pdat1* double mutant of Arabidopsis is not viable (Zhang et al., 2009), so to determine if reducing AtPDAT1 activity in the *dgat1* CL7 RcDGAT2 RcPDAT1A line might further increase HFA accumulation, we employed an artificial microRNA (amiRNA) approach. A 21-mer sequence targeting the 3’ untranslated region of the *AtPDAT1* mRNA was designed and used to replace the stem loops in the MIR319a precursor (Palatnik et al., 2003; Ossowski et al., 2008). The *pdat1-amiRNA* was cloned in a multigene vector behind the glycinin seed-specific promoter and *RcPDAT1A*
was cloned behind the oleosin promoter in the same vector, which expresses the DsRed selectable marker. The resulting Atpdat1-amiRNA/RcPDAT1A construct was transformed into the dgat1 CL7 RcDGAT2 line. Most of the transgenic, DsRed T1 seeds were wrinkled and had a low FA content. This resembles the observations made when AtPDAT1 was suppressed in the dgat1 background (Zhang et al., 2009). Germination of the T1 seeds was reduced to less than 50%, but 45 T1 plants were grown successfully. T2 seeds from these plants were analyzed and lines with high levels of HFA and showing Mendelian segregation patterns, indicating a single functional insertion allele, were selected for further analysis. Two segregating populations of lines with the highest HFA levels were planted (50 plants of each line) and the HFA levels in T3 seeds were determined. Fig. 5 shows the data for one line. The HFA level in the dgat1 CL7 RcDGAT2 Atpdat1-amiRNA RcPDAT1A segregants (32.2 ± 0.53% HFA) was not statistically different from that of the dgat1 CL7 RcDGAT2 RcPDAT1A line (31.4 ± 1.12% HFA). There were also no significant changes in the µg FA and HFA per seed (Fig. 3A,B). A possible explanation for this result is that T1 seeds with the strongest suppression of AtPDAT1 expression were inviable and did not germinate. Consistent with this possibility, quantitative RT-PCR results indicated that AtPDAT1 transcript levels in dgat1 CL7 RcDGAT2 pdat1-amiRNA RcPDAT1A plants were more than 40% of those measured in the dgat1 CL7 RcDGAT2 RcPDAT1A controls. These experiments indicate that RcPDAT1A and RcDGAT2 cannot fully replace the function of AtPDAT1 and AtDGAT1 during seed development. A table of numerical data for the seed fatty acid compositions of the four different transgene combinations investigated is included as Supplemental Table S1.

**Analysis of TAG Composition and Regiochemistry**

To determine the biochemical basis for the changes in HFA levels observed in our transgenic lines, the composition of seed TAG was investigated. TAG was extracted from the seeds of the transgenic plant lines and separated by TLC into molecular species with 0, 1, 2 or 3 HFA. Quantitative FA analysis by gas chromatography was used to determine the relative amounts of the four molecular species. Introduction of the dgat1 null mutation in the CL7 RcDGAT2 background increased the level of 2-HFA-TAG from 16.6 ± 0.60% to 26.3% ± 0.85% of total seed TAGs (a 59% increase), caused no change in 1-HFA-TAG and decreased the amount of 0-HFA-TAG from 40.8 ± 1.62% to 28.8 ± 0.51% (a 29% decrease) (Fig. 6A). This change in TAG species composition is most likely due to a difference in substrate preference between AtDGAT1 and RcDGAT2. Burgal et al., (2008) showed that
RcDGAT2 preferentially uses HFA-DAG as a substrate compared to 18:1- and 18:2-DAG. The decrease in 0-HFA-TAG is probably caused by acylation of 0-HFA-DAG with a HFA at the sn-3 position by RcDGAT2. The fact that this does not result in an increase in 1-HFA-TAG levels might be caused by acylation of sn-2 HFA-DAG with a HFA at the sn-3 position in order to produce 2-HFA-TAG (Fig. 6A).

Expression of RcPDAT1A in the dgat1 CL7 RcDGAT2 background further increased 2-HFA-TAG from 26.3 ± 0.85% to 39.5 ± 0.21% (a 50% increase), decreased 1-HFA-TAG levels from 42.1 ± 1.05% to 32.4 ± 0.33% (a 30% decrease) and caused a small (10%) decrease from 28.8 ± 0.51% to 26.0 ± 0.14% in the 0-HFA-TAG levels (Fig. 6A). A possible explanation for these observations could be that RcPDAT1A might preferentially acylate HFA-DAG over normal DAG. The decrease in 1-HFA-TAG is probably caused by RcPDAT1A transferring HFA from the sn-2 position of PC to HFA-DAG. Consistent with this interpretation, regiochemical analysis of TAG by incubation with Rhizomucor lipase (van Erp et al., 2011) indicated that increases in HFA seen in our lines was predominantly found at the Sn-1/3 position of the TAG molecule (Fig. 6B).

Suppression of AtPDAT1 in the dgat1 CL7 RcDGAT2 RcPDAT1A background did not lead to any significant additional change in TAG species composition, except that 1-HFA-TAG slightly decreased and 0-HFA-TAG slightly increased (Fig. 6A). This is consistent with the low level of suppression of expression of AtPDAT1 in the viable pdat1-amiRNA lines described above.

**Substrate Competition Between AtDGAT1 and VfDGAT2 Limits the Accumulation of Eleostearic Acid**

To determine if substrate competition between endogenous acyltransferases and introduced transgenic enzymes limits the accumulation of other unusual FAs, we investigated the synthesis of eleostearic acid (ESA) in Arabidopsis seeds. To examine how ESA accumulation was affected by competition between endogenous and transgenic enzymes, the fad3 fae1 double mutant of Arabidopsis (Smith et al., 2003) was first transformed with a construct containing the tung fatty acid conjugase FADX (Dyer et al., 2002), driven by the strong seed-specific phaseolin promoter (Slightom et al., 1983). Using this line as a starting point, we explored the ability of tung DGAT2 (VfDGAT2) to increase ESA content in Arabidopsis seeds when coexpressed with tung FADX. A DGAT2 was chosen for this role based on previous studies of VfDGAT2 in yeast (Shockey et al., 2006), which showed that this enzyme has a strong preference for ESA-containing substrates, and similar findings
regarding the substrate selectivities of other related DGAT2 enzymes (Burgal et al., 2008; Li et al., 2010). T5 FADX plants grown from homozygous transgenic T4 plants producing 7.5±0.1% ESA were retransformed with VfDGAT2 transgene under control of the seed-specific At2S-3 promoter (Guerche et al., 1990), either alone, or in combination with a seed-specific AtDGAT1 RNAi cassette driven by the soybean β-conglycinin α’-subunit promoter (Doyle et al., 1986). Multiple transgenic T1 seeds were selected by observation of fluorescence from the DsRed marker included in the binary constructs. Segregating seed samples were harvested from mature T1 plants and their lipids analyzed by gas chromatography. Nineteen independent transgenic events yielded an average of 9.01% ESA at this stage, representing a 20% increase relative to the parental FADX plants. Twenty-two transgenic lines co-expressing the combination of VfDGAT2 and AtDGAT1 RNAi averaged 11.1 ± 1.29% ESA (a 48% increase relative to the parental lines) (Fig. 7).

To ascertain the full degree of change effected by expression of VfDGAT2 overexpression and AtDGAT1 silencing, two T2 lines for each of the double transformants were chosen for analysis in the T3 generation. Seeds were harvested from between 18 and 32 progeny from each of the chosen T2 lines. Several homozygous samples (consisting of 100% red seeds) and FADX parental revertants (displaying uniform brown seeds devoid of fluorescence) from each line were analyzed. All four sets of brown FADX parental revertant seeds averaged between 8.49 - 8.85% ESA. Individual FADX plants homozygous for VfDGAT2 averaged 11.6 ± 0.71% and 11.7 ± 0.62% ESA respectively (Fig. 8), including individual plants whose seeds contained as much as 12.47 and 12.64% ESA. As in the T2 sample distributions, FADX plants co-expressing both VfDGAT2 and AtDGAT1 RNAi produced higher levels of the novel fatty acid, both in terms of plants with the highest seed ESA (14.9 and 16.1%) and average ESA across all homozygous plants (13.8 ± 0.69% and 14.7 ± 0.85%) (Fig. 8). Complete FA analyses of seeds of these eight lines are included in Supplemental Table S2.

To provide additional data on the seed oil composition of the three different transgene combinations, we analyzed new seed batches of line #5 red (homozygous VfFADX VfDGAT2), line #9 red (homozygous VfFADX VfDGAT2 AtDGAT1 RNAi), along with brown segregants (homozygous VfFADX) as controls. The data obtained confirmed the increases in percent ESA in the oil conferred by expression of VfDGAT2 and VfDGAT2 + AtDGAT1 RNAi (Supplemental Fig. S2A). These increases were associated with increases in ESA per
mg of seed weight (Supplemental Fig. S2B), while there was no significant change in the total FA/mg seed (Supplemental Fig. S2C).

DISCUSSION

AtDGAT1 and AtPDAT1 are the two enzymes that are responsible for the acylation of the sn-3 position of DAG in seeds and some other Arabidopsis tissues. When expression of AtPDAT1 was suppressed in the atdgal background, most of the pollen was not viable, likely due to a severe reduction in pollen storage lipid, and the reduced number seeds that did develop had very low oil content (Zhang et al., 2009). These results indicate that in Arabidopsis DGAT1 and PDAT1 have an essential, redundant role and that other enzymes, such as AtDGAT2 and AtDGAT3 do not contribute significantly to acylation of the sn-3 position of DAG in Arabidopsis seed lipids. AtDGAT1 has the quantitatively more significant role, as indicated by the reduction in oil content in the atdgal background (Katavic et al., 1995; Routaboul et al., 1999; Zou et al., 1999). By contrast, characterization of an atpdatl null mutant showed no reduction in seed oil content (Mhaske et al., 2005).

The goal of our investigation was to determine if substrate competition between transgenic castor or tung enzymes and their endogenous Arabidopsis counterparts is a limiting factor for the accumulation of HFA or ESA in our transgenic plant lines. This possibility arises because the endogenous Arabidopsis enzymes are known or proposed to favor substrates containing FA normally found in Arabidopsis seed TAG, while the castor and tung enzymes have been shown to have specificity for HFA- or ESA-containing substrates, respectively (Shockey et al., 2006; Burgal et al., 2008). We chose to focus on potential competition between AtDGAT1 and either RcDGAT2 or VfDGAT2, because these enzymes are known to play major roles in TAG synthesis in the respective plant species (Stahl et al., 2004; Kroon et al., 2006; Shockey et al., 2006; Burgal et al., 2008; Zhang et al., 2009; van Erp et al., 2011). In order to test if substrate competition between AtDGAT1 and RcDGAT2 is limiting for accumulation of HFA, the atdgal-2 mutation was crossed into the CL7 RcDGAT2 line. Homozygous dgatl CL7 RcDGAT2 plants had significantly higher HFA levels than the CL7 RcDGAT2 segregants (Fig. 2). Subsequent analyses of the derived dgatl and wild-type DGAT1 lines indicated that there was no reduction in seed oil content associated with the loss of the DGAT1 enzyme (Fig. 3). These results indicate that eliminating competition from the endogenous DGAT enzyme results in a 20% increase in HFA accumulation in the seed oil, compared with the parental CL7 RcDGAT2 line.
In order to determine if substrate competition is a more general problem for the engineering of unusual FAs, seed-specific overexpression of tung DGAT2, with or without accompanying silencing of AtDGAT1 expression by RNAi, was also performed in an established homozygous transgenic line producing ESA. Analysis of T2 and T3 lines showed VfDGAT2 expression resulted in expected increases in ESA levels compared to the parental lines. However, a further 22% increase in ESA was observed when AtDGAT1 expression was reduced by RNAi. As with the results for castor DGAT2, the tung DGAT2 data clearly indicates that AtDGAT1 supports incorporation of normal FA into TAG, and that AtDGAT1 and transgenic VfDGAT2 compete to mobilize different DAG and acyl-CoA substrates into TAG.

Arabidopsis does not use the classic Kennedy pathway for the synthesis of TAG, but instead uses PC as an intermediate in TAG synthesis (Fig. 1). It is PC that is the substrate for FA desaturation and modification enzymes (including RcFAH12 and VfFADX). Thus, DAG and acyl-CoA derived from PC will contain HFA or ESA, and these in turn become available for TAG synthesis via DGAT and PDAT activities (Bates and Browse, 2011). In transgenic Arabidopsis seeds expressing RcFAH12, this pathway creates a metabolic bottleneck for the incorporation of HFA into HFA-TAG. In these plants, 1-HFA-DAG is synthesized but not efficiently converted into PC. Radiolabeling experiments indicate that the 1-HFA-DAG is converted into 2-HFA-TAG and degraded (Bates and Browse, 2011) and the metabolic bottleneck also results in feedback inhibition of FA synthesis and reduced seed oil content (Bates et al., 2014). Both RcDGAT2 and RcPDAT1A expression can increase incorporation of HFA into TAG and partially alleviate the reduction of seed oil in RcFAH transgenic lines (van Erp et al., 2011; Bates et al., 2014). Consistent with these previous findings, our dgat1 CL7 RcDGAT2 RcPDAT1A transgenics showed a significant further increase in seed HFA content (Figs. 4 and 3B). These plants also had a very substantial increase in the proportion of 2-HFA TAG species, relative to the dgat1 CL7 RcDGAT2 parental line (Fig. 6). However, the elimination of AtDGAT1 and coexpression of RcDGAT2 and RcPDAT1A did not significantly change the proportion of 3-HFA TAG.

Our attempts to reduce competition from the endogenous AtPDAT1 isozyme were evidently unsuccessful. The pdat1-amiRNA/RcPDAT1A lines that we were able to recover had seed characteristics that are indistinguishable from the RcPDAT1A lines lacking the pdat1-amiRNA construct (Figs. 4 and 5) and contained levels of AtPDAT1 mRNA that were at least 40% of the levels in parental control lines. Many of the dgat1 CL7 RcDGAT2 pdat1-amiRNA RcPDAT1A T1 seeds were shrunken and did not germinate either on soil or on agar
medium supplemented with 1% sucrose. It is possible that these inviable seeds included transgenics with strong suppression of AtPDAT1 expression.

Our results indicate that substrate competition between endogenous and transgenic acyltransferases may be a general problem in the engineering of unusual FAs in heterologous plant systems. The >20% increases in HFA and ESA that we observed indicate that reducing enzyme competition can provide new avenues for metabolic engineering. Hopefully this strategy will bring us a step closer to the engineering of crop plants with high levels of unusual FAs for industrial or health purposes.

While our results characterize examples of isozyme competition that lead to reduced HFA or ESA accumulation, it is also possible for endogenous and transgenic isozymes to act synergistically. The \textit{ROD1} (\textit{Reduced Oleate Desaturation1}) gene encodes phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) that interconverts PC and DAG (Lu et al., 2009). PDCT is required for efficient HFA incorporation into TAG in transgenic Arabidopsis expressing the castor hydroxylase since the proportion of HFA in seed oil is reduced by >50% in \textit{rod1} mutants compared to \textit{ROD1 RcFAH12} controls (Hu et al., 2012). In additional experiments, these authors demonstrated that \textit{RcROD1} expression could compensate for the loss of the Arabidopsis enzyme, when transformed into \textit{rod1} mutants expressing \textit{RcFAH12}, or could lead to increased HFA, when expressed in an \textit{RcFAH12}-transgenic line that is wild-type at the \textit{ROD1} locus. Importantly, expression of \textit{RcROD1} also provided an increase in HFA (from 24.7% to 28.5% of seed FA) when expressed in the CL7 \textit{RcDGAT2} background used in our experiments (Hu et al., 2012). These results point to the potential for obtaining further increases in HFA accumulation in seeds through the production of higher-order multitransgenic lines.

\textbf{MATERIALS AND METHODS}

\textbf{Plant Growth Conditions and Transformation}

The \textit{Arabidopsis thaliana} (Arabidopsis) lines and growth conditions, were similar as described in vanErp et al. (2011) unless otherwise mentioned. Plant transformation was achieved using the floral dip method (Clough and Bent, 1998). All lines are in the Col-O wild-type background, except that \textit{dgat1-2} is in the Ws background.

For experiments comparing genotypes plants of the different lines were randomly distributed across the pots and trays used. Plants were grown in environmentally controlled
chambers under continuous fluorescent illumination of 120-150 µmol quanta m$^{-2}$ s$^{-1}$, 70% relative humidity and 22°C.

**Genotyping of Plants and RT-PCR Analysis**

For genotyping of AtDGAT1, plant material was ground (30 Hz, for 30 s) (Tissuelyser II, QIAGEN), followed by extraction of genomic DNA using a QIAcube (QIAGEN). A portion of AtDGAT1 was amplified with gene-specific primers (Supplemental Table S3) flanking both sides of the point mutation and the amplified fragment was gel-purified with a QIAcube. Sequencing was performed (Eurofins MWG Operon) to determine which plants were wild-type, heterozygous or homozygous for the point mutation in AtDGAT1. To prepare RNA, young siliques were harvested approximately 8-12 days-after-flowering (DAF) from plants, flash-frozen in liquid nitrogen and stored at -80°C. Developing seeds were scraped from each silique on a petridish on dry ice and collected in 1.5 ml eppendorf tubes kept in liquid nitrogen. For RT-PCR analysis, total RNA was extracted using the RNeasy Plant Mini Kit (Qiagen). Samples were treated with RNase-free DNase (Qiagen) using the on-column DNase digestion method according to the manufacturer protocol. cDNA was synthesized using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen/Life Technologies). In order to confirm that the transgenic plants were expressing their respective transgenes, RT-PCR was performed using primers listed in Supplemental Table S3. This confirmed that the transgenic lines expressed the genes of interest.

**Design and Cloning of Artificial microRNA (amiRNA) Against AtPDAT1**

In order to suppress expression of AtPDAT1 in our transgenic plant lines a 21-mer sequence targeting AtPDAT1 was designed [WMD3 – Web MicroRNA Designer, Arabidopsis thaliana cDNA (TAIR9), minimum number of included targets: 1, accepted off-targets: none (Ossowski S, Fitz J, Schwab R, Riester M and Weigel D, personal communication)]. In order to prevent suppression of expression other Arabidopsis genes in our plant lines a Target Search was performed against Arabidopsis cDNAs with the 21-mer sequences (TAIR9_cdna_20090619, number of mismatches 5) and no targets were found. To prevent suppression of the *Ricinus communis* (castor) genes in our transgenic plant lines (RcFAH12, RcDGAT2 and RcPDAT1A) a Target Search was performed against castor mRNAs with the 21-mer sequences [*Ricinus communis* PUT v163a (PGDB), number of mismatches 5] and no targets were found. The best 21-mer with no other targets in Arabidopsis and castor was selected for further analysis.
(5’-TTGCGGGTTATACGTAGTGA-3’, hybridization energy -40.49 kcal/mol). This 21-mer targets the AtPDAT1 mRNA in the 3’UTR.

Cloning of the pdat1-amiRNA and RcPDAT1A in a Multigene Vector

The 21-mer sequence was used to replace the stem loops in the MIR319a precursor (Ossowski et al., 2008). Primer sequences used for cloning are described in Supplemental Table S1. The pdat1-amiRNA construct was cloned in the RS3GSeed DsRed vector in between the glycinin promoter and the glycinin 3’UTR. The pdat1-amiRNA construct was digested with EcoRI and XbaI. The RS3GSeed DsRed vector was digested with EcoRI and XbaI and dephosphorylated with CIP (calf intestinal phosphatase, New England Biolabs). The EcoRI/XbaI pdat1-amiRNA fragment was ligated into the linearized vector using the Quick Ligation Kit (NEB). RcPDAT1A was cloned in the pdat1-amiRNA vector behind the oleosin promoter and followed by the oleosin 3’UTR. The RcPDAT1A cDNA was PCR amplified with primers containing NotI sites (Supplemental Table S1) and digested with NotI HF (New England Biolabs). The PCR product was cloned in the NotI side of the CIP treated pKMS2 vector behind the oleosin promoter. The oleosin promoter RcPDAT1A oleosin 3’UTR construct was cut out of the pKMS2 vector using AscI. This construct was ligated into the AscI site of the pdat1-amiRNA vector in order to generate the pdat1-amiRNA RcPDAT1A vector.

Construction of Vernicia fordii (tung) Gene Vectors and Expression in Plants

The ORFs for tung FADX, and tung DGAT2, as well as the AtDGAT1 RNAi construct, were assembled in various components of a flexible set of cloning vectors and plant binary plasmids prior to transformation into Agrobacterium tumefaciens (Agrobacterium). The cDNA for tung FADX (Dyer et al., 2002) was PCR amplified using a forward primer containing a NotI site, and a reverse primer containing a SacII site adjacent to the stop codon. This product was digested with NotI and SacII, and ligated into plasmid pK8 that had been similarly treated. pK8 contains the strong seed-specific promoter from the Phaseolus vulgaris gene (Slightom et al., 1983), and the cauliflower mosaic virus 35S transcriptional terminator, both flanked on their respective distal ends by AscI sites. The resulting plasmid was named pB190. The AscI cassette from pB190 was transferred to the AscI site of the plant binary plasmid pB9, which carries a kanamycin resistance gene for bacterial selection and a gene for basta (glufosinate ammonium) herbicide resistance for selection in plants. The resulting VfFADX binary plasmid, designated pE181, was
transformed into Agrobacterium strain GV3101; kanamycin and gentamycin-resistant colonies were cultured in liquid media and used to transform the fad3fauel double mutant line of Arabidopsis (Smith et al., 2003) by floral dip.

A seed-specific shuttle plasmid containing an N-terminal myc epitope-tagged tung DGAT2 was generated by PCR amplification of the native DGAT2 ORF with a forward primer in which the initiator methionine codon has been replaced by a KasI site, and a reverse primer containing a SacII site adjacent to the stop codon. Following KasI/SacII digestion, this product was ligated into similarly-digested pB50, a shuttle plasmid containing the Arabidopsis 2S-3 promoter (Guerche et al., 1990) the soybean glycinin G1 subunit transcriptional terminator (Sims and Goldberg, 1989), and a multiple cloning site containing sites that allow for production of N-terminal myc epitope fusions. The Ascl fragment (representing the promoter-gene-terminator cassette) from this plasmid, pB240, was transferred into the corresponding site of the plant binary vector pB110, to form plasmid E278. In turn, pE278 was modified by MluI digestion, and ligation of the Ascl fragment of pJ6, which contains the Glycine max beta-conglycinin promoter (Sato et al., 2004) driving the expression of an RNAi hairpin for AtDGAT1. The RNAi portion of this plasmid contains an intron from the 5’ untranslated region of AtFAD2, flanked by a 592 bp region of the AtDGAT1 ORF (bp 303-894) cloned in inverted orientations. The binary plasmid carrying both the tung DGAT2 overexpression cassette and the AtDGAT1 RNAi cassette is called pE290. The sequences of all primers used to generate these plasmid constructs are included in Supplemental Table S1.

Eleostearic acid production stabilized at approximately 8% in the T4 generation of E181 plants. T5 plants from this line were re-transformed with Agrobacterium bearing either pE278 or pE290. Red T1 seeds were sown on soil and grown to maturity, followed by seed lipid extraction and analysis for determination of eleostearic acid content. Two lines, one representing the highest T2 eleostearate producer and one representing a level near the average of the T2 population, were selected for each double transgenic and carried forward to the T3 generation. Seed samples from T3 plants producing either uniformly red or uniformly brown seeds were analyzed by gas chromatography.
**Fatty Acid Analysis by Gas Chromatography**

Analysis of HFA was performed as described in van Erp et al. (2011). Lipids from Arabidopsis seed containing ESA were extracted as follows. Approximately 30 mg seeds and 3 – 4 2.3 mm chrome steel beads (BioSpec Products, Inc., Bartlesville, OK) were added into a 2 mL Eppendorf tube with 500 µL hexane, followed by 5 min agitation on a Bead Beater. The extract was centrifuged (13000xg/2 min) to remove debris and a portion (300 µL) of the extract was transferred to a 13x100 mm glass Corning culture tube. Hexane (700 µL) and sodium methoxide in methanol (400 µL) were added, followed by 10 min incubation at room temperature with intermittent shaking. The reactions were quenched by addition of 2 mL hexane and 2 mL saturated NaCl solution and the phases were separated by centrifugation in a tabletop swinging bucket centrifuge. Two mL of the upper layer was transferred to vials, capped and analyzed immediately or stored at -20º until needed. Gas chromatography was conducted as described in Shockey, et al. (2011). Whenever possible, all samples containing ESA were prepared using amber-colored glassware to reduce exposure of ESA to light. Regiochemical analysis of TAG was performed as described (van Erp et al., 2011).

**Determination of Seed FA Content**

Seed FA content was determined according to the protocol of (Li et al., 2006), except that 200 µl of toluene was added to each sample, BHT (butylated hydroxytoluene) was omitted, and 20 seeds were used for each measurement. See weights were determined by counting seed numbers in samples of 1-2 mg of seed.

**Lipid Extraction and Characterization of TAG Species**

Lipid extraction and characterization of TAG species was performed as described in van Erp et al. (2011).

**Supplemental Data**

**Table S1.** Fatty acid compositions of samples of T3 seeds of transgenic lines accumulating hydroxyl fatty acids (HFA) characterized in this study.

**Table S2.** Fatty acid compositions of samples of T3 seeds of transgenic lines accumulating eleostearic acid (ESA) characterized in this study.

**Table S3.** Primers used. F= forward primer, R = reverse primer. All primers are in the 5’ to 3’ direction.
**Figure S1.** Expression of *RcDGAT2* transcript in the parental CL7 RcDGAT2 line (1) and in the *dgat1* CL7 RcDGAT2 line (2) obtained by crossing. Results of RT-PCR with 30 or 40 cycles of PCR are shown. The *PP2A* (protein phosphatase 2A) gene was used as a control. (See Materials and Methods for details).

**Figure S2.** Extended analysis of lines accumulating eleostearic acid (ESA). A, ESA as percent of total FA in lines expressing *VfFADX* alone (brown) *VfFADX + VfDGAT2* and *VfFADX + VfDGAT2 + AtDGAT1* RNAi. B, ESA per mg seed weight for these lines. C, Total FA per mg seed weight for these lines.

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Figures Legends

Figure 1. Overview of TAG biosynthesis in transgenic Arabidopsis seeds overexpressing FAH12 or FADX. These enzymes respectively modify 18:1-PC and 18:2-PC in order to generate HFA-PC or ESA-PC (m indicates FAs with these modifications). The modified FAs (as well as 18:2 and 18:3) are subsequently incorporated into TAG by the various enzymes involved in the synthesis of this storage lipid. (FAH12, fatty acid hydroxylase 12; FADX, fatty acid desaturase X; LPCAT, acyl-CoA:lysophosphatidycholine acyltransferase; PDCT, PC:DAG cholinephosphotransferase; PLC, phospholipase C; GPAT, acyl-CoA:G3P acyltransferase; LPAT, acyl-CoA:LPA acyltransferase; PAP, PA phosphatase; PDAT, phospholipid:diacylglycerol acyltransferase; DGAT, acyl-CoA:DAG acyltransferase. Substrate abbreviations: PC, phosphatidylcholine; G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; LPC, lysophosphatidylcholine.)

Figure 2. Seed FA composition of CL7 RcDGAT2 and dgat1 CL7 RcDGAT2 lines. The data represent the average of nine individual plants ± SE. Two-tailed t-test: **P < 0.01, * P < 0.05.

Figure 3. Results of extended analysis of mature seeds from the transgenic lines. A, Total FAs per seed. B, HFA per seed. C, seed weight. D, percent oil content. The data represent the average of 5-27 individual plants ± SE. Two-tailed t-test: **P < 0.01, * P < 0.05.

Figure 4. Seed FA composition of dgat1 CL7 RcDGAT2 and dgat1 CL7 RcDGAT2 RcPDAT1A lines. The data represent the average of 5-11 individual plants ± SE. Two-tailed t-test: **P < 0.01, * P < 0.05.

Figure 5. Seed FA composition of dgat1 CL7 RcDGAT2 and dgat1 CL7 RcDGAT2 pdat1-amiRNA RcPDAT1A lines. The data represent the average of 11 individual plants ± SE. Two-tailed t-test: **P < 0.01, * P < 0.05.

Figure 6. Analysis of TAG compositions of mature seeds from the transgenic lines. A, TAG molecular species; 0-, 1-, 2- and 3-HFA represent molecular species with zero, one, two or
three HFA respectively. B, Percent HFA at the sn-2 and sn-1/3 positions of TAG. The data represent the average of three replicates ± SE. Two-tailed $t$-test: **$P < 0.01$, * $P < 0.05$.

**Figure 7.** Distribution of eleostearic acid levels in transgenic plants expressing tung $FADX$ alone or with tung $DGAT2$ or tung $DGAT2$+$AtDGAT1$-RNAi. A parental $FADX$ line was re-transformed with $VfDGAT2$ or $VfDGAT2$+$AtDGAT1$-RNAi. Seed pools from segregating T1 plants representing independent transgenic events were analyzed by gas chromatography; the corresponding eleostearic acid (ESA) levels are shown. The data is plotted as a scatter plot, with the mean and standard error indicated. Differences between the three data sets are statistically significant (two-tailed $t$ test, $P < 0.001$).

**Figure 8.** Determination of eleostearic acid levels in homozygous transgenic plants co-expressing $FADX$ and $VfDGAT2$+$AtDGAT1$-RNAi. Seeds from two high-performing lines from each of the transgenic genotypes were sown on soil and grown to maturity. Seed samples from homozygous transgenic (red) and non-transgenic (brown) plants were collected separately and analyzed by gas chromatography. Mean eleostearic acid (ESA) levels ± SE are shown. Lower case letters indicate different statistically significant groups (student’s $t$-test, $P < 0.01$)
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