

# Reducing Isozyme Competition Increases Target Fatty Acid Accumulation in Seed Triacylglycerols of Transgenic Arabidopsis<sup>1[OPEN]</sup>

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One goal of green chemistry is the production of industrially useful fatty acids (FAs) in crop plants. We focus on hydroxy fatty acids (HFAs) and conjugated polyenoic FAs ( $\alpha$ -eleostearic acids [ESAs]) using Arabidopsis (*Arabidopsis thaliana*) as a model. These FAs are found naturally in seed oils of castor (*Ricinus communis*) and tung tree (*Vernicia fordii*), respectively, and used for the production of lubricants, nylon, and paints. Transgenic oils typically contain less target FA than that produced in the source species. We hypothesized that competition between endogenous and transgenic isozymes for substrates limits accumulation of unique FAs in Arabidopsis seeds. This hypothesis was tested by introducing a mutation in Arabidopsis *diacylglycerol acyltransferase1* (*AtDGAT1*) in a line expressing castor FA hydroxylase and acyl-Coenzyme A:*RcDGAT2* in its seeds. This led to a 17% increase in the proportion of HFA in seed oil. Expression of castor phospholipid:diacylglycerol acyltransferase 1A 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 tree FA conjugase/desaturase in Arabidopsis produced approximately 7.5% ESA in seed lipids. Coexpression of *VfDGAT2* increased ESA levels to approximately 11%. Overexpression of *VfDGAT2* combined with suppression of *AtDGAT1* increased ESA accumulation to 14% to 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.

Production of vegetable oils in the form of triacylglycerols (TAGs) 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). Other than these common FAs, numerous uncommon FAs are produced in nature, such as hydroxy fatty acids (HFAs), conjugated FAs, epoxy FAs, 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). 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 compared with 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; Bursal et al., 2008; Li et al., 2010; Kim et al., 2011; van Erp et al., 2011).

In this article, we focus on the engineering of HFAs (including ricinoleic acid) and  $\alpha$ -eleostearic acid (ESA) in heterologous plant systems. HFAs 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 castor (*Ricinus communis*). However, castor is not suitable for industrialized agriculture and produces the toxic protein ricin as well as other proteins that can cause allergic reactions in humans. Currently,

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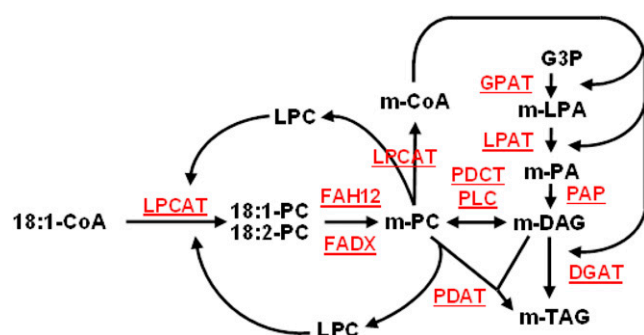
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most of the cultivation of castor for the production of HFAs occurs in China, India, and Brazil. ESA is used in industrial applications, such as inks, coatings, and resins, and produced in the seeds of tung tree (*Vernicia fordii*; formerly *Aleurites fordii*; Sonntag, 1979). Tung tree also has problematic agronomic characteristics and can only be grown in limited areas of the United States that are prone to damage from hurricanes. 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 *FATTY ACID HYDROXYLASE12* (*RcFAH12*). This enzyme hydroxylates the  $\Delta 12$  position of oleic acid esterified to the *stereospecifically numbered2* (*sn-2*) position of phosphatidylcholine (PC) and is a homolog of *FATTY ACID DESATURASE2* (*FAD2*; Van de Loo et al., 1995). Tung tree Fatty Acid Conjugase/Desaturase (*FADX*; Dyer et al., 2002) is responsible for the synthesis of ESA, and also, it is an *FAD2* homolog. It converts PC-bound linoleoyl groups to eleostearate (18:3 $\Delta 9$ cis, 11trans, and 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 article will be discussed here (a more exhaustive description is in 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) to generate TAG. The lysophosphatidylcholine generated by this reaction can be used by acyl-CoA:lysophosphatidylcholine acyltransferase to regenerate PC. PC can be converted into DAG by phospholipase C or phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT). Phospholipase C is



**Figure 1.** Overview of TAG biosynthesis in transgenic *Arabidopsis* seeds overexpressing *FAH12* or *FADX*. These enzymes modify 18:1-PC and 18:2-PC, respectively, to generate HFA-PC or ESA-PC, respectively (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. GPAT, Acyl-CoA:glycerol-3-P acyltransferase; G3P, glycerol-3-P; LPA, lysophosphatidic acid; LPAT, acyl-CoA:lysophosphatidic acid acyltransferase; LPC, lysophosphatidylcholine; LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; PA, phosphatidic acid; PAP, phosphatidic acid phosphatase; PLC, phospholipase C.

involved in the removal of the choline head group of PC to generate DAG with the same FA composition as PC. PDCT exchanges the choline head group between PC and DAG. The DAG formed by these enzymes can subsequently be used by PDAT or acyl-CoA:diacylglycerol acyltransferase (DGAT) to generate TAG. A reverse activity of lysophosphatidylcholine acyltransferase can hydrolyze the modified FAs from the *sn-2* position of PC to generate acyl-CoA and lysophosphatidylcholine. These acyl-CoAs can subsequently be used by the acyltransferase enzymes of the Kennedy pathway (acyl-CoA:glycerol-3-P acyltransferase, acyl-CoA:lysophosphatidic acid acyltransferase, and DGAT) to generate TAG-containing modified FAs.

Expression of an *RcFAH12* complementary DNA (cDNA) in *Arabidopsis* (*Arabidopsis thaliana*) 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, 2003; Lu et al., 2006) compared with approximately 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% to 28% of total seed FAs, whereas 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 melon (*Momordica charantia*; another species containing high levels of ESA in seeds) in an *fad3 fatty acid elongase1* (*fae1*) *Arabidopsis* line resulted in 7% to 13% ESA (Cahoon et al., 2006). Two types of DGAT from tung tree showed significantly different affinities toward ESA-containing substrates when expressed in yeast (*Saccharomyces cerevisiae*)-fed tung oil FAs (Shockey et al., 2006), but the effects of these and other tung tree 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 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 article, 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* isozymes and the transgenic counterparts from castor and tung tree. We focused on substrate competition between *AtDGAT1* and/or *AtPDAT1* and either *RcDGAT2* and *RcPDAT1A* from castor or *VfDGAT2* from tung tree. To increase synthesis of HFA, we attempted to replace *AtDGAT1* and *AtPDAT1* with *RcDGAT2* and *RcPDAT1A* by introducing a null mutation in *AtDGAT1* and suppressing the expression of *AtPDAT1* with artificial microRNAs (amiRNAs). 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 approximately 8% ESA in total seed lipids) was retransformed with either *VfDGAT2* alone or *VfDGAT2* together with a seed-specific *AtDGAT1* RNA interference (RNAi) construct. Analysis of seed lipids from homozygous double-transgenic versus parental single-transgenic *FADX* lines showed higher levels of ESA when tung tree *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.

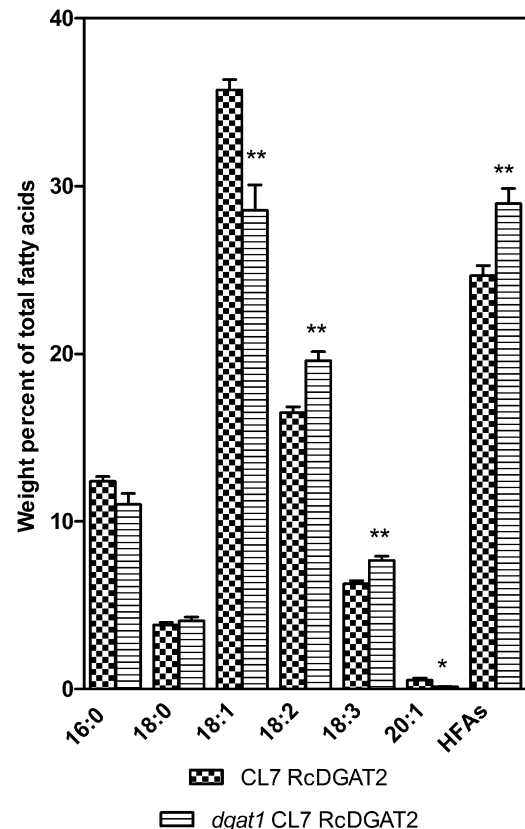
## RESULTS

### Eliminating *AtDGAT1* Increases HFA Accumulation in an *FAH12 RcDGAT2* Transgenic Line

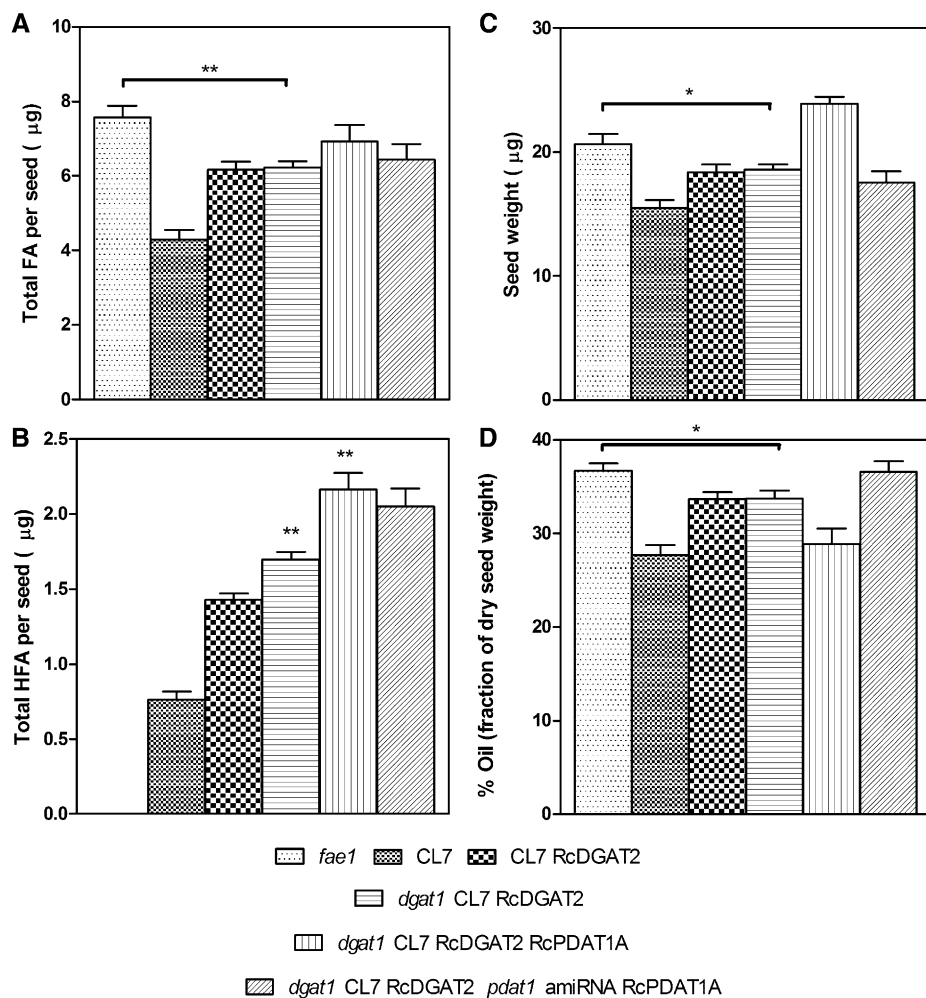
*AtDGAT1* and *AtPDAT1* catalyze the final step in the synthesis of TAG in *Arabidopsis* seeds (Zhang et al., 2009). *AtDGAT1* and *AtPDAT1* preferentially incorporate common FAs into TAG, whereas *RcDGAT2* and *RcPDAT1A* prefer HFA (Burgal et al., 2008; van Erp et al., 2011). We hypothesize that substrate 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 (Routaboul et al., 1999) was crossed with the Chaofu Lu7 (CL7) *RcDGAT2* line 544 #5 (Burgal et al., 2008) followed by selfing of F1 plants. The CL7 line expresses *RcFAH12* in seeds of the *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 FA 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 ( $n = 60$ ) was 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  $24.7\% \pm 0.61\%$  in the *DGAT1* wild-type plants to an average of  $29.0\% \pm 0.9\%$  in the *dgat1* CL7 *RcDGAT2* line (Fig. 2; Supplemental Fig. S1), 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 additional reduction in total FA/seed or percentage of oil relative to CL7 *RcDGAT2* controls (Fig. 3, A and 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 percentage of oil are reduced by 14%, 10%, and 8%, respectively (Fig. 3). Results from previous studies have indicated that *fae1* is comparable with the wild type in all of these parameters (Kunst et al., 1992; van Erp et al., 2011).



**Figure 2.** Seed FA composition of CL7 *RcDGAT2* and *dgat1* CL7 *RcDGAT2* lines. The data represent the average of nine individual plants  $\pm$  se. Two-tailed Student's *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .



**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, Percentage of oil content. The data represent the average of five to 27 individual plants  $\pm$  SE. Two-tailed Student's *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

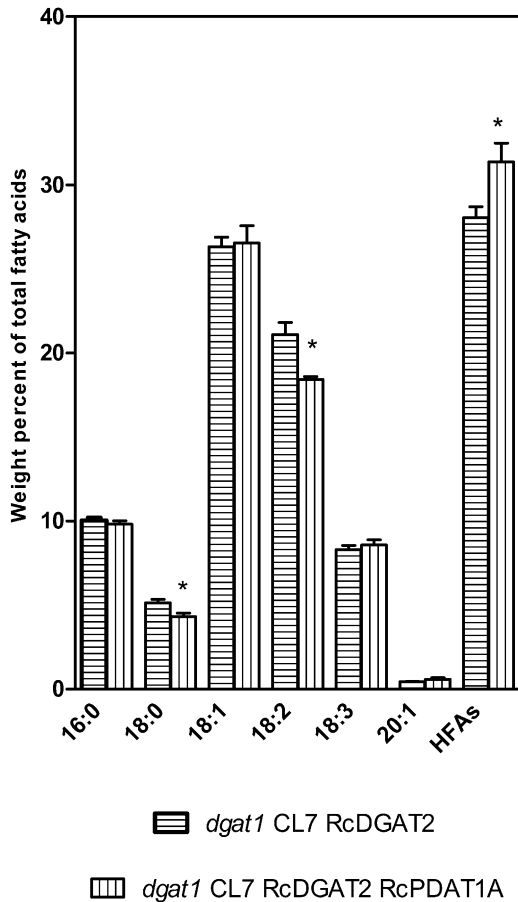
#### Expression of RcPDAT1A in the *dgat1* CL7 RcDGAT2 Background Further Increases HFA Levels

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 *Discosoma* spp. red fluorescent protein (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 segregating 1:3 for brown: red seeds, indicating a single functional insertion allele, were selected. To determine if the increase in HFA levels is caused by the presence of RcPDAT1A, a T2 population of 40 plants segregating for RcPDAT1A was planted. The four tested lines gave similar results, and Figure 4 shows data for one of these lines. HFA levels in T3 seeds increased from an average of  $28.0\% \pm 0.72\%$  in the *dgat1* CL7 RcDGAT2 plants to an average of  $31.4\% \pm 1.12\%$  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 with the *dgat1* CL7 RcDGAT2 segregants. These increases are somewhat larger than observed previously (van Erp et al., 2011) 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 Further Increase HFA Levels

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); therefore, to determine if reducing AtPDAT1 activity in the *dgat1* CL7 RcDGAT2 RcPDAT1A line might further increase HFA accumulation, we used an amiRNA approach. A 21-mer sequence targeting the 3'-untranslated region (UTR) of the *AtPDAT1* mRNA was designed and



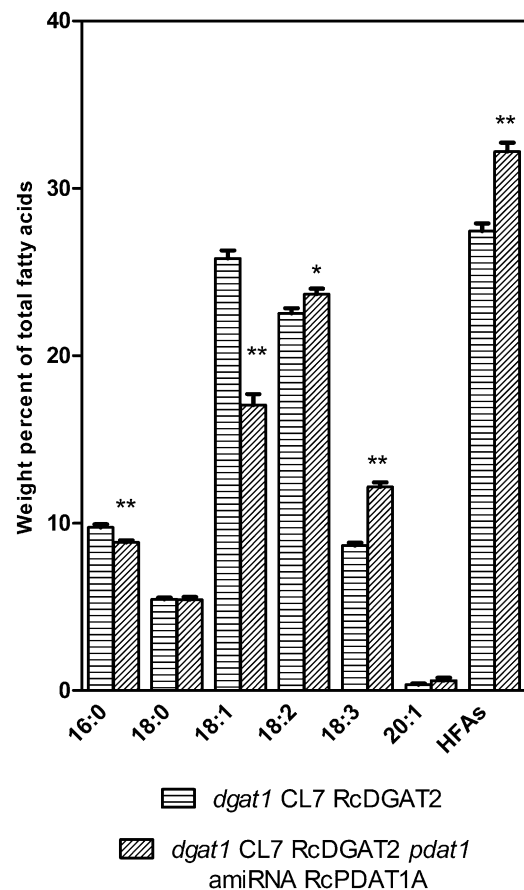
**Figure 4.** Seed FA composition of *dgat1* CL7 RcDGAT2 and *dgat1* CL7 RcDGAT2 RcPDAT1A lines. The data represent the average of five to 11 individual plants  $\pm$  se. Two-tailed Student's *t* test. \*,  $P < 0.05$ .

used to replace the stem loops in the micro-RNA 319a (MIR319a) precursor (Palatnik et al., 2003; Ossowski et al., 2008). The *pdat1-amiRNA* was cloned in a multi-gene 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 *pdat1-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 showing Mendelian segregation patterns, indicating a single functional insertion allele, were selected for additional 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. Figure 5 shows the data for one line. The HFA level in the *dgat1* CL7 RcDGAT2 *pdat1-amiRNA* RcPDAT1A segregants ( $32.2\% \pm 0.53\%$  HFA) was not

statistically different from that of the *dgat1* CL7 RcDGAT2 RcPDAT1A line ( $31.4\% \pm 1.12\%$  HFA). There were also no significant changes in the micrograms of FA and HFA per seed (Fig. 3, A and 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 reverse transcription (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. Numerical data for the seed FA compositions of four different transgene combinations investigated are included in 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



**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  $\pm$  se. Two-tailed Student's *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

extracted from the seeds of the transgenic plant lines and separated by thin-layer chromatography into molecular species with zero, one, two, or three HFAs. Quantitative FA analysis by gas chromatography was used to determine the relative amounts of four molecular species. Introduction of the *dgat1* null mutation in the CL7 RcDGAT2 background increased the level of 2-HFA-TAG from 16.6%  $\pm$  0.60% to 26.3%  $\pm$  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%  $\pm$  1.62% to 28.8%  $\pm$  0.51% (a 29% decrease; Fig. 6A). This change in TAG species composition is most likely caused by a difference in substrate preference between AtDGAT1 and RcDGAT2. Burgal et al., 2008 showed that RcDGAT2 preferentially uses HFA-DAG as a substrate compared with 18:1- and 18:2-DAG. The decrease in 0-HFA-TAG is probably caused by acylation of 0-HFA-DAG with an 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 an HFA at the *sn*-3 position to produce 2-HFA-TAG (Fig. 6A).

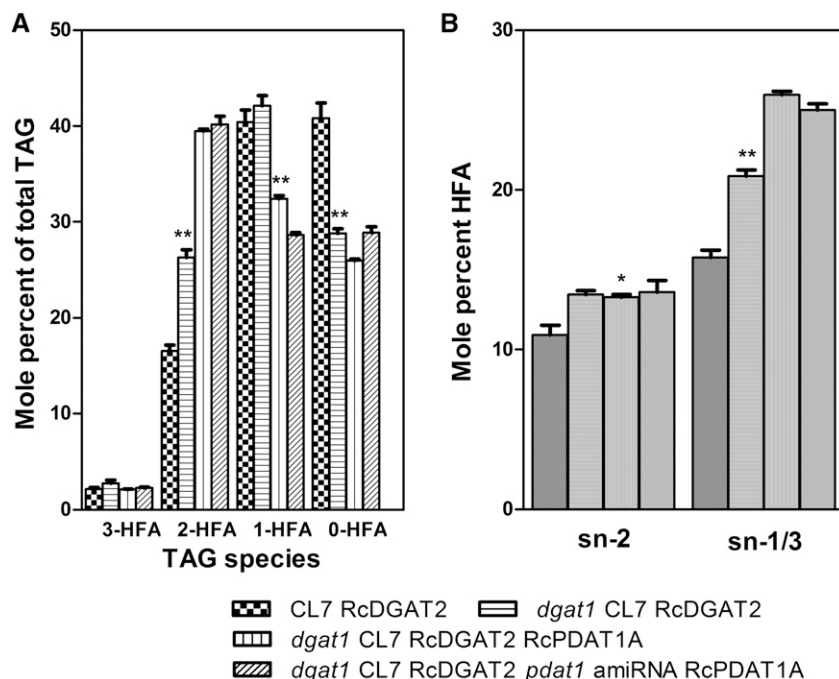
Expression of RcPDAT1A in the *dgat1* CL7 RcDGAT2 background further increased 2-HFA-TAG from 26.3%  $\pm$  0.85% to 39.5%  $\pm$  0.21% (a 50% increase), decreased 1-HFA-TAG levels from 42.1%  $\pm$  1.05% to 32.4%  $\pm$  0.33% (a 30% decrease), and caused a small (10%) decrease from 28.8%  $\pm$  0.51% to 26.0%  $\pm$  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 miehei* 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 ESA

To determine if substrate competition between endogenous acyltransferases and introduced transgenic enzymes limits the accumulation of other unusual FAs, we investigated the synthesis of 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 tree FA 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 VfDGAT2 to increase ESA content in Arabidopsis seeds when coexpressed with tung tree 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%  $\pm$  0.1% ESA were retransformed



**Figure 6.** Analysis of TAG compositions of mature seeds from the transgenic lines. A, TAG molecular species; 0-HFA to 3-HFA represent molecular species with zero to three HFAs, respectively. B, Percentage of HFAs at the *sn*-2 and *sn*-1/3 positions of TAG. The data represent the average of three replicates  $\pm$  SE. Two-tailed Student's *t* test. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ .

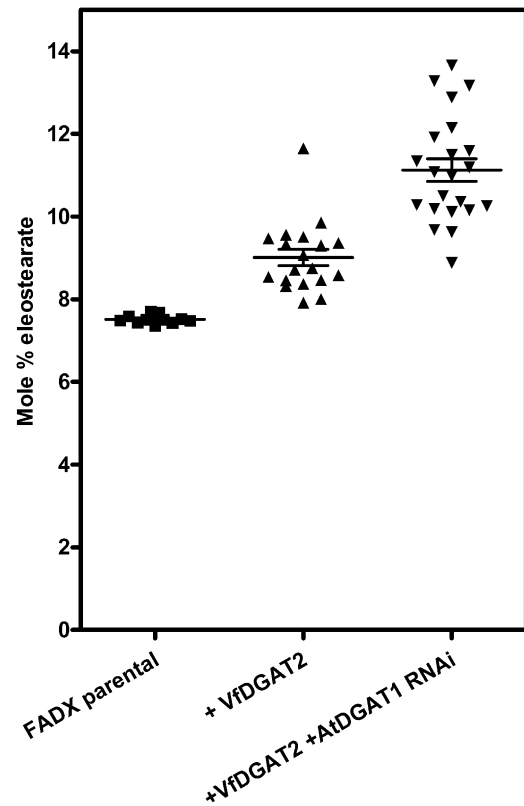
with *VfDGAT2* transgene under control of the Arabidopsis 2*S seed storage protein3* (*At2S-3*) promoter (Guerche et al., 1990) either alone or combined with a seed-specific *AtDGAT1* RNAi cassette driven by the soybean (*Glycine max*)  $\beta$ -conglycinin  $\alpha'$ -subunit promoter (Doyle et al., 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 were 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 coexpressing the combination of *VfDGAT2* and *AtDGAT1* RNAi averaged  $11.1\% \pm 1.29\%$  ESA (a 48% increase relative to the parental lines; Fig. 7).

To ascertain the full degree of change affected 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% and 8.85% ESA. Individual *FADX* plants homozygous for *VfDGAT2* averaged  $11.6\% \pm 0.71\%$  and  $11.7\% \pm 0.62\%$  ESA, respectively (Fig. 8), including individual plants with seeds that contained as much as 12.47% and 12.64% ESA, respectively. As in the T2 sample distributions, *FADX* plants coexpressing both *VfDGAT2* and *AtDGAT1* RNAi produced higher levels of the unique FA in terms of both plants with the highest seed ESA (14.9% and 16.1%, respectively) and average ESA across all homozygous plants ( $13.8\% \pm 0.69\%$  and  $14.7\% \pm 0.85\%$ , respectively; 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 three different transgene combinations, we analyzed additional seed batches of line #5 red (homozygous *VfFADX VfDGAT2*) and line #9 red (homozygous *VfFADX VfDGAT2 AtDGAT1* RNAi) along with brown segregants (homozygous *VfFADX*) as controls. The data obtained confirmed the increases in percentage of 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 milligram of seed weight (Supplemental Fig. S2B), whereas there was no significant change in the total FA per milligram of 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 *atdga1* background, most of the pollen was not viable, likely

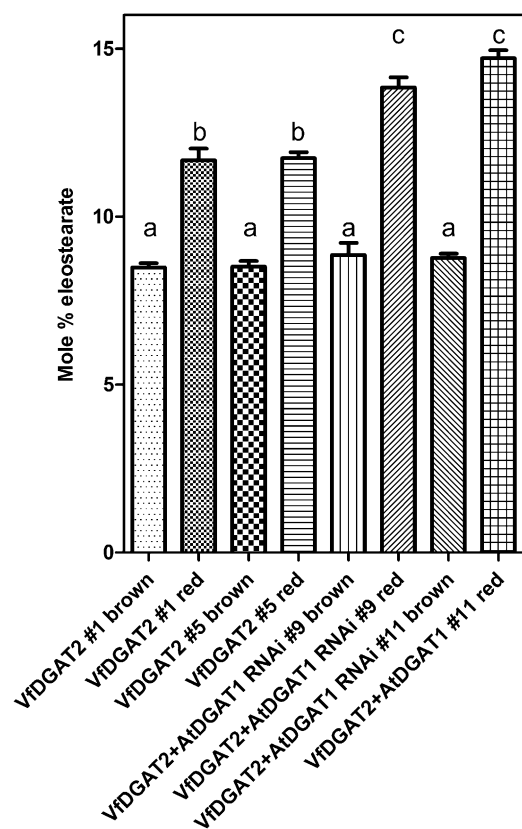


**Figure 7.** Distribution of ESA levels in transgenic plants expressing tung tree *FADX* alone or with tung tree *DGAT2* or tung tree *DGAT2 + AtDGAT1* RNAi. A parental *FADX* line was retransformed with *VfDGAT2* or *VfDGAT2 + AtDGAT1* RNAi. Seed pools from segregating T1 plants representing independent transgenic events were analyzed by gas chromatography; the corresponding ESA levels are shown. The data are plotted as a scatterplot, with the mean and SE indicated. Differences between the three data sets are statistically significant (two-tailed Student's *t* test;  $P < 0.001$ ).

because of a severe reduction in pollen storage lipid, and the reduced number of 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, which was indicated by the reduction in oil content in the *atdga1* background (Katavic et al., 1995; Routaboul et al., 1999; Zou et al., 1999). By contrast, characterization of an *atpd1* 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 tree 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, whereas the castor and tung tree enzymes have been shown to have





**Figure 8.** Determination of ESA levels in homozygous transgenic plants coexpressing *FADX* and *VfDGAT2* with or without *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 nontransgenic (brown) plants were collected separately and analyzed by gas chromatography. Mean ESA levels  $\pm$  SE are shown. Lowercase letters indicate different statistically significant groups (Student's *t* test;  $P < 0.01$ ).

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). To test if substrate competition between *AtDGAT1* and *RcDGAT2* is limiting for accumulation of HFA, the *atdgat1-2* mutation was crossed into the CL7 *RcDGAT2* line. Homozygous *dgat1* CL7 *RcDGAT2* plants had significantly higher HFA levels than the CL7 *RcDGAT2* segregants (Fig. 2). Subsequent analyses of the derived *dgat1* 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.

To determine if substrate competition is a more general problem for the engineering of unusual FAs, seed-specific

overexpression of tung tree *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 that *VfDGAT2* expression resulted in expected increases in ESA levels compared with the parental lines. However, an additional 22% increase in ESA was observed when *AtDGAT1* expression was reduced by RNAi. As with the results for castor *DGAT2*, the tung tree *DGAT2* data clearly indicate 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 through *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 1-HFA-TAG 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* expressions 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 additional increase in seed HFA content (Figs. 3B and 4). 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 the 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 on either soil or agar medium supplemented with 1% (w/v) Suc. 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 greater than 20% increases in HFA and ESA that we observed indicate that



reducing enzyme competition can provide unique 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.

Although 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 *Reduced Oleate Desaturation1 (ROD1)* gene encodes 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, because the proportion of HFA in seed oil is reduced by >50% in *rod1* mutants compared with *ROD1 RcfAH12* controls (Hu et al., 2012). In additional experiments, Hu et al. (2012) showed that *RcROD1* expression could compensate for the loss of the Arabidopsis enzyme when transformed into *rod1* mutants expressing *RcfAH12* or could lead to increased HFA when expressed in an *RcfAH12* transgenic line that is the wild type at the *ROD1* locus. Importantly, expression of *RcROD1* also provided an increase in HFA (from 24.7% to 28.5% of seed FA) when expressed in the CL7 *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.

## MATERIALS AND METHODS

### Plant Growth Conditions and Transformation

The Arabidopsis (*Arabidopsis thaliana*) lines and growth conditions were similar to those described in van Erp et al. (2011) unless otherwise mentioned. Plant transformation was achieved using the floral dip method (Clough and Bent, 1998). All lines are in the Columbia-0 wild-type background, except that *dgat1-2* is in the Wassilewskija background.

For experiments comparing genotypes, plants of the different lines were randomly distributed across the pots and trays that were used. Plants were grown in environmentally controlled chambers under continuous fluorescent illumination of 120 to 150  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$  with 70% relative humidity at 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 the wild type, heterozygous, or homozygous for the point mutation in *AtDGAT1*. To prepare RNA, young siliques were harvested approximately 8 to 12 d after flowering from plants, flash frozen in liquid nitrogen, and stored at -80°C. Developing seeds were scraped from each silique on a petri dish 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's protocol. cDNA was synthesized using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen/Life Technologies). 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 amiRNA against *AtPDAT1*

To suppress expression of *AtPDAT1* in our transgenic plant lines, a 21-mer sequence targeting *AtPDAT1* was designed (WMD3 Web MicroRNA Designer, Arabidopsis cDNA [The Arabidopsis Information Resource 9]; minimum no. of included targets, one; accepted off targets, zero; S. Ossowski, J. Fitz, R. Schwab, M. Riester, and D. Weigel, personal communication). To prevent suppression of expression of other Arabidopsis genes in our plant lines, a Target Search was performed against Arabidopsis cDNAs with the 21-mer sequences (The Arabidopsis Information Resource 9\_cdna\_20090619; no. of mismatches, five), and no targets were found. To prevent suppression of the castor (*Ricinus communis*) genes in our transgenic plant lines (*RcfAH12*, *RcDGAT2*, and *RcPDAT1A*), a Target Search was performed against castor mRNAs with the 21-mer sequences (castor PUT v163a [Plant Genome Database]; no. of mismatches, five), and no targets were found. The best 21 mer with no other targets in Arabidopsis and castor was selected for additional analysis (5'-TTGCGGGTATACGTAGTGTA-3'; hybridization energy = -40.49 kcal mol<sup>-1</sup>). 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 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* site of the calf intestinal phosphatase-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 to generate the *pdat1-amiRNA RcPDAT1A* vector.

### Construction of Tung Tree Gene Vectors and Expression in Plants

The open reading frames (ORFs) for tung tree (*Vernicia fordii*) FADX and tung tree DGAT2 as well as the *AtDGAT1* RNAi construct were assembled in various components of a flexible set of cloning vectors and plant binary plasmids before transformation into *Agrobacterium tumefaciens*. The cDNA for tung tree 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 are 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 VtFADX binary plasmid, designated pE181, was transformed into *Agrobacterium* spp. strain GV3101; kanamycin- and gentamycin-resistant colonies were cultured in liquid media and used to transform the *fad3 fae1* double-mutant line of Arabidopsis (Smith et al., 2003) by floral dip.

A seed-specific shuttle plasmid containing an N-terminal myelocytomatosis viral oncogene homolog (myc) epitope-tagged tung tree DGAT2 was generated by PCR amplification of the native DGAT2 ORF with a forward primer in which the initiator Met codon has been replaced by a *KasI* site and a reverse primer containing a *SacII* site adjacent to the stop codon. After *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 (*Glycine max*) 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 *AscI* 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 *AscI* fragment of pJ6, which contains the soybean  $\beta$ -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'-UTR of *AtFAD2* flanked by a 592-bp region of the *AtDGAT1* ORF (base pairs 303–894) cloned in inverted orientations. The binary plasmid carrying both the tung tree *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.

ESA production stabilized at approximately 8% in the T4 generation of E181 plants. T5 plants from this line were retransformed with *Agrobacterium* spp. 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 ESA content. Two lines, one representing the highest T2 18:3 $\Delta$ 9cis, 11trans, and 13trans 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.

## FA 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 of seeds and three to four 2.3-mm chrome steel beads (BioSpec Products, Inc.) were added into a 2-mL Eppendorf tube with 500  $\mu$ L of hexane followed by 5 min of agitation on a Bead Beater. The extract was centrifuged (13,000g for 2 min) to remove debris, and a portion (300  $\mu$ L) of the extract was transferred to a 13-  $\times$  100-mm glass Corning culture tube. Hexane (700  $\mu$ L) and sodium methoxide in methanol (400  $\mu$ L) were added followed by 10 min of incubation at room temperature with intermittent shaking. The reactions were quenched by the addition of 2 mL of hexane and 2 mL of saturated NaCl solution, and the phases were separated by centrifugation in a tabletop swinging bucket centrifuge. Two milliliters of upper layer was transferred to vials, capped, and analyzed immediately or stored at  $-20^{\circ}$  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 in Li et al. (2006), except that 200  $\mu$ L of toluene was added to each sample, butylated hydroxytoluene was omitted, and 20 seeds were used for each measurement. Seed weights were determined by counting seed numbers in samples of 1 to 2 mg of seeds.

## Lipid Extraction and Characterization of TAG Species

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

## Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Expression of *RcDGAT2* transcript in the parental CL7 *RcDGAT2* line and the *dgat1* CL7 *RcDGAT2* line obtained by crossing.

**Supplemental Figure S2.** Extended analysis of lines accumulating ESA.

**Supplemental Table S1.** FA compositions of samples of T3 seeds of transgenic lines accumulating HFAs characterized in this study.

**Supplemental Table S2.** FA compositions of samples of T3 seeds of transgenic lines accumulating ESA characterized in this study.

**Supplemental Table S3.** Primers used.

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