Two Acyltransferases Contribute Differently to Linolenic Acid Levels in Seed Oil

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Acyltransferases are key contributors to triacylglycerol (TAG) synthesis and, thus, are of great importance for seed oil quality. The effects of increased or decreased expression of ACYL-COENZYME A:DIACYLGLYCEROL ACYLTRANSFERASE1 (DGAT1) or PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE (PDAT) on seed lipid composition were assessed in several Camelina sativa lines. Furthermore, in vitro assays of acyltransferases in microsomal fractions prepared from developing seeds of some of these lines were performed. Decreased expression of DGAT1 led to an increased percentage of 18:3n-3 without any change in total lipid content of the seed. The tri-18:3 TAG increase occurred predominantly in the cotyledon, as determined with matrix-assisted laser desorption/ionization-mass spectrometry, whereas species with two 18:3n-3 acyl groups were elevated in both cotyledon and embryonal axis. PDAT overexpression led to a relative increase of 18:2n-6 at the expense of 18:3n-3, also without affecting the total lipid content. Differential distributions of TAG species also were observed in different parts of the seed. The microsomal assays revealed that C. sativa seeds have very high activity of diacylglycerol-phosphatidylcholine interconversion. The combination of analytical and biochemical data suggests that the higher 18:2n-6 content in the seed oil of the PDAT overexpressors is due to the channeling of fatty acids from phosphatidylcholine into TAG before being desaturated to 18:3n-3, caused by the high activity of PDAT in general and by PDAT specificity for 18:2n-6. The higher levels of 18:3n-3 in DGAT1-silencing lines are likely due to the compensatory activity of a TAG-synthesizing enzyme with specificity for this acyl group and more desaturation of acyl groups occurring on phosphatidylcholine.

Camelina sativa (false flax or gold-of-pleasure) is an ancient oil crop grown in Europe since the Bronze Age (Zubr, 1997). C. sativa production declined after the Second World War, and it is not currently grown as a large-scale crop for oil production (Zubr, 1997). However, the interest in this plant is now reinvigorated due to its high potential for biotechnological applications. In recent years, C. sativa has emerged as a model crop plant for oilseed research and field-scale production (Feussner, 2015; Vollmann and Eynck, 2015; Bansal and Durrett, 2016; Berti et al., 2016). In addition to its history as an oilseed crop, the genetic similarity to the well-studied model species Arabidopsis (Arabidopsis thaliana; Kagale et al., 2014) and the ease of genetic transformation (Lu and Kang, 2008) have pushed C. sativa into the forefront of oilseed research and trait development. The C. sativa genome was sequenced recently (Kagale et al., 2014) and is openly available (http://www.camelinadb.ca/). Transcriptome data sets have been mapped (Liang et al., 2013; Nguyen et al., 2013; Mudalkar et al., 2014; Wang et al., 2015; Abdulllah et al., 2016), and it is possible to search the seed transcriptome (http://www.camelinagenome.org/), providing an excellent starting point for crop research. Moreover, the seed yield is adequate to facilitate the rapid production of oil in quantities large enough for small-scale testing and product development. In recent years, several different oil traits have been developed in C. sativa seeds, such as high 18:1n-9 (Kang et al., 2011; Horn et al., 2013), high 16:0 (Horn et al., 2013), and high ω-7 fatty acids (Nguyen et al., 2015), as well as the production of diverse extrinsic lipid compounds, such as...
wax esters (Iven et al., 2016), fish oils (Petrie et al., 2014; Ruiz-Lopez et al., 2014), nervonic acid (Huai et al., 2015), and acetyl glyceride oils (Liu et al., 2015).

*C. sativa* seeds are about 10 times the size of Arabidopsis seeds, enabling biochemical studies of the seed, which are not as easily performed on Arabidopsis. Wild-type *C. sativa* has a high content of α-linolenic acid (18:3n-3), 25% to 41% depending on the variety and growth conditions (Zubr, 1997; Rodriguez-Rodriguez et al., 2013), and also a high content of gondoic acid (20:1n-9) compared with other oilseed crops. The reasons for this high 18:3n-3 content is not clear.

With strategies to generate new fatty acid compositions in *C. sativa* seeds, the production will be highly dependent on intrinsic lipid biosynthesis pathways and the enzymes involved therein. The intrinsic enzymes may cause bottlenecks in developing novel oils with optimal quality for certain industrial applications (Bates and Browse, 2011; Guan et al., 2014). Thus, an increased understanding of the *C. sativa* lipid biosynthesis machinery will provide a fundamental knowledge base for future tailor-made seed oil products as well as provide important information about the metabolic regulation of fatty acid composition in *C. sativa* seeds.

In most seeds, the major lipid storage form is triacylglycerol (TAG), known to be produced by acyl-CoA: diacylglycerol acyltransferase (DGAT) and phospholipid:diacylglycerol acyltransferase (PDAT; Zhang et al., 2009). DGAT1 has been shown in Arabidopsis to be the major, but not the sole, enzyme to contribute to the accumulation of seed TAGs, working together mainly with PDAT (Zhang et al., 2009). While overexpression of intrinsic Arabidopsis *PDAT* influenced neither total lipid content nor the fatty acid profile (Ståhl et al., 2004), *PDAT* from flax (*Linum usitatissimum*), another species with even higher 18:3n-3 content than *C. sativa*, was shown to increase the 18:3n-3 content when overexpressed in Arabidopsis (Pan et al., 2013). Therefore, it has become apparent that the same enzyme homolog from different species can have quite different substrate specificities, which may affect the fatty acid profile of the seed oil of specific species. Thus, in this study, we examined the role of *C. sativa* PDAT and DGAT1 in fatty acid accumulation in developing seeds and in different parts of the embryo.

PDAT and DGAT1 were overexpressed or downregulated using the expression of artificial microRNA (amiRNA), and combinations of both down-regulation and overexpression were studied. As a complement to these studies, microsomal fractions from developing seeds of selected lines were prepared, and the effect on lipid classes formed was measured in vitro with different acyl-CoA substrates. Finally, the spatial distribution of TAG and phosphatidylcholine (PC) species of homozygote transgenic seeds was investigated using matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS), as it was shown previously that molecular species of PC and TAG are distributed unequally throughout the embryo (Horn et al., 2013). Changing the expression levels of DGAT1 and PDAT resulted in large changes in both fatty acid lipid composition and the spatial distribution of TAG and PC species within the *C. sativa* seed.

## RESULTS

### C. sativa Seeds Incorporate Preferentially 18:3n-3 and 20:1n-9 into TAG during Development

In order to understand lipid biosynthesis in wild-type *C. sativa* seeds in more detail, we first characterized the temporal changes in fatty acid composition during seed development. Seed development occurred over about 31 d under our conditions. While the formation of the seed took about 10 d, seed filling lasted for about 14 d, and seed desiccation was completed after a further

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**Figure 1.** Development of *C. sativa* seeds. A, Weight of the seeds in milligrams. B, Number of lipids per seed. C, Relative amount of major fatty acids at different days after flowering (DAF).
7 d (Fig. 1). The fatty acid profile started to change in parallel with the onset of seed filling, where 18:2n-6 decreased from 50 to 20 mol % and 18:3n-3 increased from 16 to 38 mol % from 7 to 24 DAF. Fatty acid 20:1n-9 accumulation followed the trend of a seed storage product, accumulating with the onset of lipid deposition at 7 DAF until it reached its maximum at 18 DAF. Levels of 18:1n-9 stood out in comparison with the other fatty acids, as it peaked 11 DAF at 20 mol %. The relative amounts of 18:0 and 16:0 remained constant throughout seed development.

To get a deeper understanding of the fatty acid composition in different lipid classes, a more comprehensive lipidomics approach was used to quantify the lipid molecular species present at different developmental time points. These lipidomics data are summarized in Figure 2, together with an overview of the TAG synthesis pathways, and show changes of the relative amounts of major molecular species present in TAG, PA, DAG, and PC lipid classes during seed development. Furthermore, a schematic relationship between the different molecular species is shown, where it is indicated if these lipid species had an increasing or decreasing (or combination thereof) trend during seed development. PA, DAG, and PC were chosen because they are the intermediate lipids most directly involved in TAG biosynthesis. The acyl composition of TAG molecular species showed a relative increase in TAG species containing 18:3n-3 in two or in all three positions. Furthermore, the relative amount of 18:3/18:3 initially increases in PC and DAG, but in contrast to TAG, they reached their relative maximum at 18 DAF.

Figure 2. Overview of the TAG synthesis pathway and the change of relative major lipid species during seed development. A, Overview of the main steps of the TAG biosynthesis pathway of oilseeds. Enzymes are shown in boldface, and cellular compartments are underlined. Lipid species abbreviations are as follows: DAG, diacylglycerol; FA, fatty acid; G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; LPC, lysosphosphatidylcholine; PA, phosphatidic acid. Enzyme abbreviations are as follows: LPCAT, acyl-CoA:lysophosphatidylcholine acyltransferase; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase. B, Relative amounts of specific lipid species at four time points. Relative amount is determined within each lipid class. Molecular species were determined by ultra-performance liquid chromatography-nano-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS), except PA which was measured by direct-infusion nano-ESI-MS/MS. C, Trends of the relative amounts of specific lipid species during seed development. The symbol > corresponds to lipid species with a declining trend over development, whereas < indicates lipid species with an increasing trend over development; >> denotes decrease followed by increase, and < > indicates increase followed by decrease. Note that the sn position is not determined.

[Diagram of TAG synthesis pathway with molecular species trends]
and decreased somewhat thereafter. Interestingly, 20:1n-9 accumulated in TAG but not in DAG during seed development. However, in contrast to 18:3n-3, 20:1n-9 was found mostly in a single sn position of the TAG molecules. The complete data set, including galactolipids, other phospholipids, and lysophospholipids, is shown in Supplemental Table S1.

As acyl-CoAs are central substrates in most steps of TAG biosynthesis, the acyl-CoA pool was determined next (Supplemental Fig. S1). The acyl-CoA pool was dominated by very long-chain saturated species, and these levels remained relatively constant during development (Supplemental Table S2). Only a moderate and transient increase in monounsaturated acyl-CoAs of 20 carbons and longer at 18 DAF was observed, which was at the expense of long-chain saturated acyl-CoAs.

Overexpression of PDAT Increases 18:2n-6 and Silencing of DGAT1 Increases 18:3n-3 in C. sativa Seeds

Next, we analyzed how the two enzymes, DGAT1 and PDAT, contribute to TAG accumulation in C. sativa seeds. Using amiRNA or gene overexpression constructs, DGAT1 and PDAT were seed specifically silenced and overexpressed, respectively. In addition to single constructs, combinations of overexpressor and silencing constructs also were made (Supplemental Fig. S2). To investigate the effectiveness of the overexpression and silencing approaches, the transcript levels were examined using qRT-PCR on selected homozygote, single-insertion T3, developing seeds. qRT-PCR data are reported as normalized to the housekeeping gene actin (set to 1; Supplemental Fig. S3). Since C. sativa has a hexaploid genome (Hutcheon et al., 2010; Kagale et al., 2014), all genes may occur in up to three copies. It should be noted that, due to the high homology between genes in the subgenomes, neither the amiRNAs nor the primers used for this qRT-PCR experiment could distinguish between these different copies. In the studied samples, notable but incomplete silencing of DGAT1 was observed. The overexpression of PDAT, however, was highly significant, with up to 200-fold expression compared with expression in the control. In addition to DGAT1 and PDAT, the expression was monitored of other possible terminal acyltransferases, DGAT2, DGAT3, and genes here called PDAT2 and PDAT-like (homologs of AT3G44830 and AT3G05510, respectively). For these additional enzymes, no significant changes were observed (Supplemental Fig. S3).

The fatty acid profiles of pooled T2 seeds, representing a mixture of single and multiple insertion lines, were examined. Single insertion lines with constructs that showed significant differences in fatty acid profiles were selected for propagation for T3 seeds. In amiDGAT constructs, the amount of 18:3n-3, and on a smaller scale 20:3 and 22:3, was increased significantly (P < 0.05) at the expense of 18:1n-9, 18:2n-6, and 20:1n-9. Levels of 18:3n-3 up to 56 mol % were observed in one individual line with the amiDGAT1.3 construct (Fig. 3), with less effect in the amiDGAT1.2 construct lines. Interestingly, overexpression of DGAT1 had no major effect on the overall fatty acid profile (Supplemental Fig. S4). The opposite relationship was observed with PDAT. Small differences in fatty acid composition were
observed when PDAT was silenced, while larger differences in the fatty acid profile resulted from PDAT overexpression (Fig. 3). PDAT overexpression significantly increased the amount of 18:2n-6. In homozygous lines, the increase in 18:2n-6 also was accompanied by a significant decrease of 18:3n-3. Additionally, PDAT down-regulation in homozygous lines (amiPDAT.3) resulted in an increase in the relative amounts of 18:1n-9 and 20:1n-9 and a decrease in 18:2n-6 ($P < 0.05$) without affecting 18:3n-3 (Fig. 3). The total fatty acid profiles of all T2 lines, including minor fatty acids, are shown in Supplemental Figure S4.

Here, in contrast to Arabidopsis (Jako et al., 2001), the changes in the fatty acid profiles were not accompanied by any significant changes in lipid yield per seed in the amiDGAT1 lines. The silencing of DGAT1 resulted in a lower lipid content per gram of seed and in a higher seed weight, which balanced each other, thus giving no significant change in amount of lipid per seed (Supplemental Fig. S5).

**PDAT Overexpression Increases Microsomal TAG Production**

To investigate the effects of the enzymes more thoroughly, selected lines that showed differences in their fatty acid profiles were used for microsomal preparations. These were a DGAT1 silencing line, amiDGAT1.2, a PDAT overexpression in combination with DGAT1 silencing line, PDAT+amiDGAT1.2, and a control line. Lines chosen were single insertion homozygous lines and had fatty acid profiles that were stable in both T3 (Fig. 3) and T4 generations (data not shown).

Microsomal preparations were fed with [14C]G3P and nonradioactive acyl-CoA (18:1n-9, 18:2n-6, or 18:3n-3). The relative incorporation of radiolabeled glycerol into DAG, TAG, PC, and LPC is shown in Figure 4. Several general trends were observed in all lines. First, there was a high incorporation of radiolabeled substrate into PC, suggesting high PDCT activity (Lu et al., 2009). Second, TAG increased over the time

**Figure 4.** Time course of relative amounts of acylated compounds in C. sativa developing seed microsomal preparations after feeding with [14C]G3P and the corresponding acyl-CoA. Different microsomal preparations are shown in different rows: control (A), amiDGAT1.2 (B), and PDAT+amiDGAT1.2 (C). Each column corresponds to feeding with the indicated acyl-CoA. Shown are mean values and SD; $n = 3$. Assay conditions are described in “Materials and Methods.”
course at the expense of DAG levels. In the microsomal membranes where PDAT was overexpressed, the proportion of TAG was significantly higher, with less DAG as well as more LPC compared with both control and amiDGAT1.2 microsomal preparations. The highest proportion of TAG incorporation into these microsomal membranes was seen when 18:2n-6 acyl-CoA was used.

In another experiment, a mixture of equal amounts of $^{14}$C-labeled 18:1n-9, 18:2n-6, and 18:3n-3-CoA together with unlabeled G3P was fed to the microsomal preparation. Again, the microsomal membranes from PDAT+amiDGAT1.2 had the highest proportion of $^{14}$C incorporation into TAG (Fig. 5). Looking at the fatty acid profiles of the respective lipid classes (Table I), the amiDGAT1.2 microsomal preparations incorporated significantly more 18:3n-3 into TAG, whereas the PDAT+amiDGAT1.2 microsomal preparations had a higher amount of 18:3n-3 remaining in DAG but the same amount in TAG compared with the control.

The Spatial Distribution of TAG and PC in the Seeds Differs with Different Levels of DGAT1 and PDAT Expression

As recent studies suggest, the lipid molecular species within a C. sativa seed are not distributed evenly (Horn et al., 2013). Here, we utilized MALDI-MS imaging to investigate the spatial distributions of PC and TAG molecular species in our lines with altered DGAT1 and PDAT levels. Seeds from four different lines were investigated: control, PDAT+amiDGAT1.2, amiPDAT.2, and a later-propagated DGAT1-silencing line, amiDGAT1.3, which demonstrated drastically increased levels of 18:3n-3 compared with amiDGAT1.2. Parts of the C. sativa seed are labeled in Figure 6 for convenient reference to MALDI-MS images.

First, we compared the molecular distribution of individual TAG species confirmed in our amiDGAT1.3 line. Silencing of DGAT1 led to markedly increased levels of TAG-52:6 (16:0/18:3/18:3), which was distributed homogeneously in the cotyledons and embryonic axis tissues, and TAG-54:9 (tri-18:3), which was localized almost exclusively to the cotyledons (Fig. 7). Furthermore, the distributions of the likely precursor PC molecular species to these TAGs were distributed similarly in the embryo: PC-34:3(18:3/18:6) was distributed homogeneously, whereas PC-36:6(18:3/18:3) was localized mostly in the cotyledons (Fig. 8). Consistent with the results from the lipid extracts presented above, the mass spectrometry (MS) images of DGAT1-silenced lines showed increased relative levels of TAG molecular species containing 18:3, which appeared to be at the expense of 18:2-, 18:1-, and 20:1-containing TAG molecular species (Fig. 7).

Notable differences regarding where TAG molecular species were compartmentalized also were observed in the PDAT+amiDGAT1.2 line, which, as noted previously, had increased levels of 18:2n-6 (Fig. 3). Principal TAG molecular species containing 18:2n-6, TAG-54:6(18:2/18:2/18:2), TAG-54:7(18:3/18:2/18:2), and TAG-54:8(18:3/18:3/18:2), were enriched in the upper cotyledons and embryonic axis, distributed homogeneously, and in the cotyledons, respectively. However, for the longer chain molecular species of PC, PC-38:4(20:1/18:3) and PC-38:3(20:1/18:2), the levels were increased in the cotyledons compared with the control (Figs. 7 and 8). In general, overexpression of PDAT in the DGAT1-silenced background restored the spatial distribution of the 18:3-containing PC and TAG to that of the control seeds, whereas 18:2-containing and 20:1-containing PCs and TAGs were mislocalized relative to the controls, or at least their heterogenous distributions were accentuated over the controls.

When PDAT was silenced (amiPDAT.3 line), there were no appreciable differences in the distributions of 52C and 54C TAG molecular species relative to the controls, whereas the 56C TAG molecular species were enriched to a greater extent in the cotyledons in comparison with the control line (Fig. 7). For the PC pool in this line, PC-36:6(18:3/18:3), PC-36:5(18:3/18:2), PC-36:4(18:2/18:2), and PC-38:3(20:1/18:3) had elevated levels in the cotyledons relative to the control. Interestingly, PC-36:6, PC-36:5, and PC-38:4 were localized in the outer cotyledon relative to the inner cotyledon (Fig. 8). Complete analyses of all TAG and PC species investigated with MALDI-MS are provided in Supplemental Figures S6 to S13.

The relative mol % lipid profiles of PC and TAG calculated from the MALDI-MS images were in good agreement with the mol % values obtained from total lipid extracts quantified by triple quadrupole electrospray ionization-mass spectrometry (ESI-MS; Fig. 9; Supplemental Fig. S14).
DISCUSSION

Lipid Accumulation during Seed Development

For an oilseed crop, the oil content and the fatty acid profile of the oil are the two most important characteristics of its seeds. Both are established during seed filling, which occurs during the second half of seed development (Hills, 2004). The lipid accumulation in *C. sativa* seeds presented in this study is similar, overall, to what has been shown previously (Rodriguez-Rodriguez et al., 2013; Pollard et al., 2015; Quéro et al., 2016). Minor differences between these studies are likely attributed to different growth chamber conditions and differences between *C. sativa* varieties. In our conditions, lipid accumulation appeared to be completed at an earlier time point (Fig. 1), which was expected as the *C. sativa* variety (CAM139) was chosen for, among other traits, its short life cycle and, thus, suitability for laboratory work. The lipid content of the mature seeds was still similar to what was shown by Rodriguez-Rodriguez et al. (2013). Accumulation of 20:1n-9, due to the activity of the enzyme responsible for the elongation, FAE1, has been suggested as a marker of oil accumulation (Girke et al., 2000), which our study supports. Also in accordance with previous studies on *C. sativa* (Rodriguez-Rodriguez et al., 2013; Pollard et al., 2015), 18:3n-3 accumulated at the highest rate and was the major fatty acid at the end of development. The general increase in 18:3n-3 and 20:1n-9 during development was seen in both total lipids and major TAG molecular species. Interestingly, 18:3/18:3-containing PC and DAG molecular species were, in contrast to TAG, at their highest levels at an earlier time point (18 DAF; Fig. 2), indicating that, in the later stages of lipid accumulation, the capacity to transfer 18:3n-3 from PC to TAG is higher than its production rate.

The increase in 18:3n-3 in total lipids and TAG during development is not matched with an increase of the corresponding acyl-CoA, 18:3n-3-CoA remaining low throughout development (Supplemental Table S2). Additionally, 18:2n-6 and 18:1n-9-CoA contributed to minor amounts of the acyl-CoA pool throughout seed development. Since the acyl-CoA pool is totally dominated by saturated and mostly very-long-chain saturated species that do not participate in the G3P pathway to TAG, it is clear that the main species in the acyl-CoA pool is not connected to the TAG biosynthetic pathway. This cast some doubts on the relevance of these analyses regarding seed oil biosynthesis.

**DGAT1 Down-Regulation**

The last steps of TAG synthesis as they are known today are catalyzed by either a DGAT or a PDAT enzyme (Fig. 2). DGAT uses DAG and acyl-CoA as substrates to form TAG, whereas PDAT uses DAG and an acyl group from PC to form TAG and LPC. Currently, there are three different known types of DGATs: DGAT1, DGAT2, and DGAT3. DGAT1 is localized in the endoplasmic reticulum (ER), and the enzyme contributes significantly to oil accumulation in Arabidopsis seeds (Zhang et al., 2009). DGAT2 has been shown to be important in plants accumulating unusual fatty acids in their seeds, such as castor bean (*Ricinus communis*; Kroon et al., 2006) and tung tree (*Vernicia fordii*; Shockey et al., 2006). However, when DGAT2 is knocked out in Arabidopsis, neither the oil content nor the fatty acid profile is affected, and they show no difference compared with the mutant of DGAT1 when both genes are knocked out (Zhang et al., 2009). DGAT3 is cysteolic and, to date, has not been shown to contribute to TAG accumulation of seeds (Hernández et al., 2012).

In Arabidopsis, the knockout mutation *dgat1* leads to decreased seed lipid content of about 30% and a changed fatty acid composition, with increased 18:3n-3 content and decreased 18:1n-9 and 20:1n-9 (Katavic et al., 1995; Zou et al., 1999). In this study, we studied...
the down-regulation of DGAT1. We engineered three different amiRNA constructs to down-regulate DGAT1, as the success rate of this type of construct has been reported to vary depending on the target gene (Schwab et al., 2006). DGAT1 silencing in C. sativa, as for the Arabidopsis dga1 mutant, gave a substantial increase in 18:3n-3 (Fig. 3). However, no change in seed lipid content was seen in the DGAT1 down-regulation lines. This shows that, contrary to the situation in Arabidopsis, another enzyme is capable of fully compensating for the reduced DGAT1 activity. The reason for this could be that some DGAT1 activity remains in the C. sativa DGAT1-silencing lines. No transcriptional up-regulation of other (possible) terminal acyltransferases (DGAT2, DGAT3, PDAT, PDAT2, or PDAT-like) was seen in the DGAT1-silencing lines (Supplemental Fig. S3). This could be due to the incomplete silencing but may suggest that the innate enzymes are sufficiently expressed and also play an important role in wild-type C. sativa. Moreover, their regulation could be on the posttranslational level: C. sativa PDAT, like all MBOAT proteins, contains putative phosphorylation sites, and putative phosphorylation sites also are found for the other enzymes (http://www.cbs.dtu.dk/services/NetPhos/).

In the DGAT1-silencing line, 16:0/18:3 and 18:3/18:3 PC species are increased significantly (Figs. 8 and 9), implying that FAD3 desaturation is increased. Desaturation by FAD2/FAD3, adding a second and third double bond to the acyl group, is known to occur on PC species within the ER (Li-Beisson et al., 2013). PC is not a direct precursor for DGAT1. However, the microsomal assays (Fig. 4) showed a very high activity for PDCT, since feeding radiolabeled G3P resulted in higher initial amounts of radiolabeled PC compared with DAG. Thus, it is very likely that a higher amount of 18:3/18:3 is present also in the DAG by the reverse reaction of PDCT. This is supported by our data showing similarities of DAG and PC profiles over time in developing wild-type C. sativa seeds (Fig. 2). This greater desaturation occurring on PC is likely an important reason for the increased 18:3n-3 content, suggesting that the enzymes taking over the TAG production from DGAT1 are allowed to access acyl groups at a later stage of desaturation, due to a relatively higher activity of FAD2/FAD3 or localization to

Figure 7. Spatial distribution of TAG species as determined by MALDI-MS. Images are representative of seed sections that were analyzed in triplicate (three different seeds). A, Control. B, amiDGAT1.3. C, PDAT+amiDGAT1.2. D, amiPDAT.3. Scale bars represent mol %; bars in bright-field images = 200 μm.
different subdomains within the ER. A previous study has shown that DGAT1 and DGAT2 of tung tree localizes to different subdomains of the ER (Shockey et al., 2006), whereas it is currently not known if this is true also for PDAT. Our microsomal data, however, suggest that there is more to it than increased desaturation in the DGAT1-silencing lines. Feeding the amiDGAT1.2 microsomal preparations with a mixture of acyl-CoAs still shows significantly more 18:3 \( n-3 \) in the TAG and less in DAG compared with the control (Table I). Thus, it is likely that the enzyme that is taking over for TAG assembly when DGAT1 is down-regulated also has a preference for 18:3\( n-3 \).

DGAT1 Overexpression

The seed-specific overexpression of DGAT1 in Arabidopsis in some studies has led to increases in lipid content of about 10% (Jako et al., 2001). In our study, no increase in seed oil content could be seen when DGAT1 was overexpressed. Kim et al. (2016) overexpressed all three C. sativa DGAT1 homologs in C. sativa with varying results, despite the homologs differing in only a few amino acids. Some of these lines showed significant increases in seed oil content. In our study, the DGAT1 homolog used is almost the same as the one showing the strongest effect in increasing oil content in that study (only one amino acid substitution, S→T32) and they were also expressed under the control of the same promoter (napin). Also in the previous study, there are lines without significant effect on the oil content. The effects of the overexpression of genes depend on where in the genome the gene may be inserted, as chromatin structure affects the expression level (Jenuwein and Allis, 2001). We screened 10 independent transgenic lines, and none of them showed the previously reported effect (Kim et al., 2016). Perhaps we would have found overexpressor lines with effects on fatty acid profile and oil content also with this construct if we investigated more lines. Another explanation could be some differences in expression in different C. sativa varieties.

**Figure 8.** Spatial distribution of PC species as determined by MALDI-MS. Images are representative of seed sections that were analyzed in triplicate (three different seeds). A, Control. B, amiDGAT1.3. C, PDAT+amiDGAT1.2. D, amiPDAT.3. Scale bars represent mol %; bars in bright-field images = 200 μm.
PDAT Overexpression

PDAT was discovered much later than DGAT1 (Dahlqvist et al., 2000). For Arabidopsis, neither PDAT overexpression nor knockout leads to any changes in total lipid content or fatty acid composition of the seed oil (Ståhl et al., 2004; Mhaske et al., 2005; Banas et al., 2014). Overexpression of flax PDAT in Arabidopsis, on one hand, gives an increase in 18:3 \( \text{n-3} \) as well as, to a lesser degree, 18:2 \( \text{n-6} \) (Pan et al., 2013). PDAT overexpression in \textit{C. sativa}, on the other hand, leads to a large increase in 18:2 \( \text{n-6} \) both alone and in combination with amiDGAT1 constructs (Fig. 3). In homozygous PDAT+amiDGAT1.2 lines, this increase is seen at the expense of 18:3 \( \text{n-3} \). As there are no differences in fatty acid changes between PDAT when overexpressed alone and in combination with an amiDGAT1 construct, the changes discussed for the homozygote PDAT+amiDGAT1.2 are thought to be mainly the result of PDAT overexpression. The transcription of PDAT was up to 200-fold compared with the control (Supplemental Fig. S3) but the effect on DGAT1 transcription levels was low, supporting the idea that the phenotype in the PDAT+amiDGAT1.2 lines may be considered the sole effect of the PDAT overexpressor and will be discussed here as such.

\textit{C. sativa} PDAT overexpression affects the fatty acid profile differently than the overexpression of Arabidopsis and flax PDAT in Arabidopsis (Ståhl et al., 2004; Pan et al., 2013), suggesting different specificities of the enzymes in different species. The increase in 18:2 \( \text{n-6} \) at the expense of 18:3 \( \text{n-3} \) suggests that the usage of 18:2 \( \text{n-6} \) by PDAT supersedes that of FAD3, and 18:2 \( \text{n-6} \) is to a high degree removed from PC by PDAT before it is desaturated to 18:3 \( \text{n-3} \). Thus, in \textit{C. sativa}, FAD2/FAD3 heterodimer formation to form 18:3 \( \text{n-3} \) directly from 18:1 \( \text{n-9} \) without the intermediate release of 18:2 \( \text{n-6} \) (Lou et al., 2014) does not seem to be a major pathway. The reason for 18:2 \( \text{n-6} \) being increased dramatically in TAG could be that the enzyme has a specificity for either or both 18:2 \( \text{n-6} \) and 18:3 \( \text{n-3} \) will have a similar effect on the fatty acid profile. High activity of PDAT will generate high rates of \textit{sn-1}-LPC formation, which, in turn, will be acylated at a high rate with 18:1-CoA derived from the plastid. The newly acylated acyl group on PC also will be removed rapidly by PDAT by acylation to DAG, which might limit the time for the desaturation of acylated 18:1 \( \text{n-9} \) to 18:3 \( \text{n-3} \) at PC. Here, we argue that the high 18:2 \( \text{n-6} \) content is due to a combination of these two properties.
In favor of the acyl specificity of *C. sativa* PDAT for 18:2n-6 are the microsomal assay results. The microsomal assays of developing seeds where PDAT is strongly overexpressed show that feeding with 18:2-CoA gives a higher proportion of TAG formed than feeding 18:3n-3-CoA (Fig. 4), supporting the idea that PDAT indeed prefers 18:2n-6 at least as one of its two substrates. However, whether the specificity is for 18:2n-6 in DAG or PC cannot be determined with clarity here. Looking at the molecular species from the MALDI-MS images and the ESI-MS data, 20:1n-9-containing PC is increased in the PDAT overexpressor and PC 36:4 is decreased compared with the control. The TAG species, on the other hand, show that PDAT overexpression results in more 18:2 containing PC is increased in the MALDI-MS images and the ESI-MS data, 20:1n-9-containing TAG species. This strongly suggests that PDAT excludes 20:1n-9-containing PC species as a substrate. As PC 36:4 was found mostly in the form of 18:2/18:2 during seed development (Supplemental Table S1), the decrease is most likely due to the increased usage of 18:2n-6-containing PC by PDAT. There is no change in PC containing 18:3/18:3 compared with the control; thus, sufficient desaturation occurs to keep normal levels of 18:3n-3 in PC, also supporting the idea that PDAT preferentially uses 18:2n-6 as a substrate.

**PDAT Down-Regulation**

The plants with the amiPDAT construct show some changes in total fatty acid profile of the seed compared with the control (Fig. 3). In homozygote T3 seeds, a small significant (*P < 0.05*) increase in 20:1n-9 and 18:1n-9 is seen, whereas 18:2n-6 is decreased slightly. Furthermore, changes are seen in molecular TAG species but not in any molecular PC species (Fig. 9). This indicates that PDAT, despite its low expression, plays a role in wild-type *C. sativa* TAG production. The decrease in 18:2n-6 with no effect on 18:3n-3 supports the idea that PDAT prefers 18:2 substrates over 18:3. That 20:1n-9 is increased in the seed oil of this line but decreased in molecular TAG of the PDAT overexpressor clearly shows that PDAT excludes 20:1n-9 as a substrate. When PDAT is silenced, presumably DGAT1, the major TAG-forming enzyme in Arabidopsis (Zhang et al., 2009), compensates for the decreased PDAT activity.

**Spatial Distribution of Lipids**

Conventionally, lipid profiles of seed oils are analyzed by MS approaches from solvent-based extractions; however, during the process of extraction, the localization of where the lipids originated is lost. Recently, MALDI-MS imaging made it possible to study lipid spatial distributions in situ directly from tissue sections (Sturtevant et al., 2016). Previous studies utilizing MALDI-MS imaging in oilseeds of cotton, *C. sativa*, and Arabidopsis demonstrated that lipid molecular species are not distributed equally between different seed tissues (Horn et al., 2012, 2013; Sturtevant et al., 2017). The previous study on *C. sativa* seeds, as well as this one, shows that there are large differences between the embryonic axis, the cotyledon, and the peripheral endosperm of the seed. In some cases, differences between the inner and outer cotyledons also can be seen (Figs. 7 and 8). More specifically, in several of the seeds, higher mol % of PC-36:6, PC-36:5, and PC-38:4, all containing at least one 18:3 acyl chain, were seen in the outer cotyledon compared with the inner cotyledon (Fig. 8). This suggests that perhaps FAD3 could be expressed differentially in the inner and outer cotyledons. However, these differences between the cotyledons are not mirrored in the TAG species.

The localization of the lipid species in both the control seeds and the seeds with altered enzyme levels gives clues to the localization of the enzymes involved. Looking at the control, monounsaturated acyl chain (18:1n-9 or 20:1n-9)-containing TAG and PC species were localized primarily in the cotyledon. Therefore, we suggest that DGAT1 predominates in cotyledon tissues, resulting in the accumulation of longer chain species with less desaturated TAG. Looking at the silencing or overexpression lines, the changes seen were significant, but in general, the distribution of the particular TAG species between cotyledons and the embryonic axis did not change in the transgenic lines compared with the control, but the amount within one tissue type did change. When DGAT1 was down-regulated, 18:3n-3-containing TAG and PC species levels were increased in the cotyledones, whereas when PDAT was up-regulated, levels of 18:2n-6-containing TAG species (TAG-54:8, TAG-54:7, and TAG-54:6) were increased in the whole seed, but with more desaturated TAG species in the cotyledons and less desaturated ones in the embryonic axis (Fig. 7). This suggests that desaturation in these DGAT1-silencing lines may be more exaggerated in the cotyledon. Looking at the spatial distribution, a promoter effect cannot be excluded in the transgenic lines. Napin is expressed specifically in the whole embryo and in fully developed seeds, and no apparent difference is seen in its expression within the embryo (Ellerström et al., 1996). However, there are differences during seed development. At the heart stage, napin is expressed in the cotyledons but not in the embryonic axis, and at the torpedo stage, napin is expressed more strongly in the cotyledons than in the embryonic axis (Stålberg et al., 1993). It has been suggested that, for wild-type *C. sativa*, DGAT1 activity is strongest in the cotyledons, whereas PDAT is more important in the embryonic axis than in the cotyledons (Horn et al., 2013). Also, the data in this study support this suggestion. Using a seed-specific promoter that has a stronger effect in the cotyledon, such as napin, increases the likelihood of the overexpression of genes not normally expressed in the cotyledons or the down-regulation of genes normally expressed in the cotyledons. This could be an additional...
factor to why we see large effects in the PDAT overexpression and DGAT1-silencing lines whereas only small effects are seen for the opposite lines. Hence, supposedly, a promoter more strongly expressed in the embryonic axis at early time points could have a larger effect on DGAT1 overexpression and PDAT silencing than was seen with napin. The seed oil content of C. sativa with DGAT1 overexpressed under the control of the napin promoter was related to the size of the cotyledons (Kim et al., 2016), but possibly even higher oil contents could be reached if TAG-producing enzymes also were overexpressed in the embryonic axis.

CONCLUSION

This study shows that changes in DGAT1 and PDAT expression in C. sativa seeds can significantly alter fatty acid composition. Here, we showed that silencing of DGAT1 led to drastically increased 18:3n-3 levels, something that is likely due to the compensatory activity of a TAG-synthesizing enzyme with specificity for this acyl group, perhaps in combination with the increased desaturation of acyl chains on PC. The effect was seen for total lipids, but also specific molecular species of TAG and DAG with 18:3n-3 acyl groups were increased. Interestingly, some of these species were increased in the whole embryo, whereas others were dominant in the cotyledons. PDAT overexpression increased the 18:2n-6 content in the seed in total lipids as well as TAG species specifically. The combination of analytical and biochemical data suggests that the increased 18:2n-6 content in the seed of PDAT overexpressors is due to fatty acids being channeled from PC into TAG before desaturation to 18:3n-3. These data suggest that this channelling is the result of both high PDAT activity in general and a specificity for 18:2n-6. Our data also show the importance of studying the molecular species compartmentalization, as many of the changes seen were localized to specific parts of the embryo. Moreover, the results presented here support the concept that TAG assembly may be dominated by DGAT1 in the cotyledonary tissues. PDAT1 may compensate for TAG synthesis in the absence of DGAT1 in cotyledons with a change in fatty acid composition but with no penalty in seed oil content.

MATERIALS AND METHODS

Materials, Plants, and Growth Conditions

Wild-type Camelina sativa, accession CAM139 (ACCID 243618; IPK), was grown in a growth chamber under long-day conditions, 16 h of light/8 h of dark, at a constant temperature of 23°C.

Unless stated otherwise, all standard chemicals and organic solvents were obtained from Sigma-Aldrich or Roth. As a TAG internal standard, tripenta-decanoic (tri-15:0) from Sigma-Aldrich was used. Ultrapure water used for MS analysis was obtained from a Sartorius artum pro system. [U-13C]G3P and 1-13C-labeled fatty acids were from PerkinElmer. Acyl-CoA (13C labeled and unlabeled) were prepared according to Sánchez et al. (1973) with CoA and fatty acids purchased from Larodan.

Seed Development

Flowers of wild-type C. sativa were marked using a thin slice of colored scotch tape around the stele. Capsules were harvested and seeds isolated at 4, 7, 11, 18, 24, and 31 DAF, then subsequently weighed, frozen in liquid nitrogen, and stored at −80°C until further analysis.

Plasmid Construction

The amiRNA sequences were designed using Web microRNA Designer (http://wmd3.weigelworld.org), and constructs were made using the plasmid pRS500 (Addgene plasmid 22840) as a template (Schwab et al., 2006). C. sativa DGAT1 and PDAT (GenBank accession nos. KY263957 and KY263958) were cloned from cDNA made from RNA extracted from developing wild-type C. sativa seeds (−11−18 DAF) as described below for qRT-PCR. Primers used are listed in Supplemental Table S3. DGAT1, PDAT, and amiRNA sequences were cloned into modified pUC1BENTR2 vectors (Heilmann et al., 2012) with napin as a promoter. Final constructs were made by combining four different pUC1BENTR2 vectors (empty or containing a gene/amiRNA) using Gateway technology (Invitrogen) with a modified pCAMBIA3331 vector (Hornung et al., 2005). An overview of the pUC1BENTR2 vectors used for the different constructs is shown in Supplemental Figure S2.

Plant Transformation and Screening for Single Insertions and Homozygote Plants

C. sativa plants were transformed using vacuum-assisted Agrobacterium tumefaciens-mediated floral dipping as described previously (Lu and Kang, 2008). Screening for transformed plants was done by spraying 4- to 6-d-old seedlings with 0.025% Basta solution, with a second spraying 6 to 7 d after the first. Surviving plants were deemed Basta resistant, and these plants were transferred to new pots. In the next generation, seedlings were sprayed with Basta as described, and a survival rate of 70% to 80% was considered a single insertion (theoretically, three-fourths should survive), whereas survival rates of 85% and higher were consistent with multiple insertions. One generation further, similar testing was done to see if the single insertions were homozygote (100% survival rate) or heterozygote.

Fatty Acid Analysis

 Mature seeds (18–22) were dried at 105°C overnight, whereas developing seeds were freeze dried. In both cases, dry weight was determined gravimetrically. Seeds were crushed manually using a glass rod, and the weight was determined. For the analysis of fatty acid composition, fatty acid methyl esters (FAMEs) were generated using acidic methanolysis (Miquel and Browse, 1992). Therefore, 1 mL of a methanolic solution containing 2.75% (v/v) H2SO4 (95%–97%) and 2% (v/v) dimethoxypropan was added to the crushed seeds. Gas chromatography analysis of the resulting FAMEs was performed with an Agilent 6890 gas chromatograph fitted with a capillary DB-23 column (15 m × 0.18 mm, 0.2-μm coating thickness; J&W Scientific, Agilent). Helium was used as the carrier gas at a flow rate of 0.8 mL min−1. The temperature gradient was 160°C for 1 min, increased from 160°C to 200°C at 10°C min−1 and then from 200°C to 250°C at 25°C min−1, and held at 250°C for 6 min. The identification of FAMEs was performed by comparing the retention times with authentic standards. Quantification was done using tri-15:0 TAG as an internal standard, added before the methylation.

Lipidomic Analysis

The extraction of lipids for lipidomic analysis was performed with propanol-2-ol/hexane/water (60:26:14, v/v/v) as described previously by Markham et al. (2006). Depending on the developmental stage, about 50 to 100 mg of freeze-dried seeds was crushed and used for extraction.

All lipid species except PA were analyzed by means of ultra-performance liquid chromatography-nano ESI-MS/MS as described by Tarazona et al. (2015). For PA analysis, an aliquot of each sample was resolved in chloroform:methanol (1:2, v/v) containing 5 mM ammonium acetate. The analysis was performed based on a modified method described by Welti et al. (2002) using direct-infusion MS with the nano-ESI-MS/MS part of the analytical system described by Tarazona et al. (2015). For negative nano-ESI, voltage was applied directly-infusion MS with the nano-ESI-MS/MS part of the analytical system performed based on a modified method described by Welti et al. (2002) using direct-infusion MS with the nano-ESI-MS/MS part of the analytical system described by Tarazona et al. (2015). For negative nano-ESI, voltage was applied...
[M–H], and target SRM transitions were diagnostic for the acyl chain composition of the molecular species by the formation of fatty acid-related fragments.

**Acyl-CoA Determination**

For the extraction of acyl-CoA, 50 mg of frozen and crushed developing seeds was used. To this, 1 nmol of 17:0-CoA internal standard was added. Acyl-CoAs were isolated to the interphase of a chloroform/methanol extraction, and the interphase was extracted as described by Kawelke and Feussner (2015). Derivatization of acyl-CoA and detection by HPLC were done according to Larson and Graham (2001).

**qRT-PCR**

RNA was isolated from developing seeds using protocol 2 from Oñate-Sánchez and Vicente-Carbayosa (2008) and RNase-free DNase I from Fermentas. Approximately 20 mg of crushed seed material was used. cDNA was made using 1 μg of RNA as a template using the RevertAid H Minus First Strand cDNA Synthesis Kit with Oligo(dT)18 primer from Thermo Scientific. Primers were designed using Primer 3 (Untergasser et al., 2012; Supplemental Table S3). Amplification and quantification of cDNA were done with the iCycler/MyQ System from Bio-Rad. The amplification mix consisted of 1× NH4 reaction buffer (Bioline), 2 mM MgCl2, 100 μM of each of four deoxyribonucleotide triphosphates, 0.4 μM primers, 0.25 units of BIOTaq DNA polymerase (Bioline), 10 mM fluorescein (Bio-Rad), 100,000× diluted SYBR Green I solution (Cambrex), and 1 μl of 1×106 cDNA dilution in a total volume of 25 μl. Thermocycling conditions were 2.5 min at 95°C followed by 39 cycles of 20 s at 95°C, 20 s at 55°C, and 40 s at 72°C. Calculations were done according to the 2–ΔΔct method (Schmittgen and Livak, 2008) using the actin gene as a reference.

**Microsomal Preparation and Activity Assays**

Microsomal preparations were prepared from developing *C. sativa* seeds according to Bafor et al. (1991). A mixture of developing seeds of an approximate age of 12 to 18 DAF was used. Aliquots of microsomal preparations were flash frozen in liquid nitrogen and stored at −80°C until analysis. The enzymatic activity in the microsomal preparations was investigated using a slightly modified method from Stobart and Stymne (1985). Each reaction of 50 μl was performed in triplicate. A mixture of developing seeds of an approximate age of 12 to 18 DAF was used. Aliquots of microsomal preparations were flash frozen in liquid nitrogen and stored at −80°C until analysis. The enzymatic activity in the microsomal preparations was investigated using a slightly modified method from Stobart and Stymne (1985).

**MALDI-MS Imaging and Analysis**

**Cryosectioning for MALDI-MS Imaging**

All seed genotypes were embedded in 2% (w/v) gelatin (Sigma-Aldrich; G2500) solution. Embedded seeds were frozen at −80°C overnight and then transferred to −20°C for 72 h. Seeds were sectioned using a cryomicrotome (CM1950; Leica Microsystems) set to a temperature of −16°C and a section thickness of 30 μm. Sections were thaw mounted on charged glass slides and then lyophilized for 18 h. Bright-field images of seed sections were taken after lyophilization with an SXX12 stereomicroscope (Olympus) equipped with a DFC425C camera (Leica Microsystems). Tissue sections were stored in a desiccator, under vacuum, until matrix deposition. All sections were imaged within 24 h of sectioning.

**ELISA of Transgenic Lines**

Total lipids were extracted from all mutant lines and the background control line using a method adapted from Chapman and Moore (1993) using hot 70°C isopropanol (n = 3), where one replicate equals an extract from two seeds. Internal standards of 27 μg of TAG (tri-15:0) and 2 μg of PC (di-14:0) were added to each extract for quantification. For MS analysis, extracts were prepared and analyzed in a method adapted from Devaiah et al. (2006). Extracts were analyzed on a Quadrupole Applied Biosystems/Sciex API 3000 mass spectrometer. Samples were injected at a rate of 20 μl/min and a spray voltage of +5.5 kV. PC molecular species were analyzed using precursor fragment scans detecting for a precursor of m/z +184.07. TAG molecular species were identified from full MS scans. Data were processed and quantified against internal standards using the open-source software LipidomeDB (Zhou et al., 2011).

**Statistics**

All data are expressed as means ± SD and unless stated otherwise based on triplicate assays. Statistical analysis was performed using OriginPro 2016. The statistical tests used was one-way ANOVA followed by Tukey’s test for comparisons of means.

**Accession Numbers**

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers KY263957 and KY263958.

**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** Relative contents of acyl-CoA.

**Supplemental Figure S2.** Overview of all constructs.

**Supplemental Figure S3.** DGAT1, DGAT2, DGAT3, PDAT, PDAT2, and PDAT-like RNA expression in developing homozygote T3 seeds.

**Supplemental Figure S4.** Relative amounts of fatty acids in different *C. sativa* T2 seeds.

**Supplemental Figure S5.** Lipids and weights of T2 seeds.

**Supplemental Figure S6.** MALDI-MS images of principal TAG molecular species in sections of control seeds.
ACKNOWLEDGMENTS

We thank Corrina Thurow for technical assistance with the qRT-PCR measurements, Ellen Hornung for support with the cloning, and Paulina Wasserruth for technical assistance with the lipidomics of developing seeds.

Received December 9, 2016; accepted February 22, 2017; published February 24, 2017.

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Supplemental Figure S7. MALDI-MS images of principal PC molecular species in the amiDGAT1.3 line.

Supplemental Figure S8. MALDI-MS images of principal TAG molecular species in the amiDGAT1.3 line.

Supplemental Figure S9. MALDI-MS images of principal PC molecular species in the PDAT+amiDGAT1.2 line.

Supplemental Figure S10. MALDI-MS images of principal TAG molecular species in the PDAT+amiDGAT1.2 line.

Supplemental Figure S11. MALDI-MS images of principal PC molecular species in the PDAT+amiDGAT1.2 line.

Supplemental Figure S12. MALDI-MS images of principal TAG molecular species in the amiPDAT.2 line.

Supplemental Figure S13. MALDI-MS images of principal PC molecular species in the amiPDAT.2 line.

Supplemental Figure S14. Lipid profiles of DGAT and PDAT mutants from MALDI-MS.

Supplemental Table S1. Total lipidomics data.

Supplemental Table S2. Relative amounts of acyl-CoA from developing seeds.

Supplemental Table S3. Overview of primers and amiRNA target sequences.


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