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Triacylglycerol synthesis by PDAT1 in the absence of DGAT1 activity is dependent on re-acylation of LPC by LPCAT2

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Running Title: LPCAT2 is required to assist PDAT1 in oil synthesis in AS11

Keywords: dgat1 mutant AS11; LPCAT1; LPCAT2; PDAT1; Oil biosynthesis

Seed lines from Nottingham Arabidopsis Stock Centre: WT (ecotype Columbia-0); dgat1, AS11 (CS3861); A7 (SALK_039456); lpcat1 (SALK_123480); lpcat2 (SAIL_357_H01) (all in a Columbia background)

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Summary

The Arabidopsis thaliana dagt1 mutant, AS11 (Kataev et al., 1995), has an oil content which is decreased by 30%, and a strongly increased ratio of 18:3/20:1, compared to wild type. Despite lacking a functional DGAT1, AS11 still manages to make 70% of WT seed oil levels. Recently, it was demonstrated that in the absence of DGAT1, PDAT1 was essential for normal seed development, and is a dominant determinant in Arabidopsis TAG biosynthesis (Zhang et al., 2009). In this study, through microarray and RT-PCR gene expression analyses of AS11 vs WT mid-developing siliques, we observed consistent trends between the two methods. FAD2 and FAD3 were up-regulated and FAE1 down-regulated, consistent with the AS11 acyl phenotype. PDAT1 expression was up-regulated by ca 65% while PDAT2 expression was up-regulated only 15%, reinforcing the dominant role of PDAT1 in AS11 TAG biosynthesis. The expression of LPCAT2 was up-regulated by 50-75%, while LPCAT1 expression was not significantly affected. In vitro LPCAT activity was enhanced by 75-125% in microsomal protein preparations from mid-developing AS11 seed vs WT. Co-incident homozygous knockout lines of dagt1/lpcat2 exhibited a severe penalty on TAG biosynthesis, delayed plant development and seed set, even with a functional PDAT1; the double mutant dagt1/lpcat1 showed only marginally lower oil content than AS11. Collectively, the data strongly support that in AS11 it is LPCAT2 up-regulation which is primarily responsible for assisting in PDAT1-catalyzed TAG biosynthesis, maintaining a supply of PC as co-substrate to transfer sn-2 moieties to the sn-3 position of the enlarged AS11 DAG pool.

250 words
Introduction

Triacylglycerols (TAGs) are the major storage lipids which accumulate in developing seeds, flower petals, anthers, pollen grains, and fruit mesocarp of a number of plant species (Stymne and Stobart, 1987; Murphy and Vance, 1999). TAGs are thought to be not only the major energy source for seed germination but also essential for pollen development and sexual reproduction in many plants (Wolters-Arts et al., 1998; Zheng et al., 2003). In oil seeds, TAG bioassembly is catalyzed by the membrane-bound enzymes of the Kennedy pathway that operate in the endoplasmic reticulum (Stymne and Stobart, 1987). The biosynthesis of TAGs catalyzed by the sequential acylation of the glycerol backbone involving three acyltransferases: glycerol-3-phosphate acyltransferase (GPAT), lyso-phosphatidic acid acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT). DGAT catalyses the final acylation of DAG to give TAG, which has been suggested being the rate-limiting step in plant lipid accumulation.

In the traditional Kennedy pathway DGAT was thought to be the only enzyme that is exclusively committed to TAG biosynthesis using acyl-CoA as its acyl donor. The first DGAT gene was cloned from mouse and is a member of the DGAT1 family, which has high sequence similarity withsterol:acyl-CoA acyltransferase (Cases et al., 1998).

We had previously characterized an EMS-induced mutant of Arabidopsis, designated AS11, which displayed a decrease in stored TAG, delayed seed development, and an altered fatty acid composition (Katavic et al., 1995). We analyzed WT vs. AS11 lipid pools and Kennedy pathway enzyme activities in fractions isolated from green mid-developing seed, and performed parallel labeling of intact seeds at this developmental stage, with [14C] acetate. We found that compared to WT, there was an increase in all fatty acids in the DAG pool of AS11 seeds at mid-development, and, to a lesser extent, there was an associated backup of fatty acids in the PC pool. DAG was elevated from 1% in WT to 10-12% in AS11 and PC pools were elevated from about 2% in WT, to 8-12% in AS11. Cell-free fractions from WT and AS11 green seeds at mid-development were compared for their ability to incorporate [14C]-18:1-CoA into glycerolipids in the presence of G-3-P. Proportions of labeled LPA and PA formed during the incubation period were similar in WT vs AS11, indicating that the activities of the Kennedy pathway enzymes GPAT and LPAAT (EC 2.3.1.51) were relatively unaffected in the AS11 mutant. However, the proportion of labeled TAG was much lower and that of DAG was much higher in AS11. The TAG/DAG ratio was therefore consistently 3- to 5-fold lower in AS11 compared to WT at all developmental stages (early-, mid- and late development) (Katavic et al., 1995).

Cumulatively, this data suggested a lesion in DGAT1 which was subsequently proven upon cloning the mutated gene from AS11. There is an 81 bp in-frame insertion consisting entirely of exon 2 in the transcript from AS11. The exon 2 in the repeat is properly spliced, thus the alteration of the transcript does not disturb the reading frame. However, this additional exon 2
sequence in the AS11 transcript would result in an altered DGAT protein with a 27 amino acid insertion (131SHAGLFNLCVVLIAVNSRLIIENLMK157) (Zou et al., 1999). Two other labs independently and simultaneously cloned the A. thaliana DGAT1 (Hobbs et al., 1999; Routaboul et al., 1999).

Earlier studies of DGAT1 indicated that it plays a strong role in determining oil accumulation and fatty acid composition of seed oils. Thus, there was implied utility in manipulating the expression of this gene for improving oil content and perhaps, altering fatty acid composition. To this end, we demonstrated that expression of the Arabidopsis DGAT1 cDNA in a seed specific manner in the AS11 mutant restored wild type levels of TAG and VLCFA content. The acyl distribution, specifically, the sn-3 composition of the TAGs, was also restored to WT proportions. Furthermore, over-expression of the Arabidopsis DGAT1 in wild type plants led to an increase in seed oil content and seed weight (Jako et al., 2001). Subsequently, DGAT1 expression has been genetically manipulated to produce Brassica oilseed prototypes containing increased oil (Weselake et al., 2008; Taylor et al., 2009).

A second family of DGAT genes (DGAT2) was first identified in the oleaginous fungus Morteriella ramanniana, which has no sequence similarity with DGAT1 (Lardizabal et al., 2001). A human DGAT2 and several plant DGAT2s have since been characterized (Cases et al., 2001; Shockey et al., 2006; Kroon et al., 2006). The putative DGAT2 from Arabidopsis has been studied by several labs including ours; functional expression in yeast has not been successful, and therefore whether it is a true DGAT is still in question.

A novel class of acyl-CoA-dependent acyltransferases, wax ester synthase/acyl-CoA: diacylglycerol acyltransferase (WS/DGAT) was recently identified and purified from the bacterium Acinetobacter sp. Strain ADP1, which can utilize both fatty alcohols and diacylglycerols as acyl acceptors to synthesize wax esters and TAGs, respectively (Kalscheuer et al., 2004; Stoveken et al., 2005). Other proposed additions to the traditional scheme of TAG assembly pathways include demonstrations that in developing castor and safflower seeds, TAG can also be generated from two molecules of DAG via a DAG:DAG transacylase (with MAG as a co-product) and that the reverse reaction participates in remodeling of TAGs (Lehner and Kuksis, 1996; Mancha and Stymne, 1997; Stobart et al, 1997). However, genes encoding the latter enzymes have not been identified in the Arabidopsis genome.

In some species, it is clear that TAG can also be formed by an acyl-CoA-independent enzyme, phosphatidylcholine:diacylglycerol acyltransferase (PDAT), in which the transfer of an acyl group from the sn-2 position of PC to the sn-3 position of DAG yields TAG and sn-1 lyso-PC (Dahlqvist et al., 2000; Banas et al., 2000). In yeast, PDAT1 is a major contributor to triacylglycerol (TAG) accumulation during the exponential growth phase. The two closest homologs to the yeast PDAT gene have been identified in Arabidopsis: PDAT1 At5g13840 and PDAT2 At3g44830 (Stahl et al, 2004). Mhaske et al (2005) isolated and characterized a knockout
mutant of Arabidopsis thaliana L. which has a T-DNA insertion in the PDAT1 locus At5g13640 (PDAT1, EC 2.3.1.158). Lipid analyses were conducted on these plants to assess the contribution of PDAT1 to seed lipid biosynthesis; surprisingly, and in contrast to the situation in yeast, the fatty acid content and composition in seeds did not show significant changes in the mutant. At the time, these results were interpreted to indicate that PDAT1 activity as encoded by At5g13640 is not a major determining factor for TAG synthesis in Arabidopsis seeds.

Nonetheless, because the Arabidopsis DGAT1 mutant AS11 shows only a 20-30% decrease in oil content (Katavic et al., 1995; Routaboul et al., 1999), it was apparent that other enzymes must contribute to oil synthesis in the developing seed (Lu et al., 2003).

An examination of the contribution of DGAT2, PDAT2 or PDAT1 to oil deposition in an AS11 background was studied by performing double mutant crosses with AS11 (Zhang et al., 2009). While the dcat2-ko line has no oil phenotype, homozygous double mutants from cross of AS11 with dcat2-ko mutant showed an oil fatty acid profile similar to AS11. We observed the same pattern with the pdat2-ko mutant alone and in crosses of the pdat2-ko mutant with AS11. In contrast, while the pdat1-ko has no oil or fatty acid composition phenotype, crosses of the pdat1-ko with AS11 were embryo-lethal in the double homozygous condition; only heterozygous lines produced by having expression of the pdat1 or dcat1 gene partially inhibited using RNAi, allowed an examination of the double mutants. These detailed studies resulted in the finding that DGAT1 and PDAT1 have overlapping functions in both embryo development and TAG biosynthesis in the developing seed and pollen. When DGAT1 is compromised in AS11, it is PDAT1 and not DGAT2 or PDAT2 that is responsible for the remaining 65-70% of TAG which is synthesized. This finding suggested a major, perhaps dominant role of PDAT1 in this process (Zhang et al., 2009).

Recently, a castor bean-specific PDAT, PDAT1-2, was cloned and found to be highly expressed in developing seeds and localized in the ER, similar to the castor FAH12 hydroxylase. Transgenic Arabidopsis co-expressing the castor PDAT1-2 and FAH12 showed enhanced ricinoleate accumulation to up to 25% in TAGs (compared to 17% in FAH12 –only transgenics) (Kim et al., 2011; van Erp, 2011). This study may lead to a discovery that specialized PDATs may play a significant role in channeling PC-synthesized unusual fatty acids such as ricinoleic (from castor), or epoxy fatty acids (from Vernonia galamensis), into TAGs.

Here we report the further genetic and biochemical characterization of the AS11 mutant. During the course of microarray and qRT-PCR studies of AS11 vs WT gene expression in mid-developing siliques, we found that LPCAT2, encoding acyl-CoA:lysophosphatidylcholine acyltransferase 2 (EC 2.3.1.23), was up-regulated while LPCAT1 was not affected. By a series of biochemical studies and key crosses of AS11 with either ipc1 or ipc2, we identified that LPCAT2 (and not LPCAT1) is critical for TAG synthesis in the AS11 mutant, primarily to maintain the PC pool for TAG assembly primarily catalyzed by PDAT1.
Results and Discussion

Summary of the AS11 mutant developmental and oil phenotypes

The AS11 mutant line was about one week behind WT in bolting and entering the generative phase and thus, under our growing conditions, AS11 seed set was also delayed to four weeks instead of three, as typically observed in WT. For comparison, we studied another DGAT1 mutant, which we designated A7, which is a homozygous SALK line (Salk 039456) with a T-DNA insertion in the last exon of the same DGAT1 gene (At2g19450). A7 shows a developmental delay similar to that exhibited by AS11 (Fig S1, Supporting information).

Using protein fractions prepared from WT and AS11 mid-developing seeds we were able to determine the relative changes of TAG assembly activity in the mutant line. Seed material was pooled from stage 3 to stage 6 siliques, as defined by Zou et al. (1996). TAG synthesis capacity was measured in WT and AS11 lines with 14C-labeled diolein and unlabeled oleoyl-CoA as co-substrates; the 14C-labeled triolein product was measured by radio-HPLC as described previously (Taylor et al., 1992b). As shown in Fig. 1, there was a 30-37% decrease in the acylation of radiolabeled DAG in AS11, a finding which was strongly correlated with the 30-35% reduction in oil content in mature AS11 seed (Katavic et al., 1995; Jako et al., 2001).

Heterologous Expression of mutated Arabidopsis DGAT1 from both A7 and AS11 mutant in Yeast

The altered fatty acid and low TAG phenotype in AS11 seed raised questions as to how the AS11 mutant still manages to make 65-70% of WT levels of seed oil. Because AS11 has a reduced TAG phenotype, it was essential to determine whether the DGAT1 in AS11 was merely mutated and exhibited reduced activity as we initially suggested (Katavic et al., 1995), or whether it is, in fact, non-functional. This has not heretofore been confirmed. The importance of doing so will become apparent in the PCR assessment of some genetic crosses in the current study, and discussed below. The cDNA from the AS11 (with a 81bp repeat insertion in the second exon) was cloned into a yeast expression vector pYES2.1 under the control of the galactose-inducible GAL1 promoter, and the construct was used to transform a yeast mutant strain H1246MATa, which lacks all four genes, ARE1, ARE2, DGAT1 and LRO1, which were found to contribute to TAG synthesis (Sandager et al., 2002). H1246MATa yeast cells harboring an empty pYES2.1 vector plasmid or transformed with WT DGAT1 cDNA were used as a negative and positive controls, respectively. A western blot of the microsomal membrane fractions from the induced yeast cells showed that both the mutated and WT DGAT1 proteins were indeed expressed. However, the AS11 DGAT1 could not compensate for the inability to produce TAG in this yeast quadruple mutant. Equally, when we assayed the transformed yeast microsomal protein fractions in vitro for
AtDGAT1 activity using $^{14}$C-labeled oleoyl-CoA (18:1) as an acyl donor, and unlabelled sn-1,2 diolein (18:1) as acceptor, enzyme activity was not detected in the yeast strain harboring the mutated DGAT1 cDNA from the AS11 mutant and empty control pYES2.1 vector, but was found in the positive control. This indicated, perhaps not unexpectedly, but for the first time, that the mutated DGAT1 from AS11 is non-functional; equally, the A7 T-DNA mutated DGAT1 was shown to be non-functional (Fig. 2).

These results unequivocally demonstrated that the (radiolabeled) TAG formation observed in protein fractions from developing seed of AS11 was coming from another path and not via reduced DGAT1 catalysis.

Given the importance of PDAT1 in oil biosynthesis in Arabidopsis as we earlier defined (Zhang et al., 2009), and combining this new information with the $^{14}$C TAG biosynthesis results in assays of WT vs AS11 protein fractions reported above, it raised the question that, if not from DGAT1, DGAT2 nor PDAT2, what biochemical steps besides PDAT1 may be critical for TAG biosynthesis?

AS11 microarray and qRT-PCR analyses

These cumulative findings prompted us to examine a broader inventory of gene transcripts/encoded proteins that may be involved in regulating lipid biosynthesis in AS11 when DGAT1 activity is compromised, compared to their corresponding expression pattern in WT. Thus we performed a microarray analysis of gene expression in mid-developing seeds of AtDGAT1 mutant AS11 and WT Arabidopsis. Based on selected probable lipid assembly-related transcripts (Beisson et al., 2003) differentially expressed through the microarray study, we complemented this with a semi-quantitative qRT-PCR analysis. While neither method is truly quantitative, the qualitative trends in each study were highly consistent (Fig. 3).

Some general observations from these combined gene expression studies follow: In AS11, FAE1 is down-regulated, FADs 2 & 3 are up-regulated; this is consistent with the AS11 acyl composition profile (reduced 20:1 and elevated 18:3). DGAT1 expression was not significantly affected. Interestingly, LPCAT2 was up-regulated (by an average of 65%) while LPCAT1 expression was indifferent. PDAT2 was only marginally affected, but PDAT1 expression was up-regulated by an average of 62% compared to WT.

Given the critical role of PDAT1 in embryo development and TAG deposition in both pollen and seeds (Zhang et al., 2009), we were interested in the relative differences in LPCAT1, LPCAT2 between the AS11 mutant and WT. We hypothesized that an acyl-CoA-dependent LPCAT may be critical to maintain the PC pool as one of the co-substrates for PDAT1-catalyzed TAG synthesis, particularly in the absence of a functional DGAT1 and performed a series of metabolic and genetic studies to examine these relationships.
Metabolic studies:

We performed *in vitro* LPCAT assays in a time course incubation of protein fractions from AS11 and WT mid-developing seed, in the presence of sn-1 palmitoyl-\(^3\)H LPC + \(^{14}\)C-18:1-CoA and followed the \(^3\)H and \(^{14}\)C labeling patterns in PC. Based on the proportion of sn-2 \(^{14}\)C oleoyl moieties incorporated into PC, the LPCAT activity was consistently 40-60% higher in AS11 at all time points (Fig 4A). The \(^3\)H pattern showed that PC was rapidly synthesized from \(^3\)H LPC at a rate that was 3.5-fold higher in AS11 than WT within 20 min (Fig.4B). Equally, the proportion of \(^3\)H in LPC concomitantly decreased in AS11 relative to WT over this period (data not shown). This indicated that in AS11, the LPCAT activity was strongly enhanced relative to WT.

We also performed a pseudo-*in situ* feeding study wherein we supplied \(^{14}\)C acetate to bolted stems containing pods with mid-developing seeds of both AS11 and WT plants, and then analyzed the label patterns in various lipid fractions in the mid-developing seeds after a chase period of 7, 24 and 30 hours. Two major differences were immediately obvious (Fig. 5): AS11 showed higher relative incorporation of \(^{14}\)C into PC and a lower relative incorporation of \(^{14}\)C into TAG over the time course. These trends were entirely consistent with an elevated LPCAT activity and reduced TAG synthesis in AS11 as shown in the *in vitro* LPCAT (Fig. 4) and DGAT1 (Fig. 1) assays.

Based on these trends we were confident that in addition to DGAT1 and PDAT1, LPCAT plays a significant role in TAG biosynthesis, and postulated that it is important not only for membrane development and acyl turnover therein, but also to replenish the supply of PC for PDAT1-catalyzed TAG biosynthesis in the developing seed. To resolve this question we needed to study what occurs when both DGAT1 and LPCAT(s) expression are co-disrupted.

**Genetic Crosses**

To examine the relationship between LPCATs and TAG biosynthesis we performed crosses of *AS11* with *lpca1* or *lpca2* T-DNA knock-out mutants and characterized the hemizygous/homozygous and double knockout seed oil profiles.

The *lpca1* mutant is devoid of any significant oil phenotype compared to its null segregant or to WT (Fig. 6A). Crosses of *AS11* with the *lpca1* yielded progeny homozygous for both mutations. The doubly homozygous mutant showed normal plant development and seed set. Zhang et al (2009) showed that without DGAT1, the PDAT1 route contributed approximately 75% to oil synthesis in AS11; thus even without combined contributions from [DGAT1 + LPCAT1], the PDAT1 route could still provide up to 70% of the TAG synthesized in the developing AS11 seeds. In other words, the co-incident loss of LPCAT1 with DGAT1 reduced the capacity for PDAT1-catalyzed oil synthesis by only 5%. With respect to seed weight, the *lpca1* mutant showed no
significant difference from its null segregant, and the double knockout did not show any penalty in this regard (Fig. 6B). The fatty acid profile from the lpcat1 mutant is identical to that of its null segregant (Fig. 7). The AS11 x lpcat1 double knockout lines show an AS11-like profile (low 18:1 and 20:1; high 18:3); the 18:3 proportion is about 2-5% lower than in AS11 alone. The latter is not inconsistent with the fact that LPCATs are involved in the shuttling of 18:1 moieties to the PC backbone for desaturation by FADs 2 and 3.

The lpcat2 mutant does not have a significant oil phenotype compared to its null segregant- i.e. it is similar to WT (Fig. 8). The fatty acid composition is essentially identical to WT and to that exhibited by the LPCAT2 null segregant. When we performed the AS11 x lpcat2 crosses, the AS11/ lpcat2 He/H shows only a small reduction in oil content which suggests that when TAG1 is partially expressed (He), and LPCAT2 is knocked out (H), there is a reduced capacity for compensation in TAG biosynthesis, probably because PDAT is insufficiently "fueled" with PC. In these lines there is a penalty on seed development; there are many gaps in developing siliques with aborted embryos (Figs. S2A and S2B). Relative to the LPCAT2 NS and WT, seed weight is decreased by about 30-35% (Fig. 9). The acyl composition was again essentially like the LPCAT2 NS (Fig. 10). In contrast, the AS11/ lpcat2 H/He have an AS11-like reduction in oil content which is about 70% of WT and the LPCAT2 null segregant (Fig. 8). Interestingly, there is no developmental penalty in this heterozygous combination and seed set is normal as in the AS11 background, provided expression of LPCAT2 is in the heterozygous state (Fig. S3). Not unexpectedly, the acyl composition of the oil in these lines is consistent with the AS11 profile (Fig. 10).

The results were quite different in the AS11 x lpcat2 double homozygous mutant. Shown in Fig. 11 is a PCR confirmation of the genetic makeup of putative H/H lines # 6-3-10-19, # 6-3-20-1 compared to WT, AS11 and lpcat2 lines. AS11, and dcat1/lpcat2 H/H lines # 6-3-10-19 and # 6-3-20-1 have the expected 147 bp insert indicating they are homozygous for the insertion mutation resulting in a non-functional DGAT1 (as shown in Fig. 2, above), while the WT and lpcat2 samples do not. T-DNA screening was done on the samples of the same lines to test for the presence of LPCAT2. Samples were amplified using [left primer + right primer] or with [right primer + left border 2] primer. The lpcat2 and the dcat1/lpcat2 H/H lines # 6-3-10-19 and # 6-3-20-1 have a homozygous knockout of the LPCAT2 gene. Biochemically, in the double homozygous mutant (AS11/ lpcat2 H/H) there is a severe penalty on TAG synthesis; there is a 65% relative reduction in oil content. Mature seeds have only about 30% of WT and LPCAT2 null segregant oil (Fig. 8). Without DGAT1 (AS11), we calculated that PDAT1 contributes about 70-75% to oil (Zhang et al., 2009). Without both LPCAT2 and DGAT1, PDAT1 contributes about 35% to oil. Thus, the loss of LPCAT2 strongly reduces the capacity of PDAT1 to synthesize oil. This suggests a strong link between PDAT1 and its supply of PC as a co-substrate in TAG synthesis; clearly LPCAT2 plays a strong role in supplying this PC. AS11/ lpcat2 H/H shows a fatty acid
profile closer to AS11 (low 18:1, 20:1 and high 18:3). Thus we can conclude that when the DGAT1 mutation is homozygous, the AS11 acyl profile dominates regardless of LPCAT2 mutation condition (H vs He) (Fig. 10).

Plant growth and development is also delayed in the double homozygous mutant (AS11/ lpcat2 H/H), as it is in AS11: the seedlings are small and slow to develop (Fig. 12A). Once transferred to soil, bolting to enter the generative phase is delayed in AS11/ lpcat2 H/H (Fig. 12B), but is normal in dgat1/lpcat2 H/He (Fig. 12C). Seed development is poor, as in the AS11/ lpcat2 He/H lines; there are many gaps in developing siliques and seed set is only about 25-33% of that normally observed in WT or LPCAT2 NS. Thus, it is clear that some level of LPCAT2 expression is necessary for normal seed development, especially in an AS11 background.

Collectively, the data from the crosses of AS11 with lpcat1 and lpcat2 mutants strongly suggest that in an AS11 background it is LPCAT2 up-regulation (and not LPCAT1), which is primarily responsible for assisting in PDAT1-catalyzed TAG biosynthesis by supplying an enhanced pool of PC as co-substrate to transfer sn-2 moieties to the sn-3 position of the enlarged AS11 DAG pool. In its absence, oil synthesis and seed set are severely affected. Metabolic and microarray studies in AS11 support this hypothesis as LPCAT2 expression and LPCAT activity are up-regulated. In addition, the acyl profiles observed in these crosses indicate that any altered oil composition phenotype is predominantly controlled by the absence of DGAT1 activity.

Conclusions

Based on the cumulative results of these studies, we summarize our current hypothesis regarding the TAG biosynthesis pathway in WT vs. that when DGAT1 is compromised as in AS11 (Fig. 13). When both DGAT1 and PDAT1 are operating they both, perhaps equally, contribute to TAG synthesis. When DGAT1 is eliminated, the PDAT1 pathway becomes dominant and accounts for 65% of WT TAG synthesis, as supported by the results of Zhang et al. (2009). PDAT1 acylates the sn-3 position of DAGs which are accumulating (in the absence of DGAT1), to give TAG. The acyl composition of AS11 with highly enhanced PUFAs and reduced VLCFAs at the sn-3 position (Katavic et al., 1995) supports the donation of acyl groups from the sn-2 position of PC.

This adjustment in the TAG assembly route in AS11 is aided by enhanced LPCAT2 activity to supply the additional PC required by PDAT1. Given these findings and the implicit plasticity of Arabidopsis TAG assembly mechanisms to overcome critical bottlenecks, it will be
interesting to determine the relative roles played by DGAT1 and [PDAT1 + LPCAT2] during TAG assembly in higher oilseeds (e.g. canola, soybean, sunflower, safflower, flax).
Experimental Procedures

Plant Materials and Growth Conditions

*Arabidopsis* lines, WT (ecotype Columbia-0) and AS11 (CS3861), A7 (SALK_039456), lpca1 (SALK_123480) and lpca2 (SAIL_357_H01) mutant lines (all in a Columbia background) were obtained from the Salk Institute via the Nottingham Arabidopsis Stock Centre (University of Nottingham, UK). Seeds of these lines and progeny from genetic crosses were grown in a growth chamber at 22°C with photoperiod of 16 h light (120 μE·m⁻²·s⁻¹) and 8 h dark. Arabidopsis siliques containing mid-green developing seeds (pooled siliques stages 3-6 inclusive, as described by Zou et al., 1996) were harvested from embryos and frozen at -80°C for lipid analyses, enzyme assays and DNA and RNA extraction.

Lipid Analyses

Preparation of total lipid extracts (TLEs) and study of lipid classes, determination of oil content and acyl composition in seeds of WT and the *AS11*, *lpca1* and *lpca2* mutant lines and progeny from crosses were performed as described previously (Taylor et al., 1991, 1992a; Katavic et al., 1995). In all cases, the data represent the averages of three to five determinations.

Preparation of Arabidopsis Protein Fractions

In general, enzyme preparations were made from 200 *Arabidopsis* siliques of AS11 and WT containing mid-green developing seeds, and immediately powdered with liquid nitrogen in a mortar and pestle. Grinding medium (100 mM HEPES-KOH, pH 7.4 containing 0.32 M Sucrose, 1 mM EDTA, and 1 mM dithiothreitol; 8 mL/50 siliques) along with 65 mg polyvinylpolypyrrolidone were immediately added, and grinding continued on ice for 5 min. The slurried cell-free homogenate was filtered through two layers of Miracloth (Caltbiochem, La Jolla, CA), centrifuged at 3,000 x g for 5 min, the pellet discarded and the supernatant re-centrifuged at 15,000 x g for 30 min. The supernatant was re-centrifuged at 100,000 x g for one hour and the resultant pellet was resuspended in 2 mL of grinding medium, probe-sonicated on ice for 30 sec and protein concentrations were determined using BioRad™ reagent based on the method of (Bradford 1976). Protein concentrations were normalized to the same value for WT and AS11 in each experiment.
Assay of TAG synthesis activity in Arabidopsis mid-developing seeds

TAG assembly assays were conducted at pH 7.4, with shaking at 100 rev/min in a water bath at 30°C for 60 min. Assay mixtures (500 µL final volume) contained 100-300 µg protein normalized as described above, 90 mM HEPES-NaOH, 0.5 mM ATP, 0.5 mM CoASH, 1 mM MgCl₂ in the presence of 100 µM [1-¹⁴C] sn-1,2 diolein in 0.02% Tween-20 (specific activity 10 nCi/nmol; pre-purified by TLC on 5% borate silica G plates) and 18 µM unlabeled 18:1-CoA. Reactions were stopped using isopropanol:CH₂Cl₂ (2:1) v/v, and the TLE prepared as described previously (Taylor et al., 1991, 1992a). The ¹⁴C-labelled products were resolved by TLC on silica gel G plates developed in hexane:diethyl ether:acetic acid (70:30:1 v/v/v), the ¹⁴C-triolein band visualized on a Bioscan AR-2000 radio-TLC scanner using Win-Scan 2D© software (Bioscan Inc., Washington DC, USA) and the band scraped and quantified on a scintillation counter.

Expression of mutated AtDGAT1 in yeast

The mutated AtDGAT1 (from AS11 as well as A7) in pYES2.1/NT B plasmid were transformed into a quadruple yeast mutant H1246MATα (Sandager et al., 2002) using the S.c. EasyComp™ Transformation Kit (Invitrogen). Yeast cells transformed with pYES2.1/NT B plasmid containing the WT DGAT1 or with empty plasmid were used as positive or negative controls, respectively. Transformants were selected by growth on synthetic complete medium lacking uracil (SC-ura), supplemented with 2% (w/v) glucose. The colonies were transferred into liquid SC-ura with 2% (w/v) glucose and grown at 30°C overnight. The overnight culture was diluted to an OD 0.4 in induction medium (SC-ura + 2% Galactose + 1% Raffinose), and were induced for 24-36 hours at 30°C. The yeast cells were collected and broken with glass beads using a Beadbeater™. The protein concentrations of the yeast cell lysates were normalized using the Biorad™ assay and assayed for DGAT activity. DGAT assays were conducted at pH 7.4, with shaking at 100 rev/min in a water bath at 30°C for 60 min. Assay mixtures (500 µL final volume) contained 100 µg of lysate protein, 90 mM HEPES-NaOH, 100 µM sn-1,2 diolein or sn-1,2 dierucin (pre-purified by TLC on 10% borate silica H plates and emulsified in 0.02% Tween-20), and 18 µM ¹⁴C 18:1-CoA (specific activity 10 nCi/nmol) as the acyl donor. The ¹⁴C-labelled TAGs were isolated and counted as described previously (Taylor et al., 1991, 1992a).

Immunodetection

The yeast cell lysates were run on a 10% Tris-HCl SDS-PAGE gel, the proteins were then transferred to a nitrocellulose membrane (Nitrobind, Fisher). The membrane was blocked in
PBST (phosphate buffered saline containing 0.5% Tween 20) containing 4% skim milk for 60 min, and then incubated with the primary antibody, Anti-Xpress (epitope-tagged) antibody (Invitrogen) diluted to 1:5000 with PBST containing 2% skim milk, for 60 min. The membrane was submitted to three washes with PBST followed by three washes with PBS to remove any unbound antibody. Next, the membrane was incubated with a goat anti-mouse IgG peroxidase antibody (Sigma, A2554), diluted to 1:5000 with PBST containing 2% skim milk, for 60 min. The membrane was washed three times with PBST followed by three times with PBS, then the proteins were detected using the Amersham ECL Plus Western Blotting Detection Kit (GE Healthcare Life Sciences).

LPCAT assays

LPCAT assays were conducted at pH 7.4, with shaking at 100 rev/min in a water bath at 30°C in a time course of 5, 10, 20, 40 and 80 min. Assay mixtures (500 μL final volume) contained 300 μg protein normalized as described above, 90 mM HEPES-NaOH, 0.5 mM ATP, 0.5 mM CoASH, 1 mM MgCl₂ in the presence of 6 μM L-α-palmitoyl- [1- ³H methyl] lyso-3-phosphatidylcholine (specific activity 60 μCi/nmol; 1.48-2.22 TBq/mmol) and 18 μM [1-¹⁴C] 18:1-CoA (specific activity 10 nCi/nmol; 0.37GBq/mmol). Reactions were stopped at each time point by adding isopropanol: CH₂Cl₂ (2:1) v/v, and the TLE prepared as described previously (Taylor et al., 1991, 1992a). The ¹⁴C and ³H-labelled products were resolved by 3D TLC performed as follows: The first 2 dimensions were run as described by Yokoyama et al., (2000)-1st D: CHCl₃: MeOH: Formic Acid (88%):H₂O 60: 30 : 9 : 2; 2nd D: CHCl₃: MeOH: Ammonia solution (28%): H₂O 50: 40: 7: 3; 3D: The 3rd D: 100% ethyl ether. Radiolabeled spots corresponding to standards of LPC and PC were identified using a radio-TLC scanner and the bands scraped and counted on a scintillation counter with a dual ³H/¹⁴C isotope measurement program.

In vivo Feeding experiments

Twenty bolted stems with mid-developing siliques of both WT and AS11 plants were harvested under water and then immediately placed in a solution containing 1 μCi of ¹⁴C sodium acetate in 100 μL of water and incubated at room temperature in a fume hood. Once most of this solution was taken up, the plants were supplied with equal aliquots of distilled water during the chase period. At 0, 7, 24 and 30 hours, 5 bolted stems were harvested, siliques counted and weighed and then ground with a polytron and a TLE extraction performed as described by Taylor et al.(1991). The lipid extract was resuspended in 1 mL CHCl₃:MeOH 2:1. 10 μL of the solution was counted and the remainder spotted on TLC plates, 3D TLC performed, radiolabeled spots resolved on a radio-TLC scanner and scraped and counted.
Semi-quantitative RT-PCR comparison of AS11 and WT expression of key lipid genes.

Total RNA was extracted from mid-developing AS11 and WT Arabidopsis siliques as described by Wang and Vodkin (1994). One microgram of total RNA was reverse-transcribed using SuperScript® II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. The resulting cDNA was then amplified by PCR using Taq DNA polymerase (Invitrogen). PCR conditions comprised an initial cycle of 94°C for 3 min; followed by 30 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 1 min; then 72°C for 10 min to complete the reaction. Concentrations for the individual samples within each group (AS11 and WT) were normalized using 18S rRNA levels. The gene-specific primers (listed in Table S1) were designed according to the target gene sequences as annotated in Genbank™ (http://www.ncbi.nlm.nih.gov/genbank/). Amplified PCR products ranging from 400-600 bp and spanning at least one intron to eliminate any contamination by genomic DNA, were resolved by electrophoresis on 1% agarose gels. Gel photos were taken by the integrated camera installed on the ULTRA LUM electronic dual light transilluminator (ULTRA LUM Inc. paramount, CA 90723). The densities of the PCR bands were analyzed using ImageJ™ software (computer), and quantified relative to the 18S rRNA signal. Then ratio of AS11:WT gene expression level was calculated.

Affymetrix Microarray Analysis

Total RNA was extracted from mid developing AS11 and WT Arabidopsis siliques as described by Wang and Vodkin (1994). Affymetrix microarray hybridizations using the Ath1 whole genome array, containing probe sets representing ~22,800 genes, were performed using three biological replicate samples for each genotype. Labeling, hybridization, and scanning were performed by the Affymetrix Gene Chip Facility at the University of Toronto (http://www.esb.utoronto.ca/resources/facilities/affymetrix-genechip). Data analysis was performed using GeneSpring™ software version 7.2. To identify key lipid genes that were differentially expressed between the two genotypes, a per-gene normalization was applied to the values and a parametric test was performed. Genes that exhibited a false discovery rate of \( p < 0.05 \) and passed the minimum signal and fold-change threshold were determined to be differentially expressed. Reported candidate genes were selected by comparing those listed by Beisson et al., (2003) with our microarray and qRT-PCR data.
Mutant Crosses

Confirmation of *lpcat1* and *lpcat2* T-DNA insertion mutation lines

Putative *Arabidopsis* insertion mutation lines (Alonso et al., 2003) SALK_123480 for *LPCAT1* (At1g12640) and SAIL_357_H01 for *LPCAT2* (At1g63050) were identified in the Salk Institute T-DNA insertion library database (signal-.salk.edu/cgi-bin/tdnaexpress), and seeds were obtained from the Nottingham Arabidopsis Stock Centre (University of Nottingham, UK). According to annotation in the database SALK_123480 contains a T-DNA insertion in the middle of the 7th exon of the *LPCAT1* gene and SAIL_357_H01 a T-DNA insertion in the 6th exon of the *LPCAT2* gene. Individual plants homozygous for a T-DNA insertion in each the *LPCAT1* or *LPCAT2* genes were identified by PCR screening using primers SALK_123480LP, SALK_123480RP and SALK_LBb1 (Table S2) for *LPCAT1* and SAIL_357_H01LP, SAIL_357_H01RP and SAIL_LB2 (Table S2) for *LPCAT2*. Individual plants from each mutant line lacking a T-DNA insertion in the *LPCAT1* or *LPCAT2* genes (null segregants) were also identified in the same PCR primer set. Annotation of lines from each set of crosses are as designated in Table S3.

Creating double mutants of *dgat1 X lpcat1* and *dgat1 X lpcat2*

Crosses between the AS11 mutant and *lpcat1* and *lpcat2* T-DNA insertion mutation lines, respectively, were made and F1 plants heterozygous for AS11 and the insertion mutations were identified by PCR using primers listed in Table S2. F2 seeds segregating for the mutations were planted and screened by PCR. All PCR screening was done as above for *LPCAT1* and *LPCAT2* and for the AS11 mutation vs WT using primers TAG1-mut-primerA and TAG1-mut-primerB (Table S2), as designed by Zou et al. (1999). Identification of individuals homozygous for both the AS11 mutation and lpcat2 insertion mutation was only possible after growing a segregating F2 seed population (homozygous for AS11 and heterozygous for lpcat2 mutant) on agar media containing a 1/3 strength MS and 1% sucrose (Table S3).

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