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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)

Word Count: 9,331

Summary

The *Arabidopsis thaliana dgat1* mutant, *AS11* (Katavic 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 *dgat1/lpcat2* exhibited a severe penalty on TAG biosynthesis, delayed plant development and seed set, even with a functional PDAT1; the double mutant *dgat1/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 with sterol: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 [^{14}C] 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 [^{14}C]-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 (131SHAGLFNLCVVVLIAVNSRLIIENLMK157) (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* At5g13640 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 *dgat2-ko* line has no oil phenotype, homozygous double mutants from cross of *AS11* with *dgat2-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 *dgat1* 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:*lyso*phosphatidylcholine 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 *lpcat1 or lpcat2,* 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 ^{14}C-labeled diolein and unlabeled oleoyl-CoA as co-substrates; the ^{14}C-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 *H1246MATα*, which lacks all four genes, *ARE1*, *ARE2*, *DGAT1* and *LRO1*, which were found to contribute to TAG synthesis (Sandager et al., 2002). *H1246MATα* 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 *lpcat1* or *lpcat2* T-DNA knock-out mutants and characterized the hemizygous/homozygous and double knockout seed oil profiles.

The *lpcat1* mutant is devoid of any significant oil phenotype compared to its null segregant or to WT (Fig. 6A). Crosses of *AS11* with the *lpcat1* 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 *lpcat1* 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 *dgat1/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 *dgat1/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 *lpcat*1 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), lpcat1 (SALK_123480) and lpcat2 (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 $\mu E \cdot m^{-2} \cdot s^{-1}$) and 8 h dark. Arabidopsis siliques containing mid-green developing seeds (pooled silique 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*, *lpcat1* and *lpcat 2* 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 (Calbiochem, 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-centriguged 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™ reageant 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 $MgCl_2$ in the presence of 100 µM [1-^{14}C] *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:CH_2Cl_2 (2:1:) v/v, and the TLE prepared as described previously (Taylor et al., 1991, 1992a). The ^{14}C-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 ^{14}C-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 ^{14}C 18:1-CoA (specific activity 10 nCi/nmol) as the acyl donor. The ^{14}C-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 60min, 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 $MgCl_2$ in the presence of 6 µM L-α-palmitoyl- [1- ^{3}H methyl] *lyso*-3-phosphatidylcholine (specific activity 60 µCi/nmol; 1.48-2.22 TBq/mmol) and 18 µM [1-^{14}C] 18:1-CoA (specific activity 10 nCi/nmol; 0.37GBq/mmol). Reactions were stopped at each time point by adding Isopropanol: CH_2Cl_2 (2:1:) v/v, and the TLE prepared as described previously (Taylor et al., 1991, 1992a). The ^{14}C 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: $CHCl_3$: MeOH: Formic Acid (88%):H_2O 60: 30 :9 :2; 2nd D: $CHCl_3$: MeOH: Ammonia solution (28%): H_2O 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 ^{3}H/^{14}C 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 ^{14}C 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 $CHCl_3$: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 integrating 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.csb.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 F_1 plants heterozygous for *AS11* and the insertion mutations were identified by PCR using primers listed in Table S2. F_2 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 F_2 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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