Running Title

Triacylglycerol as a dynamic fatty acyl pool

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Title: A cytosolic acyltransferase contributes to triacylglycerol synthesis in sucrose-rescued Arabidopsis seed oil catabolism mutants

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ABSTRACT

Triacylglycerol (TAG) levels and oil bodies persist in sucrose-rescued *Arabidopsis thaliana* seedlings disrupted in seed oil catabolism. This study set out to establish if TAG levels persist as a metabolically inert pool when downstream catabolism is disrupted, or if other mechanisms, such as fatty acid (FA) recycling into TAG are operating. We show that TAG composition changes significantly in sucrose rescued seedlings compared to that found in dry seeds, with 18:2 and 18:3 accumulating. However, 20:1 FA is not efficiently recycled back into TAG in young seedlings, instead partitioning into the membrane lipid fraction and diacylglycerol (DAG). In the lipolysis mutant *sdp1* and the β-oxidation double mutant *acx1acx2*, levels of TAG actually increased in seedlings growing on sucrose. We performed a transcriptomic study and identified up-regulation of an acyltransferase gene, *DGAT3*, with homology to a peanut cytosolic acyltransferase. The acyl-CoA substrate for this acyltransferase accumulates in mutants that are blocked in oil breakdown post-lipolysis. Transient expression in *Nicotiana benthamiana* confirmed involvement in TAG synthesis and specificity towards 18:3 and 18:2 FAs. Double mutant analysis with the peroxisomal ABC transporter mutant *pxal* indicated involvement of DGAT3 in the partitioning of 18:3 into TAG in mutant seedlings growing on sucrose. Fusion of the DGAT3 protein with GFP confirmed localization to the cytosol of *N. benthamiana*. This work has demonstrated active recycling of 18:2 and 18:3 FAs into TAG when seed oil breakdown is blocked in a process involving a soluble cytosolic acyltransferase.
INTRODUCTION

In many plant species triacylglycerols (TAGs) are the major storage lipids, serving as an important energy reserve in seeds for subsequent germination and seedling development. TAGs are also essential for pollen development and sexual reproduction (Slocombe et al., 1994; Wolters-Arts et al., 1998; Zheng et al., 2003). Plant-derived storage lipids are a major feedstock for the food and feed industries as well as the oleochemical and biofuel industries and there continues to be much interest in understanding the regulation of their synthesis in planta (Weselake et al., 2005; Durret et al., 2008; Dyer et al., 2008).

In oilseeds TAG bioassembly is catalysed by the membrane-bound enzymes of the Kennedy pathway that operate in the endoplasmic reticulum (Stymne and Stobart, 1987). Diacylglycerol acyltransferase (DGAT; EC 3.2.1.20) catalyses the final acylation of sn-1,2 diacylglycerol (sn-1,2 DAG) to give TAG and has been suggested as one of the rate-limiting steps in plant storage lipid accumulation (Ichia et al., 1988). The first DGAT gene, a member of the DGAT1 family, was isolated from mouse and was followed by the identification of other DGAT1 members from a number of plant species, including Arabidopsis thaliana (Bouvier-Navé et al., 2000; Hobbs et al., 1999; Routaboul et al., 1999; Zou et al., 1999). A second family of DGAT genes (DGAT2) was first identified in the oleaginous fungus Mortierella ramanniana, but these have no sequence similarity with DGAT1 (Lardizabal et al., 2001) and appear to have a non-redundant function in TAG biosynthesis. Two enzyme activities catalyzing acyl-CoA-independent synthesis of TAG have also been described: phospholipid:diacylglycerol acyltransferase (PDAT) (Dahlqvist et al., 2000) and diacylglycerol:diacylglycerol transacylase (DGTA), which catalyses a transacylation reaction in which the free hydroxyl group of a DAG molecule is acylated with a fatty acid (FA) moiety from a second DAG molecule, forming monoacylglycerol (MAG) and TAG (Mancha and Stymne, 1997; Stobart et al., 1997). To date, however, a DGTA gene has not been isolated from any source. In addition to the membrane-bound pathway for TAG synthesis, an alternate pathway was proposed in peanut. This pathway takes place in the cytosol and involves the acylation of MAG to DAG and the further acylation of DAG to TAG by the action of a cytosolic DGAT (Saha et al., 2006; Tumaney et al., 2001).

Upon germination and early post-germinative growth, FAs derived from storage TAG are converted to sucrose to provide metabolic energy and carbon skeletons for seedling
growth. The mobilization of storage oil involves the coordinated induction of a number of biochemical pathways in different subcellular locations. The first step in oil breakdown is catalyzed by lipases which hydrolyze TAG to produce free FA and glycerol (Huang, 1992). The FA then enters single membrane-bound organelles termed peroxisomes (glyoxysonsomes) where β-oxidation and part of the glyoxylate cycle occurs (Cooper and Beevers, 1969). β-oxidation converts FA to acetyl-CoA, which is subsequently condensed into 4-carbon compounds via the glyoxylate cycle. These 4-carbon compounds are then transported to the mitochondria, where they can either be converted to malate and transported to the cytosol for gluconeogenesis, or used as substrates for respiration.

Disruption of a number of genes involved in oil catabolism results in a partial or complete block in TAG breakdown, defects in seed germination and an inability to establish photosynthetic competence (Graham, 2008). These genes include SUGAR DEPENDENT1 (SDP1), a patatin-like TAG lipase associated with oil body membranes (Eastmond, 2006), PEROXISOMAL ABC TRANSPORTER1 (PX1), involved in the transport of free fatty acids (Fulda et al., 2004; van Roermund et al., 2012) and/or acyl-CoA esters across the peroxisome membrane (Zolman et al., 2001) and those encoding any of the core reactions of the peroxisomal β-oxidation pathway. The first step of β-oxidation is carried out by the acyl-CoA oxidase (ACX) family, which comprises isoenzymes with distinct FA chain-length specificities. Six ACX genes have been identified in the Arabidopsis genome (Graham and Eastmond, 2002). Single mutants disrupted in each of the six ACX genes have been described and seedling establishment and lipid breakdown were unaffected, probably due to the overlapping substrate specificities of the gene products (Adham et al., 2005; Eastmond et al., 2000; Rylott et al., 2003). However, the acx1acx2 double mutant, which lacks the medium- to long-chain and the long-chain acyl-CoA oxidases respectively, shows a sucrose-dependent seedling establishment phenotype, indicating that long-chain acyl-CoA oxidases are essential for seedling establishment (Pinfield-Wells et al., 2005). The second step of peroxisomal β-oxidation consists of four separate reactions, two of which (2-trans enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase) are core activities required for the catabolism of all FA. Both activities are contained on MULTIFUNCTIONAL PROTEIN (MFP). Arabidopsis contains two isoforms of MFP. AIM1 is expressed at a low level in germinating seedlings and the aim1 mutant exhibits normal germination and seedling establishment but does have an altered meristem mature plant phenotype (Richmond and Bleecker, 1999). In contrast, MFP2, the gene encoding the
second isoform, is strongly induced during post-germinative seedling growth (Eastmond and Graham, 2000). The Arabidopsis mfp2 mutant requires an exogenous supply of sucrose for seedling establishment and is compromised in storage oil breakdown (Rylott et al., 2006). The enzyme 3-ketoacyl-CoA thiolase (KAT; EC 2.3.1.16) catalyzes the last step of FA β-oxidation. The Arabidopsis genome contains three loci that encode KAT enzymes, annotated as KAT1, KAT2 and KAT5. KAT2 is the only one of the three KAT genes expressed at significant levels during seed germination in Arabidopsis, and the kat2 mutant is blocked in storage oil breakdown and is dependent on exogenous sucrose for seedling establishment (Germain et al., 2001).

TAG oil bodies persist in sucrose-rescued seedlings of these various mutants but it is not known if the total amount and composition of TAG molecular species in the oil bodies is the same as those in mature seeds or if they reflect the FA composition of green tissues as is the case with de-novo synthesized TAG in leaves (Kunz et al., 2009; Slocombe et al., 2009). If there is feedback inhibition of lipolysis then TAG composition reflecting that in mature seed would be expected to persist in sucrose-rescued seedlings. If, on the other hand there is a mechanism for TAG recycling distinct from de novo synthesis in developing seeds, TAG composition might be expected to change. We report here that FA recycling does operate when FA breakdown is blocked post-lipolysis and this recycling is mediated to a significant extent by a previously uncharacterised cytosolic route for TAG synthesis involving an acyl-CoA dependent cytosolic acyltransferase.

RESULTS

Triacylglycerol synthesis and recycling in sucrose-rescued mutant seedlings

Total TAG content in sucrose-rescued mutants disrupted in TAG breakdown (sdp1; Eastmond, 2006), FA transport across the peroxisome membrane (pxa1; Zolman et al, 2001) and the core reactions of β-oxidation (acx1acx2; Pinfield-Wells et al, 2005, mfp2; Rylott et al, 2006 and kat2-2; Eastmond, 2006) are shown in Figure 1. While TAG was
almost completely catabolized in Col0 seedlings maintained on sucrose at 5 Days After Imbibition (DAI), the total TAG content in 5-DAI sucrose-rescued mutant seedlings showed smaller changes compared to dry seed (DS) and in some cases increases (Fig. 1). These results are consistent with published data on total FA composition and eicosenoic acid levels (a marker for seed storage TAG) and confirm these mutants are severely impaired in their ability to catabolize seed storage TAG (Eastmond, 2006; Germain et al., 2001; Pinfield-Wells et al., 2005; Rylott et al., 2006; Zolman et al., 2001). The increased TAG levels in sdp1 and acx1acx2 5-DAI seedlings suggest de novo TAG synthesis is occurring in these mutant seedlings. The slight decrease in mfp2 5-DAI seedlings compared to DS is consistent with this mutant not being completely blocked in FA breakdown as previously reported based on eicosenoic acid levels (Rylott et al., 2006).

Compositional analysis of individual TAG species confirmed that for Col0 all of the identified TAGs decreased to near undetectable levels in 5-DAI sucrose-rescued seedlings compared to DS, in marked contrast to the oil breakdown mutants (Fig. 2). In sdp1 and acx1acx2 sucrose-rescued 5-DAI seedlings the levels of most of the TAG species including those containing 20:1 actually increased. In kat2-2 sucrose-rescued 5-DAI seedlings the majority of TAG species were at similar levels compared to the corresponding DS and in mfp2 TAG species content was slightly lower than in DS. Interestingly, in pxa1 sucrose-rescued 5-DAI seedlings we observed an increase in TAG species containing 18:3 and to a lesser extent 18:2 (Fig. 2C). These results indicate that blocking different steps in TAG catabolism affects not only TAG content in 5-DAI seedlings, but also TAG composition.

In germinating seedlings the FAs released from oil bodies are activated to acyl-CoAs before they are catabolized by peroxisomal FA β-oxidation (Fulda et al., 2004). To investigate how the acyl-CoA pool size and composition changes in concert with the apparent decrease in TAG breakdown observed in the mutants, we analyzed the acyl-CoA content in seedlings. In sdp1 the total acyl-CoA content in 5-DAI seedlings was similar to Col0 (Fig. 3A). In contrast, we observed that in pxa1 and all β-oxidation mutants there was an accumulation of acyl-CoAs in 5-DAI seedlings compared to Col0. Furthermore, the acyl-CoA profile showed a relative enrichment in long-chain acyl-CoAs, especially 20:1-CoA, in pxa1 and β-oxidation mutants (Fig. 3B). These results confirm that sdp1 is impaired in lipolysis, as has been reported by Eastmond (2006), while in pxa1 and the β-oxidation mutants lipolysis is not impaired and FAs are activated to acyl-CoAs but further catabolism is compromised. In the case of pxa1, the data suggests efficient incorporation of
de novo or recycled 18:2 and 18:3 FA/acyl CoAs into TAG but poor utilization of accumulated 20:1 acyl-CoA.

An alternative pathway for TAG synthesis in pxa1 during seedling establishment.

The observation that impairing the peroxisomal ABC transporter function affects TAG and acyl-CoA pool content and composition suggests that other lipid pools may also be affected. To investigate this we analyzed DAG and polar lipid composition in Col0 and pxa1 DS and 5-DAI seedlings. The only difference we observed in DS was a slightly higher DAG content in pxa1 relative to Col0 (Fig. 4A). However, in 5-DAI seedlings there was a general increase in microsomal lipids and a decrease in galactolipids in pxa1 compared to Col0 (Fig. 4B). The lipid FA composition in pxa1 5-DAI seedlings showed an increase in 20:1 content in DAG, phosphatidic acid (PA), phosphatidylcholine (PC) and phosphatidyethanolamine (PE) compared to Col0, while there were no significant differences in 18:2 and 18:3 content between pxa1 and Col0 in any of the polar lipids analysed (Table 1).

In Arabidopsis, diacylglycerol acyltransferase 1 (DGAT1) has been described as the main enzyme responsible for TAG synthesis and accumulation during seed development (Lu et al., 2003; Lung and Weselake, 2006) and 20:1 is specifically found in TAG (Lemieux et al., 1990). However, our results imply that in pxa1 germinating seeds, where FA are released from TAG and activated to acyl-CoA but not catabolized via β-oxidation, 20:1-CoA is transferred to microsomal lipids, while 18:2-CoA and 18:3-CoA are preferentially incorporated into TAG. This finding suggests that an alternative pathway that prefers 18:2 and 18:3 over 20:1 for TAG synthesis is operating during pxa1 seedling establishment.

To further investigate the putative alternative pathway for TAG synthesis during pxa1 seedling establishment, we performed a transcriptomic study of Col0 and pxa1-1 germinating seeds at 12 hours after imbibition (HAI) and 5-DAI seedlings using the Affymetrix ATH1 microarray. Table 2 shows the expression levels of genes identified to be involved in lipid synthesis and catabolism (Li-Beisson et al., 2010). Only three genes show a greater than 2-fold change in pxa1 compared to Col0: LPEAT2, ACXI and DGAT3. Of these the DGAT3 gene (At1g48300) is potentially interesting since it shows greater homology to a peanut cDGAT, than to AtDGAT1 or AtDGAT2 (Fig. S1). Quantitative RT-
PCR analysis showed that while DGAT1, DGAT2 and PDAT1 gene expression levels in pxa1 were similar to Col0 during seed germination and seedling establishment, DGAT3 expression in pxa1 12-HAI seeds was 2-fold more abundant than in Col0 (Fig. S2). These data suggest that DGAT3 could have a role in an alternative pathway for TAG synthesis during pxa1 seedling establishment. Analysis of publicly available Affymetrix data revealed DGAT3 expression throughout seed development and seedling establishment in Col0 (Fig. S3A). Although DGAT1 showed the highest expression levels in developing seeds during the period of maximum TAG accumulation, DGAT3 expression levels were higher than DGAT1, DGAT2 or PDAT1 during seed germination and the latter stages of seedling establishment. Therefore it is possible that in pxa1 the block in the import of FA into the peroxisome leads to an increase of acyl-CoA substrate for the putative DGAT3, and the incorporation of acyl-CoA into TAG, with a preference for 18:2 and 18:3 over 20:1. We used the Genevestigator Development tool (Hruz et al., 2008) to compare the expression levels of DGAT1, DGAT2, DGAT3 and PDAT1 in Affymetrix datasets in the public domain. We found that DGAT3 shows strong expression across ten development stages that is consistently higher than the other three genes apart from the senescence stage, where DGAT1 is more strongly expressed (Fig. S3B).

A novel cytosolic diacylglycerol acyltransferase is encoded by At1g48300

To test whether the putative DGAT3 gene encodes for a cytosolic DGAT, we performed Agrobacterium-mediated transient expression in Nicotiana benthamiana leaves, using AtDGAT1 as a positive control. Five days after Agrobacterium infiltration, leaves were sampled and TAGs isolated and analyzed directly from fresh-frozen tissue. The results showed a significant increase in TAG content in N. benthamiana leaves expressing either DGAT3 or DGAT1 genes, compared to leaves from the empty vector (ev) negative control (Fig. 5A). These results support our homology-based assumption that the DGAT3 gene (At1g48300) encodes a DGAT enzyme, although its overall activity appears lower than DGAT1. Trilinolenin (LnLnLn) stood out in this analysis as the only TAG accumulating to higher levels in DGAT3 than DGAT1 (Fig 5B). Indeed, TAG composition analysis revealed an enrichment of TAGs containing 18:3 for DGAT3 compared to DGAT1, with the latter appearing to preferentially incorporate FA other than 18:3 into TAG (Fig. 5B).
We obtained, from the Nottingham Arabidopsis Stock Centre (NASC), an Arabidopsis GABI-Kat insertion line (GABI_696F08) which carries a T-DNA at the position 336 of the *DGAT3* ORF. Homozygous plants were obtained by self-pollination and confirmed by PCR. No obvious morphological or developmental differences compared to Col0 were observed under standard growth room conditions. Quantification of TAG, DAG and galactolipids in DS and 5-DAI sucrose-grown seedlings of this mutant, which we now refer to as *dgat3*, showed no differences compared with Col0 (Fig. S4). However, compared to *pxa1*, a *pxa1dgat3* double mutant did show a significant decrease in the amount of 18:3 FA derived from TAGs in 5-DAI sucrose-grown seedlings (Fig. 6), providing further evidence that DGAT3 contributes to recycling 18:3 into TAG.

To compare the localization of DGAT3 with that of DGAT1, which is known to be localized to the endoplasmic reticulum (Browse and Somerville, 1991), the genes were cloned and fused at their C-termini with green fluorescence protein (GFP). The fused genes were transiently expressed in *N. benthamiana* leaves, which were analysed for fluorescence patterns using confocal microscopy. As shown in Fig. 7, the transiently expressed gene products presented a different pattern. While leaves expressing DGAT1-GFP displayed strong fluorescence that localized to a reticular network characteristic of the endoplasmic reticulum, the more diffuse fluorescence pattern observed for the transiently expressed DGAT3-GFP can be taken as evidence that this enzyme is mainly cytosolic. There is precedence for this ER vs cytosolic labeling pattern in *N. benthamiana* leaves (Grefen et al., 2008).

These results indicate that there is a cytosolic route for TAG synthesis, where the DGAT3, encoded by At1g48300, preferentially incorporates 18:3 and to a lesser extent 18:2 into TAG.

**DISCUSSION**

Storage oil mobilization during seed germination involves the action of TAG-lipases, FA transport across the peroxisome membrane and peroxisomal FA β-oxidation to produce acetyl-CoA, which ultimately provides the carbon skeletons and energy necessary to drive post-germinative growth (Graham, 2008). It is well established that Arabidopsis mutants
disrupted in lipolysis, FA transport into the peroxisome and peroxisomal β-oxidation are unable to catabolize TAG and require exogenous sucrose for post-germinative growth and seedling establishment (Hayashi et al., 1998; Eastmond, 2006). This paper investigates how the disruption in different steps in the storage oil mobilization process affects not only overall TAG levels, but also TAG composition.

For this purpose, we analyzed Arabidopsis mutants with defects in TAG and FA breakdown during seed germination and seedling establishment. All the mutants studied were severely impaired in their ability to catabolize TAG as reported previously (Eastmond, 2006; Germain et al, 2001; Pinfield-Wells et al, 2005; Rylott et al, 2006; Zolman et al, 2001). Among the various mutants analysed we only observed a slight decrease in TAG levels in mfp2 seedlings grown for 5 days on sucrose compared to DS. This partial block in TAG breakdown in mfp2 is likely because additional hydratases and dehydrogenases are contributing to MFP activity during seedling establishment. (Rylott et al., 2006). In sdp1 the patatin-like TAG lipase associated with oil body membranes is blocked, and therefore TAGs are not hydrolyzed (Eastmond, 2006). In the pxa1 and β-oxidation mutants FA breakdown is impaired due to FA transport into the peroxisome or FA β-oxidation inside the peroxisome being disrupted, respectively.

Inhibition of lipolysis is one obvious explanation for the persistence of TAGs in sucrose-rescued mutant seedlings. TAG-lipase inhibition has been reported previously by Eastmond (2007) in the sdp2 mutant, which is disrupted in the peroxisomal membrane isoform of monodehydroascorbate reductase (MDAR4). The main role of MDAR4 appears to be to prevent H₂O₂ from escaping beyond the outer surface of the peroxisomal membrane; the consequence of H₂O₂ escape being inactivation of SDP1. While we cannot rule out some degree of post-transcriptional feedback inhibition at the level of lipolysis of TAGs, our results clearly demonstrate that available FA are being actively sequestered into TAG in sucrose-rescued mutant seedlings.

The first indication that de novo TAG synthesis is occurring during post-germinative seedling growth on sucrose came from our finding that TAG levels actually increased in 5-DAI seedlings compared to DS in both the sdp1 and acx1acx2 mutants (Fig.1A). This TAG synthesis may use a combination of FA and acyl-CoA substrates made available from partially catabolised TAGs and turnover of other glycerolipids, and de novo FA synthesis.
Sucrose has been shown to induce DGAT activity in cell suspension cultures of *Brassica napus*, with relatively little alteration in FA composition (Nykiforuk *et al*., 2002; Weselake *et al*., 1998). In addition, glucose has been reported to up-regulate DGAT1 gene expression levels when Arabidopsis seedlings are grown on supplemented medium (Lu *et al*., 2003). Sugar-induced TAG synthesis could be operating here but it is not clear why the effect is specific to *sdpl* and *acxlacx2* and is not seen in *pxa1* and *kat2-2* which are similarly blocked in FA breakdown.

Evidence for TAG recycling comes from the modifications that we see in *pxa1* 5-DAI seedling TAG composition, which indicates that FAs are released from and re-incorporated into TAG during seed germination and post-germinative growth, possibly with additional *de novo* synthesized 18:2 and 18:3 FA. Although long-chain acyl-CoA levels were increased in *pxa1* and the β-oxidation mutants we did not observe evidence, in the form of an altered TAG composition, for FA recycling in the latter. This result could be explained by the assumption that β-oxidation mutants accumulate acyl-CoAs inside the peroxisome, and therefore they are not available for TAG synthesis, whilst *pxa1* accumulates acyl-CoAs in the cytosol. Graham and Eastmond (2002) proposed that the increase in peroxisome size in *pedl* and *kat2-2* mutants might be due specifically to a peroxisomal accumulation of acyl-CoAs. This is in agreement with Pinfield-Wells *et al*. (2005) and Rylott *et al*. (2006) who observed an accumulation of long-chain acyl-CoAs and an increase in peroxisome size in *acxlacx2* and *mfp2* seedlings, respectively. On the other hand, the peroxisome size and structure remains normal in *cts* and *ped3* seedlings (Footitt *et al*., 2002; Hayashi *et al*., 2002), which are allelic to *pxa1*, further supporting the assumption that accumulation of acyl-CoA in *pxa1* occurs in the cytosol, where they are available for TAG synthesis.

The most striking observation with regard to specificity is that 18:3, and to a lesser extent 18:2, are preferentially incorporated into TAG in *pxa1* seedlings, whereas the acyl-CoA pool in the cytosol is enriched in 20:1 acyl-CoA. Interestingly, this 20:1 acyl-CoA is instead incorporated into microsomal lipids, which are increased in *pxa1* seedlings compared to Col0. One possible explanation for this could be that in *pxa1* seedlings an enzyme that prefers 18:3 over 20:1 is actively incorporating cytosolic acyl-CoAs into TAG. Lung and Weselake (2006) described that DGAT1 is the main enzyme responsible for TAG synthesis during seed development, with DGAT1 gene expression peaking during the period of maximum TAG accumulation. However, DGAT1 gene expression is not
restricted to developing seeds as it is also expressed albeit at lower levels during seed germination and seedling establishment and it has been shown to be involved in the production of TAG in mature leaf tissue (Slocombe et al., 2009). In Arabidopsis 20:1 is specifically found in storage TAG in seeds (Lemieux et al., 1990), indicating that DGAT1 preferentially incorporates these FAs into TAG. In addition, in as11 which carries an EMS mutation in the TAG1 gene, the reduction in TAG accumulation during seed development is accompanied by decreased 20:1 and 18:1 levels and an increase in 18:3 (Katavic et al., 1995). In agreement with these results, Andrianov et al. (2010) reported that the overexpression of AtDGAT1 in tobacco leaves leads to an increase in TAG content and a shift in the FA composition, with an increase in 18:1 and a decrease in 18:3 levels. Taken together, these data suggest that an alternative pathway for TAG synthesis is responsible for the incorporation of 18:3 into TAG, since DGAT1 shows a preference for FAs other than 18:3.

Transcriptomic analysis in Col0 and pxa1 revealed an up-regulation of the AtDGAT3 (At1g48300) gene in 12 HAI seeds of pxa1. This gene is a homolog of the gene encoding the cytosolic DGAT from peanut (Saha et al., 2006) and is therefore a candidate for involvement in partitioning acyl-CoAs to TAG in pxa1 seedlings. In peanut, the cytosolic DGAT catalyzes the acylation of sn-1,2 DAG (Saha et al., 2006). AtDGAT3 is expressed in Col0 germinating seed and young seedlings at higher levels than other genes involved in TAG synthesis, such as DGAT1, DGAT2 and PDAT1. In addition, we observed that the transiently expressed cytosolic DGAT in N. benthamiana leaves has higher preference for 18:2 and 18:3, the main FA in young seedlings, than DGAT1. Consistent with this, the pxa1dgat3 double mutant had significantly decreased levels of 18:3 in TAG compared with pxa1 in 5 day old seedlings grown on sugar. The remaining levels of 18:3 in pxa1dgat3 were still significant suggesting an additional pathway for incorporation into TAG is also working. Zang et al. (2009) showed that PDAT1 is the gene responsible for most of the TAG synthesis in the dgat1-1 mutant, indicating that DGAT1 and PDAT1 have overlapping functions for TAG synthesis in seed and pollen of Arabidopsis. The fact that the dgat1-1 mutant showed a 20 to 30% decrease in oil content (Katavic et al., 1995) while no changes of oil in pdat1-1 were observed (Mhaske et al., 2005) might suggest that DGAT1 can completely compensate for the lack of PDAT1 function, whereas PDAT1 only partially complements the function of DGAT1 in developing seeds. Partial complementation of DGAT3 by PDAT1 could be occurring in the pxa1dgat3 double
mutant, where PDAT1 would be responsible for the incorporation of 18:3 into TAG in germinating seeds and young seedlings. Despite numerous attempts, we were unable to isolate pdat1pxa1 double mutants by selfing plants that were either homozygous for pxa1 and heterozygous for pdat1 or vice versa. We previously showed that DGAT1 plays a major role in the partitioning of FAs to TAG in mature and senescing leaves of Col0 and pxa1 plants (Slocombe et al., 2009). The double mutant disrupted in DGAT1 and PXA1 is severely compromised in vegetative growth highlighting the negative effect that perturbation of these pathways can have on plant growth (Kunz et al., 2009; Slocombe et al., 2009). We have been able to uncover the in-vivo function of DGAT3 because pxa1 seedlings accumulate acyl-CoAs and this together with an induction of DGAT3 expression, leads to the synthesis of TAG species containing 18:3 and 18:2 FAs. The incorporation of 20:1 into microsomal lipids in young seedlings of pxa1 suggests that DGAT1 activity is not operating as it does in developing seeds or mature leaves. It therefore appears that the relative contribution of the different routes for incorporation of FAs into TAG varies in different tissues.

Taken together these data allow us to propose an in-vivo role for the cytosolic DGAT in recycling of 18:3 to TAG via a previously uncharacterized cytosolic pathway. The consistently high level of expression of DGAT3 across various developmental stages suggests a housekeeping function associated with regulating flux between the cytosolic acyl CoA pool and TAG. In young seedling the biosynthetic machinery is directed primarily towards the production of membrane lipids. In such a case we propose that TAGs are involved as a dynamic fatty acyl pool with DGAT3 playing a role in regulating acyl-CoA pool size and composition in response to the needs of membrane lipid biosynthesis. Cytosolic oil droplets are well documented in the literature including for example a field survey of 302 angiosperm species which found that 24% had conspicuous cytosolic oil droplets in leaves (Lersten et al., 2006) and we have also reported TAG present in oil droplets in Arabidopsis leaves (Slocombe et al., 2009). A role for cytosolic leaf TAG in carbon storage and/or membrane lipid re-modeling has previously been proposed (Murphy and Parker, 1984; Murphy, 2001; Kaup et al., 2002; Lin and Oliver, 2008). Our data suggest a cytosolic pathway to TAG involving DGAT3 plays a key role in this important aspect of lipid metabolism.
METHODS

Plant Material and growth conditions

Wild-type *Arabidopsis thaliana* ecotype Columbia (Col-0) and mutants (Col-0 background) seeds were surface-sterilized and germinated in half-strength Murashige and Skoog (½ MS) media (Murashige and Skoog, 1962) containing 1% (w/v) agar and 20 mM sucrose. After a cold treatment of 72 h at 4°C in the dark, plates were transferred to a growth room at 20°C with continuous light (70 µM m⁻² sec⁻¹). Twelve hours after imbibition seeds and 5 days old seedlings were harvested, frozen with liquid nitrogen and stored at -80°C. *sdp1* and *kat2* in the Col-0 background (herein referred to as *kat2*-2) were isolated in a sugar-dependent screen (Eastmond, 2006). *pxa1*-1 and *acx1*-2*acx2*-1 seeds were kindly donated by Dr Bonnie Bartel and *mfp2* was obtained from the Salk collection (http://signal.salk.edu/cgi-bin/tdnaexpress). *dgat3* is a GABI-kat insertion line (GABI_696F08) obtained from NASC, which carries a T-DNA in the At1g48300 gene. The homozygous *pxa1*-1*dgat3* double mutant was confirmed for the *pxa1* mutation by the *pxa1* phenotype of failure to undergo successful seedling establishment without sucrose (Zolman et al., 2001) and for the *dgat3* mutation by PCR using *DGAT3* specific primers LH9 and LH10 (Table S1) and T-DNA specific primer GABI-left: 5’-CCCATTTGGACGTGAATGTAGACAC-3’.

Lipid analysis

Lipid extraction and neutral lipid analysis by LC/MS/MS were performed as previously described (Burgal et al., 2008). Polar lipids were separated by two-dimensional TLC (Hernandez et al., 2008). Fatty acid methyl esters (FAMEs) of individual lipids classes were produced by acyl-catalysed trans-methylation (Browse et al., 1986) and analysed by gas-chromatography with flame ionization detection (GC8000 Top, Thermoquest Separation Products, Manchester, UK), fitted with a 30 m long 0.25 mm ID SGE BPX70 column (SGE, Milton Keynes, UK). Helio was used as a carrier gas at 1 ml min⁻¹ with a 30:1 split ratio. The oven was run isothermally at 110°C for 1 min, then ramped to 180°C at 20°C min⁻¹ then to 221°C at 2.5°C min⁻¹.
The acyl-CoA profile was measured using the method of Larson and Graham (2001) with modifications described in Larson et al. (2002).

**RNA purification and cDNA synthesis**

Total RNA isolation from dry and imbibed seeds was performed using solutions previously treated with diethyl pyrocarbonate (DEPC) to inhibit RNases. Approximately 200 seeds were ground with liquid nitrogen using a blue pestle. After homogenization, 150 µl of extraction buffer (0.2 M sodium borate decahydrate, 30 mM EGTA, 1% (w/v) SDS and 1% (w/v) sodium deoxycholate), 10mM dithiothreitol, 2% (w/v) polyvinyl pyrrolidone and 1% (v/v) IGEPAL were added. After adding 6 µl of proteinase K (Roche Diagnostics, Indianapolis, USA), samples were mixed and incubated at 42°C for 90 min. After the incubation, 12 µl of 2M KCl were added, samples were mixed and incubated on ice for 60 min. To remove debris samples were centrifuged at 15000 g for 20 min at 4°C. The supernatant was transferred to a fresh tube and 54 µl of 8M LiCl were added. Samples were mixed and incubated at -20°C for 3h. After the incubation, samples were centrifuged at 15000 g for 20 min at 4°C and the RNA pellet was dissolved in 100 µl RNase-free water. RNA was purified by RNeasy Plant Mini Kit (QIAGEN).

Total RNA was isolated from 5 days old seedling using the RNeasy Plant Mini Kit (QIAGEN).

The quality of RNA was verified by demonstration of intact ribosomal bands following agarose gel electrophoresis in addition to the absorbance ratios (A_{260/280} and A_{260/230}). Contaminating DNA was removed from RNA samples (1 µg) using the TURBO DNA-free kit (Ambion, USA). First strand cDNA was synthesized from 0.5 µg DNA-free total RNA using the SuperScript™ III First-Strand Synthesis System (Invitrogen, USA) with oligo (dT)_{20} primer, following the manufacturer’s instructions.

**Affymetrix Genechip experiment and data analysis**

Isolated RNA was used for cDNA synthesis and biotin-modified RNA amplification using the MessageAmp™ III RNA Amplification Kit (Ambion, Austin, TX, USA). Three
biological replicates per sample were hybridized independently to the Affymetrix ATH1 array, washed, stained and scanned following the procedures described in the Affymetrix technical manual. The expression levels of genes were measured by signal intensities using the Micro Array Suite 5.0 software with a target signal of 500. Public domain Affymetrix ATH1 data sets were obtained from NascArrays (http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl) and TAIR (http://www.arabidopsis.org/). The Genevestigator V4 Classic Metaprofile analysis tool (available at https://www.genevestigator.com) was used to compare expression levels of DGAT1 (At2g19450), DGAT2 (At3g51520), DGAT3 (At1g48300) and PDAT1 (At5g13640) across ten different developmental stages.

**Quantitative real time PCR**

Gene expression analysis was performed by quantitative real time PCR (qRT-PCR) using an ABI Prism 7000 thermal cycler and the “SYBR-Green PCR Master Mix” (Applied Biosystems, Foster City, CA, USA). Primers for gene-specific amplification (Table S1) were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to generate a product of 100-200 bp, and to have a Tm (melting temperature) of 60±1 °C and a length of 19-23 bp. Reaction mix (25 μl per well) contained 1 x SYBR-Green PCR Master Mix, 400 nM forward and reverse primers, and 1 μl of cDNA diluted ten times, which was selected according to the primers amplification efficiency. The thermal cycling conditions consisted of an initial denaturation step of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The specificity of the PCR amplification was monitored by melting curve analysis following the final step of the PCR. PCR efficiencies (E) of all primers were calculated using dilution curves with four dilution points, and the equation $E = \left[10^{(-1/slope)}\right] - 1$. The CITRATE SYNTHASE 3 gene (CSY3) was used as the endogenous reference since it has similar levels of expression as the genes of interest in both wild type and pxa1. The real time PCR data were calibrated relative to the corresponding gene expression level in Col-0, following the $2^{-\Delta\Delta Ct}$ method for relative quantification (Livak and Schmittgen, 2001). The data is presented as means ± SD of three biological replicates, each having three replicates per plate.

**Transient expression in Nicotiana benthamiana**
For *Agrobacterium*-mediated CaMV35S-driven transient expression, the *DGAT3* and *DGAT1* coding sequences were PCR-amplified using the specific primers LH47, LH48 and LH35, LH36, respectively (Table S1) and subcloned into the GATEWAY-compatible binary vector pH2GW7 (for TAG analysis) or pK7FWG2 (for subcellular localization) (Karimi et al., 2002). The resulting constructs and the empty vector used as a control were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cultures (OD$_{600}$ ≈ 0.8) according to Voinnet et al. (2003) omitting the acetosyringone treatment. Samples were collected five days after infiltration, ground in liquid nitrogen and stored at –80 ºC until TAG analysis.

For subcellular localization, fresh leaf imaging was carried out on a Zeiss LSM 510 META laser scanning confocal equipped with a Zeiss Axioplan 2 microscope (Carl Zeiss Ltd, Welwyn Garden City, UK). Images were acquired using a Plan-Neofluar 20x/0.5 or a Plan-Apochromat 63x/1.4 Oil immersion DIC objective. Imaging of GFP emission was performed by sequential scanning. GFP was excited with the 488nm line of a 30mW argon laser and the emission collected through a 505-530bp emission filter. Images were taken at Nyquist resolution with 8 line averaging.

**Supplemental Material**

**Figure S1.** Comparison of the deduced amino acid sequences of *Arachis hypogaea DGAT3* and Arabidopsis *DGAT1* (At2g19450), *DGAT2* (At3g51520) and *DGAT3* (At1g48300) genes.

**Figure S2.** Quantitative gene expression profiles of TAG synthesis genes during seed germination and seedling establishment in *pxa1*-1 relative to Col0.

**Figure S3.** Affymetrix ATH-1 Selected TAG synthesis gene expression levels in Col0

**Figure S4.** Total TAG, DAG and MGDG content from Col0 and *dgat3* DS and 5-DAI seedlings.

**Table S1.** Locus names and sequences of primers pairs used for gene expression analysis by qRT-PCR in the present study.
ACKNOWLEDGEMENTS

We thank Dr Peter Eastmond for providing seeds of *sdp1* and Dr Bonnie Bartel for providing seeds of *pxa1*-1 and *acx1*-2*acx2*-1. We thank the University of York Department of Biology Technology Facility for assistance with confocal microscopy.

Distribution of materials

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the Journal policy described in the Instructions for Authors (http://www.plantphysiol.org) is: Ian A. Graham (ian.graham@york.ac.uk).

REFERENCES:


Cooper TG and Beevers H (1969) β-oxidation in glyoxysomes from castor bean endosperm. J. Biol. Chem 244: 3514-3520


Hayashi M, Toriyama K, Kondo M and Nishimura M (1998) 2,4-Dichlorophenoxybutyric acid-resistant mutants of Arabidopsis have defects in glyoxyosomal fatty acid β-oxidation. Plant Cell 10: 183-195


Murphy DJ (2001) The biogenesis and functions of lipid bodies in animals, plants and microorganisms. Prog Lipid Res 40: 325–438


Zhang M, Fan J, Taylor DC and Ohlrogge JB (2009) DGAT1 and PDAT1 acyltransferases have overlapping functions in Arabidopsis triacylglycerol biosynthesis and are essential for normal pollen and seed development. Plant Cell 21: 3885-3901


Figure Legends

Figure 1. Total TAG content of Col0 and mutant dry seeds (DS) and 5-days after imbibition (DAI) seedlings. Values are means ± SD of measurements from five separate batches of 50 seeds or 30 seedlings. Significant differences (Student’s t-test, p < 0.05) are indicated by an asterisk.

Figure 2. TAG composition of Col0 and mutant dry seeds (DS) and 5-days after imbibition (DAI) seedlings. Values are means ± SD of measurements from five separate batches of 50 seeds or 30 seedlings. TAG molecular species are given as three concatenated FA codes, with FAs coded as follows: P=16:0, H=16:3, S=18:0, O=18:1, L=18:2, Ln=18:3, Ar=20:0, G=20:1, Gi=20:2, Be=22:0, Er=22:1. Coding order reflects molecular composition only and does not imply regiospecific arrangements. Significant differences (Student’s t-test, p < 0.05) were performed for selected pxa1 TAGs and are indicated by an asterisk.

Figure 3. Total acyl-CoA content in Col0 and mutant dry seeds (DS) and 5-days after imbibition (DAI) seedlings (A) and acyl-CoA composition in 5-DAI seedlings (B). Values are means ± SD of measurements from five separate batches of 30 seedlings.

Figure 4. Glycerolipid content of Col0 and pxa1 dry seeds (DS) (A) and 5-days after imbibition (DAI) seedlings (B). Values are means ± SD of measurements from five
separate batches of 200 seeds or 100 seedlings. Lipid classes are abbreviated as follows: DAG=diacylglycerol, PC=phosphatidylcholine, PE=phosphatidylethanolamine, PA=phosphatidic acid, DGDG=digalactosyldiacylgalactolipid, MGDG=monogalactosyldiacylgalactolipid.

**Figure 5.** Total TAG content (A) and composition (B) of *N. benthamiana* leaves transiently expressing DGAT1 or DGAT3 compared to the empty vector (ev) negative control. Values are means ± SD of measurements from five samples of ≈40 mg leaf tissue. Total TAGs (A) were analysed by ANOVA followed by pairwise *t*-tests with Bonferroni correction; significantly different groupings (*p* < 0.05) are indicated by letters above the bars. For TAG composition (B), the same tests were performed for each individual TAG; only those species where DGAT3 was significantly different from the empty vector (ev) control are labeled. The triolein (LnLnLn) TAG species denoted by an asterisk is the only one that is higher in DGAT3 compared to DGAT1.

**Figure 6.** FA composition calculated from TAG in Col0, *dgat3*, *pxa1* and *pxa1dgat3* 5-days after imbibition (DAI) seedlings. Values are means ± SD of measurements from five separate batches of 30 seedlings. Significant differences (*p* < 0.05) between *pxa1* and *pxa1dgat3* are indicated by an asterisk.

**Figure 7.** Subcellular localization of DGAT1-GFP (A) and DGAT3-GFP (B) fusion proteins by transient expression in *N. benthamiana* leaves. Scale as indicated.

**Table 1.** DAG and polar lipid fatty acid composition in 5-days after imbibition (DAI) seedlings. Values are means ± SD of measurements from five separate batches of 100 seedlings.

**Table 2.** Expression of lipid synthesis and mobilization-related genes in 12-hours after imbibition (HAI) seeds and 5-days after imbibition (DAI) seedlings using Affymetrix ATH1 microarray data.
Table 1. DAG and polar lipid fatty acid composition in 5-day after imbibition (DAI) seedlings. Values are means ± SD of measurements from five separate batches of 100 seedlings.

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Seed type</th>
<th>FATTY ACID COMPOSITION (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>16:0</td>
</tr>
<tr>
<td>DAG</td>
<td>Col0</td>
<td>19.19 ± 1.66</td>
</tr>
<tr>
<td></td>
<td>pxa1</td>
<td>7.92 ± 0.39</td>
</tr>
<tr>
<td></td>
<td></td>
<td>26.29 ± 2.28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19.17 ± 1.55</td>
</tr>
<tr>
<td>PC</td>
<td>Col0</td>
<td>27.42 ± 1.59</td>
</tr>
<tr>
<td></td>
<td>pxa1</td>
<td>21.82 ± 0.99</td>
</tr>
<tr>
<td>PE</td>
<td>Col0</td>
<td>24.83 ± 1.88</td>
</tr>
<tr>
<td></td>
<td>pxa1</td>
<td>17.65 ± 1.50</td>
</tr>
<tr>
<td>PA</td>
<td>Col0</td>
<td>13.45 ± 2.25</td>
</tr>
<tr>
<td></td>
<td>pxa1</td>
<td>11.35 ± 1.70</td>
</tr>
<tr>
<td>DGDG</td>
<td>Col0</td>
<td>2.95 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>pxa1</td>
<td>3.15 ± 0.28</td>
</tr>
</tbody>
</table>

*p Indicates significantly different from Col0 (P < 0.05) by ANOVA test
Table 2. Expression of lipid synthesis and mobilization-related genes in 12-hours after imbibition (HAI) seeds and 5-days after imbibition (DAI) seedlings using Affymetrix ATH1 microarray data.
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<table>
<thead>
<tr>
<th>Strain</th>
<th>Total TAG (ng/seed or seedling)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Col0</td>
<td></td>
</tr>
<tr>
<td>sdp1</td>
<td></td>
</tr>
<tr>
<td>pxa1</td>
<td></td>
</tr>
<tr>
<td>acx1</td>
<td></td>
</tr>
<tr>
<td>acx2</td>
<td></td>
</tr>
<tr>
<td>mfp2</td>
<td></td>
</tr>
<tr>
<td>kat2-2</td>
<td></td>
</tr>
<tr>
<td>DS</td>
<td></td>
</tr>
<tr>
<td>5-DAI seedlings</td>
<td></td>
</tr>
</tbody>
</table>
Figure 2. TAG composition of Col0 and mutant dry seeds (DS) and 5-days after imbibition (DAI) seedlings. Values are means ± SD of measurements from five separate batches of 50 seeds or 30 seedlings. TAG molecular species are given as three concatenated FA codes, with FAs coded as follows: P=16:0, H=16:3, S=18:0, O=18:1, L=18:2, Ln=18:3, Ar=20:0, G=20:1, Gi=20:2, Be=22:0, Er=22:1. Coding order effects molecular composition only and does not imply regiospecific arrangements. Significant differences (Student’s t-test, p < 0.05) were performed for selected pxa1 TAGs and are indicated by an asterisk.
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