

Dual Role for Phospholipid:Diacylglycerol Acyltransferase: Enhancing Fatty Acid Synthesis and Diverting Fatty Acids from Membrane Lipids to Triacylglycerol in *Arabidopsis* Leaves¹

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There is growing interest in engineering green biomass to expand the production of plant oils as feed and biofuels. Here, we show that PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE1 (PDAT1) is a critical enzyme involved in triacylglycerol (TAG) synthesis in leaves. Overexpression of *PDAT1* increases leaf TAG accumulation, leading to oil droplet overexpansion through fusion. Ectopic expression of oleosin promotes the clustering of small oil droplets. Coexpression of *PDAT1* with oleosin boosts leaf TAG content by up to 6.4% of the dry weight without affecting membrane lipid composition and plant growth. *PDAT1* overexpression stimulates fatty acid synthesis (FAS) and increases fatty acid flux toward the prokaryotic glycerolipid pathway. In the *trigalactosyldiacylglycerol1-1* mutant, which is defective in eukaryotic thylakoid lipid synthesis, the combined overexpression of *PDAT1* with oleosin increases leaf TAG content to 8.6% of the dry weight and total leaf lipid by fourfold. In the *plastidic glycerol-3-phosphate acyltransferase1* mutant, which is defective in the prokaryotic glycerolipid pathway, *PDAT1* overexpression enhances TAG content at the expense of thylakoid membrane lipids, leading to defects in chloroplast division and thylakoid biogenesis. Collectively, these results reveal a dual role for PDAT1 in enhancing fatty acid and TAG synthesis in leaves and suggest that increasing FAS is the key to engineering high levels of TAG accumulation in green biomass.

INTRODUCTION

In plants, fatty acids (FAs), the building blocks for membrane lipids and storage triacylglycerol (TAG), are almost exclusively synthesized in the plastid (Ohlrogge and Browse, 1995). They are incorporated into glycerolipids through two parallel pathways, with the prokaryotic pathway confined to plastids and the eukaryotic pathway involving the endoplasmic reticulum (ER) and plastids (Roughan and Slack, 1982; Ohlrogge and Browse, 1995). Both pathways begin with the stepwise acylation of glycerol-3-phosphate (G3P), leading to the generation of phosphatidic acid (PA). Dephosphorylation of PA by PA phosphohydrolase gives rise to diacylglycerol (DAG), which in the plastid serves almost exclusively as a precursor for assembly of photosynthetic membrane lipids, through the prokaryotic pathway. DAG synthesized de novo in the ER through G3P acylation is mostly used to synthesize extraplastidic membrane phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in growing leaves (Bates et al., 2007) and in developing seeds (Bates et al., 2009).

PC plays a central role in glycerolipid metabolism. It is the precursor for the synthesis of glycolipids, the predominant lipid species found in photosynthetic membranes. This synthesis

occurs through the eukaryotic pathway (Roughan and Slack, 1982; Ohlrogge and Browse, 1995; Somerville and Browse, 1996), although the extent to which the eukaryotic pathway contributes to thylakoid lipid assembly varies depending on plant species and tissue within the plant. For example, in pea (*Pisum sativum*) plants (Mongrand et al., 1998) and in developing seeds of *Arabidopsis thaliana* (Xu et al., 2005), the eukaryotic pathway of thylakoid lipid synthesis predominates, whereas in leaves of *Arabidopsis* and spinach (*Spinacia oleracea*; Browse et al., 1986), about half of the photosynthetic membrane lipids are derived from the eukaryotic pathway and half from the prokaryotic pathway. Because the substrate specificity of acyltransferases in the plastid and ER differs, lipids synthesized via the prokaryotic pathway are characterized by the almost exclusive presence of a 16-carbon (C16) FA at the *sn*-2 position of the glycerol backbone, whereas those made via the eukaryotic pathway have a C18 at the same position (Frentzen et al., 1983; Heinz and Roughan, 1983; Frentzen, 1998). The *Arabidopsis plastidic glycerol-3-phosphate acyltransferase1* (*act1*) mutants are defective in the first step of G-3-P acylation in the plastid (Kunst et al., 1988; Xu et al., 2006). As a consequence, these mutants lack prokaryotic thylakoid glycolipids and instead synthesize their thylakoid lipids almost exclusively via the eukaryotic pathway. On the other hand, the *trigalactosyldiacylglycerol1* (*tgd1-1*) mutant is deficient in eukaryotic thylakoid lipid synthesis, and most of the photosynthetic membrane lipids in this mutant are assembled via the prokaryotic pathway (Xu et al., 2003, 2005).

PC is the dominant entry point for acyl groups exported from the plastid into glycerolipids through acyl editing (Bates et al., 2007, 2009; Tjellström et al., 2012). It is the major site of the eukaryotic pathway of FA desaturation (Sperling and Heinz, 1993; Shanklin and Cahoon, 1998), an important precursor for

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the synthesis of eukaryotic thylakoid lipids (Ohlrogge and Browse, 1995), and a potential carrier of newly synthesized FAs from plastid envelope membranes to the ER (Tjellström et al., 2012). In developing seeds, PC is the predominant source of DAG for TAG synthesis via the acyl-CoA-dependent and -independent pathway catalyzed by diacylglycerol:acyl-CoA acyltransferase (DGAT) and phospholipid:diacylglycerol acyltransferase (PDAT), respectively (Chapman and Ohlrogge, 2012). TAG is packaged in a dynamic subcellular organelle termed oil droplets (ODs) or oil bodies, which are composed of a central matrix of TAG surrounded by a monolayer of phospholipids with a subset of specific embedded proteins (Huang, 1996). The most abundant proteins coating seed ODs are oleosins, and genetic studies in *Arabidopsis* mutants have suggested a key role for oleosins in preventing ODs from coalescing, thereby maintaining ODs as small discrete entities to facilitate TAG mobilization and confer freezing tolerance during postgerminative seedling growth (Siloto et al., 2006; Shimada et al., 2008).

In the model plant *Arabidopsis*, DGAT1 and PDAT1 play overlapping roles in TAG synthesis in developing seeds and pollen (Zhang et al., 2009). Both DGAT1 and PDAT1 genes are expressed in leaves, roots, and stems, in addition to developing seeds and flowers (Lu et al., 2003; Ståhl et al., 2004). DGAT1 has been implicated in TAG biosynthesis in senescent leaves (Kaup et al., 2002; Slocombe et al., 2009), but the functional role of PDAT1 in leaf tissue remains largely unknown, although knockout of PDAT1 has recently been shown to have a small negative effect on TAG accumulation in roots of *sdp1* mutants defective in SUGAR-DEPENDENT1 TAG lipase (Kelly et al., 2013). Here, we provide evidence that PDAT1 plays a critical role in TAG synthesis in *Arabidopsis* leaves. Overexpression of PDAT1 enhances both FA and TAG synthesis in leaves. The possible functional role of PDAT1 in membrane lipid turnover is also discussed.

RESULTS

The Relative Contribution of PDAT1 and DGAT1 to TAG Synthesis in Leaves

The *Arabidopsis* *dgat1-1* and *pdat1-2* mutants harbor an ethyl methanesulfonate-induced lesion in the *DGAT1* locus (Zou et al., 1999) and a T-DNA insertion in the *PDAT1* gene (Zhang et al., 2009), respectively. As the first step toward understanding the role of PDAT1 and DGAT1 in TAG synthesis in vegetative tissues, we quantified TAG levels in developing and senescing leaves of wild-type, *dgat1-1*, and *pdat1-2* mutant plants. On a dry weight (DW) basis, the TAG levels measured as fatty acid methyl esters (FAMES) were 0.04 and 0.15% in developing and senescing leaves of wild-type plants, respectively (Figure 1). Compared with the wild type, the *pdat1-2* mutant displayed a 57% decrease in TAG content in developing leaves and 39% in senescing leaves. By contrast, the *dgat1-1* mutant showed a less pronounced decrease in TAG level in developing leaves (31%) but a more severe drop in TAG content in senescing leaves (63%). These results suggest that PDAT1 has a more important role in TAG synthesis in growing leaves than DGAT1, whereas the opposite is true in senescing leaves. Consistent with this notion, analyzing the

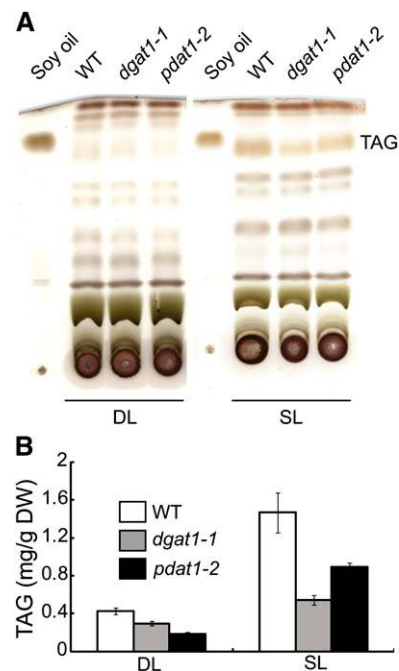


Figure 1. Roles of DGAT1 and PDAT1 in TAG Synthesis during Leaf Development.

Lipids were extracted from developing leaves (DL; the thirteenth leaf of 5-week-old plants) and senescing leaves (SL; the fifth leaf of 7-week-old plants) of the wild type (WT), *dgat1-1*, and *pdat1-2*. Values are means and SD of three to five biological replicates.

[See online article for color version of this figure.]

database of lipid-related gene expression during leaf senescence (Troncoso-Ponce et al., 2013) revealed that *PDAT1* is expressed at much higher levels than *DGAT1* during early leaf growth and development (see Supplemental Figure 1 online). In addition, the *PDAT1* transcript level decreases with age in all three microarray studies compiled in the database, whereas *DGAT1* expression tends to increase as the leaf senesces. The involvement of *DGAT1* in TAG synthesis during leaf senescence is consistent with previous reports (Kaup et al., 2002; Slocombe et al., 2009).

Overexpression of PDAT1 Enhances TAG Accumulation, Leading to OD Overexpansion in Leaves

A previous study showed that overexpressing *PDAT1* driven by the constitutive 35S cauliflower mosaic virus promoter did not affect TAG levels or membrane lipid content and FA composition in the young seedlings of three independent transgenic *Arabidopsis* lines (Ståhl et al., 2004). We extended this study by examining TAG content in the leaves of a large number of adult transgenic plants grown in soil. On average, there was a sevenfold increase in TAG content in 22 randomly chosen primary transgenic plants compared with the wild type (see Supplemental Figure 2A online). By comparison, overexpressing *DGAT1* driven by the same promoter resulted in only a marginal, if any, increase in the average leaf TAG levels in 21 randomly selected primary transformants tested. These results are in accordance with the absence of significant increases in total leaf FA content in

Arabidopsis transgenic plants constitutively overexpressing *DGAT1* as reported recently by others (Winichayukul et al., 2013). Together, these results indicate that *PDAT1* exerts a much higher impact than *DGAT1* on TAG synthesis in *Arabidopsis* leaves.

We subsequently obtained three homozygous transgenic lines overexpressing *PDAT1* for detailed characterization. On a DW basis, the TAG level in mature rosette leaves of 7-week-old *PDAT1* overexpressors grown in soil was 2.6%, a 28-fold increase compared with the wild type (Figure 2A). TAG compositional analysis showed that the predominant acyl chains in TAG derived from the leaves of *PDAT1* overexpressors were polyunsaturated FAs with 18:2 and 18:3 accounting for over 75% of the total acyl chains. By comparison, the FAs recovered from wild-type plants by analysis of the TAG band from thin layer chromatography (TLC) plates were largely saturated acyl chains (Figure 2B). In contrast with TAGs isolated from *Arabidopsis* seedlings ectopically overproducing a *Chlamydomonas reinhardtii* *DGAT* type-two enzyme (Sanjaya et al., 2013) or transcription factors involved in storage product accumulation, such as *LEAF COTYLEDON2* (*LEC2*) (Santos Mendoza et al., 2005), TAGs derived from leaves of *PDAT1* overexpressors contained very limited amounts of very-long-chain fatty acids (Figure 2B).

Consistent with the marked increase in TAG content, microscopy examination following Nile Red staining showed that, whereas only a few or no OD-like structures were seen in the leaves of wild-type plants under our growth conditions (Figures 3A and 3C), overexpression of *PDAT1* led to OD accumulation in leaves (Figure 3B). Those ODs were often irregular in shape and frequently appeared to coalesce. Ultrastructural examination using transmission electron microscopy (TEM) confirmed that overexpression of *PDAT1* led to the formation of large, irregularly shaped ODs in leaves (Figure 3D).

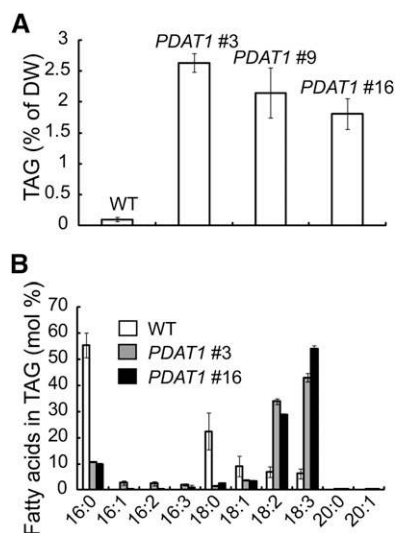


Figure 2. TAG Accumulation in Leaves of Transgenic Plants Overexpressing *PDAT1*.

(A) Leaf TAG content in 7-week-old wild type (WT) and three independent transgenic lines.

(B) FA composition of TAGs in the wild type and two independent transgenic lines. Values are means and *sd* of three biological replicates.

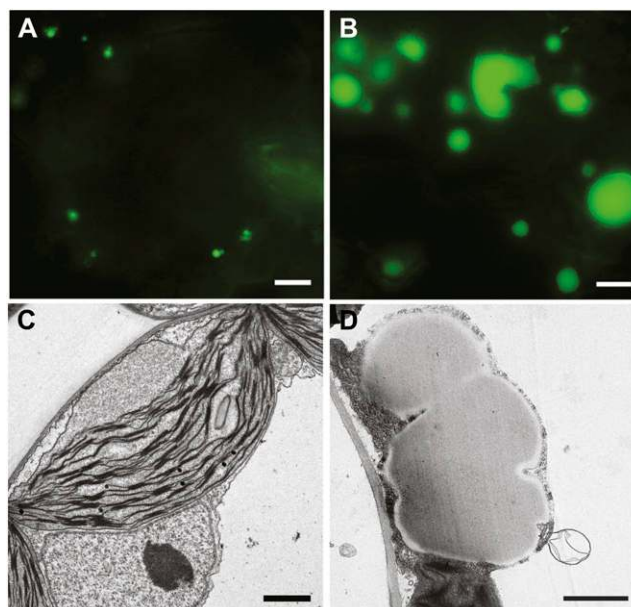


Figure 3. OD Accumulation in Leaves of Transgenic Plants Overexpressing *PDAT1*.

(A) and (B) Images of ODs in leaves of 7-week-old wild type (A) and *PDAT1*-overexpressing line 16 (B) stained with Nile red. Bars = 5 μ m.

(C) and (D) TEM images of leaf cells from 7-week-old wild type (C) and *PDAT1*-overexpressing line 16 (D). Bars = 1 μ m.

Ectopic Expression of Oleosin Promotes the Clustering of Small ODs and Boosts Oil Accumulation in *PDAT1* Transgenic Plants

Oleosins are known to play a key role in preventing ODs from coalescing in oilseeds (Siloto et al., 2006; Shimada et al., 2008). In addition, in yeast and mammalian model systems, the ectopic expression of OD-associated proteins often leads to increased TAG storage (Brasaemle et al., 2000; Froissard et al., 2009). To test the functional role of oleosins in TAG accumulation in vegetative tissues, *Oleosin1* (*OLE1*), the most abundant seed OD-specific protein of *Arabidopsis* (Huang, 1996; Shimada et al., 2008), was C-terminally tagged with green fluorescent protein (GFP), and this fusion gene was expressed in *Arabidopsis* wild-type plants under the control of the 35S cauliflower mosaic virus promoter. Three independent transgenic lines exhibiting high levels of green fluorescence signal in young seedlings were obtained. Analyzing leaf lipid extracts from 3-week-old transgenic plants revealed an up to sevenfold increase in TAG content compared with the wild type (see Supplemental Figure 2B online). TAGs isolated from *OLE1* overexpressors were mainly composed of 16:0, 18:2, and 18:3 FAs, whereas very-long-chain fatty acids, which typically accumulate in *Arabidopsis* seeds, were present at very low levels (see Supplemental Figure 2C online), suggesting that TAG accumulation in 3-week-old seedlings is not a result of reduced hydrolysis of seed oil due to the ectopic overexpression of *OLE1*.

TEM analysis showed that ectopic expression of *OLE1* induced the formation of clusters of small spherical ODs (Figure 4A), unlike the large aberrant ODs observed in transgenic plants

overexpressing *PDAT1* (Figure 3). Using confocal microscopy, we found that the *OLE1*-GFP fusion protein was exclusively associated with OD clusters and appeared as a ring-like pattern surrounding the perimeters of ODs (Figure 4B).

We next transformed the homozygous *OLE1* overexpressor line 1 with the *PDAT1* construct. Three independent *PDAT1/OLE1* double transgenic lines were propagated to the T3 generation and used for detailed analysis. Using Fat Red 7B, a nonfluorescent dye that specifically stains neutral lipids (Brundrett et al., 1991), we found that the double transgenic seedlings were stained red in both roots and leaves (Figure 5A), whereas the wild-type seedlings did not stain. On soil, the *PDAT1/OLE1* double transgenic lines displayed no obvious growth or developmental abnormalities (see Supplemental Figure 3A online). Quantification of TAG levels in the rosette leaves of 7-week-old soil-grown double transgenic plants showed an up to 74-fold increase compared with the wild type. On

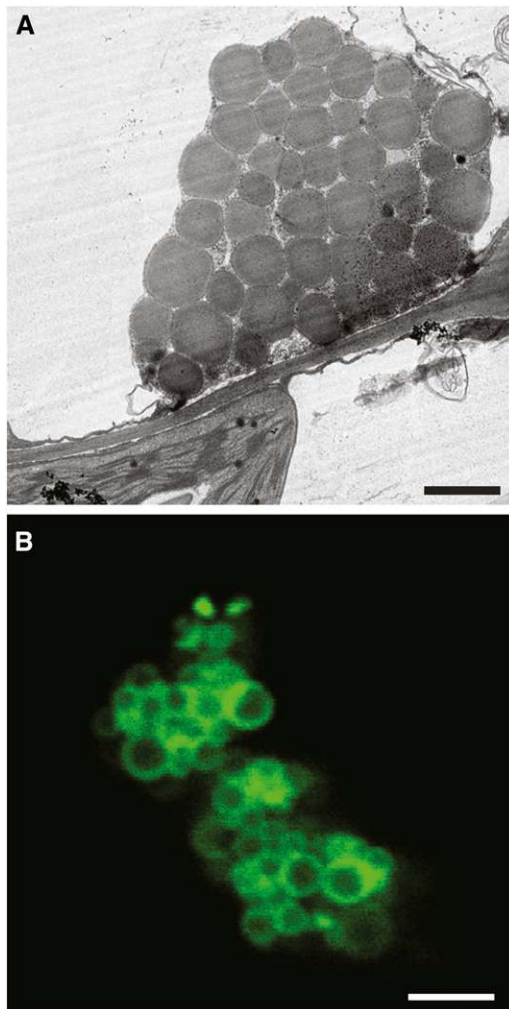


Figure 4. Ectopic Expression of *OLE1* Promotes the Clustering of Small ODs.

(A) TEM imaging of ODs in leaves of the *OLE1*-GFP-overexpressing line 1. Bar = 1 μ m.
(B) Confocal microscopy of OD clusters in leaves of the *OLE1*-GFP-overexpressing line 1. Bar = 2 μ m.

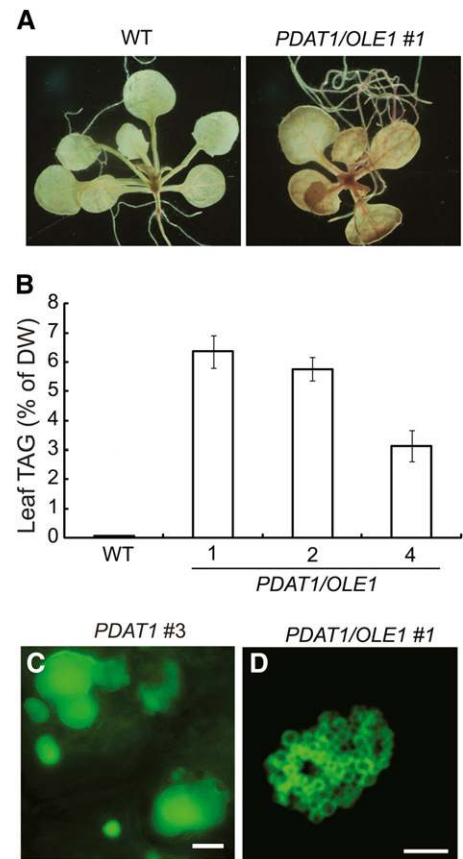


Figure 5. TAG Accumulation in Transgenic Lines Coexpressing *PDAT1* and *OLE1*.

(A) Fat Red 7B staining of 3-week-old seedlings of the wild type (WT), *PDAT1* and *OLE1*-GFP double transgenic line 1 (*PDAT1/OLE1* #1).
(B) TAG content in leaves of 7-week-old plants of the wild type and three transgenic lines coexpressing *PDAT1* and *OLE1*-GFP (*PDAT1/OLE1*). Values are means and so of three biological replicates.
(C) Images of ODs in leaves of 7-week-old *PDAT1*-overexpressing line 3 (*PDAT1* #3) stained with Nile red. Bar = 5 μ m.
(D) Confocal imaging of ODs in leaves of 7-week-old soil-grown transgenic line 1 coexpressing *PDAT1* and *OLE1*-GFP (*PDAT1/OLE1* #1). Bar = 5 μ m.

a DW basis, the TAG content in wild-type leaves was 0.05%, while in the three *PDAT1/OLE1* double transgenic lines, it was between 5.7 and 6.4% (Figure 5B). There was also an up to twofold increase in the total leaf FA content in 7-week-old transgenic plants coexpressing *PDAT1* and *OLE1* compared with the wild type (see Supplemental Figure 3B online). In addition, TAG accumulated up to 1.5% per DW in the stems of the double transgenic plants, a 30-fold increase compared with the wild type (see Supplemental Figure 3C online). Microscopy analysis showed that, unlike large and often irregularly shaped ODs in plants carrying the *PDAT1* transgene only (Figure 5C), the double transgenic plants contained clusters of spherical ODs in leaves (Figure 5D), similar to those seen in plants ectopically expressing *OLE1* alone (Figure 4), thus confirming the role of *OLE1* in promoting the clustering of ODs.

The FA Composition of Membrane Lipids Is Altered in *PDAT1* Transgenic Plants

To investigate the potential impact of *PDAT1* overexpression on membrane lipids, we first surveyed the FA profiles of total polar lipids in leaves of three independent transgenic lines overexpressing *PDAT1*. The relative proportions of 16:3, 18:1, and 18:2 increased, with a concomitant decrease in the proportion of 18:3 (see Supplemental Figure 4A online). We next separated individual polar lipids by TLC. Analysis by gas chromatography of the FAMES revealed no discernible differences in levels of the individual classes of major polar lipids between *PDAT1* overexpressor 16 and the wild type (see Supplemental Figure 4B online). However, there was a substantial decrease in 18:3 with a corresponding increase in 18:1 and 18:2 in both extraplastidic (PE and PC) and thylakoid (digalactosyldiacylglycerol [DGDG] and monogalactosyldiacylglycerol [MGDG]) lipids (see Supplemental Figures 4C to 4F online). The marked decrease in 18:3 in PC and PE may reflect the substrate preference of *PDAT1* as demonstrated by *in vitro* studies by Ståhl et al. (2004). This is supported by the particular enrichment of 18:3 in TAG accumulated in leaves of *PDAT1* transgenic plants (Figure 2B). On the other hand, the decrease in the proportion of 18:3 in galactolipids, particularly in DGDG, may reflect the fact that PC is the precursor of galactolipids synthesized by the eukaryotic pathway. In addition to changes in C18 FAs, the levels of C16 acyl chains tended to decrease in PC but increase in DGDG and MGDG when compared with the wild type (see Supplemental Figures 4C to 4F online).

Overexpression of *PDAT1* Alters the Positional Distribution of FAs in Galactolipids

The increase in C16 FA content in galactolipids may suggest that the prokaryotic pathway of thylakoid lipid synthesis is enhanced in *PDAT1* overexpressors. To test this possibility, the positional distribution of FAs in galactolipids was analyzed following *Rhizopus* lipase digestion. As shown in Figure 6, the relative proportion of C16 FAs at the *sn*-2 position of MGDG increased from 80% in the wild type to 94% in *PDAT1* transgenic line 16. In the case of DGDG, the increase was from 34 to 57%. The finding that *sn*-2 C16 FA content increased to a greater degree in DGDG than in MGDG reflects the fact that the prokaryotic pathway makes a much smaller contribution to the synthesis of DGDG than to MGDG in the leaves of wild-type plants (Browse et al., 1986); consequently, DGDG is more affected than MGDG when prokaryotic thylakoid lipid synthesis is enhanced.

Rate of FA Synthesis Is Enhanced in *PDAT1* Transgenic Plants

The accumulation of large amounts of TAG without a concomitant decrease in membrane lipid content in *PDAT1* transgenic plants suggests an increase in fatty acid synthesis (FAS). To test this possibility, we conducted ^{14}C -acetate feeding experiments with leaf strips from expanding leaves for a period of 1 h, which was within the linear range of label incorporation (see Supplemental Figure 5 online). Because acetate incorporation into FAs can be influenced by levels of endogenous metabolites (Cronan et al.,

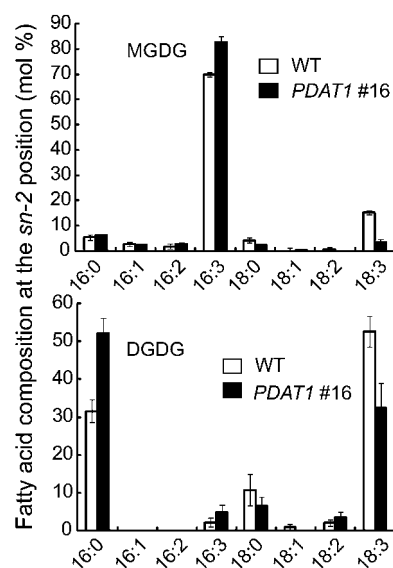


Figure 6. FA Composition Exclusively at the *sn*-2 Position of the Glycerol Backbone of Galactolipids Isolated from Leaves of 7-Week-Old Wild-Type (WT) and *PDAT1*-Overexpressing Line 16 (*PDAT1* #16).

Values are means and sd of three biological replicates.

1975; Nunn et al., 1977), the labeling experiments were performed in the presence of 1 mM unlabeled acetate in an attempt to eliminate potential variations in endogenous substrate pools between the transgenics and the wild type. We detected 72 and 44% increases in radiolabeled lipids in the *PDAT1* overexpressor 9 and 16, respectively, compared with the wild type (Figure 7A), suggesting an increase in the rate of FAS due to *PDAT1* overexpression. Analysis of label distribution showed that overexpression of *PDAT1* significantly increased the label incorporation into galactolipids, phosphatidylglycerol (PG), and TAG, whereas the proportions of label in PC, PE, and sulfoquinovosyldiacylglycerol (SL)/phosphatidylinositol (PI) decreased (Figure 7B). These results are consistent with an increase in acyl fluxes toward galactolipids and *PDAT1*-mediated TAG synthesis in response to *PDAT1* overproduction.

Rate of FAS Strongly Correlates with Leaf TAG Content in Transgenic Plants

The leaf TAG levels in double transgenic plants coexpressing *PDAT1* and *OLE1* (Figure 5B) were much higher than in transgenic plants carrying *PDAT1* alone (Figure 2A). To begin to dissect the biochemical basis for the increased TAG accumulation in double transgenic lines, growing leaves of *PDAT1* single and *PDAT1/OLE1* double transgenic plants were labeled with ^{14}C -acetate. Compared with the transgenic plants overexpressing *PDAT1* alone (Figure 7A), the initial rate of acetate incorporation into total lipids was much higher for the *PDAT1/OLE1* double transgenic lines (Figure 8A). In addition, in five additional independent *PDAT1/OLE1* double transgenic lines tested, there was a strong correlation between the rate of FAS (Figure 8B) and TAG content (Figure 8C). These results suggest that increased

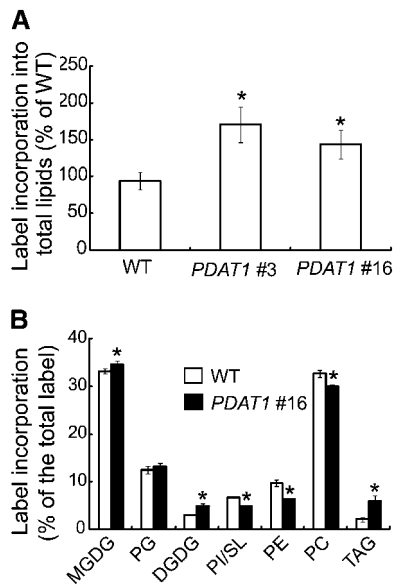


Figure 7. Overexpression of *PDAT1* Enhances the Rate of FAS in Leaves.

(A) Initial rates of FAS in growing leaves of 7-week-old wild-type (WT), *PDAT1*-overexpressing line 3 (*PDAT1* #3), and *PDAT1*-overexpressing line 16 (*PDAT1* #16) measured by ^{14}C -acetate labeling.

(B) The distribution of label into TAG and polar lipids after labeling of detached leaves with ^{14}C -acetate for 60 min.

Values in **(A)** and **(B)** are means and SD of three biological replicates. Asterisks indicate statistically significant differences from the wild type based on Student's *t* test ($P < 0.05$).

TAG accumulation in leaves of *PDAT1/OLE1* double transgenic lines may be due to enhanced FAS. On the other hand, no apparent difference in acetate incorporation into total lipids was found between the wild type and transgenic plants overexpressing the *OLE1* single transgene (Figure 8A), suggesting that the increased TAG accumulation in transgenic plants carrying *OLE1* may be attributed to decreased TAG turnover, likely because of the shielding of ODs by *OLE1* from the access of cytosolic lipases, in a manner similar to that observed for mammalian OD-associated protein perilipins (Brasaemle et al., 2000).

Disruption of the Eukaryotic Thylakoid Lipid Pathway Enhances TAG Accumulation in Double Transgenic Plants Coexpressing *PDAT1* and *OLE1*

In *Arabidopsis*, the prokaryotic and eukaryotic glycerolipid pathway each consumes about half of acyl chains synthesized de novo in the plastid (Browse et al., 1986). Disruption of either glycerolipid pathway may therefore be expected to increase acyl chains for TAG synthesis. To test this possibility, the *tgdl-1* mutant, which is defective in the eukaryotic pathway of thylakoid lipid synthesis (Xu et al., 2003), was crossed with the *PDAT1/OLE1* double transgenic line 1. Analysis of leaf lipid extracts from mature leaves of 7-week-old soil-grown plants showed that, on a DW basis, TAG content in the *PDAT1/OLE1* double transgenic line in the *tgdl-1* background was increased to 8.6%

and total lipid content to 16.2%, a 97-fold and fourfold increase, respectively, compared with *tgdl-1* (Figure 9A). As expected, coexpression of *PDAT1* with *OLE1* in *tgdl-1* induced the formation of OD clusters in leaves (Figure 9B). In addition to leaves, TAG also accumulated in stems of *tgdl-1/PDAT1/OLE1* transgenic plants to $3.0\% \pm 0.18\%$ per DW ($n = 3$, \pm SD), a 60-fold increase relative to *tgdl-1* ($0.05\% \pm 0.01\%$ per DW, $n = 3$, \pm SD). Remarkably, despite a large increase in TAG and total lipid levels, the growth and development of the double transgenic plants were only slightly affected (see Supplemental Figure 6A online). Furthermore, no apparent difference in total polar lipid content was found between *tgdl-1/PDAT1/OLE1* and *tgdl-1* (see Supplemental Figure 6B online).

Disruption of *PDAT1* Partially Alleviates the Phenotype of the *act1* Mutant

We next constructed a double mutant between *pdat1-2* and *act1* defective in the prokaryotic thylakoid lipid pathway. When

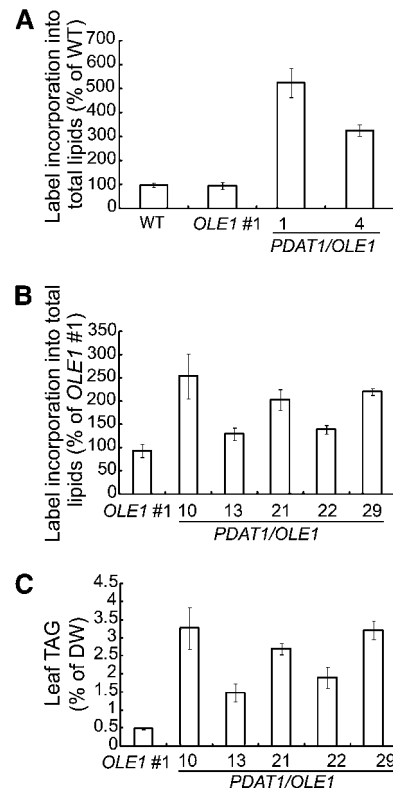


Figure 8. TAG Accumulation Positively Correlates with the Rate of FAS.

Initial rates of FAS in growing leaves of 5-week-old wild-type (WT), *OLE1-GFP*-overexpressing line 1 (*OLE1* #1), and *PDAT1* and *OLE1-GFP* double transgenic line 1 (*PDAT1/OLE1* #1) and 4 (*PDAT1/OLE1* #4) measured by ^{14}C -acetate labeling is shown in **(A)**. Initial rates of FAS in growing leaves of 5-week-old *OLE1* #1 and five double transgenic lines coexpressing *PDAT1* and *OLE1-GFP* (*PDAT1/OLE1*) measured by ^{14}C -acetate labeling is presented in **(B)**. TAG content in leaves of 7-week-old *OLE1* #1 and five double transgenic lines coexpressing *PDAT1* and *OLE1-GFP* (*PDAT1/OLE1*) is shown in **(C)**. Values are means and SD of three biological replicates.

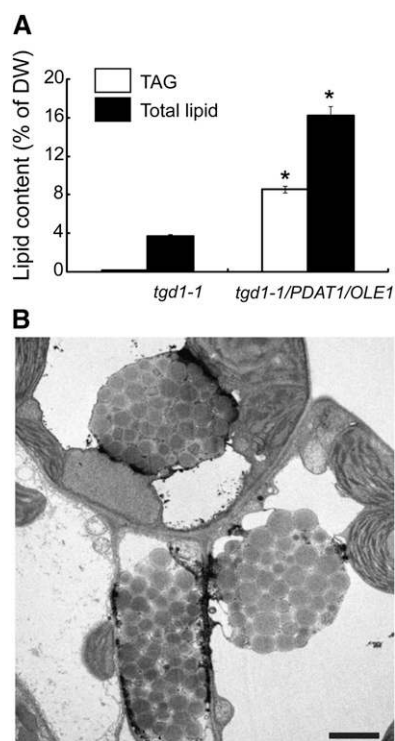


Figure 9. Disruption of the Eukaryotic Thylakoid Lipid Pathway Enhances TAG Accumulation in Transgenic Plants Coexpressing *PDAT1* and *OLE1*.

(A) TAG and total lipid levels in leaves of 7-week-old *tgd1-1* and transgenic plants coexpressing *PDAT1* and *OLE1-GFP* in the *tgd1-1* background (*tgd1-1/PDAT1/OLE1*). Values are means and *SD* of three biological replicates. Asterisks indicate statistically significant differences from *tgd1-1* based on Student's *t* test ($P < 0.05$).

(B) TEM imaging of ODs in leaves of 7-week-old *tgd1-1/PDAT1/OLE1* plants. Bar = 2 μ m.

grown in soil, the *act1 pdat1-2* double mutant seedlings were visibly darker in color compared with the *act1* mutant (Figure 10A). Consistent with the dark-green phenotype, the total chlorophyll content was increased by 9.8%, from 0.90 ± 0.02 in *act1* to 0.99 ± 0.04 mg/g fresh weight (FW) ($n = 3$, \pm SD) in the double mutant. Lipid analysis showed that disruption of *PDAT1* in *act1* resulted in small but significant increases in the levels of thylakoid lipids MGDG and PG (Figure 10B). In addition, the combined amount of SL and PI was slightly increased, whereas the level of PC, PE, and DGDG stayed largely unaltered in the *act1 pdat1-2* double mutant relative to *act1*. The FA composition of all polar lipid classes examined did not show noticeable deviations from those in *act1*, with the exception of PC, which exhibited a significant increase in relative proportions of 18:2 and 18:3 with a corresponding decrease in 18:1 (Figure 10C). The mutation in *act1* has been shown to result in decreases in the relative levels of chloroplast lipids PG, SL, and MGDG and increases in the relative amounts of 18:1 in extrachloroplast lipids, particularly in PC (Kunst et al., 1988), and a slight reduction in the amount of chlorophyll on a FW basis (Kunst et al., 1989). Thus, our lipid data,

together with the increased chlorophyll content in *act1 pdat1-2* plants, suggest that disruption of *PDAT1* partially alleviates the phenotypes of the *act1* mutant, presumably as a consequence of the increased diversion of FAS from TAG to eukaryotic thylakoid lipids in the double mutant.

Overexpression of *PDAT1* in *act1* Enhances TAG Accumulation at the Expense of Thylakoid Lipids

Next, the *PDAT1* construct was introduced into *act1* by *Agrobacterium tumefaciens*-mediated transformation. When grown in soil, transgenic plants overexpressing *PDAT1* in *act1* (*act1/PDAT1*) were frequently found to be pale in color and stunted in growth (Figure 11A). Consistent with the pale phenotype, the amount of the major thylakoid lipids MGDG, DGDG, and PG was reduced by 25, 46, and 31%, respectively, in *act1/PDAT1* line 7 compared with *act1*, whereas the levels of PC, PE, and SL/PI remained largely unchanged (Figure 11B). On a FW basis, the

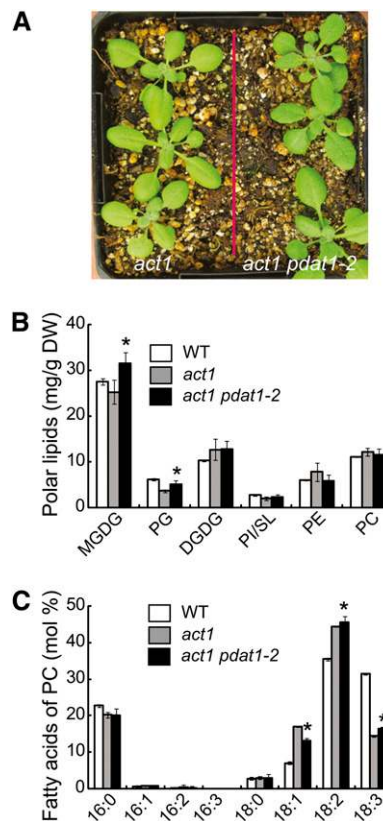


Figure 10. Disruption of *PDAT1* Partially Complements the Phenotype of the *act1* Mutant.

Images of 4-week-old *act1* and *act1 pdat1-2* plants are shown in **(A)**. Polar lipid content in leaves of *act1* and *act1 pdat1-2* plants is shown in **(B)**. FA composition of PC isolated from leaves of 4-week-old wild-type (WT) *act1* and *act1 pdat1-2* plants grown on agar plates is shown in **(C)**. Values are means and *SD* of three biological replicates. Asterisks indicate a statistically significant difference from *act1* based on Student's *t* test ($P < 0.05$).

[See online article for color version of this figure.]

sum of MGDG, DGDG, and PG decreased from 2826 in *act1* to 2065 $\mu\text{g/g}$ FW in *act1/PDAT1* line 7, resulting in a net loss of 761 $\mu\text{g/g}$ FW, while the amount of TAG increased from 57.3 \pm 9.8 to 760.9 \pm 164.6 $\mu\text{g/g}$ FW ($n = 3$, $\pm\text{SD}$), a net gain of 704 $\mu\text{g/g}$ FW. These results suggest that TAG accumulation in *act1 PDAT1* overexpressing lines is largely accounted for by the increased diversion of acyl groups from thylakoid membrane lipid synthesis to TAG. Consistent with this, the total leaf FA content was comparable in *act1/PDAT1* line 7 (5.61% \pm 0.61% per DW, $n = 3$, $\pm\text{SD}$) and *act1* (5.91% \pm 0.84% per DW, $n = 4$, $\pm\text{SD}$).

To further investigate the nature of the pale-green phenotype in *act1* overexpressing *PDAT1*, microscopy examination of leaf cross sections was performed. Compared with *act1*, the transgenic *act1* plants displayed increased leaf thickness and increased cell size, but decreased cell number (see Supplemental Figure 7 online). The chloroplasts of the transgenic line appeared to be larger. The number of chloroplasts per cell cross section was reduced from 6.63 \pm 0.05 in *act1* to 3.33 \pm 0.05 ($n = 4$, $\pm\text{SD}$) in the *act1/PDAT1* line 7. At the ultrastructural level, the amount of thylakoid membranes and thylakoid membrane stacking per chloroplast in mature leaves of *act1* plants overexpressing *PDAT1* were greatly reduced when compared with chloroplasts from *act1* leaves at the same developmental stage (Figures 11C and 11D).

To gain more information on the metabolic changes leading to TAG accumulation due to *PDAT1* overproduction in *act1*, we

performed ^{14}C -acetate pulse-chase labeling experiments using detached leaves. During the 1-h pulse, the rates of ^{14}C -acetate incorporation were approximately threefold higher in the *act1/PDAT1* line 7 (232.0 \pm 18.9 disintegrations per minute/mg FW/min, $n = 3$, $\pm\text{SD}$) compared with *act1* (636.6 \pm 127.7 disintegrations per minute/mg FW/min, $n = 3$, $\pm\text{SD}$), suggesting an increase in FAS due to *PDAT1* overexpression in the *act1* background. Because the total leaf lipid content remained largely unchanged in *act1/PDAT1* line 7 relative to *act1*, the rate of FA degradation must be increased concurrently such that a constant lipid level is maintained. Indeed, the average label decay rate was increased by threefold in *act1/PDAT1* line 7 (22% per day) compared with *act1* (7% per day) (see Supplemental Figure 8A online). Analysis of label distribution showed that PC was the most radioactive lipid immediately following the pulse, followed by PG and PE. Galactolipids MGDG and DGDG, despite accounting for over 70% of the total membrane lipid mass, contained <10% of total initial label in *act1* (Figure 12). Overexpression of *PDAT1* led to a 19% drop in initial label in PC, no change in PE, but a 25% decline in SL/PI, 42% in DGDG, 35% in PG, and 44% in MGDG, whereas TAG label increased from 3% in *act1* to 25% in the *act1/PDAT1* line 7, thus representing a nearly fourfold increase in the initial label partitioning into TAG (Figure 12). During the chase, the PC label declined, whereas labeled MGDG and DGDG increased in both *act1* and *act1* transgenic plants, reflecting a well-documented precursor-product relationship between PC and galactolipids, but the extent of increases in label in MGDG and DGDG was smaller in *act1* transgenic plants (see Supplemental Figure 8C online) than in *act1* (see Supplemental Figure 8B online). In both *act1* and the *act1 PDAT1* overexpressor, the label in TAG slightly increased during the initial 24 h of chase. This was followed by substantial decreases in labeled TAG during the remainder of the chase, reflecting turnover of TAG in both *act1* and *act1* transgenic plants.

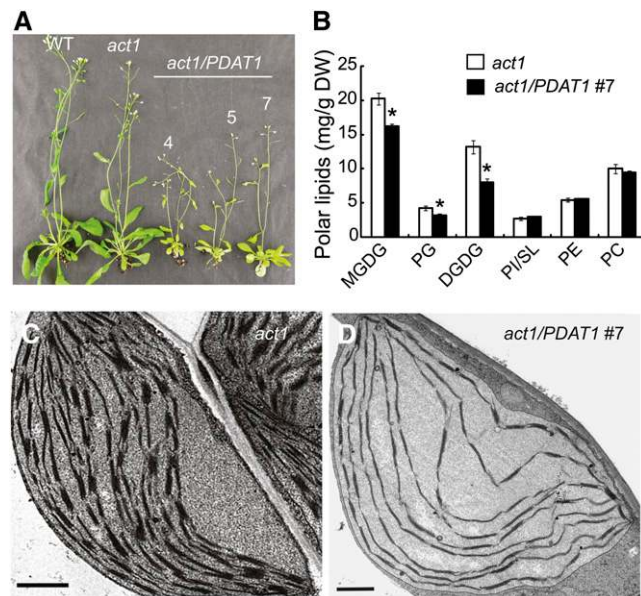


Figure 11. Overexpression of *PDAT1* in *act1* Promotes TAG Accumulation at the Expense of Thylakoid Lipids.

(A) Images of 6-week-old wild type (WT), *act1*, and three *PDAT1*-overexpressing lines in the *act1* background (*act1/PDAT1*).

(B) Polar lipid levels in leaves of *act1* and the *act1/PDAT1* #7. Values are means and SD of three to four biological replicates. Asterisks indicate statistically significant differences from *act1* based on Student's *t* test ($P < 0.05$).

(C) and (D) TEM analysis of chloroplasts in leaves of *act1* (C) and the *act1/PDAT1* #7 (D). Bars = 1 μm .

[See online article for color version of this figure.]

DISCUSSION

PDAT Is Functionally Conserved among Yeast, Microalgae, and Plants

In plants as well as in microalgae, mammals, and fungi, the terminal and committed step in the pathway of TAG biosynthesis is catalyzed by multiple DAG acyltransferases encoded by distinct gene families. Although the basic enzymes involved in the DAG esterification reaction have been extensively characterized at the biochemical and molecular level in several model systems, fundamental questions remain regarding the roles of the individual DAG acyltransferases and the functional significance of highly redundant activities. At least three classes of DAG acyltransferases consisting of acyl-CoA-dependent DGAT1 and DGAT2 and acyl-CoA-independent PDAT, contribute to TAG synthesis in yeasts (Kohlwein, 2010), microalgae (Liu and Benning, 2013), and plants (Chapman and Ohlogge, 2012). Studies in both yeast (Oelkers et al., 2000, 2002) and the green alga *C. reinhardtii* (Yoon et al., 2012) have indicated that PDAT plays a major role in TAG synthesis during phases of active cell growth and division, while DGATs appear to be more important in cells entering the stationary phase of growth (Oelkers et al., 2002; Sandager et al., 2002). In

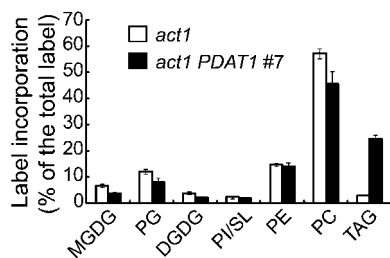


Figure 12. The Distribution of Radioactivity in Polar Lipids and TAG after Labeling of Detached Leaves with ^{14}C -Acetate for 60 min in *act1* and *act1 PDAT1*-Overexpressing Line #7 (*act1/PDAT1 #7*).

Values are means and SD of three biological replicates.

Arabidopsis, most evidence suggests that DGAT1 is a major contributor to TAG synthesis in developing seeds (Katavic et al., 1995; Routaboul et al., 1999; Jako et al., 2001) and in leaves during senescence (Kaup et al., 2002; Slocombe et al., 2009). On the other hand, although PDAT1 is known to have an overlapping role in TAG synthesis in seeds and pollen (Zhang et al., 2009), neither disruption (Ståhl et al., 2004; Mhaske et al., 2005) nor overexpression of PDAT1 (Ståhl et al., 2004) appears to affect lipid content and FA composition in seeds and in young seedlings. Through extensive analyses of PDAT1 knockout mutants and overexpressing lines, particularly in the *act1* mutant background, this work provides insight into the biological functions of PDAT1. We show that PDAT1 plays an important role in TAG synthesis in leaves; overexpression of *PDAT1*, but not *DGAT1*, increased leaf TAG content, whereas disruption of PDAT1 substantially decreased TAG levels with the most pronounced decrease occurring in rapidly growing leaves and the least in senescing leaves. Collectively, these results point to evolutionary conservation of PDAT functions associated with rapid cell growth and membrane proliferation in yeast, microalgae, and plants.

According to current knowledge about glycerolipid metabolism in plants, most of the acyl chains exported outside the plastid are first incorporated into PC, the substrate of PDAT, through acyl editing in growing leaf cells (Bates et al., 2007, 2009; Tjellström et al., 2012). Because lipid metabolism in growing leaf cells is directed primarily toward membrane lipid synthesis, the acyl chains released as a result of acyl editing of PC are primarily channeled into G3P acylation reactions to generate DAG for de novo PC synthesis (Bates et al., 2007) and therefore may be limiting for DGAT1-mediated TAG synthesis. As leaf tissues age, the rate of FAS declines (Bao et al., 2000; Hellgren and Sandelius, 2001) and fewer nascent FAs are fluxed through PC and more are used directly in the chloroplast to sustain thylakoid lipid turnover (Hellgren and Sandelius, 2001). This may explain why the PDAT1-mediated acyl-CoA-independent route is more involved in TAG synthesis in growing but not in senescing leaves of *Arabidopsis* than the DGAT1-catalyzed acyl-CoA-dependent reaction.

PDAT Plays a Role in Membrane Lipid Turnover

In vitro biochemical assays showed that *Arabidopsis* PDAT1 displays the highest activity toward oxygenated acyl groups, such as hydroxyl acyl chains (Ståhl et al., 2004), much as does the

enzyme from castor (*Ricinus communis*) oil seeds that accumulates high levels of hydroxyl FAs (Dahlqvist et al., 2000). It has thus been hypothesized that *Arabidopsis* PDAT1, like PDAT in castor, may play a role in cell membrane repair by removing oxygenated FAs from membrane phospholipids (Ståhl et al., 2004). However, direct evidence in support of this hypothesis is still lacking.

Recent studies in other model organisms have indicated that membrane lipid and TAG metabolism are tightly linked processes (Rajakumari et al., 2010; Horvath et al., 2011; Yoon et al., 2012). In yeast acyl-CoA-deficient mutants, disruption of PDAT blocked phospholipid deacylation, resulting in an increase in levels of PC and PE (Mora et al., 2012). Likewise, knockdown of PDAT in *C. reinhardtii* has been shown to alter the molecular species composition of major thylakoid membrane lipids (Yoon et al., 2012). In this study, we showed that disruption of PDAT1 in the *act1* mutant increased the proportions of polyunsaturated FAs in PC with a corresponding decrease in 18:1, whereas the reverse trend was found for *PDAT1*-overexpressing plants. Because PC is the site of FA desaturation, the opposite effects of PDAT1 knockout and overproduction on levels of FA unsaturation are in line with PDAT1's function in the removal of acyl groups from PC. In addition to changes in the FA composition of PC, knockout of PDAT1 increased, whereas overexpression of this gene decreased levels of thylakoid lipid content in the *act1* mutant. These results provide direct in vivo evidence that *Arabidopsis* PDAT1, like its homologs in yeast and *C. reinhardtii*, is also involved in the turnover of membrane lipids in leaves.

In the *pdat1-2* single mutant, no appreciable change in membrane lipid content and FA composition was observed, despite the marked reduction in TAG content compared with the wild type. The lack of detectable phenotype in *pdat1-2* may reflect the operation of lipid homeostatic mechanisms that fully compensate for the changes in membrane compositions resulting from the loss of PDAT1 function in the wild type. In this context, in *PDAT1*-overexpressing lines in the wild-type background, the increased diversion of acyl groups from membrane lipid synthesis to TAG was entirely compensated for by enhanced FAS and increased thylakoid lipid assembly via the prokaryotic pathway, thus resulting in little net change in the membrane lipid content and composition. Although the factors responsible for such metabolic adjustments are unknown, it is tempting to speculate that biochemical feedback regulation of FAS plays a key role. In this scenario, PDAT1-mediated TAG accumulation consumes, in the cytosol, acyl moieties destined for eukaryotic thylakoid lipid synthesis. This could (1) provide a driving force for FA export outside the chloroplast through vectorial acylation and (2) increase the demand for FAs needed for the prokaryotic thylakoid lipid synthesis. Both of these metabolic responses could lead to a depletion of 18:1-acyl carrier protein in the plastid, thereby releasing its feedback inhibition on the plastidic acetyl-CoA carboxylase (Andre et al., 2012). This could in turn lead to an increase in the rate of FAS and an increase in flow of FAs toward the prokaryotic pathway to compensate for the loss of lipid precursors normally shuttled from the ER to the chloroplast for eukaryotic thylakoid lipid synthesis.

In contrast with PDAT1 overexpressors in the wild-type background, overexpression of *PDAT1* in *act1* resulted in substantial reductions in levels of thylakoid lipids, leading to defects in

chloroplast biogenesis, despite marked increases in the rates of FAS. These results indicate that the prokaryotic pathway plays a critical role in maintaining membrane lipid homeostasis when the eukaryotic pathway is severely compromised. In this regard, we showed previously that the genetic defects in the eukaryotic pathway of thylakoid lipid synthesis in *tgdl* mutants can be almost fully compensated for by the augmented prokaryotic pathway without major growth and developmental consequences (Xu et al., 2005).

Biotechnological Implications for PDAT

FAs are the predominant component of TAG. Therefore, increasing FAS is a prerequisite for attaining high oil yield in rational genetic engineering studies aimed at enhancing oil accumulation in vegetative tissues of plants. To date, much attention has been focused on overexpression of transcription factors involved in seed storage product accumulation and seed maturation, such as WRINKLED1 (WRI1) and LEC2 (Slocombe et al., 2009; Andrianov et al., 2010; Kelly et al., 2013; Vanhercke et al., 2013). Aside from the growth and developmental defects associated with the ectopic overexpression of such seed-specific master regulators (Stone et al., 2001; Cernac and Benning, 2004; Baud et al., 2007), recent genetic evidence suggests that the WRI class of transcription factors are not involved in the regulation of FAS in vegetative tissues of *Arabidopsis* (To et al., 2012). In addition, it has been reported that WRI1-mediated TAG accumulation is dependent on the addition of soluble sugar in growth media (Cernac and Benning, 2004), and ectopic overexpression of WRI1 alone only results in marginal increases in TAG accumulation in leaves of soil-grown plants (Sanjaya et al., 2011). Foliar tissues constitute a major portion of the harvestable biomass of dedicated bioenergy crops such as switchgrass (*Panicum virgatum*). So far, however, only modest levels of leaf TAG have been achieved (Troncoso-Ponce et al., 2013), with the highest level among genetically modified *Arabidopsis* plants being 5% per DW found in the *sdp1* TAG lipase mutant coexpressing WRI1 and DGAT1 (Kelly et al., 2013). Our study shows that PDAT1 has a dual role in enhancing FAS and directing FAs from membrane lipids to TAG in *Arabidopsis* leaves. We show that the combined expression of *PDAT1* and *OLE1* increases leaf TAG to 6.4% per DW in the wild type and 8.6% per DW in *tgdl* without major negative growth consequences. Given the growing recognition of the potential benefits of maximizing TAG content in vegetative tissues of crops (Durrett et al., 2008; Ohlogge et al., 2009; Chapman et al., 2013; Troncoso-Ponce et al., 2013), this clarification of the role of PDAT1 in plants may enable new strategies for future genetic engineering efforts aimed at enhancing oil accumulation in biomass crops used for biofuel production.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* plants used in this study were of the Columbia ecotype. The *tgdl* mutant was previously described (Xu et al., 2003), as were *dgat1-1* and *pdatt1-2* (Zhang et al., 2009). For growth on plates, surface-sterilized seeds of *Arabidopsis* were germinated on 0.6% (w/v) agar-solidified half-strength Murashige and Skoog (MS) (Murashige and

Skoog, 1962) medium supplemented with 1% (w/v) Suc in an incubator with a photon flux density of 80 to 120 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a light period of 16 h (22°C), and a dark period of 8 h (18°C). For growth in soil, plants were first grown on MS medium for 10 d and then transferred to soil and grown under a photosynthetic photon flux density of 150 to 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 22/18°C (day/night) with a 16-h-light/8-h-dark period.

Generation of Plant Expression Vectors and Plant Transformation

The full-length coding regions of *PDAT1* and *DGAT1* were amplified by RT-PCR using the primers 5'-GCGTGGTACCATGCCCCCTTATTCATCGGA-3' and 5'-ACGTCTGCAGTCACAGCTTCAGGTCAATACGCTC-3' for *PDAT1* and 5'-ACCTGGAGCTCATGGCGATTTGGATTCTGC-3' and 5'-CCGAGG-TACCTCATGACATCGATCCTTTTCGGT-3' for *DGAT1*. The resulting *PDAT1* and *DGAT1* PCR products were digested with *KpnI*-*PstI* or *SacI*-*KpnI*, respectively, and inserted into the respective sites of a binary vector derived from pZP212 (Hajdukiewicz et al., 1994). To generate the *OLE1*-*GFP* fusion construct, the entire genomic DNA encoding the *Arabidopsis* *OLE1* was amplified using the primers 5'-ATGGCGGATACAGCTAGAG-3' and 5'-AGTAGTGTGCTGGCCACC-3' and ligated into pCR8 topo-cloning entry vector (Invitrogen). The gene was then fused in frame with GFP at the C terminus through the LR reaction to the destination vector pGKPGWG (Zhong et al., 2008). After confirming the integrity of the constructs by sequencing, plant stable transformation was performed according to Clough and Bent (1998). Transgenic plants were selected in the presence of the respective antibiotics for the vectors on MS medium lacking Suc.

Lipid and FA Analyses

Plant tissues were frozen in liquid nitrogen, and total lipids were extracted by homogenization in chloroform/methanol/formic acid (1:1:0.1, by volume) and 1 M potassium chloride-0.2 M phosphoric acid according to Dörmann et al. (1995). Neutral and total polar lipids were separated on silica plates (Si250 with a preadsorbant layer; Mallinckrodt Baker) by TLC using a solvent system of hexane/diethyl ether/acetic acid (70:30:1, by volume). Polar lipids were separated using a solvent system consisting of acetone/toluene/water (91:30:7, by volume). Lipids were visualized by spraying 5% H_2SO_4 followed by charring. For quantitative analysis, lipids were visualized by brief exposure to iodine vapor and identified by cochromatography with lipid standards. Individual lipids were scraped from the plate and used to prepare FAMES. Separation and identification of the FAMES was performed on an HP5975 gas chromatography-mass spectrometer (Hewlett-Packard) fitted with 60 m \times 250- μm SP-2340 capillary column (Supelco) with helium as a carrier gas. The methyl esters were quantified using heptadecanoic acid as the internal standard as described by Fan et al. (2011). The TAG content was calculated as described previously (Li et al., 2006). The FA composition at the *sn*-2 position of the glycerol backbone was determined by *Rhizopus arrhizus* lipase digestion as described by Härtel et al. (2000). Pigments were quantified according to Lichtenthaler (1987).

In Vivo Acetate Labeling

In vivo labeling experiments with ^{14}C -acetate were done according to Koo et al. (2005). Briefly, rapidly growing leaves of 7-week-old plants were cut in strips and then incubated in the light (60 $\mu\text{mol m}^{-2} \text{s}^{-1}$) at 22°C with shaking in 10 mL of medium containing 1 mM unlabeled acetate, 20 mM MES, pH 5.5, one-tenth strength of MS salts, and 0.01% Tween 20. The assay was started by the addition of 0.1 mCi of ^{14}C -acetate (106 mCi/mmol; American Radiolabeled Chemicals). At the end of the incubation, leaf strips were washed three times with water and blotted onto filter paper. For the chase period, leaf tissue was incubated in the same medium lacking ^{14}C -acetate under the same conditions as used for the pulse. Total lipids were extracted and separated as described above and radioactivity

associated with total lipids or different lipid classes was determined by liquid scintillation counting or phosphorimaging, respectively.

Staining with Fat Red 7B

Seedlings were stained with a 0.1% (w/v) solution of Fat Red 7B (Sigma-Aldrich) overnight essentially as described by Brundrett et al. (1991). Following brief rinsing with distilled water, samples were visualized with a Wild Heerbrugg dissecting microscope.

Microscopy

For OD imaging, leaf tissues were stained with a neutral lipid-specific fluorescent dye, Nile red (Sigma-Aldrich), at a final concentration of 10 µg/mL and observed under a Zeiss epifluorescence microscope (Carl Zeiss Axiovert 200M) with a GFP filter. For TEM, leaf tissues were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for 2 h and then postfixed with 1% osmium tetroxide in the same buffer for 2 h at room temperature. After dehydration in a graded series of ethanol, the tissues were embedded in EPON812 resin (Electron Microscopy Sciences) and sectioned and stained with 2% uranyl acetate and lead citrate before viewing under a JEOL JEM-1400 LaB6 120KeV transmission electron microscope. For light microscopy observation, the sections were stained with 1% toluidine blue and examined using a Zeiss epifluorescence microscope as described above.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *DGAT1*, At2g19450; *OLE1*, At4g25140; and *PDAT1*, At5g13460.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Changes in Levels of *DGAT1* and *PDAT1* Transcript during Leaf Development.

Supplemental Figure 2. TAG Levels in Transgenic Plants Overexpressing *DGAT1*, *PDAT1*, or *OEL1*.

Supplemental Figure 3. Growth and Lipid Phenotypes of the Transgenic Lines Coexpressing *PDAT1* and *OEL1*.

Supplemental Figure 4. FA Composition of Individual Membrane Lipids from Leaves of *PDAT1* Overexpressors.

Supplemental Figure 5. Time Course of ¹⁴C-Acetate Incorporation into Total Lipids of Leaf Strips from 7-Week-Old Wild-Type Plants Grown in Soil.

Supplemental Figure 6. Growth Phenotype and Total Leaf Polar Lipid Content of Transgenic Plants Coexpressing *PDAT1* and *GFP-OLE1* in the *tgdl-1* Background.

Supplemental Figure 7. Overexpression of *PDAT1* in *act1* Leads to Defects in Chloroplast Division and Thylakoid Biogenesis.

Supplemental Figure 8. Pulse-Chase Acetate Labeling of FAs Associated with Individual Lipids of the *act1* Mutant and *act1 PDAT1*-Overexpressing Line.

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AUTHOR CONTRIBUTIONS

C.X. and J.F. designed the experiments. C.X., J.F., C.Y., and X.Z. performed the research. C.X. and J.F. analyzed the data and wrote the article.

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