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Arabidopsis Lipins, PDAT1 Acyltransferase, and SDP1 Triacylglycerol Lipase Synergistically Direct Fatty Acids toward β-Oxidation, Thereby Maintaining Membrane Lipid Homeostasis[C][W]

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Triacylglycerol (TAG) metabolism is a key aspect of intracellular lipid homeostasis in yeast and mammals, but its role in vegetative tissues of plants remains poorly defined. We previously reported that PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE1 (PDAT1) is crucial for diverting fatty acids (FAs) from membrane lipid synthesis to TAG and thereby protecting against FA-induced cell death in leaves. Here, we show that overexpression of PDAT1 enhances the turnover of FAs in leaf lipids. Using the *trigalactosyldiacylglycerol1-1* (*tgd1-1*) mutant, which displays substantially enhanced PDAT1-mediated TAG synthesis, we demonstrate that disruption of SUGAR-DEPENDENT1 (SDP1) TAG lipase or PEROXISOMAL TRANSPORTER1 (PXA1) severely decreases FA turnover, leading to increases in leaf TAG accumulation, to 9% of dry weight, and in total leaf lipid, by 3-fold. The membrane lipid composition of *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2* double mutants is altered, and their growth and development are compromised. We also show that two *Arabidopsis thaliana* lipin homologs provide most of the diacylglycerol for TAG synthesis and that loss of their functions markedly reduces TAG content, but with only minor impact on eukaryotic galactolipid synthesis. Collectively, these results show that *Arabidopsis* lipins, along with PDAT1 and SDP1, function synergistically in directing FAs toward peroxisomal β-oxidation via TAG intermediates, thereby maintaining membrane lipid homeostasis in leaves.

INTRODUCTION

Intracellular lipid homeostasis is vital for normal membrane structure and function and for cell survival in response to lipid metabolism perturbations resulting from environmental stresses or other causes (Zhang and Rock, 2008; Hermansson et al., 2011; Holthuis and Menon, 2014). To date, although most of the enzymatic steps in lipid biosynthesis are defined at the molecular-genetic level in several model organisms (Nohturfft and Zhang, 2009; Chapman and Ohlrogge, 2012; Henry et al., 2012), the signals and mechanisms regulating intracellular lipid homeostasis are less well defined (Nohturfft and Zhang, 2009; Hermansson et al., 2011, Holthuis and Menon, 2014), particularly in plants (Bonaventure et al., 2004; Kunz et al., 2009; Zhang et al., 2009; Fan et al., 2013a; Park et al., 2013).

Studies in yeast and mammals suggest that triacylglycerol (TAG) and sterol esters can serve as a buffer for fatty acids (FAs) playing a key role in intracellular lipid homeostasis and energy storage. Under conditions of acute lipid overload, excess FAs are converted into biologically inert neutral lipids and packaged in specific subcellular organelles named lipid droplets (LDs) (Listenberger et al., 2003; Petschnigg et al., 2009; Kohlwein, 2010; Murphy, 2012; Kohlwein et al., 2013). In yeast (*Saccharomyces cerevisiae*), a critical enzyme involved in TAG synthesis (Han et al., 2006) and LD formation (Adeyo et al., 2011) is the Mg^{2+}-dependent phosphatidic acid phosphohydrolase (Pah1), the homolog of the mammalian lipin protein family. Yeast mutant cells lacking Pah1 display elevated phospholipid levels (Han et al., 2006), a massive proliferation of endoplasmic reticulum (ER) and nuclear membranes (Santos-Rosa et al., 2005), and increased sensitivity to exogenous FA-induced cell death (Fakas et al., 2011). Similarly, inactivation of the *Caenorhabditis elegans* lipin homolog has also been shown to cause decreased fat storage and altered ER membrane structure (Golden et al., 2009). *Arabidopsis thaliana* lipins, PAH1 and PAH2, have been implicated in the synthesis of thylakoid membrane lipids (Kobayashi et al., 2007) and in the regulation of phospholipid synthesis and ER membrane organization, but surprisingly, disruption of PAH1 and PAH2 causes only a small reduction in seed oil content (Eastmond et al., 2010). The role of PAH1 and PAH2 in TAG biosynthesis in vegetative tissues is currently unknown.

Likewise, disruption of membrane phospholipid synthesis in mammalian cells (Caviglia et al., 2004; Leonardi et al., 2009) or yeast (Malanovic et al., 2008) or a block in intracellular lipid trafficking in yeast (Gaspar et al., 2008) or plants (Xu et al., 2005, 2008) causes increased lipid fluxes toward TAG synthesis and

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storage. By contrast, increasing TAG synthesis by overexpression of *ACYL-COA:DIACYLGLYCEROL ACYLTRANSFERASE1* (*DGAT1*) has been shown to cause a decrease in membrane phospholipid content in human cells (Bagnato and Igal, 2003). Interplay between TAG metabolism and membrane lipid synthesis is also evident from the observations that storage TAG hydrolysis can function as a source of lipid precursors for the synthesis of membrane phospholipids and signaling lipids in both yeast and mammals (Igal and Coleman, 1996; Kohlwein et al., 2013). Deficits in acylglycerol recycling from TAG to phospholipids are the likely cause of the neutral lipid storage disease in humans caused by mutations in COMPARATIVE GENE IDENTIFIER58 (CGI58) (Igal and Coleman, 1996). Disruption of the *Arabidopsis* CGI58-LIKE (CGI58L; Kelly et al., 2011) results in increased TAG accumulation, decreased jasmonate production, and altered responses to auxin, implying a role for CGI58L in lipid turnover and signaling (James et al., 2010; Park et al., 2013).

In oilseeds, TAG serves as a major FA source for energy production and gluconeogenesis during germination and seedling establishment (Graham, 2008; Theodoulou and Eastmond, 2012). The catabolism of TAG begins with the release of its fatty acyl chains by the action of an evolutionarily conserved family of patatin domain-containing lipases (Eastmond, 2006). There are several patatin-like TAG lipase candidates in *Arabidopsis*, including SUGAR-DEPENDENT1 (SDP1), SUGAR-DEPENDENT1-LIKE (SDP1L), and ADIPOSE TRIGLYCERIDE LIPASE-LIKE (ATGLL) (Eastmond, 2006). In plants and yeast, the major pathway for metabolic breakdown of FAs is β-oxidation in peroxisomes (Graham, 2008; Theodoulou and Eastmond, 2012), and FAs as CoA esters are transported into this organelle by PEROXISOMAL TRANSPORTER1 (PXA1) in *Arabidopsis* (De Marcos Lousa et al., 2013). Recent genetic studies have implicated PXA1, SDP1, and SDP1L in TAG breakdown in nonseed tissues (Slocombe et al., 2009; Kelly et al., 2013), but the physiological function of TAG turnover and the regulatory aspects of the FA β-oxidation pathway in leaves remain largely unknown.

In addition to TAG metabolism, biochemical and genetic studies have shown that changes in the balance between glycerolipid pathways contribute to the maintenance of membrane lipid homeostasis. In photosynthetic tissues, two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), constitute the bulk of cellular membrane lipids, and their synthesis in *Arabidopsis* involves two distinct pathways, commonly referred to as the prokaryotic and eukaryotic pathways (Ohlrogge and Browse, 1995). Inactivation of either pathway can be offset by upregulation of the other, so that the overall membrane lipid content and composition remain largely unchanged. This is best exemplified by the *plastidic glycerol-3-phosphate acyltransferase1* (*act1*) (Kunst et al., 1988) and *trigalactosyldiacylglycerol1-1* (*tgd1-1*) (Xu et al., 2003) mutants, which are defective in the prokaryotic and eukaryotic pathways of thylakoid lipid synthesis, respectively. Likewise, diversion of FAs from eukaryotic thylakoid lipid synthesis to TAG synthesis upon overexpression of *PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE1* (*PDAT1*) is accompanied by an increase in FA flux toward prokaryotic lipid synthesis without affecting the membrane lipid content (Fan et al., 2013b). Although the exact mechanism underlying this homeostasis remains unclear, we recently showed that FA synthesis is increased in both the *tgd1-1* mutant and *PDAT1*-overexpressing lines (Fan et al., 2013a, 2013b). In addition, the rate of FA turnover is markedly enhanced in leaves of the *tgd1-1* mutant. Here, we systematically tested several candidate genes for their involvement in FA degradation in *tgd1-1*. The results suggest that two *Arabidopsis* lipins, PDAT1 and SDP1, function synergistically in regulating FA flow from membrane lipid synthesis toward peroxisomal β-oxidation through a transient TAG pool and that blocking FA turnover alters membrane lipid content and composition and compromises plant growth and development in the *tgd1-1* mutant.

RESULTS

Overexpression of *PDAT1* Enhances Both the Synthesis and Turnover of FAs

We previously showed that overexpression of *PDAT1* enhances both FA and TAG synthesis in leaves (Fan et al., 2013b). In rosette leaves of three independent transgenic lines, the rates of FA synthesis, measured as the rate of [^{14}C]acetate incorporation into total FAs, were 61% to 82% higher in leaves of three 5-week-old transgenic lines overexpressing *PDAT1* relative to the wild type (Figure 1A). Since the overall membrane lipid content remained largely unaltered (Fan et al., 2013b), we reasoned that the increased rates of FA synthesis must be accompanied by corresponding increases in the rates of FA turnover. To test this hypothesis, detached leaves were labeled with [^{14}C]acetate for 1 h, and the changes in the concentration of labeled FAs were then chased for 3 d. In the wild type, the decline in labeled FAs during the 3-d chase period was 13% (Figure 1B). This corresponds to an FA turnover rate of 4.3% per day, a value similar to that previously measured in wild-type *Arabidopsis* plants based on an isotope dilution method (Bao et al., 2000) but higher than that reported by Bonaventure et al. (2004). By comparison, the average decay rate of labeled FAs was increased to 6.7% per day in PDAT1 overexpressors (Figure 1B). These results suggest that overexpression of *PDAT1* enhances the rates of both the synthesis and degradation of FAs in leaves.

FA Turnover Involves SDP1 and PXA1

Recent genetic studies have demonstrated a role for SDP1 in TAG breakdown in vegetative tissues of *Arabidopsis* (Kelly et al., 2013). To determine the role of SDP1 in FA turnover in leaves of transgenic plants overexpressing *PDAT1*, genetic crosses were conducted between the PDAT1 overexpressor 3 and *sdp1-4* (Eastmond, 2006), and plants expressing the *PDAT1* transgene in the *sdp1-4* genetic background were subsequently obtained. On a dry weight basis, the leaf TAG levels were almost doubled in the PDAT1 overexpressor 3 in the *sdp1-4* genetic background (1.12% ± 0.22% per dry weight; $n = 3$) compared with the PDAT1 overexpressor in the wild-type background (0.57% ± 0.13% per dry weight; $n = 3$). These results suggest that TAG turnover in PDAT1 overexpressors involves SDP1.

Besides SDP1, the *Arabidopsis* genome contains at least two additional putative patatin-like TAG lipases, SDP1L and ATGLL (Eastmond, 2006). In addition, *Arabidopsis* CGI58L, a member of

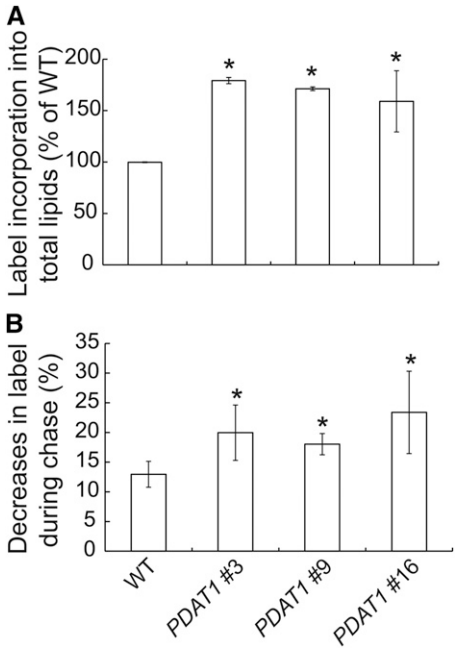

Figure 1. Overexpression of *PDAT1* Enhances the Synthesis and Turnover of FAs.

Initial rates of FA synthesis **(A)** and rates of FA turnover **(B)** in growing leaves of 5-week-old wild-type plants and three transgenic lines overexpressing *PDAT1* in the wild-type background. Data are means of the three replicates with SD. Asterisks indicate statistically significant differences from the wild type based on Student's *t* test ($P < 0.05$).

the α/β-hydrolase family of proteins, also exhibited TAG lipase activity in addition to lysophosphatidic acid acyltransferase activity (Ghosh et al., 2009). To systematically test the in vivo functions of these candidate TAG lipases in FA turnover in leaves, we performed double mutant analysis taking advantage of *tgd1-1*, which accumulates TAG in leaves (Fan et al., 2013a), along with T-DNA insertion mutants in *SDP1* (*sdp1-4*), *SDP1L* (*sdpL-2*; Kelly et al., 2011), *CGI58L* (*cgi58L*; James et al., 2010), and *ATGLL* (*atglL*; Kelly et al., 2011) genes. In addition, we generated a double mutant of *tgd1-1* and *pxa1-2* (Kunz et al., 2009) that contains a T-DNA insertion in the *PXA1* gene to test whether TAG turnover in *tgd1-1* is mediated via β-oxidation in peroxisomes.

Compared with the *tgd1-1* single mutant, no obvious differences in leaf TAG content were found in *tgd1-1* disrupted in SDP1L, CGI58L, or ATGLL (Figure 2A). By contrast, inactivation of SDP1 or PXA1 in *tgd1-1* led to a marked increase in TAG accumulation in leaves. On a dry weight basis, the amounts of leaf TAG in 7-week-old plants grown on soil increased from 0.6% in *tgd1-1* to 8.8% in *tgd1-1 sdp1-4* and 8.1% in *tgd1-1 pxa1-2* (Figure 2B). The increased TAG level was accompanied by a corresponding increase in total leaf FA content to 14.2 and 12.3% per dry weight in *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2*, respectively (Figure 2C), suggesting that TAG accumulation results from decreased hydrolysis rather than a mere redistribution of FAs from membrane lipids to TAG. FA compositional analysis showed that the predominant FAs in TAG from the leaves of both *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2* are polyunsaturated FAs, with 18:2 and 18:3, which account for over 70% of the total TAG acyl chains (Figure 2D). The FA composition of TAG from leaves of *tgd1-1 sdp1-4* was similar to that of *tgd1-1*, except for a small increase in the relative proportion of 16:3 at the expense of 18:0 in the double mutant. In TAG from leaves of *tgd1-1 pxa1-2*, there was a marked increase in 18:3 balanced by a decrease in saturated FAs and 18:2 (Figure 2D).

Microscopic examination of leaves stained with Nile red revealed a few small LD-like structures for the wild type (Figure 3A) and the *sdp1-4* and *pxa1-2* single mutants (Figures 3B and 3C). By contrast, leaves of *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2* double mutants (Figures 3E and 3F) showed marked increases in both the size and number of LDs compared with the *tgd1-1* single mutant (Figure 3D). Ultrastructural imaging using transmission electron microscopy confirmed the presence of LDs larger than 8 μm in diameter in leaf mesophyll cells of the *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2* double mutants (Figures 3H and 3I).

The amounts of TAG appeared to increase slightly in leaves of the single mutant disrupted in SDP1 or PXA1 compared with the wild type, whereas no obvious difference in leaf TAG content was found in the single mutant carrying T-DNA insertion in the *SDP1L*, *CGI58L*, or *ATGLL* gene (Supplemental Figure 1). This lack of effect of CGI58L disruption on TAG content under our growth conditions may reflect the dynamic nature of LD accumulation in *cgi58L*, being apparent only at certain stages of leaf development (James et al., 2010).

Disruption of SDP1 or PXA1 Affects Plant Growth and Development in *tgd1-1*

Homozygous single mutants of both *sdp1-4* and *pxa1-2* displayed similar growth and developmental patterns to the wild type (Supplemental Figure 2). The *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2* double mutants, on the other hand, were stunted in both aerial portions of seedlings in addition to roots, particularly the *tgd1-1 pxa1-2* double mutant compared with *tgd1-1*, when grown on agar-solidified Murashige and Skoog (MS) plates supplemented with 1% sucrose (Figure 4A). The growth defects also extended to plants grown on soil (Figure 4B), but the final plant size and height were similar between *tgd1-1* and the double mutants (Figure 4C). The bolting time was slightly delayed in both *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2* compared with *tgd1-1*. While the seed setting appeared to be normal in *tgd1-1 sdp1-4*, the *tgd1-1 pxa1-2* plants were completely infertile. Cross-pollination of *tgd1-1 pxa1-2* double mutant plants with *tgd1-1* pollen resulted in seed production, but reciprocal crossing did not yield seeds, indicating a pollen defect in *tgd1-1 pxa1-2*. Close examination of the flowers revealed no obvious morphological differences between *tgd1-1* and *tgd1-1 pxa1-2* (Figures 5A and 5B). Assaying pollen viability by Alexander staining indicated that the mature *tgd1-1 pxa1-2* anthers contained abundant viable pollen grains (Figures 5C and 5D). However, no or only a few pollen grains from freshly anther-dehisced flowers of the *tgd1-1 pxa1-2* double mutant germinated in either in vitro (Figures 5E and 5F) or in vivo (Figures 5G and 5H) pollen germination assays. By contrast, most of pollen grains from *tgd1-1* germinated and their pollen tubes elongated

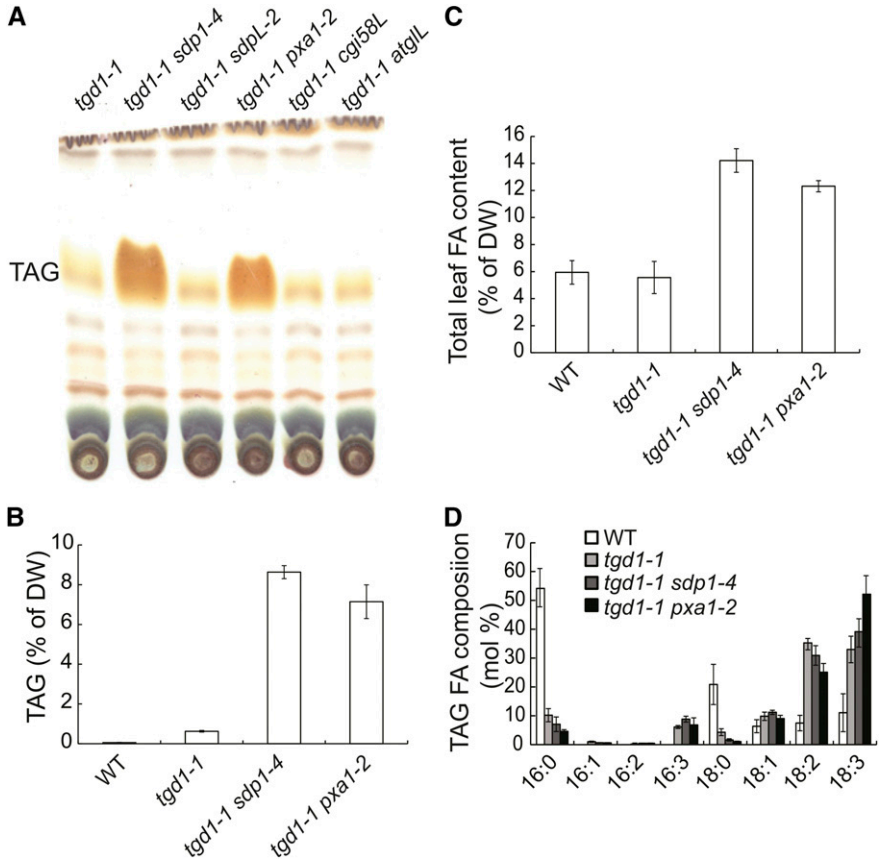

Figure 2. Disruption of SDP1 or PXA1 Boosts Leaf TAG Accumulation in *tgd1-1*.

(A) Thin layer chromatogram of neutral lipids. Lipids were visualized with 5% sulfuric acid by charring.
(B) TAG content in fully mature leaves of 7-week-old wild-type, *tgd1-1*, *tgd1-1 sdp1-4*, and *tgd1-1 pxa1-2* plants grown on soil. Data are means of the three replicates with SD. DW, dry weight.
(C) and **(D)** Total FA content **(C)** and TAG FA composition **(D)** in leaves of wild-type and mutant plants. Values are means and SD of three replicates. [See online article for color version of this figure.]

in a normal manner. These results suggest that disruption of PXA1 causes a defect in pollen germination in the *tgd1-1* background.

Disruption of SDP1 or PXA1 Blocks FA Turnover and Markedly Enhances FA Flux toward TAG Storage in the *tgd1-1* Background

To test whether FA turnover was affected in double mutants, we analyzed the decay rate of labeled FAs in [^{14}C]acetate pulse-chase experiments. The results showed that knockout of SDP1 or PXA1 resulted in significant decreases in the rates of labeled FA degradation in the *tgd1-1* background, whereas no apparent differences were found between *tgd1-1* and *tgd1-1 sdpL-2*, *tgd1-1 atglL*, or *tgd1-1 cgi58L* (Figure 6A). To determine whether the decreased rates of FA turnover were associated with specific lipids, we analyzed the radiolabel distribution in individual glycerolipids in *tgd1-1*, *tgd1-1 sdp1-4*, and *tgd1-1 pxa1-2* following the 3-d chase period. This analysis revealed that disruption of SDP1 or PXA1 led to a marked increase in radiolabel in TAG in *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2*, whereas the radioactivity in major membrane lipid classes appeared to be largely unaltered in the double mutants compared with *tgd1-1* (Figures 6B and 6C). Accordingly, as much as 48.6% ± 1.4% and 35% ± 4.1% (n = 3) of total labeled FAs were associated with TAG in *tgd1-1 sdp1* and *tgd1-1 pxa1-2*, respectively, compared with only 9.6% ± 3.4% (n = 3) of the labeled FAs in TAG in *tgd1-1*.

Previous studies have shown that SDP1 and SDP1L have overlapping functions in TAG hydrolysis in germinating seeds of *Arabidopsis* (Eastmond, 2006; Kelly et al., 2013). To test whether this is also the case in leaves, we constructed a triple mutant impaired in both SDP1 and SDP1L functions in the *tgd1-1* mutant background. Quantification of leaf TAG content revealed no significant difference (P = 0.22, n = 3) between *tgd1-1 sdp1-4* (8.2% ± 0.7% per dry weight) and *tgd1-1 sdp1-4 sdpL-2* (8.9% ± 0.4% per dry weight). The lack of change in leaf TAG content upon disrupting SDP1L is perhaps not unexpected, because the *SDP1L* transcript is minimally expressed in leaves, whereas it is highly expressed in pollen (Kelly et al., 2011).

Based on double mutant analysis, it has been shown that *PXA1* is epistatic to *SDP1* in regulating TAG content in roots (Kelly et al., 2013). To determine the genetic relationship between

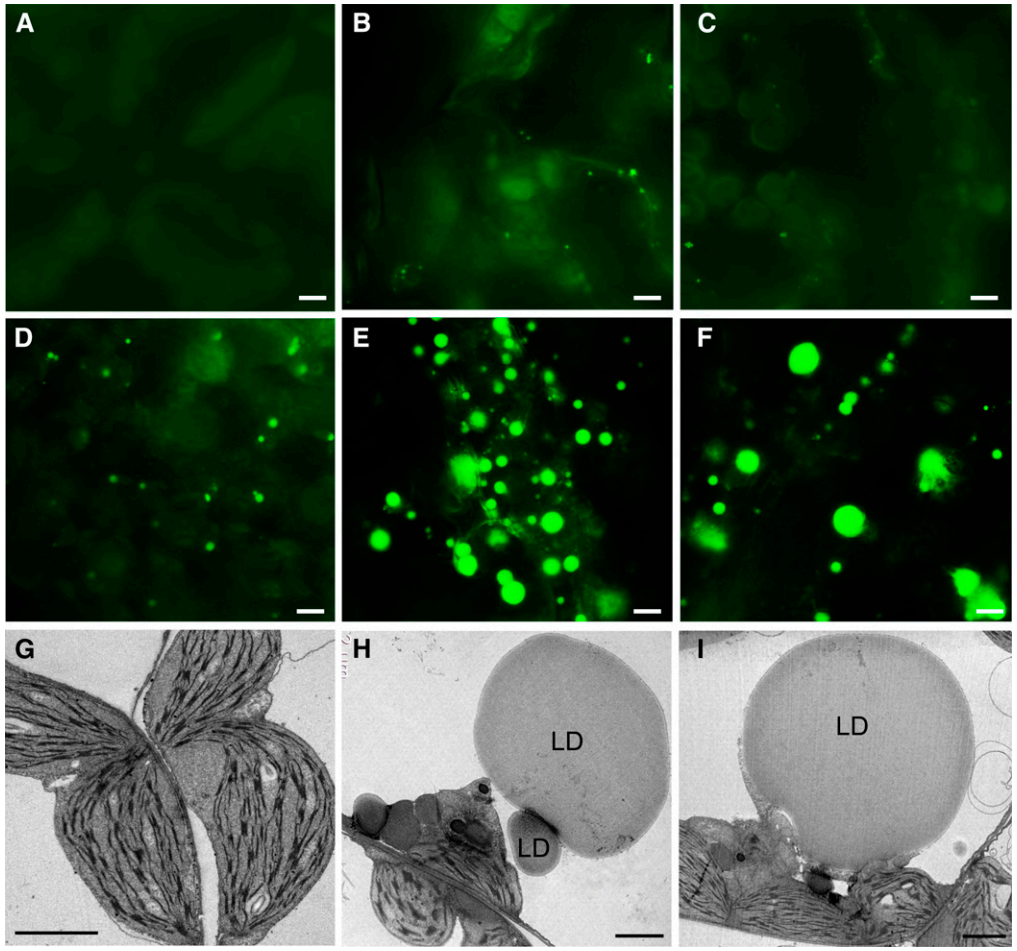

Figure 3. LD Accumulation in Leaves of *tgd1-1* Lacking SDP1 or PXA1.

(A) to **(F)** Images of LDs in wild-type **(A)**, *sdp1-4* **(B)**, *pxa1-2* **(C)**, *tgd1-1* **(D)** *tgd1-1 sdp1-4* **(E)**, and *tgd1-1 pxa1-2* **(F)** leaves stained with Nile red. Bars = 10 μm.

(G) to **(I)** Transmission electron microscopy images of leaf cells of *tgd1-1* **(G)** *tgd1-1 sdp1-4* **(H)**, and *tgd1-1 pxa1-2* **(I)**. Bars = 2 μm.

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these two genes in TAG accumulation in leaves, we generated the triple mutant *tgd1-1 sdp1-4 pxa1-2*. Compared with *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2*, no apparent difference in leaf TAG content was found between the *tgd1-1 sdp1-4 pxa1-2* triple mutant and *tgd1-1 sdp1-4* or *tgd1-1 pxa1-2* (Supplemental Figure 3A). The FA composition of TAG in the triple mutant was also indistinguishable from that of *tgd1-1 pxa1-2* (Supplemental Figure 3B). On soil, the *tgd1-1 sdp1-4 pxa1-2* triple mutant showed similar growth and developmental patterns to *tgd1-1 pxa1-2* (Supplemental Figure 3C) and was completely infertile. Taken together, these results suggest that SDP1 acts in the same pathway with PXA1 that affects leaf TAG metabolism and plant growth and development.

Disruption of SDP1 or PXA1 Alters Membrane Lipid Content and FA Composition in the *tgd1-1* Background

To further assess the impact of blocked FA turnover on lipid metabolism, we analyzed the membrane lipid composition in leaves of single and double mutants. The levels of individual membrane lipids did not show differences between the wild type, *sdp1-4*, and *pxa1-2* (Supplemental Figure 4A). In addition, there was no significant variation in FA composition of total leaf lipids between the wild type and the single mutants lacking SDP1 or PXA1 (Supplemental Figure 4B).

By contrast, disruption of SDP1 or PXA1 alters both membrane lipid content and FA composition in the *tgd1-1* mutant background. On a dry weight basis, the amounts of two major extraplastidic membrane phospholipids, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), significantly increased in leaves of both *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2* (Figure 7A). In addition, the levels of the galactolipids MGDG and DGDG were significantly elevated in *tgd1-1 sdp1-4* but not in *tgd1-1 pxa1-2* as compared with *tgd1-1*. As a consequence, the total amounts of membrane lipids increased by 35 and 12% in *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2*, respectively, relative to *tgd1-1*. Examining the FA composition of individual membrane lipids revealed

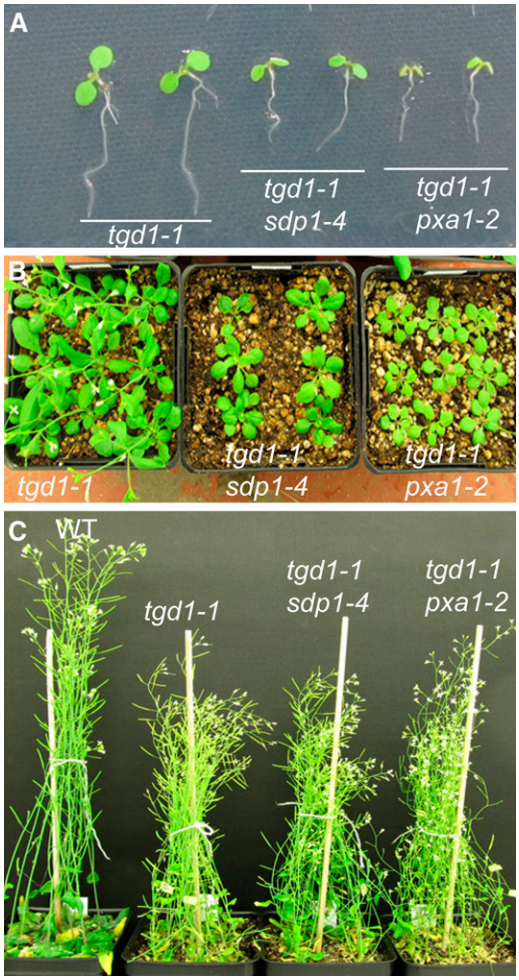

Figure 4. Disruption of SDP1 or PXA1 Affects Growth and Development of *tgd1-1*.

(A) One-week-old plants grown on agar plates in the presence of 1% Suc.
(B) Four-week-old plants grown on soil.
(C) Six-week-old wild-type and mutant plants grown on soil.
[See online article for color version of this figure.]

significant increases in the proportion of 18:2 at the expense of 18:3 in both PC and PE in *tgd1-1 sdp1-4* (Supplemental Figures 5A and 5B). In *tgd1-1 pxa1-2*, there was a significant increase in 18:3 in PC and PE, with a concomitant decrease in saturated acyl chains. The changes in the FA composition of the major thylakoid lipids MGDG and DGDG appeared to be less pronounced in the double mutants compared with *tgd1-1*. However, there was a significant increase in the relative level of 18:3 at the expense of C16 FAs in galactolipids in both *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2* relative to *tgd1-1* (Supplemental Figures 5C and 5D).

Since C16 FAs are predominantly associated with thylakoid lipids made by the prokaryotic pathway (Browse et al., 1989), the decreased C16 level may indicate a shift in the balance between two glycerolipid biosynthetic pathways. To test this hypothesis, we analyzed the FAs at the *sn-2* position of the individual galactolipids. As shown in Figure 7B, there was a significant increase in the relative proportion of C18 FAs at the *sn-2* position of both MGDG and DGDG in the double mutants relative to *tgd1-1*, indicating an increased eukaryotic pathway activity for galactolipid synthesis in the double mutants. Because the *tgd1-1* point mutant is a leaky mutation in a permease-like protein involved in ER-to-plastid lipid import, the increased eukaryotic pathway of galactolipid synthesis may be attributable to the increased availability of lipid substrates for the TGD1-associated lipid transfer system due to blocked FA turnover in the double mutants.

Overexpression of *SDP1* Decreases TAG Levels in *tgd1-1*

To provide additional evidence to support a role of SDP1, but not other lipase candidates, in TAG hydrolysis in leaves, we generated transgenic plants overexpressing *SDP1*, *SDP1L*, *CGI58L*, or *ATGLL* from the constitutive 35S promoter in the *tgd1-1* background. Multiple independent transgenic lines for each construct were tested for leaf TAG content by thin layer chromatography. Five representative lines for each transgene were selected for detailed analysis. Consistent with the results from the loss-of-function studies as shown in Figure 2A, overexpression of *SDP1*, but not other candidate lipases, significantly decreased leaf TAG content in *tgd1-1* (Supplemental Figure 6). The average leaf TAG content in 5-week-old plants grown on soil decreased by 51% in *tgd1-1* overexpressing *SDP1* compared with *tgd1-1*. By contrast, no decreases in TAG levels were noted in *tgd1-1* overexpressing *SDP1L* or *CGI58L*. Interestingly, overexpression of *ATGLL* caused a marked increase in leaf TAG in *tgd1-1*. This raises the possibility that ATGLL may mediate transacylation reactions to form TAG from monoacylglycerol and diacylglycerol (DAG), as has been reported for some mammalian lipases (Jenkins et al., 2004).

Taken together, the results from our genetic analyses are consistent with previous data (Kelly et al., 2013), showing SDP1 as a major player in TAG mobilization in vegetative tissues, while ruling out significant contributions to TAG breakdown by SDP1L, CGI58L, and ATGLL.

PAH1 and PAH2 Are Required for TAG Synthesis in Leaves

The *Arabidopsis* genome harbors two highly similar lipin homolog genes encoding Mg^{2+}-dependent PAHs that function redundantly in catalyzing the dephosphorylation of phosphatidic acid (PA) to DAG (Nakamura et al., 2009; Eastmond et al., 2010), the direct precursor of TAG and phospholipids. To test their role in TAG synthesis and FA degradation in leaves, we constructed a double mutant lacking PAH1 and PAH2 by genetic crossing. This double mutant is the same allele combination as that described previously by Nakamura et al. (2009) and Eastmond et al. (2010) and was designated as *pah1 pah2-1* by Eastmond et al. (2010). We then made genetic crosses between *pah1 pah2-1* and *tgd1-1* or *PDAT1* overexpressing line 3. The *tgd1-1 pah1 pah2-1* triple mutant and the *PDAT1*-overexpressing line in the *pah1 pah2-1* background were subsequently recovered. As shown in Figure 8A, the amount of TAG in leaves of *tgd1-1 pah1 pah2-1* was decreased by 66% compared with *tgd1-1*. In the PDAT1 overexpressor 3, disruption of two *PAH* genes resulted in an 83% reduction in leaf TAG content relative to the transgenic

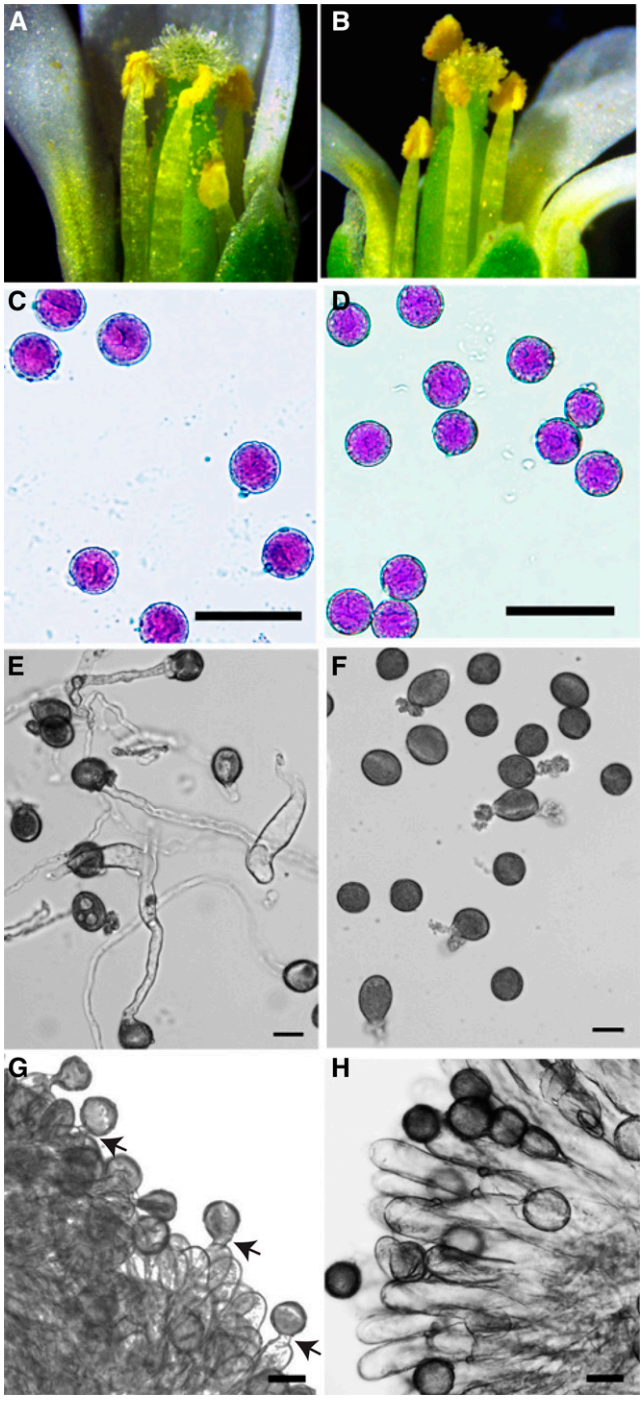

Figure 5. Disruption of PXA1 Affects Pollen Grain Germination in *tgd1-1*.

(A) and **(B)** Flower morphology of *tgd1-1* **(A)** and *tgd1-1 pxa1-2* **(B)**.
(C) and **(D)** Pollen viability in *tgd1-1* **(C)** and *tgd1-1 pxa1-2* **(D)**. Bars = 20 μm.
(E) and **(F)** Germination of mature pollen grains from *tgd1-1* **(E)** and *tgd1-1 pxa1-2* **(F)** in liquid medium. Bars = 20 μm.
(G) and **(H)** Pollen germination and pollen tube growth (arrows) of *tgd1-1* **(G)** and *tgd1-1 pxa1-2* **(H)** in the transmitting tract of the wild-type style. Bars = 20 μm.
[See online article for color version of this figure.]

lines in the wild-type background. These results suggest that, in contrast with seeds (Eastmond et al., 2010), PA dephosphorylation catalyzed by PAH1 and PAH2 is the major source of DAG for TAG synthesis in leaves.

There appeared to be a slight reduction in leaf TAG content in the *pah1 pah2-1* double mutant compared with the wild type (Figure 8A). The overall TAG levels, however, were too low to calculate a meaningful percentage difference. Therefore, we sought to assess the ability of the *pah1 pah2-1* double mutant to synthesize TAG by labeling detached leaves with [^{14}C]acetate followed by thin-layer chromatography and phosphor-imaging analysis. We detected a significant ($P = 0.01$, $n = 3$) reduction in acetate incorporation into TAG in *pah1 pah2-1* (1.90% ± 0.13% of the total label) compared with the wild type (2.97% ± 0.37% of the total label).

PAH1 and PAH2 Play a Limited Role in Eukaryotic Galactolipid Synthesis

PAH1 and PAH2 have been proposed to be involved in the eukaryotic pathway of galactolipid biosynthesis (Kobayashi et al., 2007), although this has recently been questioned (Eastmond et al., 2010). To test genetically whether PAH1 and PAH2 are involved in eukaryotic thylakoid lipid synthesis, we constructed the triple mutant *act1 pah1 pah2-1* by crossing *pah1 pah2-1* and *act1* mutants. Given that the prokaryotic thylakoid pathway is largely disabled in the *act1* mutant, we reasoned that the *act1 pah1 pah2-1* triple mutant should be nonviable or severely impaired in galactolipid synthesis and plastid biogenesis if PAH1 and PAH2 are important for the eukaryotic thylakoid lipid synthesis. In contrast with the embryo-lethal phenotype observed for the *tgd1-1 act1* double mutant (Xu et al., 2005), the *act1 pah1 pah2-1* triple mutant was capable of robust autotrophic growth on soil and producing viable seeds (Supplemental Figure 7). On a fresh weight basis, the amount of MGDG in *act1 pah1 pah2-1* was decreased by 24% compared with *pah1 pah2-1* but increased by 20% compared with *act1*, while DGDG content remained largely unchanged relative to either parent (Figure 8B). These results suggest that PAH1 and PAH2 do not play a major role in the eukaryotic pathway of thylakoid lipid synthesis in leaves.

Disruption of SENSITIVE TO FREEZING2 Affects Lipid Content and FA Turnover in the *tgd1-1* Background

The somewhat less pronounced decrease in TAG content due to PAH disruption in *tgd1-1* compared with the PDAT1 overexpressors (Figure 8A) may suggest the existence of additional enzyme activity capable of providing DAG for TAG synthesis in the mutant. One such candidate protein is SENSITIVE TO FREEZING2 (SFR2). SFR2 converts MGDG to DGDG and oligogalactolipids by transglycosylation with a concomitant production of DAG, which can be further metabolized to TAG (Moellering et al., 2010). The *tgd1-1* mutant accumulates oligogalactolipids in leaves, indicating that SFR2 is activated in this mutant (Xu et al., 2003, 2008; Awai et al., 2006). To test the role of SFR2 in lipid metabolism in *tgd1-1*, we generated a double mutant between *tgd1-1* and an *SFR2* T-DNA knockout mutant, *sfr2-3* (Moellering et al., 2010). Quantitative analysis of leaf lipid composition revealed that TAG content was 26.7%

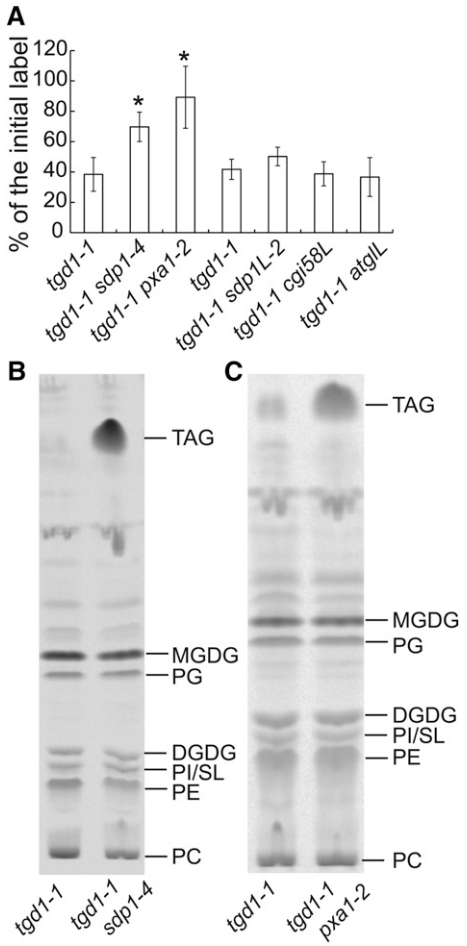

Figure 6. Disruption of SDP1 or PXA1 Enhances FA Flux toward TAG Storage in *tgd1-1*.

(A) Decreases in total labeled FAs during the chase period in *tgd1-1* and double mutants. Detached leaves were labeled with [^{14}C]acetate for 1 h, and the label was chased for 3 d. Values are means and SD of three replicates. Asterisks indicate statistically significant differences from *tgd1-1* based on Student's *t* test ($P < 0.05$).

(B) and **(C)** Autoradiographs of radiolabeled lipids separated by thin layer chromatography using a double development. PI/SL, phosphatidylinositol/sulfoquinovosyldiacylglycerol.

lower in *tgd1-1 sfr2-3* (202.5 ± 20.5 μg/g fresh weight, $n = 3$) compared with *tgd1-1* (276.2 ± 49.1 μg/g fresh weight, $n = 3$). The amounts of MGDG and DGDG were increased significantly in the double mutant, whereas the major phospholipids PC and PE remained largely unchanged (Figure 9A). The polar lipid composition in the *sfr2-3* single mutant was not significantly different from that of the wild type (Supplemental Figure 8).

We next analyzed the changes in radioactivity in individual glycerolipids in the wild type, *sfr2-3*, *tgd1-1*, and *tgd1-1 sfr2-3* following pulse-chase labeling of detached leaves with [^{14}C] acetate. During the 3-d chase in the wild type, a marked decrease in radioactivity in PC was accompanied by a 2.5-fold increase in radiolabel in DGDG (Figure 9B), reflecting the established precursor-product relationship between these two membrane lipid species, while the radioactivity remained largely unchanged in PE, phosphatidylinositol/sulfoquinovosyldiacylglycerol, phosphatidylglycerol, and MGDG. During the chase in *tgd1-1*, the radioactivity decreased by 88% in MGDG, 75% in PC, and 50 to 70% in PE, phosphatidylglycerol, and phosphatidylinositol/sulfoquinovosyldiacylglycerol and remained largely unchanged in DGDG. Since the eukaryotic pathway of the galactolipid biosynthesis pathway is severely compromised in *tgd1-1*, the substantial decrease in MGDG label may in part reflect a conversion of MGDG to DGDG and oligogalactolipids catalyzed by SFR2. In support of this possibility, disruption of SFR2 in *tgd1-1* led to increases in radiolabel in galactolipids, particularly in DGDG, while the radiolabel in major phospholipids stayed unaltered during the chase relative to the *tgd1-1* single mutant (Figure 9B). The

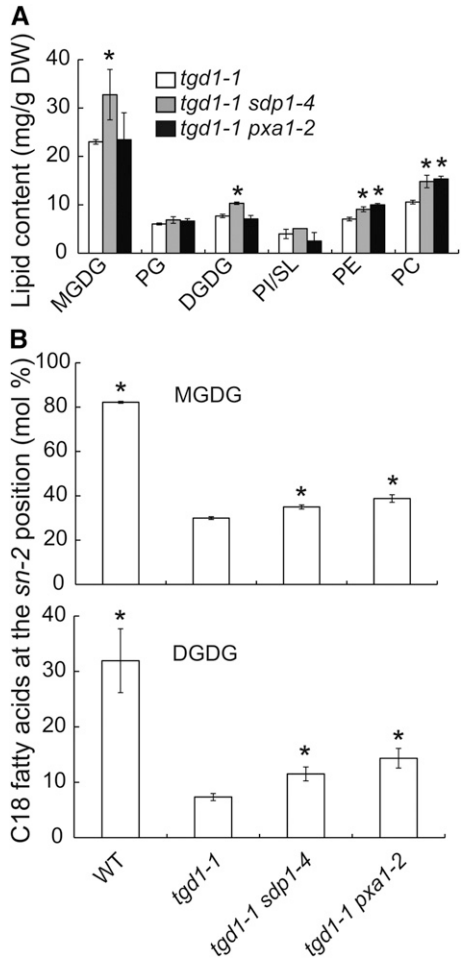

Figure 7. Disruption of SDP1 or PXA1 Alters Membrane Lipid Content and Galactolipid FA Positional Distribution in *tgd1-1*.

(A) Polar lipid content in *tgd1-1* and double mutants. DW, dry weight; PI/SL, phosphatidylinositol/sulfoquinovosyldiacylglycerol.

(B) FA composition exclusively at the *sn-2* position of the glycerol backbone of galactolipids from leaves of wild-type and mutant plants. Data are means of the three replicates with SD. Asterisks indicate statistically significant differences from *tgd1-1* based on Student's *t* test ($P < 0.05$).

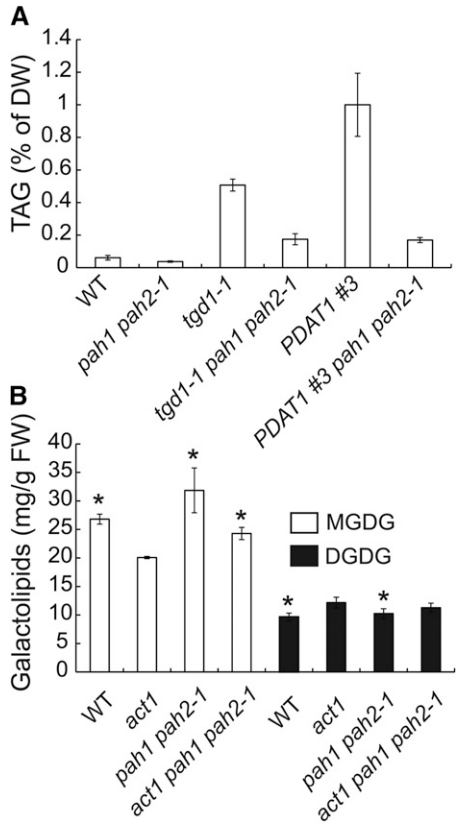

Figure 8. Disruption of PAH1 and PAH2 Compromises TAG but Not the Eukaryotic Galactolipid Synthesis.

(A) Leaf TAG accumulation in the wild type, *tgd1-1*, and *PDAT1* overexpressing line 3 (*PDAT1 #3*) disrupted in PAH1/2. Data are means of the three replicates with SD. DW, dry weight.

(B) Leaf galactolipid content in the wild type and mutants. Data are means of the three replicates with SD. Asterisks indicate statistically significant differences from *act1* based on Student's *t* test ($P < 0.05$). FW, fresh weight.

increased label accumulation in MGDG and DGDG in *tgd1-1 sfr2-3* compared with *tgd1-1* agrees well with the increased mass composition of galactolipids in the double mutant, as shown in Figure 9A. In addition to membrane lipids, the radiolabel in TAG decreased by 46% in the wild type but more than 70% in both mutants, indicating accelerated turnover of TAG in *tgd1-1* and *tgd1-1 sfr2-3*. Importantly, the rate of the decay in total labeled FAs was decreased significantly in *tgd1-1 sfr2-3* compared with the *tgd1-1* single mutant (Figure 9C), implying that SFR2 transglycosylation activity contributes to FA degradation in *tgd1-1*. The *sfr2-3* single mutant displayed no significant change in the rate of FA turnover compared with that of the wild type (Figure 9C).

The FA β-Oxidation and Glyoxylate Cycle Pathways Are Not Induced in *tgd1-1*

Because the rate of FA degradation was strongly enhanced in leaves of the *tgd1-1* mutant (Figure 9C; Fan et al., 2013a), we sought to determine the enzymatic activity of acyl-CoA oxidase (ACOX), which catalyzes the first and rate-limiting step in the β-oxidation cycle (Aoyama et al., 1994) and is a reliable indicator of peroxisomal β-oxidation capacity (Eccleston et al., 1996; Hooks et al., 1999). Compared with the wild type, there was no significant difference in ACOX activity with long-chain acyl-CoA as substrate in leaves of *tgd1-1* (Supplemental Figure 9A). In plants, the end product of FA peroxisomal β-oxidation, acetyl-CoA, can be converted to carbohydrates via the glyoxylate cycle. Accordingly, the transcript levels of isocitrate lyase and malate synthase, two unique components of the glyoxylate cycle, were

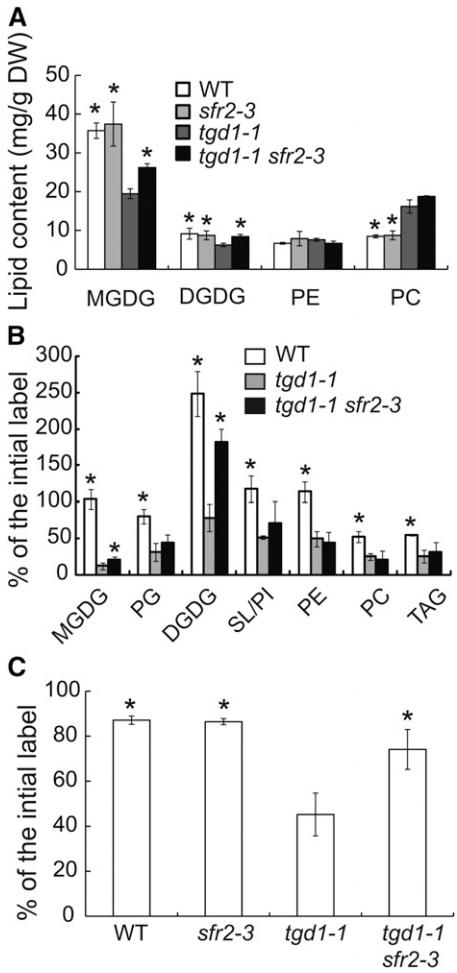

Figure 9. Disruption of SFR2 Affects Membrane Lipid Content and FA Turnover in *tgd1-1*.

Detached leaves were labeled with [^{14}C]acetate for 1 h, and the label was chased for 3 d. Values are means and SD of three replicates. Asterisks indicate statistically significant differences from *tgd1-1* based on Student's *t* test ($P < 0.05$).

(A) Membrane lipid content in leaves of wild-type and mutant plants. DW, dry weight.

(B) Changes in radiolabel in membrane lipids and TAG during the chase period. SL/PI, sulfoquinovosyldiacylglycerol/phosphatidylinositol.

(C) Changes in total radiolabeled FAs during the chase period.

quantified by quantitative PCR. Consistent with previous reports (Troncoso-Ponce et al., 2013), the expression levels of both genes were extremely low in leaves (Supplemental Figure 9B), and again, no differences were observed between the wild type and the *tgd1-1* mutant. It thus appears that the endogenous β-oxidation capacity in leaves of *Arabidopsis* is sufficient to catabolize the additional FAs produced in *tgd1-1* leaves. This lack of induction of β-oxidation is consistent with the finding that the activity of the β-oxidation cycle in vegetative tissues of *Arabidopsis* is inducible by unusual FAs but not by FAs normally associated with membrane lipids (Mittendorf et al., 1999).

DISCUSSION

In plants and fungi, peroxisomal β-oxidation is the major pathway for the breakdown of FAs to acetyl-CoA, a key metabolite for energy production and for the synthesis of carbohydrates. The molecular identity of the core β-oxidation pathway components and their physiological role in FA catabolism in postgerminative seedling growth in oilseeds are now well established (Graham, 2008; Theodoulou and Eastmond, 2012). Physiological understanding of β-oxidation in mature plant tissues has been defined in dark-induced (Kunz et al., 2009) or natural (Yang and Ohlrogge, 2009) senescence, reproductive development (Rylott et al., 2003; Footitt et al., 2007a, 2007b), with respect to the biosynthesis of auxin (Strader and Bartel, 2011) and jasmonate (Goepfert and Poirier, 2007), and during storage TAG production (Slocombe et al., 2009; Kelly et al., 2013). The results of this study provide new insights into the regulation of the peroxisomal β-oxidation pathway and its role in membrane lipid homeostasis in leaves. We show that blocking FA β-oxidation compromises plant growth and development and disrupts membrane lipid homeostasis in the *tgd1-1* mutant background in which the eukaryotic pathway of thylakoid lipid synthesis is impaired. We provide evidence that PDAT1 and SDP1 play key roles in diverting FAs from membrane lipid synthesis toward β-oxidation through a transient TAG pool. We additionally show that the majority of DAG for TAG synthesis is provided by PAH1 and PAH2, pointing to an evolutionarily conserved role for lipins in the regulation of TAG storage and glycerolipid metabolism in yeast, plants, and mammals.

PDAT1 and SDP1 Function Cooperatively in Directing FAs toward β-Oxidation in Peroxisomes

Based on the results from this and previous research, we propose a model describing the FA degradation pathway in leaves and its role in membrane lipid homeostasis in the *tgd1-1* mutant (Figure 10). In oilseeds, the first step in storage oil mobilization is primarily catalyzed by SDP1, which hydrolyzes TAG to free FAs and DAG (Eastmond, 2006). Following hydrolytic release, FAs must be translocated from oil droplets to the peroxisome, and this FA transfer process is believed to be facilitated by direct physical contact between organelle membranes (Graham, 2008; Theodoulou and Eastmond, 2012). By analogy to the FA breakdown pathway described in oilseeds (Theodoulou and Eastmond, 2012), we propose that FAs released by SDP1 along with other lipases from TAG stored in LDs, rather than nascent FAs exported from the plastid, are the direct substrates for peroxisomal β-oxidation in leaves. Several lines of evidence support an interconnection between leaf TAG metabolism and FA β-oxidation. First, according to current knowledge of acyl fluxes between glycerolipid pathways (Bates et al., 2007), FAs exported from the plastid are first incorporated into PC through an acyl editing cycle in the ER, and the acyl groups released from PC are mostly, if not exclusively, used for membrane lipid synthesis for organelle biogenesis and cell growth. In the *tgd1-1* mutant that exhibits large increases in rates of both FA synthesis and turnover, more than 90% of the [^{14}C]acetate label was initially associated with membrane lipids. During the chase, the loss of radioactivity occurred in almost all glycerolipids. Second, disruption of SDP1-dependent release of FAs from TAG largely

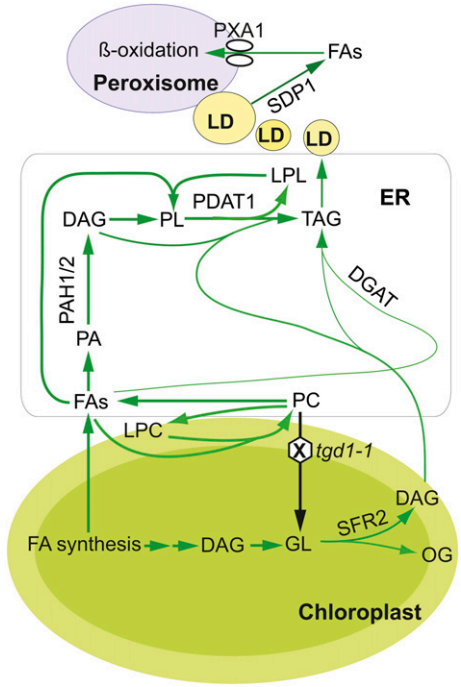

Figure 10. Model for the Proposed Pathway of FA Oxidation in Leaves of the Wild Type and the *tgd1-1* Mutant.

The model proposes that FAs released by SDP1 from TAG stored in cytosolic LDs are the direct substrates for peroxisomal β-oxidation and that PDAT1 plays a critical role in diverting FAs from membrane lipid synthesis to TAG storage. FAs exported from the plastid are first incorporated into PC via an acyl editing cycle. Acyl groups released from PC acyl editing are used predominantly for sequential acylation of glycerol-3-phosphate to produce PA. Dephosphorylation of PA by PAH1/2 generates DAG, which in the wild-type leaves is mostly used for the de novo synthesis of extraplastidic phospholipids, including PC. A major portion of PC derivatives are returned to the plastid to support the eukaryotic pathway of thylakoid glycolipid (GL) synthesis. In the *tgd1-1* mutant, a compromised (X) eukaryotic GL pathway enhances (green arrows) not only FA synthesis but also FA peroxisomal β-oxidation through PC and TAG. A substantial fraction of acyl chains in *tgd1-1* are channeled into peroxisomal β-oxidation through SFR2-mediated DAG production. LPC, lysophosphatidylcholine; LPL, lysophospholipid; OG, oligogalactolipid; PL, phospholipid.

blocks FA β-oxidation, causing increased TAG and membrane lipid accumulation in the *tgd1-1* background. Importantly, both TAG content and the rate of FA turnover are similar between *tgd1-1 sdp1-4* and *tgd1-1 pxa1-2* double mutants, implying that SDP1 is the major lipase involved in the initiation of TAG hydrolysis in leaves and that the majority of FAs destined for peroxisomal β-oxidation pass through a transient TAG pool. Third, genetic analysis indicates that *PXA1* is epistatic to *SDP1* in leaf FA breakdown, TAG accumulation, and plant growth and development, suggesting that SDP1 and PXA1 function in the same pathway, consistent with recent results from root TAG storage (Kelly et al., 2013). Fourth, the finding that TAG accumulated in leaves of *tgd1-1*, *tgd1-1 sdp1-4*, and *tgd1-1 pxa1-2* is enriched in polyunsaturated acyl chains supports the origin of FAs in TAG from membrane lipid remodeling, rather than from de novo synthesis, because polyunsaturated FAs are produced as a result of the desaturation of PC and other membrane lipids, whereas the end products of de novo FA synthesis are principally 18:1 and 16:0 (Ohlrogge and Browse, 1995). Fifth, overexpression of *SDP1* decreases TAG levels in leaves of *tgd1-1*, suggesting that the initiation of TAG breakdown is rate-limiting for peroxisomal FA β-oxidation in the mutant. In the wild type, overexpression of *PDAT1* enhances both FA synthesis and turnover in leaves, suggesting that FA degradation is limited by FA and/or TAG synthesis rather than by TAG hydrolysis. Finally, inactivation of SFR2 results in decreases in both TAG content and FA turnover, supporting the notion that TAG synthesis and FA breakdown are tightly linked metabolic processes in leaves.

Both the acyl-CoA-dependent and acyl-CoA-independent reactions catalyzed by DGAT1 and PDAT1, respectively, are involved in TAG synthesis in nonseed tissues (Fan et al., 2013a). In addition, a soluble DGAT enzyme named DGAT3 also contributes to TAG synthesis in young seedlings (Hernández et al., 2012). While the relative contributions of DGAT1 and PDAT1 to TAG synthesis vary among different stages of leaf development, PDAT1 is more involved in TAG production than DGAT1 in growing leaves (Fan et al., 2013a, 2013b), when both the FA synthesis (Kannangara et al., 1973) and the FA efflux from the plastid into PC (Andersson et al., 2001), the substrate for PDAT1-mediated TAG synthesis, are at their maximal levels. The proposed critical role of PDAT1 in TAG synthesis is also consistent with the observation that overexpression of PDAT1, but not DGAT1, enhances TAG synthesis and LD formation in *Arabidopsis* leaves (Fan et al., 2013a, 2013b), while disruption of PDAT1 decreases TAG accumulation with a simultaneous increase in membrane phospholipid levels in *act1* and *tgd1-1* (Fan et al., 2013a, 2013b). In vitro assays showed that the PDAT enzyme catalyzes the transfer of acyl groups from a wide range of glycerolipid substrates to DAG to form TAG and lysolipids (Ståhl et al., 2004; Ghosal et al., 2007; Yoon et al., 2012), supporting the idea that PDAT1 may mediate the enhanced acyl flux through several major membrane lipid classes in the *tgd1-1* mutant. However, it should be noted that PDAT1 and DGAT1 have overlapping functions in *Arabidopsis* (Zhang et al., 2009) and that DGAT1 is more important than PDAT1 in TAG synthesis at later stages of leaf development (Fan et al., 2013a, 2013b), when the β-oxidation genes are coordinately induced (Troncoso-Ponce et al., 2013). Further study is apparently needed to investigate the role of DGAT1 and possibly other acyltransferases in FA turnover and whether DGAT1-mediated TAG synthesis is also coupled with SDP1 and FA β-oxidation in leaves, although it was recently shown that disruption of SDP1 enhances leaf TAG accumulation in transgenic plants overexpressing *DGAT1* (Kelly et al., 2013).

Since the PDAT1 activity is associated with microsomal membranes (Ståhl et al., 2004) while SFR2 is situated in the outer envelope of chloroplasts (Xu et al., 2003), the use of the DAG generated by SFR2 for TAG production would entail the transfer of DAG from the outer chloroplast envelope to the ER. Although the exact trafficking mechanism remains unclear, it is possible that this transfer is facilitated by the direct physical association between the membranes of the ER and the chloroplast outer envelope. In support of this possibility, ER-chloroplast membrane contact sites have been reported in a number of microscopy studies (Wang and Benning, 2012), and interorganelle contact sites have been proposed as possible conduits for the trafficking of lipids and other metabolites in plants (Andersson et al., 2007) and other eukaryotes (Levine and Loewen, 2006; Elbaz and Schuldiner, 2011). Alternatively, DGAT1 or the soluble DGAT3 may be involved in the acylation of DAG derived from SFR2 into TAG at the outer chloroplast envelope. In this regard, it is interesting that DGAT1 has been shown to be associated with chloroplast membranes in senescing leaves of *Arabidopsis* (Kaup et al., 2002) and that the majority of acyl-CoA-dependent TAG biosynthetic activities in spinach (*Spinacia oleracea*) leaves colocalize with the galactosyltransferase activity at chloroplast envelopes (Martin and Wilson, 1984).

FA β-Oxidation Is Important for Cellular Lipid Homeostasis

Due to their amphipathic properties, free FAs are toxic to cells and their accumulation can lead to cell dysfunction and even cell death, in a process collectively termed lipotoxicity (Listenberger et al., 2003). In wild-type plants under normal growth conditions, FA synthesis is fine-tuned to the cellular demand for membrane biogenesis, and only limited amounts of FAs are channeled into TAG synthesis and hence peroxisomal β-oxidation. Disruption of the eukaryotic pathway of thylakoid lipid synthesis in the *tgd1-1* mutant causes an increase of up to 3.8-fold in the rate of FA synthesis (Fan et al., 2013a). In spite of this increase, lipid homeostasis is largely maintained in the *tgd1-1* mutant through the combined activity of TAG synthesis and FA β-oxidation. Deficiency in TAG storage due to PDAT1 disruption causes lipotoxic cell death in the *tgd1-1* mutant (Fan et al., 2013a). Likewise, blocking peroxisomal β-oxidation compromises plant growth and development and affects membrane lipid homeostasis in the *tgd1-1* mutant. In this context, it is noteworthy that FA β-oxidation was recently shown to be vital for protection against lipotoxicity in mature leaves during extended darkness (Kunz et al., 2009).

In addition to TAG, membrane phospholipid levels are significantly enhanced when FA β-oxidation is disabled in leaves of *tgd1-1*. The increase in membrane lipid content was also observed in *tgd1-1* disrupted in PDAT1 (Fan et al., 2013a) and in yeast mutants defective in TAG synthesis (Petschnigg et al., 2009; Fakas et al., 2011). Such an increase has been regarded

as an alternative means to protect against lipotoxicity due to lipid overload (Petschnigg et al., 2009; Fakas et al., 2011). Because phospholipid levels are elevated in both *tgd1-1 sdp1-4*, in which TAG hydrolysis is impaired, and *tgd1-1 pxa1-2*, in which FA degradation is impaired, it is unlikely that this increase is due to the augmented recycling to TAG into membrane lipids as a result of blocked FA turnover. It is important to note that lipid homeostatic regulation in bacteria (Zhang and Rock, 2008), yeast (Henry et al., 2012), and mammals (Hermansson et al., 2011) is complex and occurs by diverse mechanisms. While we have shown that PDAT1, SDP1 lipase, and lipins are involved in this process in leaves, it is likely that additional components will be uncovered in future research that will lead to a more global understanding of lipid homeostasis in plants.

PAH1 and PAH2 Play an Evolutionarily Conserved Role in TAG Synthesis

In *tgd1-1*, the FA composition of both DAG (Fan et al., 2013a) and PA (Xu et al., 2005) resembles that of PC. This likely reflects the facts that (1) the sequential glycerol-3-phosphate acylation leading to PA production employs recycled acyl groups derived from PC remodeling (Bates et al., 2007), (2) PC and DAG are interconvertible (Lu et al., 2009), and (3) PA can be converted to DAG by the action of PAH1 and PAH2 (Nakamura et al., 2009; Eastmond et al., 2010). In yeast, DAG pools generated by Pah1 are the major source of DAG for TAG synthesis (Han et al., 2006). Similarly, disruption of the single lipin ortholog in *Drosophila melanogaster* (Ugrankar et al., 2011) or inactivation of lipin-1 in mice (Mitra et al., 2013) or lipin-2 in human cells (Valdearcos et al., 2012) leads to a decrease in TAG storage. Here, we show that disruption of *Arabidopsis* PAH1 and PAH2 results in a substantial decrease in TAG content in leaves, suggesting that the role of lipins in TAG synthesis is evolutionarily conserved in plants. In contrast with leaf tissues, knockout of PAH1 and PAH2 only marginally affects oil content in developing seeds (Eastmond et al., 2010). The reason for this difference is not entirely clear but implies that the DAG pool used for TAG synthesis in developing seeds is not derived directly from PA dephosphorylation but from PC and DAG interconversion (Bates et al., 2009), catalyzed mainly by phosphatidylcholine:diacylglycerol cholinephosphotransferase (Lu et al., 2009), and that other PA phosphohydrolase isoenzymes are responsible for DAG synthesis in the absence of PAH1 and PAH2 (Eastmond et al., 2010)

Biotechnological Implications for TGD Proteins

Engineering TAG accumulation in vegetative tissues of plants has widely been discussed as a potential approach to extending plant oil production capacity for the production of nutrition-rich feed and renewable fuels due to their high abundance relative to oil-rich seed tissues (Durrett et al., 2008; Ohlrogge et al., 2009; Chapman et al., 2013; Troncoso-Ponce et al., 2013). To date, a major effort has focused on the ectopic expression of WRINKLED1 (WRI1), a key seed-specific transcriptional regulator (Cernac and Benning, 2004), to increase the supply of FAs for TAG assembly (Sanjaya et al., 2011; Kelly et al., 2013; Vanhercke et al., 2014). We previously reported that disruption of the eukaryotic galactolipid pathway in the *tgd1-1* mutant results in substantial increases in the rates of both FA synthesis and turnover in leaves (Fan et al., 2013a). Here, we show that blocking FA turnover in the *tgd1-1* mutant background yields leaf TAG content of up to 9% per dry weight 7 weeks after sowing, corresponding to an average rate of TAG increase of 1.3% per dry weight per week. If a similar rate of TAG accumulation could be achieved in vegetative tissues of crop plants, a higher TAG level could theoretically be reached by leaf maturation, since many crops have much longer life cycles than *Arabidopsis*. Further increases in the rate of TAG accumulation may still be possible by overexpression of WRI1, since FA synthesis is insensitive to feedback inhibition (Andre et al., 2012) in the *tgd1-1* background (Fan et al., 2013a). Therefore, TGD1 or other TGD proteins represent attractive targets for genetic engineering efforts aimed at enhancing the energy density and nutritional value of vegetative plant biomass.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana* plants used in this study were of the Columbia ecotype. The *tgd1-1* mutant was previously described by Xu et al. (2003), *act1* by Kunst et al. (1988), *sfr2-3* by Moellering et al. (2010), and the transgenic lines overexpressing *PDAT1* by Fan et al. (2013b). The T-DNA insertion mutants for *PXA1* (SALK_019334, *pxa1-2*; Kunz et al., 2009), *SDP1* (SALK_102887, *sdp1-4*; Eastmond, 2006), *SDP1L* (SALK_119557, *sdpL-2*; Kelly et al., 2011), *ATGLL* (SALK_139011, *atglL*; Kelly et al., 2011), *PAH1* (SALK_042850, *pah1*; Nakamura et al., 2009), and *PAH2* (SALK_047457, *pah2-1*; Eastmond et al., 2010) were isolated from the ABRC seed stocks at Ohio State University (Alonso et al., 2003). The homozygosity of the T-DNA lines was verified by PCR using the primer sets listed in Supplemental Table 1. The mutant line with a T-DNA insertion in *Arabidopsis CGI58-LIKE* (*cgi58L*; Kelly et al., 2011) was kindly provided by Kent Chapman.

For growth on plates, surface-sterilized seeds of *Arabidopsis* were germinated on 0.6% (w/v) agar-solidified one-half-strength MS medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose in an incubator with a photon flux density of 80 to 120 μmol m^{-2} s^{-1}, a light period of 16 h (22°C), and a dark period of 8 h (18°C). For growth on soil, plants were first grown on MS medium for 10 d, transferred to soil, and grown under a photosynthetic photon flux density of 150 to 200 μmol m^{-2} 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 *SDP1*, *SDP1L*, *CGI58L*, and *ATGLL* were amplified by RT-PCR using the primer sets listed in Supplemental Table 1. The resulting PCR products were restricted with *Xma*I/*Sal*I, *Sac*I/*Kpn*I, *Pst*I/*Kpn*I, and *Xba*I/*Kpn*I, respectively, and inserted into the respective sites of binary vectors derived from pPZP212 (Hajdukiewicz et al., 1994). 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 antibiotics for the vectors on MS medium.

Lipid and FA Analyses

Lipids were extracted and analyzed as described previously (Fan et al., 2013b). Separation and identification of the FA methyl esters were performed on an HP5975 gas chromatograph-mass spectrometer (Hewlett-Packard) fitted with a 60-m $\times$ 250-μm SP-2340 capillary column (Supelco) with helium as the carrier gas. The TAG content was calculated according to

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).

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 μmol m^{-2} 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 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. 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.

LD Imaging

For LD 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 with a Zeiss epifluorescence microscope (Carl Zeiss; Axiovert 200M) with a green fluorescent protein filter. For transmission electron microscopy, 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), sectioned, and stained with 2% uranyl acetate and lead citrate before viewing with a JEM-1400 LaB6 120-keV transmission electron microscope (JEOL).

Enzyme Activity Assays

Plant leaves were homogenized in cold extraction buffer containing 150 mM Tris-HCl, pH 7.5, 10 mM potassium chloride, 10 μM flavin adenine dinucleotide, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.01% Triton X-100, and 1% polyvinylpyrrolidone. The extracts were centrifuged at 14,000*g* at 4°C for 10 min. The supernatants were directly used for ACOX activity measurements by using 50 μM oleoyl-CoA substrate in the peroxidase-coupled reaction with *p*-hydroxybenzoic acid according to Hooks et al. (1996).

RNA Isolation and Quantitative RT-PCR

Total RNA was extracted using TRIzol reagent. MMLV reverse transcriptase (New England Biolabs) was used for first-strand cDNA synthesis. Quantitative expression analysis was performed using the iCycler Real-Time PCR System (Bio-Rad) with SYBR green master mix (Bio-Rad) following the standard procedure described by Udvardi et al. (2008). The *ICL* transcript was amplified with forward primer ICLFw and reverse primer ICLRv. The *MS* transcript was amplified with forward primer MSFw and reverse primer MSRv (Supplemental Table 1). The results were normalized to expression levels of *UBQ10*, which was amplified using forward primer UBQ10Fw and reverse primer UBQ10Rv (Supplemental Table 1).

Pollen Germination Assays

In vitro pollen germination assays were performed on growth medium containing 2 mM $CaCl_2$, 0.01% boric acid, 1 mM $MgSO_4$, 1% (w/v) agar, and 17% (w/v) sucrose (pH adjusted to 7.0 using KOH). Experiments were performed with pollen grains from freshly opened flowers. Pollen germination was examined with a Zeiss epifluorescence microscope following overnight incubation.

For in vivo pollen germination, wild-type pistils were prepared by removing other tissues from flower buds. The stigmas were then briefly brushed with anthers from freshly opened flowers of *tgd1-1* and *tgd1-1 pxa1-2* mutants. The pistils were excised the next day after pollination and examined with the Zeiss microscope.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ATGLL*, At1g33270; *CGI58L*, At4g24160; *PAH1*, At3g09560; *PAH2*, At5g42870; *PXA1*, At4g39850; *SDP1*, At5g04040; *SDP1L*, At3g57140.

Supplemental Data

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

Supplemental Figure 1. TAG Levels in Leaves of Wild-Type and Single Mutant Plants Grown on Soil.

Supplemental Figure 2. Growth Phenotype of the Single Mutants in Comparison with the Wild Type.

Supplemental Figure 3. Growth and Lipid Phenotypes of the Triple *tgd1-1 sdp1-4 pxa1-2* Mutant.

Supplemental Figure 4. Lipid Phenotypes of the Single *sdp1-4* and *pxa1-2* Mutants.

Supplemental Figure 5. Disruption of SDP1 or PXA1 Affects the FA Composition of Major Membrane Lipids in *tgd1-1*.

Supplemental Figure 6. Leaf TAG Content in *tgd1-1* Overexpressing *SDP1*, *SDP1L*, *CGI58L*, or *ATGLL*.

Supplemental Figure 7. Morphology of the 7-Week-Old Triple *act1 pah1 pah2-1* Mutant Grown on Soil.

Supplemental Figure 8. Membrane Lipid Content in Leaves of the *sfr2-3* Mutant.

Supplemental Figure 9. The FA β-Oxidation and Glyoxylate Cycle Pathways Are Not Induced in *tgd1-1*.

Supplemental Table 1. Primers Used in This Study.

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AUTHOR CONTRIBUTIONS

C.X., J.F., and J.S. designed the experiments. J.F., C.Y., R.R., and C.X. performed the research. J.F., C.Y., J.S., and C.X. participated in data analysis. C.X., J.F., and J.S. wrote the article.

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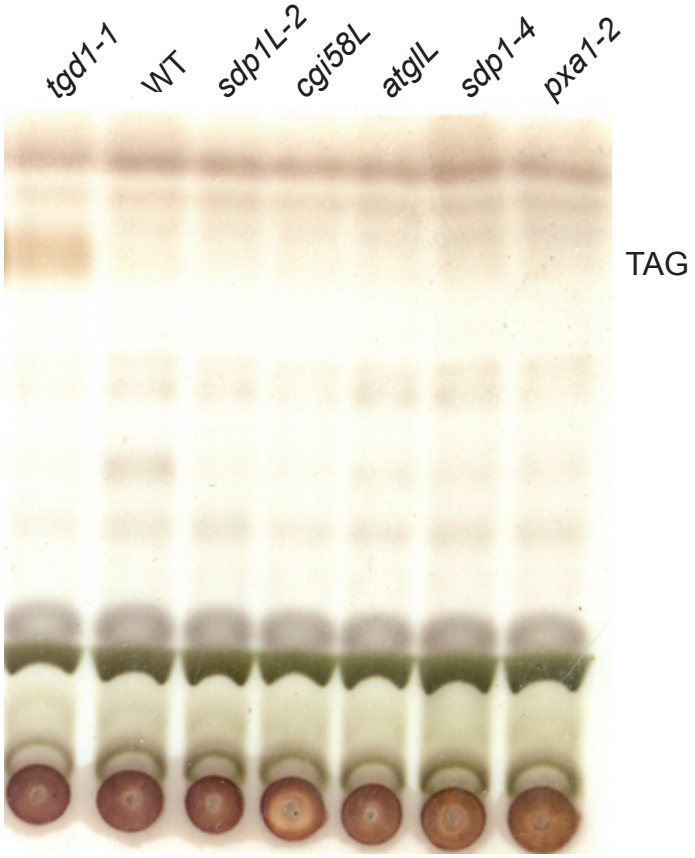
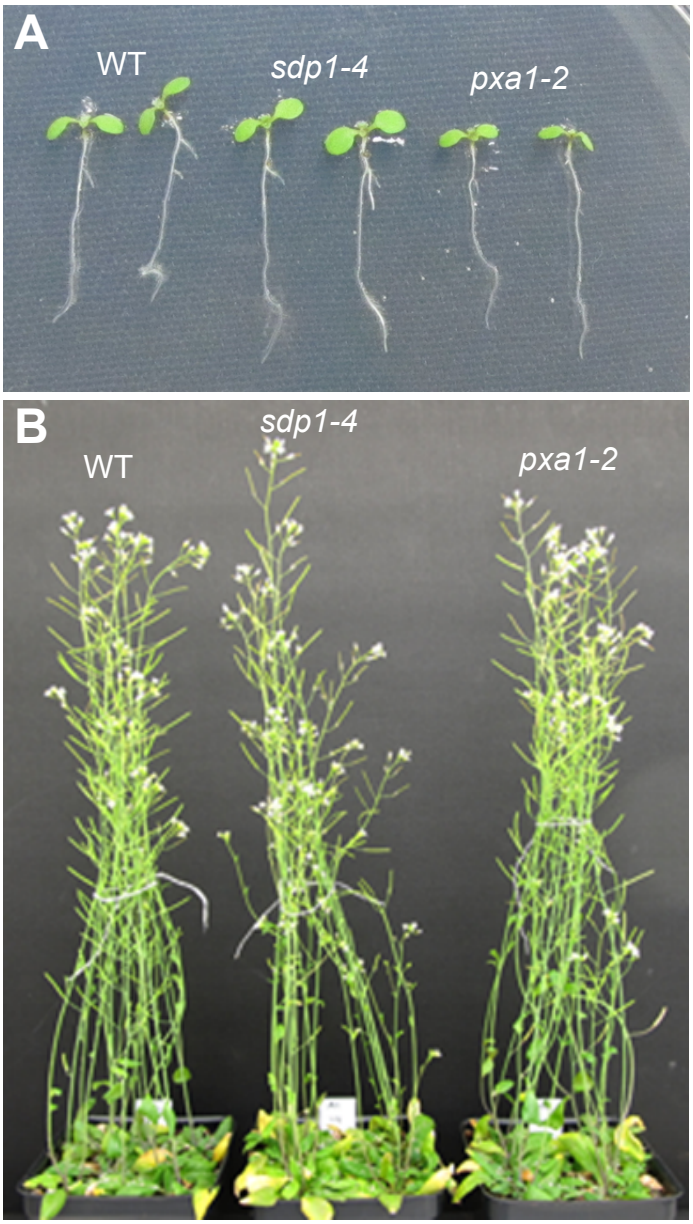
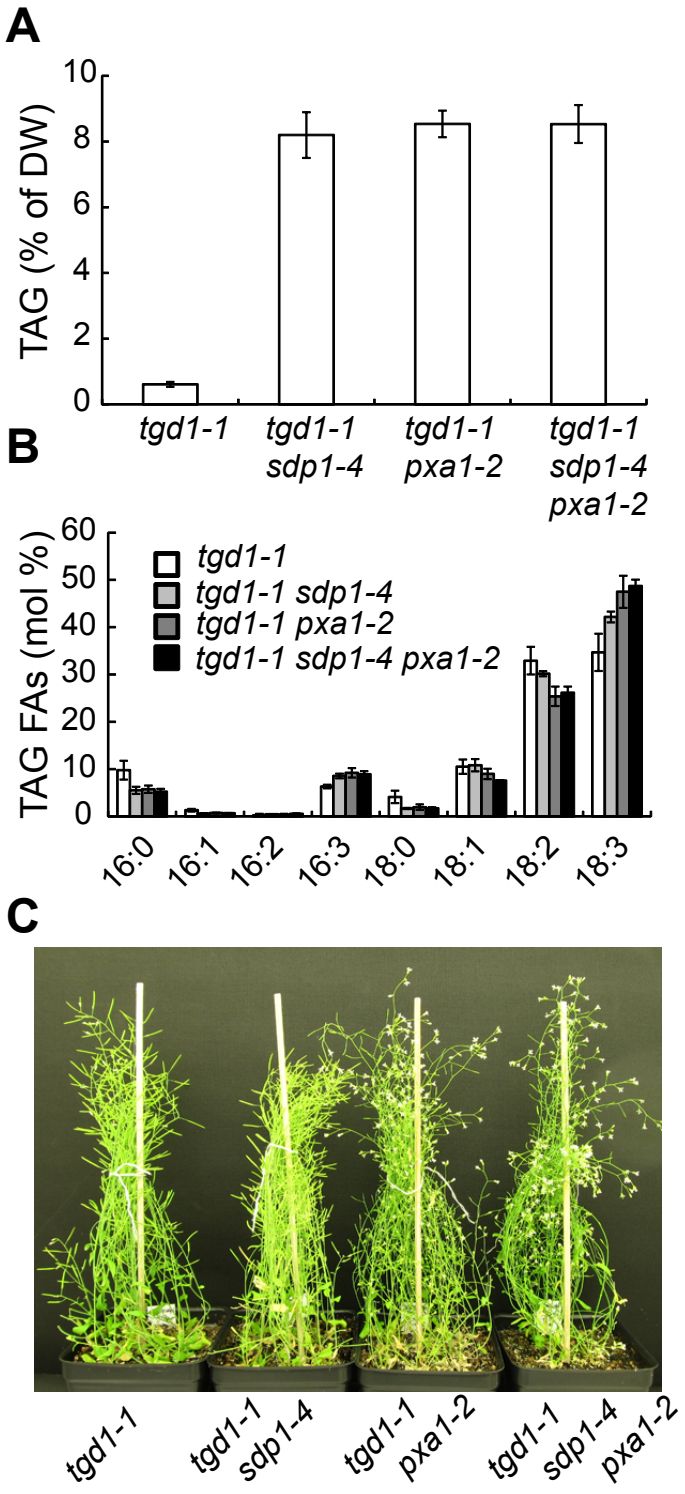


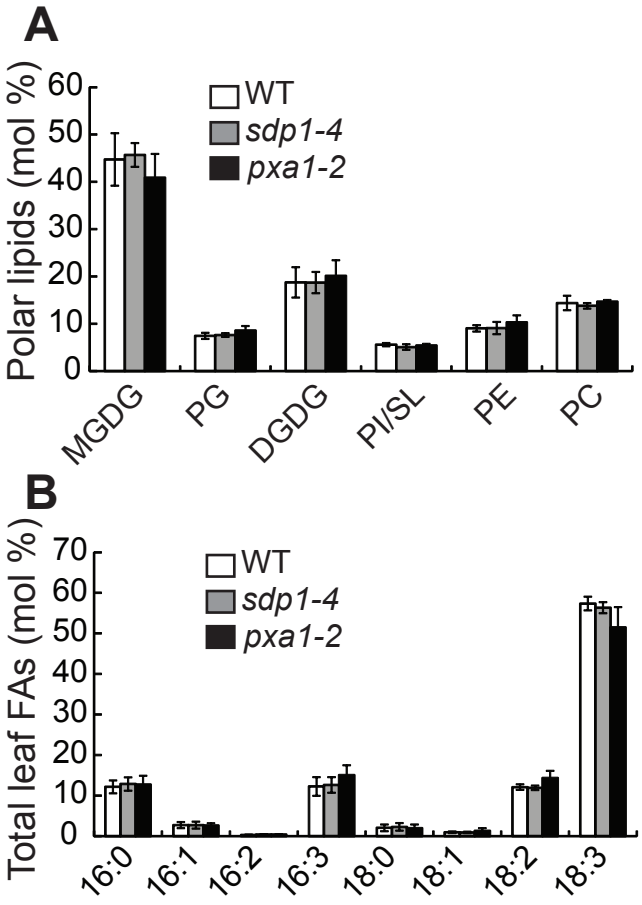
Supplemental Figure 1. TAG levels in leaves of wild-type and single mutant plants grown on soil. A thin layer chromatogram of neutral lipids is shown. Lipids were visualized with 5% sulfuric acid by charring.



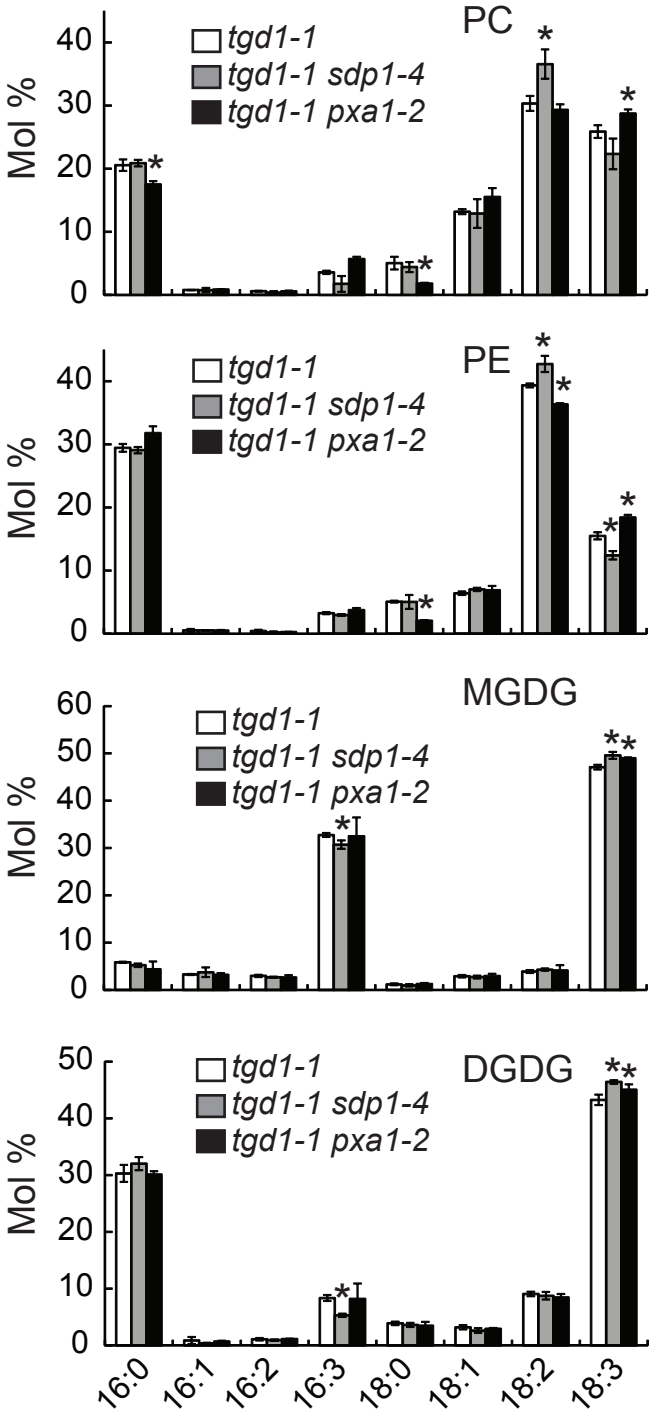
Supplemental Figure 2. Growth phenotype of the single mutants in comparison to the wild type (WT). (A) One-week-old plants grown on agar plates supplemented with 1% Suc. **(B)** Six-week-old plants on soil.



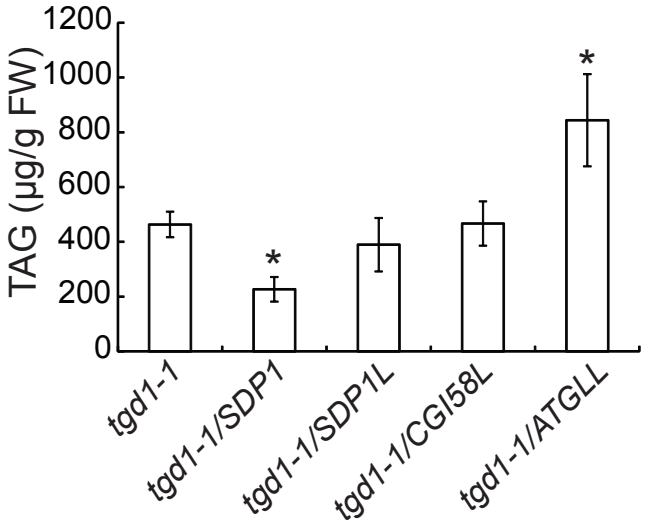
Supplemental Figure 3. Growth and lipid phenotypes of the triple *tgd1-1 sdp1-4 pxa1-2* mutant. (A) TAG content in leaves of 7-week-old plants grown on soil. **(B)** FA composition of leaf TAG. **(C)** Morphology of 7-week-old plants grown on soil. Data are the means of the three replicates. Error bars indicate SD.



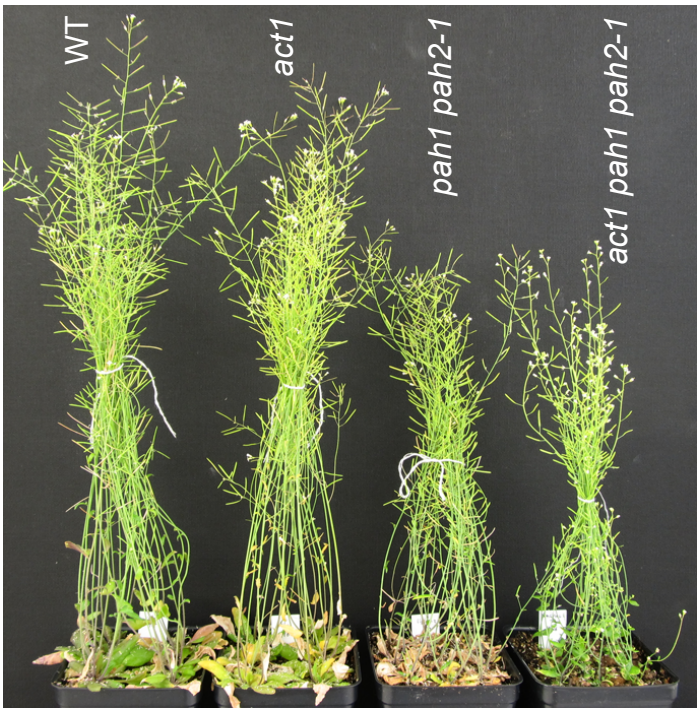
Supplemental Figure 4. Lipid phenotypes of the single *sdp1-4* and *pxa1-2* mutants. (A) Polar lipid composition in leaves of wild-type (WT) and mutant plants. **(B)** FA composition of total leaf lipids. Data are the means of the two to three replicates. Error bars indicate SD.



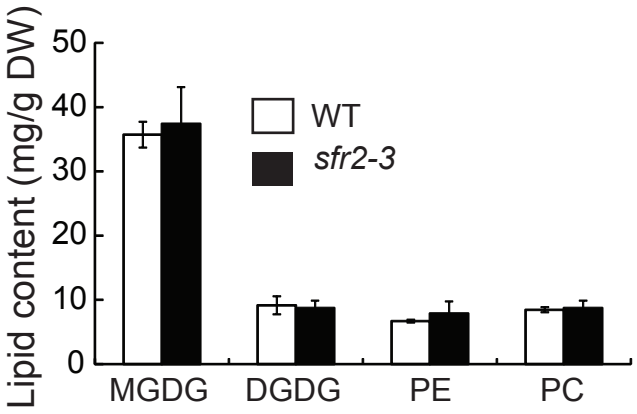
Supplemental Figure 5. Disruption of SDP1 or PXA1 affects the FA composition of major membrane lipids in *tgd1-1*. Data are the means of the three to four replicates. Error bars indicate SD. Asterisks indicate statistically significant differences from *tgd1-1* based on Student's *t* test ($P < 0.05$).



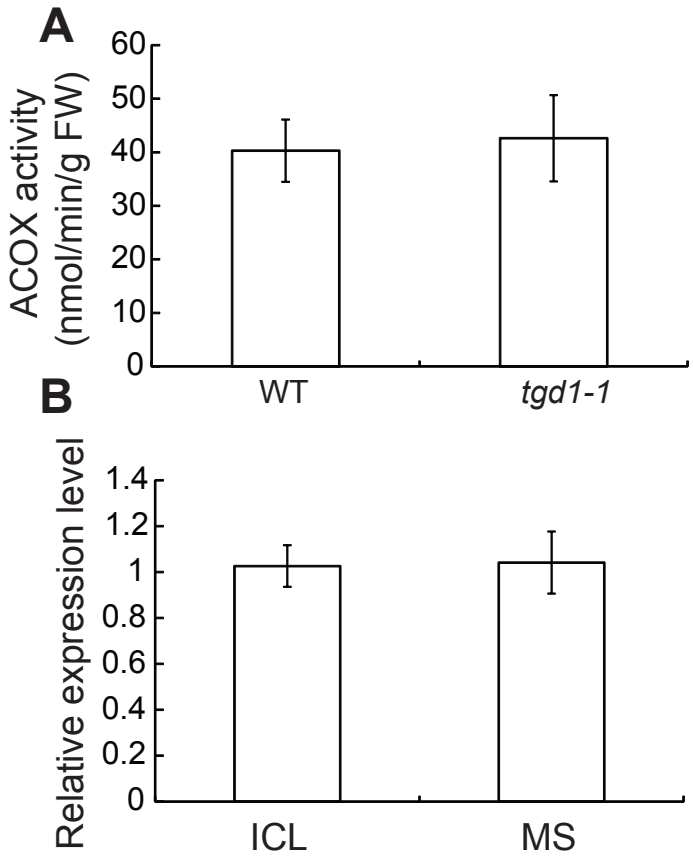
Supplemental Figure 6. Leaf TAG content in *tgd1-1* overexpressing *SDP1*, *SDP1L*, *CGI58L* or *ATGLL*. Data are the means of five independent transgenic lines. Error bars indicate SD. Asterisks indicate statistically significant differences from *tgd1-1* based on Student's *t* test ($P < 0.05$).



Supplemental Figure 7. Morphology of the 7-week-old triple *act1 pah1 pah2-1* mutant grown on soil.



Supplemental Figure 8. Membrane Lipid content in leaves of the *sfr2-3* mutant.
Data are the means of the three replicates. Error bars indicate SD.



Supplemental Figure 9. The FA ß-Oxidation and Glyoxylate Cycle Pathways Are Not Induced in *tgd1-1.* (A) Acyl-CoA oxidase (ACOX) activity in leaves of wild type (WT) and *tgd1-1*. **(B)** Transcript abundance of *isocitrate lyase* (*ICL*) and *malate synthase* (*MS*) in *tgd1-1* leaves as quantified by qPCR. The expression level for each gene is expressed as a value relative to its level in the wild type. Data are the means of the three replicates. Error bars indicate SD.

Supplemental Table 1. Primers used in this study

Primer name	Sequence
S019334LP	TAAAGCCATCGGTTGACATTC
S019334RP	AAAGCACCAAGATTTTGCATC
S102887LP	CGAGCCTGTCTTATCTCGTTG
S102887RP	CGAGATTCCTCACCAAATCAG
S119557LP	CACCACCACCACCATTTCTAG
S119557RP	AGCATATCGTGGTTGGATCTG
S139011 LP	TTTAGCAGGATCTTCTGCTGG
S139011 RP	AGCTCCATGGAACATGTTCAG
S042850LP	TAATTTTGGCTTGTTGTTGGG
S042850RP	GTTTTGGTCAGCTCTGACTGC
S047457LP	AATGCAACAGGTAGACGCAAG
S047457RP	GCAAATGCAAACAAACAGTTG
ICLFw	GCAGAGTTTGACGGTGGCGGT
ICLRv	TGACAGAAAGCAGAGGGAGG CA
MSFw	ACCGACTGAGTGATCACG
MSRv	GGAACATTCTAGGGAAGC
SDP1Fw	AATTGGTACCGAATGGATATAAGTAATGAGGC
SDP1Rv	AATTGTCGACCTAAGCATCTATAACACT
SD1LFw	GTCAGGTACCATGGATATAAGCAACGAAGC
SDP1LRv	AGCAGTCGACTTACTCATGGTCATGGAT
ATGLFw	GTCAGGTACCATGGCGATGTCTCTCCGTTC
ATGLRv	GTCACTGCAGTTAGCTAGTAGGAATCTCTT
CGI58LFw	GGGGTACCATGAACTTGAGCCGTTTTGCTTC
CGI58LRv	GCTCTAGAAACCAATCGTAGACCATCTAGG
UBQ10Fw	TCAATTCTCTCTACCGTGATCAAGATGCA
UBQ10Rv	GGTGTCAGAACTCTCCACCTCAAGAGTA