

Mutation of the TGD1 Chloroplast Envelope Protein Affects Phosphatidate Metabolism in *Arabidopsis*^{VI}

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Phosphatidate (PA) is a central metabolite of lipid metabolism and a signaling molecule in many eukaryotes, including plants. Mutations in a permease-like protein, TRIGALACTOSYLDIACYLGLYCEROL1 (TGD1), in *Arabidopsis thaliana* caused the accumulation of triacylglycerols, oligogalactolipids, and PA. Chloroplast lipids were altered in their fatty acid composition consistent with an impairment of lipid trafficking from the endoplasmic reticulum (ER) to the chloroplast and a disruption of thylakoid lipid biosynthesis from ER-derived precursors. The process mediated by TGD1 appears to be essential as mutation of the protein caused a high incidence of embryo abortion. Isolated *tgd1* mutant chloroplasts showed a decreased ability to incorporate PA into galactolipids. The TGD1 protein was localized to the inner chloroplast envelope and appears to be a component of a lipid transporter. As even partial disruption of TGD1 function has drastic consequences on central lipid metabolism, the *tgd1* mutant provides a tool to explore regulatory mechanisms governing lipid homeostasis and lipid trafficking in plants.

INTRODUCTION

Chloroplasts harbor one of the most complex membrane systems (thylakoids) in nature to conduct photosynthesis. Efforts in gaining a fundamental understanding of chloroplast biogenesis have to address questions of membrane assembly, including polar lipid biosynthesis and lipid trafficking. The predominant thylakoid membrane lipids are the galactolipids (Dörmann and Benning, 2002), which are assembled in the model plant *Arabidopsis thaliana* by the action of three sets of enzymes associated with the chloroplast envelope membranes (Benning and Ohta, 2005). A basic model of galactolipid biosynthesis in *Arabidopsis* is shown in Figure 1. In the wild type, the bulk of galactolipids is synthesized by monogalactosyldiacylglycerol (MGDG) synthase 1 (MGD1) (Shimajima et al., 1997; Jarvis et al., 2000) and digalactosyldiacylglycerol (DGDG) synthase 1 (DGD1) (Dörmann et al., 1999). Interestingly, MGD1 is associated with the inner plastid envelope (Block et al., 1983; Miegge et al., 1999; Awai et al., 2001) and DGD1 with the outer envelope (Froehlich et al., 2001), raising mechanistic questions of MGDG movement from the inner to the outer envelope and of DGDG transfer back from the outer to the inner envelope (Figure 1). The specific biological function or the evolutionary advantages, if any, of the arrangement of the galactoglycerolipid biosynthetic machinery are not well understood. To complicate matters, two parallel pathways

contribute to chloroplast lipid biosynthesis in many plants (e.g., *Arabidopsis*, Figure 1) as originally proposed by Roughan and Slack (Roughan et al., 1980; Roughan and Slack, 1982). The eukaryotic pathway for thylakoid lipid biosynthesis encompasses the export of fatty acids synthesized in the chloroplast, their incorporation into phosphatidates (PAs), and, subsequently, other phospholipids at the endoplasmic reticulum (ER). The biosynthesis of thylakoid lipids by the eukaryotic pathway necessitates the return of either PA directly and/or the return of phosphatidylcholine (PC) to the plastid, which would require the conversion of PC to PA by a phospholipase D at the outer chloroplast envelope and, subsequently, the dephosphorylation of PA by a PA phosphatase (PAP) to diacylglycerol (DAG), which is the precursor of MGDG biosynthesis at the inner chloroplast envelope. Alternatively, fatty acids are directly incorporated at the plastid inner envelope into PA, which enters MGDG biosynthesis as described for the eukaryotic pathway. This *de novo* assembly of MGDG in the plastid represents the prokaryotic pathway. According to this hypothesis, a PAP activity plays a central role in both pathways (Figure 1). While PAPs are known for *Arabidopsis* and other plants (Pearce and Slabas, 1998; Pierrugues et al., 2001), a specific isoform(s) involved in the eukaryotic or prokaryotic pathways has not yet been identified. Distinct molecular species of MGDG or any other thylakoid lipid arise from the two pathways due to the different substrate specificities of the lysophosphatidic acid acyltransferases associated with the ER or the inner chloroplast envelope, respectively, and the fatty acid composition of different MGDG molecules are diagnostic for their origin (Heinz and Roughan, 1983). While the contributions of the eukaryotic and prokaryotic pathways can be greatly different in different plant and algal species (Heinz, 1977; Mongrand et al., 1998), they are nearly equal in *Arabidopsis* (Browse et al., 1986).

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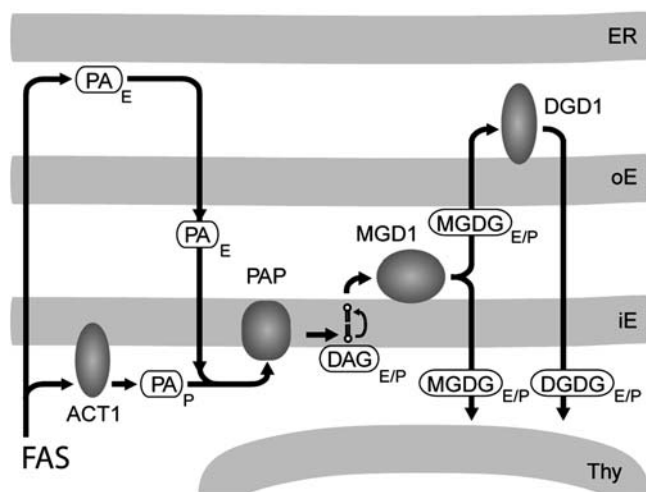


Figure 1. Two-Pathway Scheme for Galactolipid Biosynthesis.

Lipid flow is shown with solid arrows. Membranes shown in gray are as follows: iE, inner chloroplast envelope; oE, outer chloroplast envelope; Thy, thylakoids. Proteins (round shapes) shown are: ACT1, plastidic glycerol-3-phosphate acyltransferase; DGD1, major DGDG synthase; MGD1, major MGDG synthase; PAP, plastidic PAP. Lipids are encircled, and molecular species derived from the eukaryotic pathway carry the subscript "E," those derived from the prokaryotic pathway "P," and mixtures derived from both pathways "E/P."

A central metabolite of the eukaryotic and prokaryotic pathways of thylakoid lipid assembly is PA. In wild-type *Arabidopsis*, it represents only a minor fraction of the polar lipids. In fact, PA released from phospholipids by specific lipases acts as a signaling molecule in a variety of pathways in plants, and PA levels are under strict control (Lee et al., 2003; Potocky et al., 2003; Zhang et al., 2003; Li et al., 2004; Park et al., 2004; Thiery et al., 2004). It should also be noted that PA is a regulator of phospholipid metabolism in yeast (Loewen et al., 2004) and likely also in plants.

Three *trigalactosyl-diacylglycerol 1* (*tgdl*) mutant alleles of *Arabidopsis* were identified, and the locus (At1g19800) was named based on the unusual accumulation of oligogalactolipids in the mutants (Xu et al., 2003). As described here, we discovered that the *tgdl-1* mutant representative for all alleles also accumulates triacylglycerols (TAGs) and PA. The oligogalactolipids found in *tgdl-1* differed with regard to the glycosidic linkage of the head group sugars from the typical MGDGs and DGDGs of thylakoid membranes, suggesting that a processive galactosyl transferase different from the primary galactolipid biosynthetic enzymes was responsible. In addition, molecular species of galactolipids derived from the ER pathway of thylakoid lipid biosynthesis were underrepresented in *tgdl-1* such that most of the galactolipid molecules associated with chloroplast membranes were derived from the prokaryotic pathway. The gene affected in the allelic *tgdl* mutants was predicted to encode a six-membrane-spanning domain protein with similarity to the permease component of multipartite bacterial ABC transporters. The *tgdl* mutant alleles were originally isolated in the genetic background of the digalactolipid-deficient *dgd1* mutant (Dörmann et al., 1995).

However, the in-depth analysis of the complex *tgdl* mutant phenotype described here was conducted in the wild-type background following extensive backcrossing. Extensive analysis revealed subtle morphological phenotypes and a high incidence of embryo lethality due to compromised TGD1 function. Based on the cumulated results derived from the analysis of the complex *tgdl-1* phenotype and TGD1, it is suggested that TGD1 is a component of a lipid translocator at the inner plastid envelope involved in the eukaryotic pathway of thylakoid lipid biosynthesis.

RESULTS

Impairment of TGD1 Affects Growth and Development

While the *tgdl* mutants were originally isolated in the *dgd1* mutant background during a suppressor screen (Xu et al., 2003), it became quickly clear following crosses to the wild type that mutations in the *TGD1* gene cause phenotypes independent of *dgd1*, permitting a more direct analysis of *TGD1* function in the wild-type background. Because all three available point mutant alleles showed comparable biochemical and growth phenotypes, when studied in the *dgd1* mutant background, we focused primarily on the *tgdl-1* allele in this study. All three alleles were leaky, with *tgdl-1* being the most severe with regard to the developmental phenotypes described here. The growth of the *tgdl-1* mutant line was slightly reduced, and leaves and siliques could be distinguished from the wild type based on their stoutness, as summarized in Figure 2. The reduction in growth was not only visible for green tissues but extended to nongreen tissues, such as roots, as well (Figure 2C).

Because no knockout allele was readily available, we used an RNA interference (RNAi) approach to enhance the mutant phenotype of *tgdl-1* and thereby demonstrate the leakiness of the *tgdl-1* allele. Two representative RNAi lines are shown in Figure 3. The abundance of the *tgdl* mRNA was reduced in these lines (Figure 3A), resulting in further diminished growth (Figure 3B).

During the course of handling the *tgdl* mutant lines, it was noted that seed batches reproducibly contained large numbers of sterile seeds or empty seed hulls. In homozygous *tgdl-1* lines, nearly 50% of the seeds were aborted (Figures 4A and 4B), a phenotype that was already visible for immature seeds of opened siliques. The ratio of aborted seeds approached 90% (409 out of 481 total for RNAi line-1 and 519 out of 583 total for RNAi line-2; Figure 3C) for the *tgdl-1/TGD1*-RNAi lines. This seed phenotype, as all others studied for *tgdl-1*, was rescued by ectopic expression of the *TGD1* wild-type cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter or by a cosmid that harbored a genomic fragment containing the wild-type gene with its endogenous gene promoter (Figure 4B). Approximately 50% of the seeds of the *tgdl-1* mutant were aborted (Figure 4B). When embryos forming in siliques of the *tgdl-1* mutant line were directly examined from 2 to 6 d after flowering, many were found arrested at the heart stage (Mansfield and Briarty, 1991) just before the greening of the plastid was expected to begin (Figure 4C).

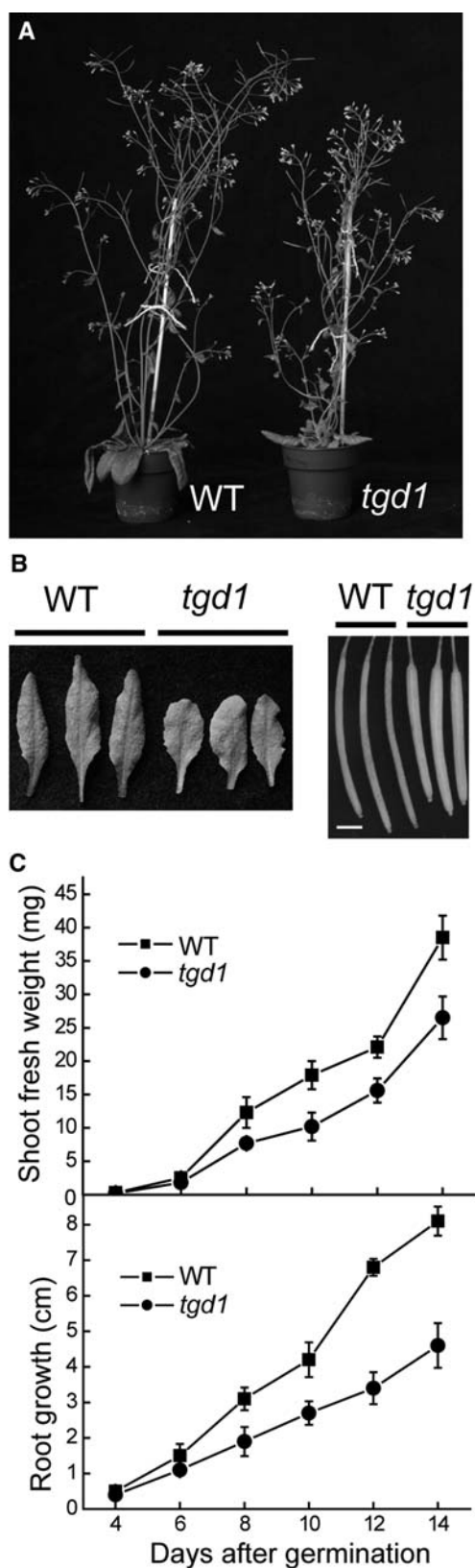


Figure 2. Impaired Growth and Development of the *tgd1-1* Mutant.

The Eukaryotic Pathway Is Predominant in Developing Seeds

Based on the previous biochemical analysis of the *tgd1* mutants (Xu et al., 2003), it was concluded that the mutation in *tgd1-1* causes an impairment of the eukaryotic (ER) pathway for thylakoid lipid biosynthesis (cf. Figure 1). To corroborate this hypothesis by genetic means, we crossed the homozygous *tgd1-1* mutant with a homozygous *act1* mutant. The *act1* mutant is deficient in the plastidic glycerol 3-phosphate acyltransferase (Figure 1) blocking the prokaryotic pathway of galactolipid biosynthesis. Its chloroplast galactolipids are derived from the eukaryotic pathway (Kunst et al., 1988). Given the deficiency of the eukaryotic pathway in the *tgd1-1* mutant mentioned above, the *tgd1-1 act1* double mutant was expected to be unable to synthesize galactolipids and to develop plastids, as galactolipids are essential components of all plastid membranes. Indeed, no viable homozygous *tgd1-1 act1* double mutants were recovered from the F₂ seeds. Screening 98 F₂ plants for the diagnostic loss of 16:3 carbon fatty acids, 17 homozygous *act1* mutants were identified. Genotyping these at the *TGD1* locus, 10 were found to be heterozygous for *tgd1-1* and 7 were homozygous wild-type at the *TGD1* locus. When developing siliques of the F₁ plants from the cross between homozygous *tgd1-1* and *act1* mutants were opened (Figure 3C), three classes of seeds or seed-like structures were discernible: green normal-looking seeds, white seeds of the same size as the wild type, and small white seed-like structures (Figure 3C, arrows). The latter appeared at a frequency of nearly 1/16 (39 out of 602, 6.45%) which is the expected frequency of double homozygous mutant seeds in this F₂ population. Furthermore, when F₃ seeds in siliques of F₂ lines homozygous for *act1* and heterozygous for *tgd1-1* were analyzed, ~28% (121 out of 432) were very small and white. We propose that these very small seed-like structures represent the double homozygous mutant incapable of developing a viable seed due to their inability to synthesize galactolipids. The white, large seeds are presumably homozygous for *tgd1-1* only.

Because embryos of *Arabidopsis* develop chloroplasts with extensive thylakoid membranes at the heart stage (Mansfield and Briarty, 1991), we investigated the contribution of the prokaryotic and eukaryotic pathways to thylakoid lipid biosynthesis in developing seeds of the *tgd1-1* mutant and wild-type lines, suspecting that the eukaryotic pathway might be more predominant in seeds than in leaves, as seed metabolism in general is largely directed toward TAG biosynthesis involving the eukaryotic pathway. All seeds (phenotypically mutant or wild-type) derived from a developing homozygous mutant silique were combined and extracted for further lipid analysis and compared

(A) Morphology of 7-week-old wild-type and *tgd1-1* plants grown on soil. **(B)** Comparison of leaves (left) and siliques (right) of the wild type and the *tgd1-1* mutant. Bar (right panel only) = 1 mm.

(C) Growth rates for shoots (aerial parts; top) and roots (bottom) of wild-type and *tgd1-1* mutant plants grown on agar-solidified Murashige and Skoog (MS) medium with 1% sucrose. For each point, 15 plants for shoot and 10 plants for root measurements were averaged. Standard errors are indicated. Squares represent the wild type, and circles represent the *tgd1-1* mutant.

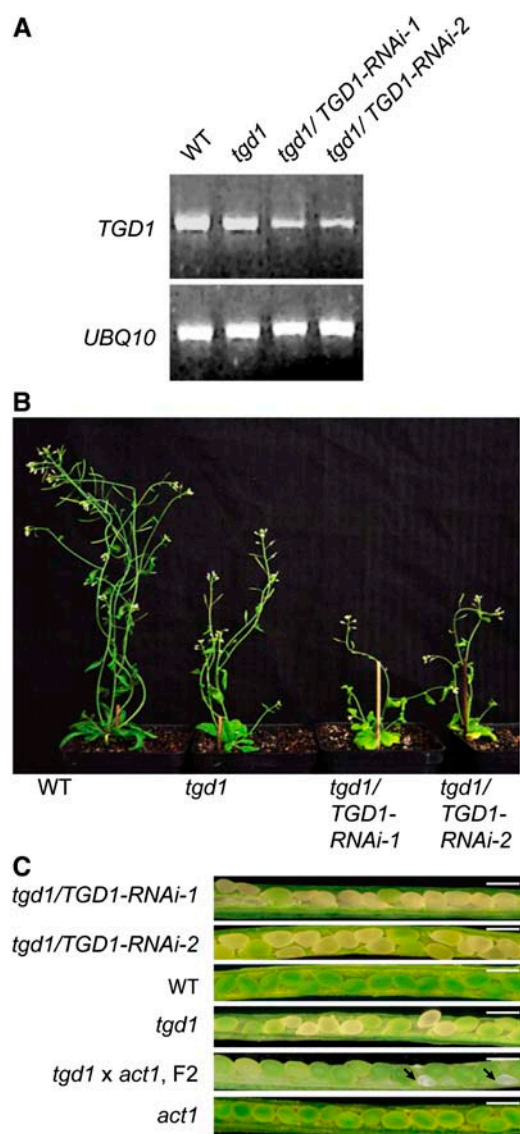


Figure 3. Enhancement of the Morphological Phenotype of *tgdl-1* in *TGD1*-RNAi Lines and a *tgdl-1 act1* Double Mutant.

(A) Reduced amounts of *tgdl-1* mRNA in two representative independent *TGD1* RNAi lines constructed in the homozygous *tgdl-1* mutant background. *UBIQUITIN10* mRNA (*UBQ10*) was used as a control for the RT-PCR experiment.

(B) Morphology of 6-week-old plants, as labeled, grown on soil.

(C) Representative siliques 7 d after flowering opened up to expose the developing seeds. Only in the *tgdl act1* F1 siliques are three seed types visible: wild type, green; mutant, white but same size as the wild type; mutant, small and white (arrow). Seed ratios are provided in the text. Bars = 1 mm.

with seed extracts of developing wild-type siliques of comparable age. As shown in Figure 4D, the polar lipid composition of mutant seeds was biased toward a decrease of chloroplast lipids, such as the two galactolipids and an increase of ER lipids, such as PC, consistent with the arrest of development of mutant embryos at the stage of chloroplast formation. Interestingly, the

relative amount of phosphatidylethanolamine, unlike PC, a lipid exclusively found in extraplastidic membranes, was not altered in the mutant. Possibly, PC is more dominant in the ER of embryos than phosphatidylethanolamine because of its precursor role in oil biosynthesis (Ohlrogge and Browse, 1995). Focusing on the exclusive chloroplast lipid MGDG, we observed that 18-carbon fatty acids were predominant in the wild-type MGDG but reduced in mutant MGDG (Figure 4E). In general, molecular MGDG species containing two 18-carbon fatty acids in their DAG backbone are thought to be derived from the eukaryotic pathway, while molecular species of MGDG with an 18-carbon fatty acid in the first position of DAG and a 16-carbon fatty acid in the second position are derived from the prokaryotic pathway (Heinz and Roughan, 1983). Given the predominance of 18-carbon fatty acids in MGDG of the wild type, it was apparent that the eukaryotic pathway dominates in developing *Arabidopsis* seeds while it contributes 50% of thylakoid lipids in leaves (Browse et al., 1986). As all results on the lipid metabolism in the *tgdl* mutant point to a general defect in the eukaryotic pathway of thylakoid lipid biosynthesis, the *tgdl-1* mutation should have severe consequences for the ability of the embryo to generate sufficient lipids for plastid membrane biogenesis. Therefore, the observed predominance of the eukaryotic pathway in wild-type seeds explains the high incidence of seed abortion in the leaky *tgdl-1* mutant and the strongly increased incidence of seed abortion in the *tgdl-1/TGD1* RNAi lines.

Oligogalactolipids, TAG, and PA Accumulate in *tgdl-1* Leaves

The *tgdl* mutants were originally identified by their accumulation of 1,6 β -linked oligogalactolipids, such as trigalactosyldiacylglycerol (TGDG). A comparison of polar lipids in the *tgdl-1* mutant and the wild type is shown in Table 1. While TGDG was not detectable in the wild type, it was present at ~ 1.4 mol % in the mutant. The relative amount of the major galactolipid MGDG was slightly reduced and that of DGDG was slightly more (by 29%). In the wild type, DGDG is mostly of eukaryotic origin presumably due to the location of the respective DGD1 glycosyltransferase on the outside of the outer envelope or the substrate specificity of this enzyme. Therefore, the short supply of molecular lipid species from the eukaryotic pathway in the *tgdl-1* mutant might explain this decrease in DGDG. The relative amounts of phospholipids overall increased in the mutant. The structural identity of purified TGDG had been previously determined by mass spectrometry and nuclear magnetic resonance spectroscopy (Xu et al., 2003).

During the ultrastructural comparison of wild-type and mutant cells, we observed peculiar osmium-dense bodies in the cytoplasm exclusively in the mutant as shown in Figure 5. Typically, tight packing of alkane chains associated with membrane lipids or oil droplets causes an enhancement of osmium staining, and we hypothesized that these bodies might be lipid droplets. When lipid extracts from leaves and roots of the mutant were analyzed for neutral lipids, a lipid cochromatographing with soy seed oil TAGs was detected (Figure 6A). Digestion of this compound isolated from mutant leaves and of soy oil TAGs with pancreatic lipase led to the formation of free fatty acids, diacylglycerol, and

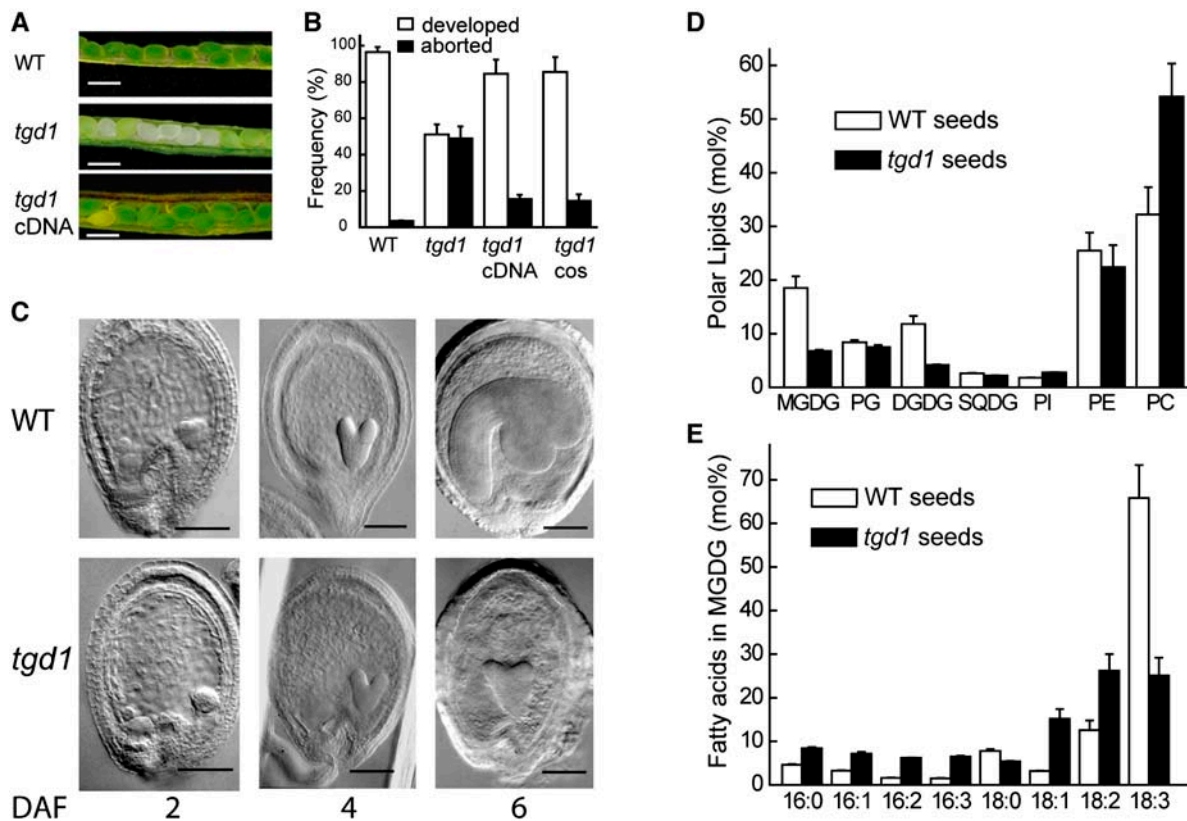


Figure 4. Seed Phenotypes of the *tgd1-1* Mutant.

(A) Representative siliques 7 d after flowering opened up to expose the developing seeds. Top, the wild type; center, the *tgd1-1* mutant; bottom, transgenic *tgd1-1* mutant complemented by expression of the *TGD1* wild-type sense cDNA under the control of the CaMV 35S promoter. Bars = 1 mm.

(B) Frequency of aborted seeds (from left) in the wild type, the *tgd1-1* mutant, the *tgd1* mutant complemented with the cDNA as described in **(A)**, and the mutant complemented with a genomic cosmid expressing the wild-type *TGD1* gene under the control of the endogenous promoter. Open bars, filled seed; closed bars, empty seeds. At least 10 siliques per line were averaged. Standard errors are indicated.

(C) Nomarski microscopy of developing seeds of the wild type (top panels) and the *tgd1-1* mutant (bottom panels) 2, 4, and 6 d after flowering (DAF). Bars = 80 μ m.

(D) Relative polar lipid content of wild-type and mutant developing seeds. Seeds were analyzed \sim 6 d after flowering. Data from three independent lipid preparations were averaged. The standard error is indicated. Open bars, wild type; closed bars, *tgd1-1* mutant seeds. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol.

(E) Fatty acid composition of the prevalent chloroplast lipid, MGDG. Fatty acids are listed according to the carbon number followed by the number of double bonds. Data from three independent lipid preparations were averaged. The standard error is indicated. Open bars, wild type; closed bars, *tgd1-1* mutant seeds.

monoacylglycerol cochromatographing in both samples, thereby confirming the identity of the compound accumulating in the mutant as TAG (see Supplemental Figure 1 online). Soy seed oil was chosen here as a standard because its fatty acid composition, unlike that of seed oil from *Arabidopsis*, resembles that of *Arabidopsis* leaf lipids. Indeed, analysis of fatty acid methyl esters derived from the TAGs accumulating in leaves of the *tgd1-1* mutant confirmed the presence of fatty acids typical for leaves (Figure 7A). Furthermore, the fatty acid composition was similar to that of PC (Figure 7B), a lipid derived from the eukaryotic pathway. It is interesting to note that a small amount of 16:3 fatty acids is present in PC as well as in PA in the *tgd1-1* mutant but not in the wild type (Figures 7B and 7C). This fatty acid is normally indicative of molecular species derived from the

prokaryotic pathway, and its presence in PC indicates a small flux of prokaryotic lipid species to the extraplastidic membranes in the *tgd1-1* mutant but not the wild type. To show that the TAG was not associated with the plastids, chloroplasts were isolated and analyzed as well. No TAGs were detectable in isolated chloroplasts (Figure 6A). Taking these data together, it is proposed that leaf cells of the *tgd1-1* mutant accumulate TAGs in droplets in the cytoplasm.

To examine whether central phospholipid metabolism was affected in the *tgd1-1* mutant, leaves were labeled with [32 P]orthophosphate to detect changes in phospholipid intermediate pools in the mutant. Two-dimensional thin-layer chromatograms (TLCs) are shown in Figure 6B. Of all the lipids detected, label in a compound comigrating with an authentic PA standard was

Table 1. Polar Lipid Composition in Leaves of the Wild Type and the *tgdl1-1* Mutant

Lipid	Wild Type	<i>tgdl1-1</i>
MGDG	43.0 ± 1.8	39.6 ± 0.3
PG	10.4 ± 0.1	12.6 ± 0.1
DGDG	18.2 ± 0.4	12.9 ± 0.5
SQDG	2.3 ± 0.2	3.4 ± 0.7
PI	1.2 ± 0.1	1.7 ± 0.2
PE	10.0 ± 0.6	11.0 ± 0.4
PC	15.1 ± 1.4	16.8 ± 1.2
TGDG	n.d.	1.4 ± 0.2

PA could not be separated by linear TLC used for this experiment and had to be analyzed by two-dimensional TLC. Values represent mol % ± SE of fatty acid methylesters derived from individual lipids. n.d., not detected. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol.

increased. Determining the relative mole fraction of fatty acid methylesters of PA over all fatty acids in the sample (wild type, 0.47 ± 0.12 mol %; *tgdl1-1*, 2.15 ± 0.55 mol %; $n = 3$, ±SD) indicated an approximate fivefold increase of relative PA amounts in the *tgdl1-1* mutant. The fatty acid composition of this PA was similar to that of PC (Figures 7B and 7C). Moreover, positional analysis of fatty acids in PA revealed an enrichment of 18-carbon fatty acids in the *sn*-2 position of the glycerol backbone (92.5 ± 5 mol %, $n = 3$, ±SD) consistent with its origin from the eukaryotic pathway (Heinz and Roughan, 1983). When intact chloroplasts were isolated from the *tgdl1-1* mutant, no PA was detected (data not shown), suggesting that PA found in total leaf lipid extracts of the mutant is accumulating in extraplastidic membranes. However, we cannot rule out that any PA present in the plastids was metabolized during chloroplast isolation.

Impaired Conversion of PA into Galactolipids by *tgdl1* Chloroplasts

Because eukaryotic PA accumulated in the *tgdl1-1* mutant and the eukaryotic pathway of galactoglycerolipid biosynthesis was generally disrupted, we hypothesized that the transport of PA and/or conversion of ER-derived PA to the eukaryotic DAG precursor of the galactolipid biosynthetic machinery (i.e., MGD1) in chloroplast envelopes was impaired. To test this hypothesis, chloroplasts were isolated from mutant and wild-type leaves, and acylcarbon-labeled lipid substrates were tested for their differential incorporation into MGDG by the two chloroplast preparations. The results of these experiments are shown in Figure 8. When DAG, the direct substrate for MGD1, was used, incorporation into MGDG was slightly increased for mutant chloroplasts (Figure 8A). The reaction was strictly dependent on the addition of the second MGD1 substrate, UDP-Gal, but was independent of phospholipase C, as expected, because DAG is not a substrate of phospholipase C. However, when labeled PC was used (Figure 8B), incorporation of label into MGDG by mutant chloroplasts was generally decreased. The reaction was UDP-Gal dependent. Stimulation by phospholipase C was small, if any, given the statistical limitations of the data set.

Phospholipase C generates DAG from PC, and stimulation was expected. Interestingly, the difference between the mutant and the wild-type chloroplasts was nearly abolished following phospholipase C treatment. A similar result as for PC was observed for PA (Figures 8C and 8D). Incorporation of PA into MGDG was nearly linear for both chloroplast preparations between the interval of 10 to 30 min, and the rate of PA incorporation was reduced for the mutant chloroplasts on a chlorophyll basis. This reaction was dependent on UDP-Gal and stimulated by additions of cytosol, which also led to an increase in the difference between the mutant and wild-type chloroplasts (Figure 8D). Addition of ER fractions was not effective. Cytosol and ER fractions were added because we wanted to test whether factors not yet identified might be needed for the transfer of PA from liposomes to the plastid envelopes.

Because it was possible that the reduced incorporation of PA into MGDG by isolated *tgdl1-1* chloroplasts is caused by a decreased activity of PAP (Figure 1), we tested the activity of this enzyme in the two chloroplast preparations. As shown in Supplemental Figure 2 online, PAP activity was similar in both preparations and can therefore not account for the observed decrease in the incorporation of PA into MGDG by isolated mutant chloroplasts (Figures 8C and 8D). Together with the documented accumulation of PA in the *tgdl1-1* mutant (Figure 6B), these results suggest an impairment of the eukaryotic pathway at the level of PA and its conversion into DAG, the direct substrate of MGDG biosynthesis.

MGD1 Activity Associated with the Inner Envelope Is Increased in *tgdl1-1*

To correctly interpret the observed rates of lipid incorporation into MGDG described above (Figure 8), it was important to know

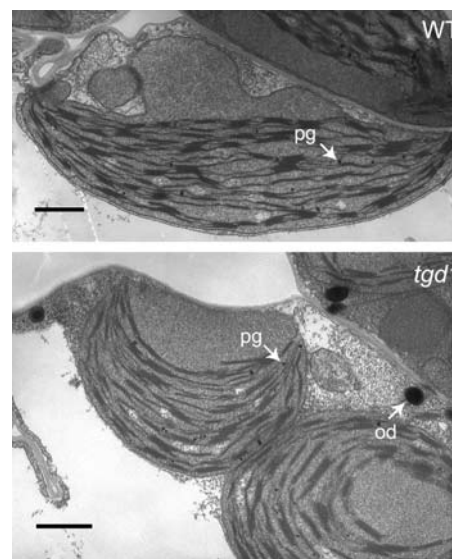


Figure 5. Electron Micrographs of Representative Cells of the Wild Type and the *tgdl1-1* Mutant.

Plastoglobuli (pg) in plastids and oil droplets (od) in the mutant cytosol are indicated by arrows. Top, wild type; bottom, *tgdl1-1* mutant. Bars = 1 μm.

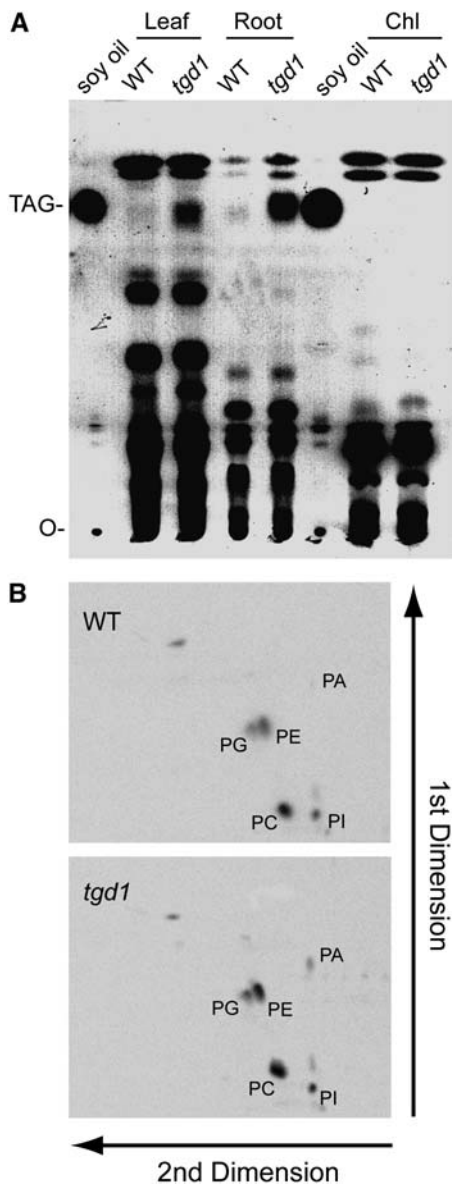


Figure 6. Accumulation of TAGs and PA in the *tgdl-1* Mutant.

(A) TLC of lipid extracts of wild-type and *tgdl-1* tissues and isolated chloroplasts (Chl) as indicated. A neutral lipid TLC system was used as described in Methods, and lipids were visualized by iodine staining. O, origin.

(B) Two-dimensional chromatogram of phospholipids from leaves of the wild type (top) and the *tgdl-1* mutant (bottom). Lipids were ^{32}P -labeled and visualized by autoradiography. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol.

whether the activity of MGDG synthase, in particular that of MGD1, which is the enzyme responsible for bulk MGDG biosynthesis in the wild type, was altered in the mutant. The activity of MGDG synthase was assayed directly using labeled UDP-Gal. No lipids were added to the mixture requiring the utilization of endogenous DAG generated in the envelope membranes. As

shown in Figure 9A, under these conditions, the rate of UDP-Gal incorporation into MGDG was increased approximately fivefold in isolated mutant chloroplasts. To corroborate that this activity was due to MGD1 and not to other described galactosyltransferase activities, chloroplasts were treated with either thermolysin or

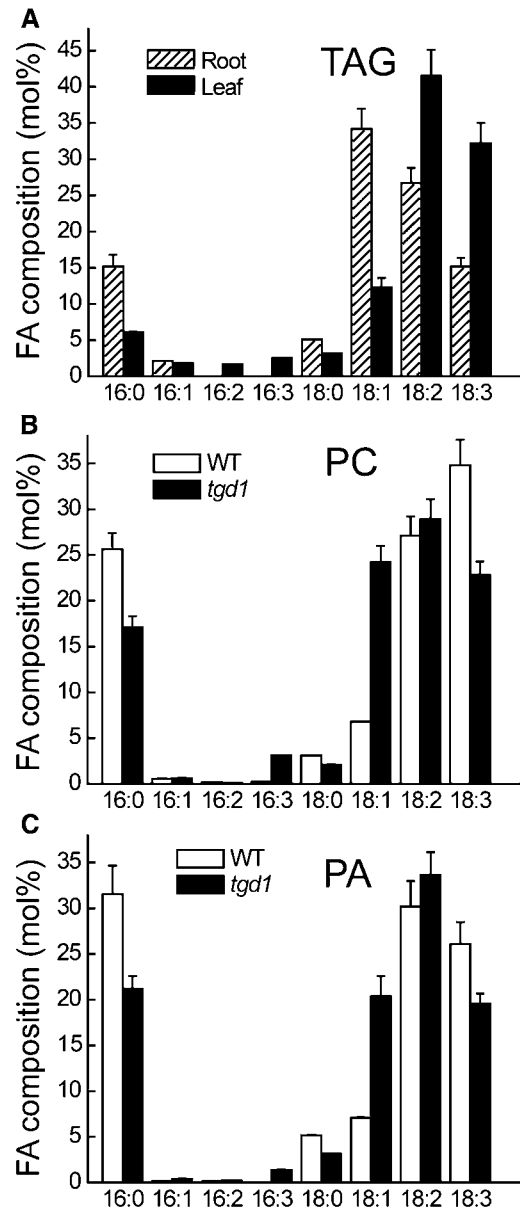


Figure 7. Fatty Acid Composition of Relevant Lipids.

(A) Fatty acid (FA) composition of TAG accumulating in the *tgdl-1* mutant in root (hatched bars) or leaf (closed bars) tissues.

(B) Fatty acid composition of PC isolated from leaves of the wild type (open bars) and the *tgdl-1* mutant (closed bars).

(C) Fatty acid composition of PA isolated from leaves of the wild type (open bars) and the *tgdl-1* mutant (closed bars).

For data in all three panels, three independent extracts were averaged, and the standard error is shown. Fatty acids are designated according to their number of carbons:number of double bonds.

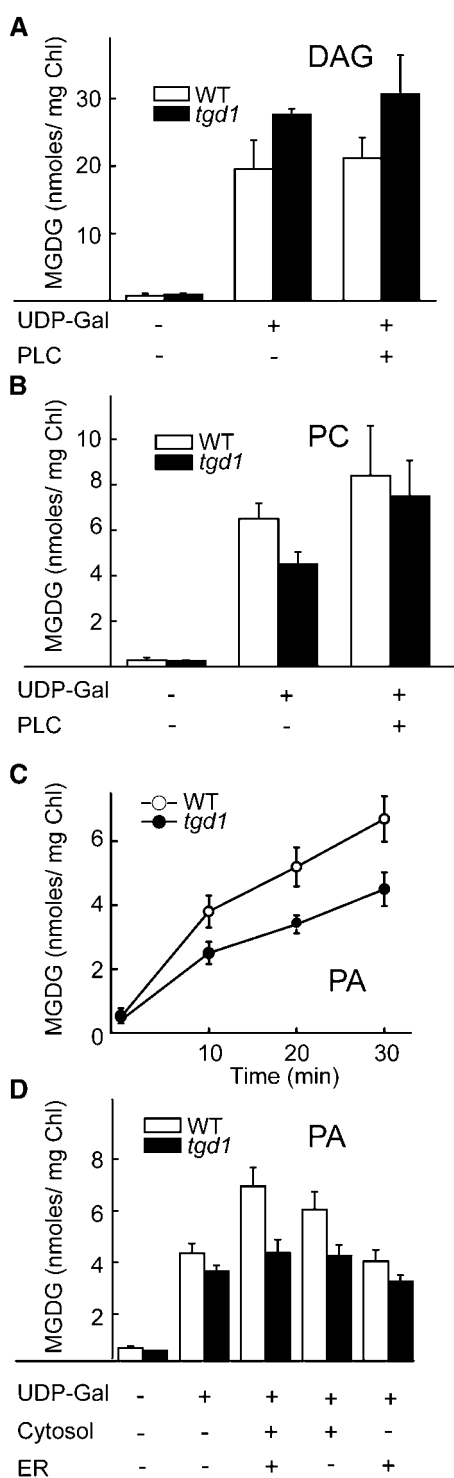


Figure 8. Incorporation of Lipid Substrates into MGDG by Isolated Wild-Type and Mutant Chloroplasts.

(A) MGDG biosynthesis from labeled DAG. Additions of UDP-galactose (UDP-Gal) and phospholipase C (PLC) are indicated.

(B) MGDG biosynthesis from labeled PC. Additions of UDP-Gal and PLC are indicated.

buffer only, reisolated, and incubated with substrate. Thermolysin can degrade surface-exposed proteins localized to the outer envelope membrane, such as processive galactosyl transferase (Xu et al., 2003), DGD1 (Froehlich et al., 2001), or MGD2 and MGD3 (Awai et al., 2001), while not penetrating into the intermembrane space and degrading proteins, such as MGD1, which is proposed to be associated with the inner envelope membrane (Miege et al., 1999; Awai et al., 2001). While the formation of DGDG (Figure 9A) and TGDG (Figure 9A) was thermolysin sensitive, the formation of MGDG was not (Figure 9A). This result was consistent with either an activation of MGD1 or greater availability of the endogenous lipid precursor of the MGD1 reaction in the *tgd1-1* mutant. We determined that the increase of MGDG formation in the mutant was not due to increased *MGD1* gene expression as shown in Figure 9B. Considering this increase in MGD1 activity, the result for the decrease of PA incorporation into MGDG by the mutant chloroplasts shown above (Figure 8) becomes even more significant.

While a substantial body of evidence suggested an association of *Arabidopsis* MGD1 with the inner chloroplast envelope membrane (Block et al., 1983; Miege et al., 1999; Awai et al., 2001), its exact topology had not been experimentally verified. We expected that an understanding of the topology of MGD1 would allow us to make a testable prediction regarding its functional cooperation with TGD1 during lipid metabolism. Toward this end, we performed import experiments using in vitro-translated MGD1 protein and isolated intact chloroplasts. After import, chloroplasts were reisolated and subjected to protease treatment with either thermolysin or trypsin in the absence or presence of detergents. As controls, ARC6 of *Arabidopsis* (Vitha et al., 2003) and a truncated version of Tic110 (tp110-110N) from pea (*Pisum sativum*; Lübeck et al., 1997) were used in parallel import and protease protection assays. It has previously been determined that ARC6 is a protein of the chloroplast inner envelope, which is thermolysin resistant but contains a trypsin-sensitive domain that protrudes into the intermembrane space (Vitha et al., 2003). By contrast, the bulk of the truncated inner envelope membrane-localized Tic110-110N faces the stroma, thus making this truncated membrane protein both thermolysin and trypsin resistant (Jackson et al., 1998). The results of the MGD1 import experiments are shown in Figure 9C. The preprotein of MGD1 (Figure 9C, pMGD1) is close in size to the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit and was therefore partially covered up by this most abundant protein in leaves (Figure 9C, top, lane 1). The MGD1 preprotein was processed under import conditions to generate mature MGD1 (Figure 9C, mMGD1) and was likewise shown to be resistant to protease degradation by post-treatment with thermolysin (Figure 9C, top, lane 3). However, mature MGD1 was partially cleaved by

(C) Time course of MGDG biosynthesis from labeled PA. UDP-Gal and cytosol were added, and PLC and ER were omitted in this assay.

(D) MGDG biosynthesis from labeled PA. Additions of UDP-Gal, cytosol fraction, and ER fraction are indicated.

Open symbols or bars represent the wild type and closed symbols or bars *tgd1-1* mutant chloroplasts. In general, three replicates were averaged, except two in **(C)**, and the standard error is shown.

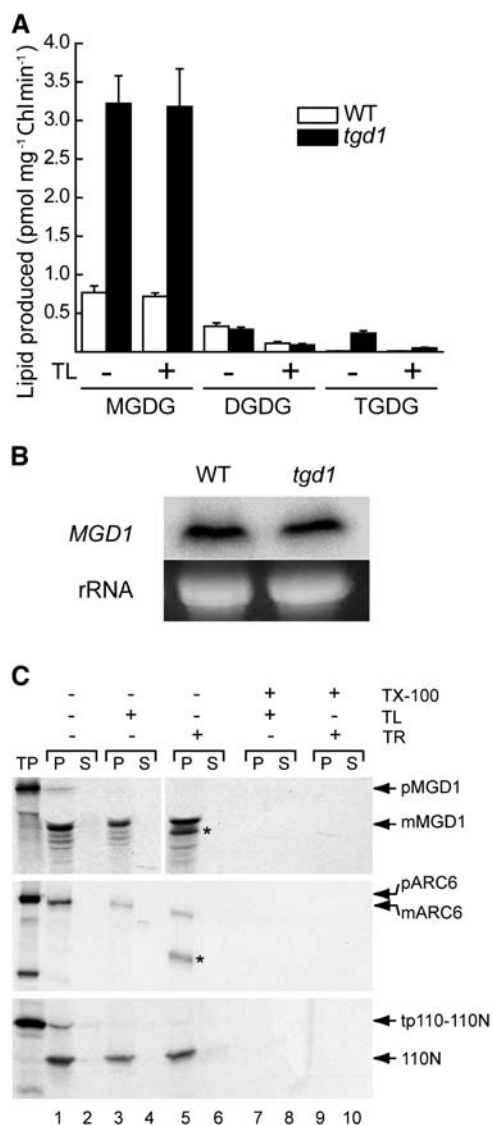


Figure 9. MGD1 Activity, Expression, and Localization in the Wild Type and the *tgd1-1* Mutant.

(A) Incorporation of labeled UDP-Gal into the three galactolipids MGDG, DGDG, and TGDG by wild-type (open bars) and *tgd1-1* mutant chloroplasts (closed bars). Samples were treated with thermolysin (TL) as indicated. Three replicates were averaged, and the standard error is shown.

(B) Abundance of *MGD1* mRNA in the wild type and the *tgd1-1* mutant. Total RNA was isolated from leaves. The top panel shows an autoradiograph of the RNA/DNA hybridization signal; the bottom panel shows one of the rRNA bands on the ethidium bromide-stained gel that was blotted.

(C) In vitro import of labeled in vitro-translated precursor proteins by pea chloroplasts. The top panel shows the import of pMGD1, the middle panel the import of pARC6, and the bottom panel the import of truncated tpTic110-110N. After import, chloroplasts were subjected to post-treatment with either thermolysin (TL) or trypsin (TR) in the absence (–) or presence (+) of Triton X-100 (TX-100). Intact chloroplasts were recovered by centrifugation through a 40% Percoll cushion and fractionated into a total membrane (P) and a supernatant (S) fraction. All fractions were analyzed by SDS-PAGE and fluorography. TP represents 10% of translation product added to an import assay. pMGD1, precursor

trypsin (Figure 9C, top, lane 5). Disruption of the chloroplast by detergent treatment made the mature MGD1 accessible to degradation by both thermolysin and trypsin (Figure 9C, top, lanes 7 to 10). Import assays involving the two control proteins, ARC6 (Figure 9C, middle), and truncated Tic110-110N (Figure 9C, bottom) behaved in agreement with previously published results as described by Vitha et al. (2003) and Jackson et al. (1998), respectively. Briefly, both control proteins were thermolysin resistant. However, while ARC6 was cleaved by trypsin, truncated Tic110-110N was completely resistant to trypsin degradation (Figure 9C, bottom, see lane 5). Taken together, the data presented here support the conclusion that MGD1 is tightly associated with either the outer or inner chloroplastic envelope in a peripheral manner with the bulk of the MGD1 protein orientated toward the intermembrane space. Determining in a more rigorous fashion whether MGD1 peripherally associates with either the outer or inner envelope was not further investigated here. However, we have demonstrated that imported mature MGD1 does associate with the total membrane fraction (Figure 9C, top, see lanes 1, 3, and 5) even though MGD1 contains no predicted transmembrane domain(s). Furthermore, we have confirmed that mature MGD1 can be removed from the envelope membrane when extracted with either high salt or sodium carbonate (see Supplemental Figure 3 online). In conjunction with results obtained by others showing directly that MGD1 and its ortholog in spinach (*Spinacia oleracea*) are associated with the inner envelope membrane (Miege et al., 1999; Awai et al., 2001), one can conclude that MGD1 is located on the outside of the inner chloroplastic envelope membrane.

TGD1 Is a Multispanning Integral Envelope Membrane Protein

To determine the subcellular localization of TGD1, we stably expressed a C-terminal fusion of TGD1 with green fluorescent protein (GFP) under the control of the CaMV 35S promoter and showed that expression of this fusion protein in *Arabidopsis* wild type resulted in a punctate fluorescence pattern at the periphery of chloroplasts (Figures 10A to 10C). No fluorescence signal was observed that could be associated with other subcellular membranes. However, one possible explanation for the pattern seen in Figures 10A to 10C might be that it is the result of an artifact due to the potentially high expression levels of the TGD1-GFP fusion protein itself. To address this concern, the abundance of the TGD1-GFP fusion protein was estimated by probing leaf protein derived from the transgenic lines with anti-GFP antibodies. We observed only a weak signal during this analysis (see Supplemental Figure 4 online), suggesting that the fusion protein was not excessively overexpressed in the transgenic lines or in the tissue examined. It should also be noted that the same TGD1-GFP construct led to a reversion of mutant phenotypes when introduced

of MGD1; mMGD1, mature processed form of MGD1; pARC6, precursor of ARC6; mARC6, mature form of ARC6; tp110-110N, N-terminal fragment of Tic110 with transit peptide; 110N, N-terminal fragment of Tic110, after loss of transit peptide. The bands in lane 5 marked with asterisks represent cleavage products following trypsin treatment.

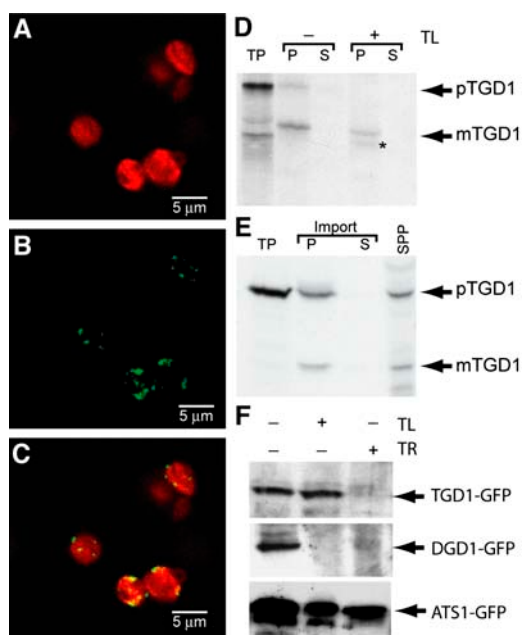


Figure 10. Subcellular Localization of TGD1 and Membrane Association.

(A) to (C) Stable transformation of *Arabidopsis* wild type with a TGD1-GFP fusion construct. Chlorophyll fluorescence (A), GFP fluorescence (B), and merged image (C).

(D) In vitro import of pTGD1 into isolated pea chloroplasts. After import of pTGD1, chloroplasts were subjected to post-treatment with thermolysin (TL). Intact chloroplasts were recovered by centrifugation through a 40% Percoll cushion and fractionated into total membrane (P) and supernatant (S) fractions. All fractions were analyzed by SDS-PAGE and fluorography. TP represents 10% of translation product added to an import assay. A fragment appearing after thermolysin treatment is marked by an asterisk. pTGD1, precursor of TGD1; mTGD1, mature form of TGD1.

(E) pTGD1 was subjected to an SPP assay. An import experiment as described in (D) is shown for comparison next to the lane showing the results of an SPP assay of pTGD1.

(F) In vivo import assay of GFP fusion proteins. Top, TGD1-GFP; middle, DGD1-GFP; bottom, ATS1-GFP. The GFP fusion constructs were transiently expressed in tobacco leaves. Chloroplasts were isolated and treated either with thermolysin (TL) or trypsin (TR) as indicated. Protein gel blots are shown on which the GFP portion of the fusion proteins was detected.

into the *tgdl-1* mutant demonstrating its correct function. Thus, we assume within reason that the punctate fluorescence pattern shown in Figure 10 accurately reflects the proper targeting of the TGD1 protein to the plastid envelope membrane.

Employing an improved in vitro translation system for TGD1, which in our initial attempts was poorly translated in vitro (Xu et al., 2003), we examined the in vitro targeting and insertion of TGD1 into the chloroplast envelope membranes. When in vitro-translated pTGD1 protein was incubated with isolated pea chloroplasts under import conditions, we observed that pTGD1 was processed to the mature form, mTGD1 (Figure 10D). After import and treatment with thermolysin, the mTGD1 form was partially cleaved, and both proteins associated with the envelope membrane fraction. This experiment was done repeatedly, and

in no instance was complete digestion of mTGD1 with thermolysin observed. It should be noted that TGD1 does not contain a predictable N-terminal chloroplast transit peptide. Thus, TGD1 may be a protein that requires a nonclassical transit peptide for its proper targeting and insertion into the envelope membrane. To further investigate whether pTGD1 is indeed processed during import, in vitro-translated pTGD1 was incubated with a crude chloroplastic stromal preparation that contained the stromal processing peptidase (SPP), which cleaves and removes transit peptides from precursor proteins (Tranel and Keegstra, 1996). Treatment of translated pTGD1 with crude SPP generated a truncated protein of similar size and mobility with that of authentic mTGD1 that was produced from a chloroplastic import assay (Figure 10E, compare import assay with SPP assay). We conclude from this comparison that pTGD1 does contain a potential nonclassical transit peptide that targets pTGD1 to the envelope membrane of chloroplasts.

Partial thermolysin sensitivity of mTGD1 observed in the in vitro import assay system continued to raise questions with regard to the subcellular localization of TGD1 within the envelope membranes. To complement our in vitro import assays (Figure 10D), an in vivo chloroplast protein import assay was developed (Figure 10F). For this new assay approach, we generated C-terminal GFP fusion constructs of TGD1, of DGD1, which localized to the outside of the outer envelope membrane (Froehlich et al., 2001), and of ATS1, the plastidic glycerol-3-phosphate acyltransferase that is localized to the stroma or on the inside of the inner chloroplast envelope (Joyard and Douce, 1977; Nishida et al., 1993). The expression of these three fusion proteins was driven by the CaMV 35S promoter. These constructs were transiently expressed in tobacco (*Nicotiana tabacum*) leaves following *Agrobacterium tumefaciens* infection. Expression was enhanced by coexpressing a viral suppressor protein construct (Voinnet et al., 2003). Once the transgenes were fully expressed, chloroplasts were isolated and were then treated with thermolysin, trypsin, or buffer (Figure 10F). The GFP tag of these three fusion proteins was detected by protein gel blot analysis using GFP antibodies. The result of this experiment is shown in Figure 10F. The TGD1-GFP fusion protein was thermolysin resistant but trypsin sensitive, suggesting that the GFP portion of the protein and consequently the C terminus of TGD1 were facing the intermembrane space. The DGD1-GFP protein was sensitive to both proteases, as expected for a protein on the outside of the outer envelope membrane, while the ATS1-GFP fusion was not cleaved by either protease, as expected for a protein localized either on the inside of the inner envelope or in the stroma. Based on these multiple lines of evidence, we conclude that pTGD1 was imported into chloroplasts, processed, and subsequently targeted to the inner chloroplastic envelope membrane.

DISCUSSION

Diversion of Lipids at the ER and the Chloroplast Envelopes in the *tgdl* Mutant

At first glance, mutations in *TGD1* represented by the chemically induced alleles *tgdl-1*, *tgdl-2*, and *tgdl-3* caused a bewildering

array of aberrant lipid phenotypes in *Arabidopsis*. Closer analysis of the *tgdl1-1* mutant revealed that plastidic as well as extra-plastidic lipid pools were affected. The aberrant production of lipids not associated with the chloroplast in leaves included TAGs (Figure 6A), which had a fatty acid composition consistent with their origin from the eukaryotic pathway of lipid assembly associated with the ER (Figure 6A). Osmium-dense structures present in the cytosol of *tgdl1-1* cells but absent from wild-type cells (Figure 5) were likely containing these TAGs and presumably represented oil droplets. These TAGs were also found in nonphotosynthetic tissues, such as roots of the *tgdl1-1* mutant (Figure 6A), supporting the notion that they were not associated with chloroplasts. This was directly shown by isolating chloroplasts and demonstrating that they lacked TAGs (Figure 6A). The substantial accumulation of TAGs in the roots of the *tgdl1-1* mutant was somewhat surprising because only proplastids are present in the roots requiring a relatively minor flux through the eukaryotic pathway of thylakoid lipid biosynthesis during growth as compared with chloroplasts in the leaves. At this time, we do not understand how TAG biosynthesis is activated and sustained in roots of the *tgdl1-1* mutant. Clarifying this aspect of the mutant will require the identification and analysis of the enzyme(s) responsible for the biosynthesis of the root TAGs.

In vegetative tissues of growing, healthy, wild-type plants, TAGs are usually not present in measurable amounts, but they accumulate during seed development in the embryo where they serve as storage compounds. These seed oil TAGs have a distinct fatty acid composition that includes diagnostic very-long-chain fatty acids (Voelker and Kinney, 2001). Very-long-chain fatty acids were absent from TAGs accumulating in *tgdl1-1* leaf tissues, distinguishing the phenomenon described here from, for example, the *de novo* synthesis of TAGs in green seedlings reprogrammed to synthesize seed TAGs by the expression of the *WR11* gene that encodes a transcription factor controlling storage oil biosynthesis (Cernac and Benning, 2004). Moreover, TAGs accumulating in leaves of *tgdl1-1* were different in their fatty acid composition from TAGs accumulating in ozone-fumigated leaves (Sakaki et al., 1990). In this case, TAGs were similar in fatty acid composition to the predominant thylakoid lipid MGDG containing substantial amounts of molecular species derived from the prokaryotic pathway, suggesting a product precursor relationship between the TAGs and MGDG. Apparently, TAG accumulation in *tgdl1-1* was not related to a stress-induced turnover of thylakoid lipids as described for ozone fumigation.

In addition to TAG accumulation, oligogalactolipids synthesized at the outer chloroplast envelope as a result of the activation of a processive galactosyltransferase accumulated in the *tgdl1-1* mutant (Table 1). These oligogalactolipids showed a fatty acid composition consistent with their origin from the prokaryotic pathway associated with the chloroplast (Xu et al., 2003). While we do not yet understand what controls the accumulation of TAGs and oligogalactolipids in the *tgdl1-1* mutant, it seems likely that eukaryotic lipids are funneled into TAGs at the ER and prokaryotic lipids into oligogalactolipids at the chloroplast envelopes. This interpretation would be consistent with a disruption of the movement of eukaryotic lipids from the ER to the chloroplast, leading to a backup of eukaryotic lipid

species at the ER and an increased ratio of prokaryotic lipid species in the chloroplast to make up for the lack of eukaryotic lipids. One uncertainty is the location of TAG biosynthesis in the *tgdl1* mutant. In seeds, TAGs are typically produced by ER-associated enzymes, and we assume that the same is the case for TAGs produced in the leaves of the *tgdl1-1* mutant. The possibility that the *tgdl1-1* leaf TAGs are synthesized at the outer plastid envelope seems highly unlikely, as most of the lipids produced at the chloroplast envelopes in the *tgdl1-1* mutant are of prokaryotic origin, while the TAGs are of eukaryotic origin based on their fatty acid composition.

PA Is an Intermediate of Eukaryotic MGDG Biosynthesis

The TGD1 protein was predicted to be an integral membrane component of a multipartite bacterial-type ABC transporter complex that was proposed to be involved in the eukaryotic pathway of thylakoid lipid biosynthesis (Xu et al., 2003). The key question was, what molecule(s) is (are) transported by the complex such that impairment of the transport complex leads to the accumulation of TAGs in the cytosol. It seems likely that TAG itself is not transported, but rather a precursor of TAGs that backs up at the ER and is funneled into TAGs in the *tgdl1-1* mutant. The biosynthesis of TAGs draws from intermediates of phospholipid metabolism, such as PA or PC (Ohlrogge and Browse, 1995). Indeed, fivefold higher levels of PA were found in the *tgdl1-1* mutant, while the relative bulk amounts of other phospholipids were slightly decreased or remained the same (Table 1). Because PA is a regulatory molecule in addition to a central metabolite, one must assume that in the *tgdl1-1* mutant, excess PA at the ER is converted primarily into TAG, which can be harmlessly sequestered into oil droplets as shown in Figure 5. Interestingly, we did not observe an accumulation of PC in the mutant (Table 1). Likewise, an obvious increase in the content of DAG, which is the intermediate in the conversion of PA to MGDG and presumably TAG in the mutant, was not apparent in most experiments. The fact that PC did not accumulate suggests that PA and not PC might be the eukaryotic lipid transported from the ER to the envelopes.

Because of the consistent increase in PA content in the *tgdl1-1* mutant, the question arose whether PA could be the substrate of the TGD1 transport complex. Comparative labeling experiments with isolated wild-type and *tgdl1-1* mutant chloroplasts showed an impairment of conversion of label from PC and PA into MGDG in mutant chloroplasts (Figure 8). The rates for PC and PA labeling were similar and were approximately half of those observed for DAG, which is the direct substrate of MGD1, the main enzyme responsible for MGDG biosynthesis in *Arabidopsis* (Jarvis et al., 2000). A caveat in the interpretation of these experiments was the increased MGD1 activity in the *tgdl1-1* mutant, visible when labeled DAG (Figure 8A) or UDP-Gal (Figure 9A) was incubated with chloroplasts. However, because PA incorporation was decreased in mutant chloroplasts in spite of this fact, it was not the activity of MGD1 but that of TGD1 that was limiting in these experiments. The reason for the activation of MGD1 in the mutant is not known, but activation might be brought about by a changed lipid environment at the inner chloroplast envelope.

Adding labeled PC and phospholipase C to the reactions, which directly generates DAG from PC, did stimulate the incorporation of label into MGDG and nearly abolished the differences between the mutant and the wild type (Figure 8B). This result suggested that PA is the intermediate in the conversion of PC to DAG, in which TGD1 plays a role. Presumably, when only labeled PC is added to chloroplast preparations, an endogenous phospholipase converts it to PA, which is further metabolized to MGDG, but at a slower rate in the mutant chloroplasts. It should be noted that in similar experiments with isolated pea chloroplasts, a stimulation of incorporation of labeled PC by cytosolic fractions was observed and was attributed to cytosolic phospholipases producing PA (Andersson et al., 2004). The stimulation of PA incorporation following addition of cytosol fractions in the wild type, but not the mutant, suggests that additional factors are required under the employed conditions for a possible transfer of PA offered in the form of liposomes to the chloroplasts. Stimulation was not observed for the mutant presumably because the activity of the TGD1 mutant protein was limiting for the incorporation of PA into MGDG or because these factors could not interact with the TGD1 mutant protein. In any case, a direct conclusion from the results of this comparative analysis was that PA mediated by TGD1 is a precursor of MGDG biosynthesis. Moreover, PA as it is synthesized at the ER could possibly be the direct substrate provided by the ER for eukaryotic MGDG biosynthesis. This latter suggestion and the possible involvement of TGD1 are supported by the accumulation of PA but not PC in the *tgdl-1* mutant. However, our current data do not rule out that PC is transferred from the ER to the outer chloroplast envelope, where it could be converted to PA by a not yet identified enzyme, making it available to the TGD1 complex. Most likely, PC would have to be transferred from the ER to the chloroplast even if PA were the preferred ER lipid for the biosynthesis of eukaryotic MGDG because there is no known PC biosynthetic activity associated with the chloroplast envelopes (Douce and Joyard, 1996).

What Is the Biochemical Function of TGD1 at the Inner Chloroplast Envelope?

To understand the role that TGD1 might play in the conversion of PA to DAG, it was essential to determine the subcellular location of this protein within chloroplasts. An analysis of the amino acid sequence of TGD1 revealed that this protein does not contain a predictable transit peptide. Previous *in vitro* import and protease protection assays showed that TGD1 is partially thermolysin sensitive, leading to the tentative conclusion that it might be localized to the outer chloroplast envelope membrane (Xu et al., 2003). However, upon reinvestigating the subcellular localization of TGD1 using several alternative approaches, we obtained multiple lines of evidence (Figure 10) suggesting that this protein most likely is located in the inner chloroplast envelope membrane. For instance, to complement our standard *in vitro* chloroplast import assays, we developed an *in vivo* import assay using the transient expression of proteins in tobacco leaves followed by the isolation and protease treatment of chloroplasts. The results presented in Figure 10F show that after import and insertion, the TGD1-GFP fusion protein was thermolysin resistant

but trypsin sensitive. This result disagrees with the results derived from the *in vitro* import assays (Figure 10D). One interpretation of this discrepancy may be that in our newly developed *in vivo* import assay system, conditions are favorable for the proper and complete insertion of the TGD1-GFP fusion protein into the envelope membrane. In contrast with our *in vitro* import assay system, chloroplasts might have difficulty importing and subsequently inserting a multispinning membrane protein into the chloroplast envelope membrane. Indeed, discrepancies between *in vitro* and *in vivo* import systems have been reported previously (Silva-Filho et al., 1997). Likewise, attempts to employ *in vitro* import assay systems to investigate membrane proteins with multiple transmembrane domains, such as chloroplast metabolite translocators, have proven to be very problematic and challenging (Schunemann et al., 1993; Fischer et al., 1994; Weber et al., 1995; Flügge, 1998). Hence, to overcome these technical difficulties, we employed several strategies to examine the import and localization of TGD1 within chloroplasts. Additionally, the *in vivo* import assays also provided added information on the possible topology of TGD1, suggesting that the C-terminal end of TGD1 may be orientated toward the intermembrane space (Figure 10F). The currently accumulated evidence suggests that TGD1 is in fact being inserted into the inner chloroplast envelope membrane. However, the partial thermolysin sensitivity of TGD1 in import assays leaves the question open whether this protein might actually cross the inner and outer envelopes, a difficult to prove hypothesis at this time.

In light of our revised localization of TGD1 and assuming that TGD1 might facilitate PA transfer through the inner envelope, the question arises why a PA transporter at the inner chloroplast envelope would be necessary for the biosynthesis of eukaryotic MGDG given that we and others have shown that the major MGDG synthase, MGD1, is tethered to the outside of the inner envelope in a peripheral manner protruding into the intermembrane space (Figure 9C; see also Miede et al., 1999; Awai et al., 2001). The answer can be found in the apparent localization of PAP on the inside of the inner envelope. Evidence for the exclusive and tight association of chloroplast PAP activity with the inner envelope has been published (Block et al., 1983; Andrews and Mudd, 1985). While these experiments could not distinguish whether PAP was on the inside or outside of the inner envelope, the fact that plastidic PAP has a pH optimum of 9.0 and is affected by Mg^{2+} similar to other stroma proteins would be in agreement with a location on the inside of the inner envelope (Block et al., 1983; Malherbe et al., 1992). As far as we know today, chloroplast envelopes lack phospholipase C activity (Roughan and Slack, 1982; Browse and Somerville, 1991; Marechal et al., 1997), which could directly produce DAG from PC. Since PA by itself cannot readily traverse the membrane, a PA transporter at the inner chloroplast envelope would be a prerequisite for the conversion of eukaryotic PA into DAG by the PAP on the inside of the inner envelope. While we have no proof at this time that TGD1 and associated factors directly transfer PA through the inner chloroplast envelope, the underlying working hypothesis implies that the eukaryotic and prokaryotic pathways share the plastidic PAP. The activity of this enzyme is much lower in plants that exclusively use the eukaryotic pathway for galactolipid

biosynthesis unlike seed plants, *Arabidopsis* included, which use both pathways (Heinz and Roughan, 1983). A critical test of this hypothesis will have to await the identification of the gene for plastidic PAP. Furthermore, as we expect that additional components of the transporter exist in *Arabidopsis* based on additional *tgdl*-like nonallelic mutants (Xu et al., 2003), a reconstitution and direct demonstration of PA transport mediated by the TGD1 complex is not yet possible, and will have to await the isolation of all components.

Bringing together the eukaryotic and prokaryotic pathways at the level of PAP is consistent with the fact that only a single MGDG synthase, MGD1, is responsible for the biosynthesis of the bulk of eukaryotic and prokaryotic lipids (Jarvis et al., 2000). How DAG produced on the inside of the inner envelope moves to the reactive center of MGD1 on the other side of the membrane is not known. Furthermore, the processes by which MGDG produced by MGD1 moves to DGD1 on the outside of the outer envelope and DGDG moves back to the inner envelope remain to be discovered.

TGD1 Function Appears to Be Essential

All the available mutant alleles of *tgdl* are leaky, yet the strongest allele, *tgdl-1*, frequently led to an abortion of developing seeds at the heart stage, when greening of the chloroplast occurs in *Arabidopsis* (Figure 4). Furthermore, this phenotype was strongly enhanced by reducing the level of *tgdl-1* mRNA in an RNAi experiment (Figure 3). Fatty acids in MGDG with 16 carbons, in particular 16:3 (carbons:double bonds), at the second position of the glycerol moiety are diagnostic for the prokaryotic origin of the respective MGDG species (Heinz and Roughan, 1983). In *Arabidopsis* leaves, ~50% of the MGDG molecules are derived from the prokaryotic pathway, and a substantial fraction of fatty acids in MGDG is of the 16-carbon type (Browse et al., 1986). Interestingly, in developing seeds, the fatty acid profile of MGDG, which is a marker lipid for the plastid, showed much less 16-carbon fatty acids than MGDG in leaves (Figure 4D), suggesting that most of MGDG in embryo chloroplasts is derived from the eukaryotic pathway. The metabolism in embryos is specialized toward the biosynthesis of TAGs, which are exclusively derived from the eukaryotic pathway (Ohlrogge and Browse, 1995). Apparently, this prevalence of the eukaryotic pathway in embryos extends to the thylakoid lipids in the chloroplasts. If TGD1 plays an essential role in the biosynthesis of eukaryotic plastid lipids, embryos affected in TGD1 function cannot produce functional plastids and abort. Alternatively, the possible accumulation of PA, a potential signaling molecule, in the *tgdl-1* mutants simply might not be tolerated. As a consequence, seed abortion was observed, although with varying penetrance in the different *tgdl-1* mutant alleles and stronger in the *tgdl-1/TGD1*-RNAi lines. Varying penetrance of seed abortion is frequently encountered in mutants affected in primary lipid metabolism (Bonaventure et al., 2004). When the amount of TAGs in mature homozygous *tgdl-1* seeds that did develop was determined, no change in oil content per seed was observed. This result was in agreement with a proposed function of TGD1 as a PA translocator at the inner chloroplast envelope, which would presumably play no role in seed TAG biosynthesis at the ER. While the

TGD1 protein seems to be essential for the developing embryo, it is also important for the growing plant because growth of the *tgdl-1* mutant was slightly reduced and more so for the *tgdl-1/TGD1*-RNAi lines. Therefore, it seems likely that the TGD1 protein plays an essential role throughout the life cycle of the plant consistent with its hypothesized involvement in the transport of a central metabolite of primary lipid metabolism and a regulatory molecule, such as PA, into the chloroplast.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana tgdl-1* mutant allele, which was originally isolated in the *dgd1* background (Xu et al., 2003), was crossed into the wild-type background (ecotype Columbia-2) and at least three times backcrossed. Unless otherwise indicated, all experiments were conducted with this line. The *act1(ats1)* mutant has been previously described and corresponds to the JB25 allele (Kunst et al., 1988). Genotyping of the *TGD1* locus was done as previously described (Xu et al., 2003). Surface-sterilized seeds were germinated on 0.8% (w/v) agar-solidified MS medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) sucrose. Typically, 10-d-old seedlings of *Arabidopsis* wild type and mutants were transferred to soil drenched with half-strength *Arabidopsis* nutrient solution (Estelle and Somerville, 1987) and grown under a photosynthetic photon flux density of 70 to 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 22/18°C (day/night) with a 14-h-light/10-h-dark period. For quantitative growth experiments, MS 1% sucrose agar plates with seedlings were placed on a single shelf of a CU-36L5 growth chamber (Percival Scientific). At each time point, aerial parts of 15 wild-type and mutant plants on matching shelf positions were weighed. For root growth assays, 10 seedlings for each genotype were grown on vertically positioned agar plates, and 10 seedlings were analyzed per time point. Standard errors were calculated.

Generation of RNAi Lines

The full-length coding region of *TGD1* was amplified by PCR using the primers 5'-AATACTAGTGGCGCGCCATGATGCAGACTTGTT-3' (*SpeI* and *AscI*; sense primer) and 5'-CCAGGATCCATTTAAATTCAAACA-CAGTTCCTT-3' (*BamHI* and *SwaI*; antisense primer) and the original plasmid as template (Xu et al., 2003). The resulting DNA fragment was cloned into the silencing vector pGSA1285 (ABRC, Ohio State University, Columbus, OH; CD3-454) in sense and antisense orientations separated by a glucuronidase intron and was expressed under the control of the CaMV 35S promoter. Plant transformation was achieved by the floral dip method (Clough and Bent, 1998). Resistant seedlings were selected on MS medium containing 40 $\mu\text{g/mL}$ of kanamycin.

Microscopy

Leaves and siliques were imaged using a Leica MZ 12.5 dissecting microscope (Leica Microsystems) equipped with a Spot Insight color camera (Diagnostic Instruments). Siliques from homozygous *tgdl-1* plants were dissected with hypodermic needles. Seeds were cleared in chloral hydrate solution (chloral hydrate:water:glycerol, 8:2:1 w/v/v) for 1 h at room temperature and were observed with a Leica DMLB microscope equipped with Nomarski optics (Leica Microsystems).

For electron microscopy, leaf tissues from 3-week-old wild-type and *tgdl-1* mutant plants grown on agar-solidified medium were fixed for 2 h at room temperature in 2.5% glutaraldehyde and 0.1 M sodium phosphate, pH 7.2, followed by a secondary fixation in 1% (w/v) osmium tetroxide in the same buffer. After this double fixation, samples were

dehydrated in a graded series of acetone, embedded in EPON812 resin (Electron Microscopy Sciences), and sectioned. The thin sections (~70 to 90 nm) were stained with uranyl acetate and lead citrate prior to examination in a JEOL 100CX electron microscope (JEOL).

For the GFP fusion protein localization study, leaf samples were mounted in water on slides and were directly examined using a Zeiss LSM5 confocal microscope. Excitation light was provided by an argon laser at 488 nm. GFP fluorescence was observed with a band-pass filter of 505 to 530 nm and chlorophyll fluorescence with a 650-nm long-pass filter. Enhanced-quality images were acquired with the LSM5 imaging system software, and postacquisition image processing was performed with the LSM5 image browser and Adobe Photoshop software. This work was performed at the Center for Advanced Microscopy (Michigan State University).

Lipid and Fatty Acid Analyses

Lipids were extracted as previously described (Dörmann et al., 1995). Polar lipid extracts were analyzed on activated ammonium sulfate-impregnated silica gel TLC plates (Si250 with preadsorbent layer; Mallinckrodt Baker) using a solvent system of acetone:toluene:water (91:30:7, v/v). Two-dimensional TLC was used to separate PA as previously described (Benning et al., 1995). Neutral lipids were separated on the same silica plates, but untreated with ammonium sulfate, using a solvent system consisting of petroleum ether:ethyl ether:acetic acid (80:20:1, by volume). Lipids were visualized with iodine vapor and identified by cochromatography with lipid extracts of known composition or commercially purchased standards. For quantitative analysis, individual lipids were isolated from TLC plates and used to prepare fatty acid methyl esters. The methyl esters were quantified by gas-liquid chromatography using myristic acid as internal standard (Rossak et al., 1997). Positional analysis of fatty acids in PA was done as previously described (Härtel et al., 2000).

For in vivo labeling experiments, detached leaves from 3-week-old *Arabidopsis* plants were incubated with 3.7 MBq carrier-free [³²P]orthophosphate (Amersham) in 20 mL of MS medium. Lipids were extracted after 3 h of incubation and separated by two-dimensional TLC as described above. Radiolabeled lipids were visualized by autoradiography.

Isolation of ER Membrane and Cytosol Fraction

ER-enriched membranes were isolated from 3-week-old seedlings according to Bessoule et al. (1995). The resulting ER membranes were suspended in incubation buffer (see below). Cytosol fraction was isolated according to Andersson et al. (2004) with some modifications. Seedlings were homogenized in 50 mL of 50 mM HEPES-KOH, pH 7.0, 10 mM KCl, 2.5 mM MgCl₂, and 5 mM ascorbate and supplemented with 1 mL of protease inhibitor cocktail for plant extracts (P 9599; Sigma-Aldrich). The homogenate was then filtered through two layers of Miracloth (Calbiochem) and centrifuged at 12,000g for 10 min, and the supernatant was centrifuged further at 100,000g for 60 min. The supernatant was concentrated 10 times with a centricon 10 kD cutoff filter (Millipore). The concentrated cytosol fraction was frozen in liquid nitrogen and stored at -80°C until further use.

Chloroplast Lipid Import Assays

Intact chloroplasts were isolated from 3-week-old *Arabidopsis* plants by discontinuous gradient (5 mL 80% and 10 mL 40%) as previously described (Xu et al., 2002). Pigments were quantified according to Lichtenthaler (1987).

Radiolabeled PC for chloroplast labeling assays was prepared after incubating 16-d-old *Arabidopsis* seedlings overnight in 20 mM MES-KOH, pH 6.0, with 50 μmol of [1-¹⁴C]-acetate (2.22 GBq/mmol; American

Radiolabeled Chemicals) according to Kelly et al. (2003). Lipids were extracted and separated by TLC. Radioactive PC was eluted from silica gel using chloroform:methanol:formic acid (10:10:1, v/v) and redissolved in chloroform:methanol (3:1, v/v). The [¹⁴C]-labeled PA and DAG substrates were prepared by digesting radiolabeled PC with phospholipase D (Type I, cabbage [*Brassica capitata*]) and phospholipase C (Type XI, *Bacillus cereus*), respectively. All enzymes were from Sigma-Aldrich. Specific radioactivity of the resulting [¹⁴C]-PC, -PA, and -DAG were determined by gas chromatography and scintillation counting and were ~42, 16, and 12 MBq/mmol, respectively.

The chloroplast labeling experiments were done according to Ohnishi and Yamada (1982) with some alterations. Total lipids, including 0.45 nmol of radiolabeled [¹⁴C]-lipids and 10 nmol of unlabeled lipids dissolved in chloroform:methanol (2:1, v/v), were added to a glass vial, and the solvent was evaporated under a steam of nitrogen. Lipids were solubilized by sonication in 100 μL of incubation buffer (0.33 M sorbitol, 50 mM HEPES-KOH, pH 7.0, 10 mM KCl, 1 mM ATP, and 2.5 mM MgCl₂). In some experiments, as indicated in the figure legends, 0.3 units of phospholipase C or unlabeled UDP-Gal at a final concentration of 2 mM were added. Cytosolic and ER fractions were added equivalent to 50 μg of total protein each. The reaction was started by addition of the chloroplast preparation (50 μg chlorophyll) and was stopped after 30 min if not otherwise indicated by extracting lipids with chloroform:methanol (2:1, v/v). Lipids were separated by TLC as described above. After staining with iodine vapor, lipids were scraped off the plate and counted by liquid scintillation.

MGD1 Activity Assays

Intact *Arabidopsis* chloroplasts were isolated as above. In some experiments, the purified chloroplasts (1 mg/mL chlorophyll) were incubated with 0.5 mg/mL thermolysin (Sigma-Aldrich) in 0.33 M sorbitol, 50 mM HEPES, pH 7.3, and 1.0 mM CaCl₂ in the dark for 30 min at 4°C. After treatment, intact chloroplasts were repurified by centrifugation through a gradient as used for the isolation. Intact chloroplasts were resuspended in 0.33 M sorbitol, 50 mM HEPES, pH 7.6, 5 mM EDTA, 1 mM MnCl₂, and 1 mM MgCl₂. Total galactolipid synthesis in intact chloroplasts (50 μg chlorophyll) was assayed by incubation with 18.5 KBq of UDP-[U-¹⁴C]-galactose (10.28 GBq/mmol; American Radiolabeled Chemicals) in 150 μL of medium as described above for resuspension. Lipids were extracted and separated by TLC, and the radiolabel was quantified as described above.

PAP Activity Assay

Mixed envelope membranes from purified wild-type and *tgdl-1* chloroplasts were prepared by the method of Froehlich et al. (2003). Activity of PAP was assayed by following PA conversion into DAG by isolated envelope membranes according to Malherbe et al. (1992). Radioactive PA was synthesized within the envelope membranes by acylation of sn-[¹⁴C]glycerol 3-phosphate (specific activity, 5.29 GBq/mmol; Amersham). Lipids were extracted from aliquots of the reaction mixture taken at different time intervals, and the neutral lipids were analyzed by TLC as described above. Radioactivity in DAG was quantified by isolating the respective silica area from the plate followed by scintillation counting.

Quantification of RNA

Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. For RNA gel blots, RNA was separated by agarose gel electrophoresis and blotted onto Hybond N⁺ membranes (Amersham) using standard procedures and standard high-stringency conditions.

For RT-PCR quantification of *TGD1* RNA, total RNA was extracted from 3-week-old plants using the RNeasy plant mini kit (Qiagen) following the manufacturer's instructions. The RNA was treated with DNase I (Promega). First-strand cDNA was synthesized using the Ominiscript reverse transcription kit (Qiagen). PCR thermocycling conditions were 94°C for 3 min followed by 29 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with a final polymerization step at 72°C for 10 min. Primers used to amplify a 726-bp fragment of the *TGD1* gene were 5'-CAAGGTACCATGATGCA-GACTTGTGTAT-3' (forward) and 5'-TCAGAAACACATGAGTGTGAC-3' (reverse). A 317-bp fragment within the ubiquitin *UBQ10* gene (GenBank At5g05320) was amplified separately as a control. The *UBQ10* forward primer was 5'-TCAATCTCTCTACCGTGATCAAGATGCA-3', and the reverse primer was 5'-GTGTCAGAACTCTCCACCTCAAGAGTA-3'.

In Vitro Chloroplast Import Assays and Stromal Peptidase Treatment

Plasmids expressing cDNAs encoding ARC6 (Vitha et al., 2003), tp110-110N (Lübeck et al., 1997), and MGD1 (Awai et al., 2001) have been described previously. These genes were transcribed/translated, and proteins were subsequently labeled with [³⁵S]-Met using the TNT-coupled reticulocyte lysate system according to the manufacturer's recommendations (Promega). The *TGD1* cDNA was PCR amplified from the original plasmid (Xu et al., 2003) and reinserted into the pGEM-T vector to allow its expression from the SP6 promoter (Promega). This *TGD1* plasmid was linearized prior to translation with the SP6 RNA Polymerase TNT-coupled reticulocyte lysate system. Pea plants (*Pisum sativum* var Little Marvel; Olds Seed Co.) were grown under natural light in the greenhouse at 18 to 20°C. Chloroplasts were isolated from 8- to 12-d-old plants as described previously (Bruce et al., 1994). Binding or import reactions were performed according to Tranel et al. (1995). Post-treatments of import reactions with either thermolysin or trypsin were performed as described previously (Jackson et al., 1998). SPP assays were performed according to Tranel and Keegstra (1996). All fractions were analyzed by SDS-PAGE (Laemmli, 1970) and fluorography (Tranel et al., 1995).

Gene-GFP Fusion Constructs

For the generation of a GFP fusion construct, the entire coding region of *ATS1* (At1g32200) was inserted into the *NcoI*-*SpeI* sites of pCambia1302 (www.cambia.org) using primers 5'-AGCCATGGTAATGACTCTCAC-GTTTTTC-3' and 5'-CGACTAGTATCCAAGTTGTGACAAAG-3'. The N-terminal part of DGD1 up to Pro³³⁷ was amplified from pBinAR-Hyg-DGD1 (Dörmann et al., 1999) using primers 5'-CACGGTACCATGG-TAAAGGAAACT-3' and 5'-CTCGGATCCAGGCTTCACAAAATC-3' and was directly cloned into pCambia1300MCS-GFP (a derivative of pCambia1302 with a more versatile multiple cloning site in the expression cassette). This N-terminal portion of DGD1 contains the correct targeting information and is inserted into the outer envelope membrane as previously shown. The full-length coding sequence of *TGD1*, including the transit peptide, was amplified by PCR using the primers 5'-CAGGAG-ATCTAATGATGCAGACTTGTG-3' and 5'-CCGGACTAGTAACACAGT-TCTTCAAAGA-3' and pBINAR-Hyg-TGD1 (Xu et al., 2003) as template. This fragment was inserted into the binary vector pCambia1302 using the *BglI* and *SpeI* restriction sites. Stable transformation of *Arabidopsis* was achieved using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected in the presence of Hygromycin B (25 μg mL⁻¹) on MS medium lacking sucrose.

In Vivo Chloroplast Import Assays

For transient expression of GFP constructs in *Nicotiana benthamiana*, the *Agrobacterium tumefaciens* strain C58C1 carrying the respective construct (see above) was injected into the abaxial air space of 3- to 4-week-

old plants according to Voinnet et al. (2003). A second construct expressing the p19 protein of tomato bushy stunt virus was used to suppress gene silencing and therefore enhance the level of transient expression of the target gene construct (Voinnet et al., 2003). *Agrobacterium* strains carrying GFP constructs or the p19 silencing plasmid were grown to an OD₆₀₀ of 0.5 and 1.0, respectively, prior to mixing of equal parts and injection. At 5 d after infiltration, leaves were harvested, and intact chloroplasts were isolated by discontinuous gradient as described above. Protease protection assays were done according to McAndrew et al. (2001). Briefly, isolated chloroplasts were treated with either trypsin or thermolysin at final concentrations of 500 or 800 μg/mL for 30 min in ice-cold reaction buffer (50 mM HEPES-KOH, pH 7.9, 0.33 M sorbitol, and 1 mM CaCl₂ in a total volume of 250 μL). Intact chloroplasts were reisolated by centrifugation through 40% Percoll (v/v), washed, and solubilized directly in sample buffer prior to analysis by SDS-PAGE.

Protein Extraction and Immunoblotting

For GFP fusion protein extraction, 0.1 g of frozen leaf tissue was extracted with 0.2 mL buffer containing 4 M urea and 100 mM DTT. Two hundred microliters of loading buffer (Laemmli, 1970) were added and the samples were boiled for 5 min. After centrifugation for 5 min at 10,000g, the proteins in the supernatant were separated by SDS-PAGE (Laemmli, 1970). For GFP immunodetection, a rabbit anti-GFP antibody (Molecular Probes) and anti-rabbit alkaline phosphatase-coupled antibody (Jackson Immuno Research Laboratories) were used at 1:1000 and 1:10,000 dilutions, respectively. The blots were developed using the Immun-Star AP detection system (Bio-Rad).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers (www.arabidopsis.org) for the *Arabidopsis* genes mentioned in the text are as follows: *ACT1(ATS1)*, At1g32200; *ARC6*, At5g42480; *DGD1*, At3g11670; *MGD1*, At4g31780; *TGD1*, At1g19800.

Supplemental Data

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

Supplemental Figure 1. Digestion of Leaf TAG and Soy Oil TAG with Pancreatic Lipase.

Supplemental Figure 2. Phosphatidate Phosphatase Activity in Isolated Chloroplasts of the *tgdl1-1* Mutant and the Wild Type.

Supplemental Figure 3. Sodium Chloride and Sodium Carbonate Extraction of Isolated Pea Chloroplast Total Membranes following Import of MGD1.

Supplemental Figure 4. GFP Immunoblot of Leaf Extracts of the Wild Type or the Strongest TGD1-GFP Expressing Transgenic Line (WT/TGD1-GFP).

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