The development of chloroplasts in Arabidopsis thaliana requires extensive lipid trafficking between the endoplasmic reticulum (ER) and the plastid. The biosynthetic enzymes for the final steps of chloroplast lipid assembly are associated with the plastid envelope membranes. For example, during biosynthesis of the galactolipids predominant in photosynthetic membranes, galactosyltransferases associated with these membranes transfer galactosyl residues from UDP-Gal to diacylglycerol. In Arabidopsis, diacylglycerol can be derived from the ER or the plastid. Here, we describe a mutant of Arabidopsis, trigalactosyldiacylglycerol4 (tgd4), in which ER-derived diacylglycerol is not available for galactolipid biosynthesis. This mutant accumulates diagnostic oligogalactoglycerolipids, hence its name, and triacylglycerol in its tissues. The TGD4 gene encodes a protein that appears to be associated with the ER membranes. Mutant ER microsomes show a decreased transfer of lipids to isolated plastids consistent with in vivo labeling data, indicating a disruption of ER-to-plastid lipid transfer. The complex lipid phenotype of the mutant is similar to that of the tgd1,2,3 mutants disrupted in components of a lipid transporter of the inner plastid envelope membrane. However, unlike the TGD1,2,3 complex, which is proposed to transfer phosphatidic acid through the inner envelope membrane, TGD4 appears to be part of the machinery mediating lipid transfer between the ER and the outer plastid envelope membrane. The extent of direct ER-to-plastid envelope contact sites is not altered in the tgd4 mutant. However, this does not preclude a possible function of TGD4 in those contact sites as a conduit for lipid transfer between the ER and the plastid.

INTRODUCTION

Photosynthesis and the production of plant biomass depend on the photosynthetic membrane, which is organized into thylakoids inside chloroplasts. Development of this intricate and expansive membrane system places high demands on the synthesis of proteins, pigments, and membrane lipids. Most, if not all fatty acids found in polar membrane lipids in plants are synthesized de novo inside chloroplasts (Ohlrogge et al., 1979). Fatty acyl groups either attached to acyl carrier proteins (inside the plastid), CoA (outside the plastid), or polar lipids must be transferred to or exchanged between the three biogenic membranes of plant cells that participate in the biosynthesis of thylakoid lipids: the inner and outer envelope membranes of the chloroplasts and the endoplasmic reticulum (ER). In many plants de novo lipid assembly occurs at the inner envelope membrane of plastids. In addition, glycerolipid moieties assembled at the ER find their way back into thylakoid membrane lipids. This two-pathway hypothesis of thylakoid lipid biosynthesis was first postulated by Roughan and colleagues based on labeling evidence (Benning et al., 2006; Jouhet et al., 2007; Benning, 2008).

Most plants do not use both pathways to the same extent. Chloroplasts can synthesize their complement of thylakoid membrane lipids de novo in only a limited number of plant species (Heinz and Roughan, 1983; Mongrand et al., 1998), including Arabidopsis (Browse and Somerville, 1991, 1994). The underlying lipid trafficking phenomena are complex, and mechanistic insights into lipid trafficking between the ER and the plastid have only recently begun to emerge based on genetic, cell biological, and biochemical evidence (Benning et al., 2006; Jouhet et al., 2007; Benning, 2008).

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DGD1, which presumably transfers a second galactosyl residue from UDP-Gal to MGDG to form DGDG (Dörmann et al., 1995, 1999; Kelly et al., 2003). Of these two enzymes, MGD1 is associated in Arabidopsis with the inner plastid envelope membrane facing the intermembrane space (Awai et al., 2001; Xu et al., 2005) and DGD1 with the cytosolic side of the outer plastid envelope membrane (Froehlich et al., 2001). It has been speculated that this arrangement is necessary to provide access to UDP-Gal synthesized in the cytosol (Joyard et al., 1993). At the same time, it necessitates the transfer of lipid precursors through and between the plastid envelope membranes.

The trigalactosyldiacylglycerol (tgd) mutants tgd1, tgd2, and tgd3 (Xu et al., 2003, 2005; Awai et al., 2006; Lu et al., 2007) of Arabidopsis are disrupted in one particular aspect of ER-to-plastid lipid trafficking. These three mutants are deficient in three components of what appears to be a microbial-type multipartite ABC transport complex of the inner plastid envelope (Benning, 2008) that is hypothesized to transport phosphatidic acid (PtdOH), making it available to the PtdOH phosphatase at the stroma-facing side of the inner envelope. Candidate genes for plastidic PtdOH phosphatase were recently proposed (Nakamura et al., 2007). The tissues of all three tgd mutants accumulate oligogalactolipidic lipids, hence their name, and triacylglycerols, and, based on multiple lines of biochemical evidence, the mutants are impaired in the synthesis of ER-derived galactolipid molecular species (Xu et al., 2003, 2005; Awai et al., 2006; Lu et al., 2007). Here, we describe the tgd4 mutant of Arabidopsis derived from the same mutant screen that produced the tgd1,2,3 mutants (Xu et al., 2003) and that is phenotypically similar to the previously described tgd mutants. Unlike the TGD1,2,3 proteins, TGD4 is not plastid localized but ER associated. This raised the question whether TGD4 might be involved in forming the ER-plastid contact zones that have recently been proposed as conduits for ER-to-plastid lipid trafficking (Kjellberg et al., 2000; Andersson et al., 2007).

RESULTS

The tgd4-1 Mutation Defines Arabidopsis Gene At3g06960

The tgd4-1 mutant was isolated during a screen for lines accumulating oligogalactolipids (Xu et al., 2003) in an ethyl methanesulfonate–mutagenized population of Arabidopsis (ecotype Columbia [Col-2]). The growth and lipid phenotypes of tgd4-1 are shown in Figure 1. The tgd4-1 plant is slightly pale green but otherwise indistinguishable from the wild type. The defining tgd4-1 phenotype is the presence of a lipid in leaf extracts that comigrates during thin layer chromatography with trigalactosyldiacylglycerol (TGDG) and stains positive for sugars. This phenotype was sufficiently robust for mapping of the tgd4-1 locus in an F2 mapping population of 1436 plants from a cross of tgd4-1 with the wild type of the Landsberg erecta (Ler) ecotype. The diagnostic oligogalactolipid phenotype segregated in the F2 population according to Mendelian ratios for recessive mutant alleles. The physical map surrounding the tgd4-1 locus and the number of recombination events between tgd4-1 and respective DNA markers are shown in Figures 1C to 1E. Cosmids containing wild-type DNA between T-DNA borders covering the locus and neighboring sequences were used for complementation analysis and narrowed down tgd4-1 to two possible genes: At3g06960 and At3g06970. Sequencing of cDNAs for both predicted genes derived from tgd4-1 detected a mutation in nucleotide position 102 of the coding sequence (GenBank accession number NM_111576) in the first predicted exon of At3g06960. The mutation leads to a Pro-to-Leu substitution in position 20 of the predicted protein (Figure 1F). Two independent T-DNA insertion lines (SAIL_760_F05, insertion at nucleotide 1908, and SAIL_133_H06, insertion at nucleotide 905 of the genomic sequence, The Arabidopsis Information Resource accession number 4010722787; Figure 1F) were available (Sessions et al., 2002). Analysis of homozygous plants for these two T-DNA lines revealed the accumulation of oligogalactolipid in lipid extracts (Figure 1B). Finally, a wild-type cDNA derived from locus At3g06960 was fused to the open reading frame of green fluorescent protein (GFP) and when expressed in the tgd4-1 mutant under the control of the cauliflower mosaic virus (CaMV) 35S promoter was able to restore the wild-type lipid phenotype, as shown in Supplemental Figure 1 online. Based on these mapping data, multiple independent alleles, and complementation data, it was concluded that At3g06960 is identical to TGD4 of Arabidopsis. From now on the point mutant allele is designated tgd4-1, the T-DNA allele corresponding to SAIL_760_F05, tgd4-2, and the T-DNA allele corresponding to SAIL_133_H06, tgd4-3.

The Phenotype of tgd4 Mutants Suggests Impaired ER-to-Plastid Lipid Trafficking

The growth phenotypes of tgd4-1, tgd4-2, and tgd4-3 are shown in Figure 1A. Plants carrying the tgd4-2 and tgd4-3 T-DNA insertion alleles were stunted, pale yellow in color, and infertile. The two T-DNA alleles had to be maintained as heterozygous lines, which limited the availability of material for some of the subsequent analyses described below. Viable seeds were obtained when wild-type pollen was transferred onto tgd4-2 or tgd4-3 pistils, but reciprocal transfer did not yield seeds, suggesting that the tgd4-2 and tgd4-3 mutations cause a pollen defect. The total leaf fatty acid content of the severe tgd4-3 mutant was not reduced (wild type, 3.1 ± 0.2, and tgd4-3, 3.1 ± 0.1 μg/mg fresh weight; n = 3, s.d. is given). A comparison of polar lipids in the wild type and the three mutant lines is shown in Table 1. The mutants have slightly reduced amounts of galactolipidic lipids and slightly increased amounts of phosphatidylglycerol (PtdGro), phosphatidylethanolamine (PtdEtn), and phosphatidylcholine (PtdCho). The galactolipidic lipids are primarily found in plastids, while PtdGro and PtdCho are present in plastid and extraplastidic membranes, and PtdEtn is exclusively in extraplastidic membranes. Therefore, these lipid changes may reflect a decreased ratio of plastid-to-extraplastidic membranes in the mutants, which is consistent with the pale green color of the mutants indicative of reduced chlorophyll and photosynthetic membranes. The TGDG content of the mutants was <1 mol % of total fatty acids in leaves (e.g., 0.5 ± 0.1 mol % for tgd4-3; n = 3, s.d. is given). Ultrastructural analysis of chloroplasts also suggested less thylakoid stacking and fewer thylakoid membranes in mutant chloroplasts (Figure 2). Similar to findings on the
previously studied tgd1-1 mutant, the tgd4-2 and tgd4-3 mutants showed osmiophilic particles in the cytoplasm resembling oil droplets (Figure 2). Analysis of neutral lipids in extracts of tgd4-2 and tgd4-3 mutants revealed the accumulation of lipids cochromatographing with triacylglycerols found also in the previously characterized tgd1-1 mutant (see Supplemental Figure 2 online). Triacylglycerol content of the mutant leaves was <1 mol % of total fatty acids (e.g., 0.5 ± 0.2 mol % for tgd4-3; n = 3, so is given).

Molecular species of galactoglycerolipids derived from the plastid pathway preferentially carry a 16-carbon fatty acyl group in the sn-2 position of the diacylglycerol moiety, and ER-derived species preferentially carry an 18-carbon fatty acyl chain (Heinz and Roughan, 1983). Analysis of acyl chains following position-specific lipase treatment of the two galactoglycerolipids from the wild type and different tgd4 mutants is shown in Table 2. Additional fatty acid analysis data for lipids in the wild type and the tgd4-2 and tgd4-3 mutants are provided in Supplemental Table 1 online. It is apparent that 16-carbon fatty acids are highly enriched in the sn-2 position in the mutants consistent with an overabundance of molecular species derived from the plastid pathway. In other words, like in the tgd1, tgd2, and tgd3 mutants, the ER pathway of galactoglycerolipid biosynthesis appears to be disrupted in the tgd4 mutants.

The TGD4-GFP Protein Is Associated with the ER

The TGD4 gene is predicted to encode a 52,822-D protein for which a molecular or biochemical function has not yet been described. Putative orthologs for this protein are exclusively present in seed plants, mosses, and green algae, as shown in an unrooted tree in Supplemental Figure 3 online. However, putative orthologs are not obvious in all completed genomes of this group (e.g., the green alga Chlamydomonas reinhardtii or the poplar tree). The protein is listed in the ARAMEMNON database of Arabidopsis membrane proteins (Schwacke et al., 2003) and is predicted by iPSORT (Bannai et al., 2002) to be ER associated.
To experimentally verify the subcellular location of TGD4, the TGD4cGFP cDNA fusion that tested positive for complementation (see Supplemental Figure 1 online) and a construct encoding a GFP protein targeted to the lumen of the ER (Batoko et al., 2000) were transiently expressed in tobacco cells under the control of the CaMV 35S promoter. The TGD4-GFP fusion protein resulting from the TGD4cGFP construct was detected in crude tobacco leaf extracts using a GFP antibody, as shown in Figure 3A. Fractionation of the cell extract by differential centrifugation indicated that the fusion protein was associated with microsomes. The association of TGD4-GFP to microsomes was quite strong as different salts and chaotropic agents did not lead to the removal of the protein from the microsome fraction (Figure 3B). However, treatment of the extract with detergent was able to solubilize the TGD4-GFP fusion protein (Figure 3C). This result suggested that TGD4 is inserted into microsomal membranes possibly as a function of the two predicted membrane-spanning domains.

Introduction of a GFP marker protein with an ER retention signal led to the visualization of the ER network (Batoko et al., 2000), as shown in Figure 3D. Likewise, when the TGD4-GFP protein was transiently produced in tobacco cells, the ER network was labeled (Figure 3E). Organelles other than the ER were not labeled under these conditions, implying that the overexpression conditions used did not result in mistargeting. Taken together, the results suggest that TGD4 is inserted into microsomal membranes possibly as a function of the two predicted membrane-spanning domains.

The Arabidopsis gene At2g44640 is a possible paralog of TGD4. The encoded protein sequence of this gene is 35% identical (171 out of 477 amino acids) to the TGD4 protein. The At2g44640 encoded protein is not predicted to contain a membrane-spanning domain but was identified in proteomics studies of the plastid envelope (Ferro et al., 2003; Froehlich et al., 2003) and the vacuole (Jaquinod et al., 2007). Since we were unable to confirm a published T-DNA insertion line (FLAG_410H05), Arabidopsis transgenic lines expressing the At2g44640 cDNA in sense and antisense orientation under the control of the CaMV 35S promoter were produced. None of the 20 transgenic lines tested had lipid phenotypes, visible growth, or morphological phenotypes distinct from the wild type. Therefore, the analysis of this gene was not further pursued in the context of ER-to-plastid lipid trafficking.

Severe Growth Defects in ats1-1 tgd4 Double Mutants

To provide genetic evidence for a disruption of ER-to-plastid lipid trafficking in the tgd4 mutants, the different tgd4 alleles were introduced into the ats1-1 mutant background. This mutant lacks most of its plastidic acyl-ACP:glycerol 3-phosphate acyltransferase activity and is blocked in the plastid pathway of

| Table 1. Leaf Polar Lipid Composition of the Wild Type, tgd4-1, tgd4-2, and tgd4-3 |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Lipids          | Wild Type       | tgd4-1          | tgd4-2          | tgd4-3          |
| MGDG            | 37.4 ± 1.7      | 38.5 ± 7.5      | 34.4 ± 1.0      | 32.6 ± 1.1      |
| PtdGro          | 8.9 ± 0.6       | 12.1 ± 1.2      | 12.3 ± 0.5      | 13.5 ± 0.2      |
| DGDG            | 19.3 ± 0.5      | 15.2 ± 1.6      | 12.9 ± 0.9      | 12.6 ± 0.1      |
| SQDG            | 3.1 ± 0.3       | 4.0 ± 0.6       | 3.5 ± 0.1       | 4.0 ± 0.2       |
| PtdIns          | 1.1 ± 0.2       | 2.5 ± 0.9       | 1.2 ± 0.4       | 1.4 ± 0.2       |
| PtdEtn          | 13.8 ± 0.1      | 10.0 ± 1.9      | 16.6 ± 0.3      | 16.8 ± 0.3      |
| PtdCho          | 15.8 ± 0.3      | 15.8 ± 4.1      | 19.6 ± 0.7      | 19.1 ± 0.9      |

Values (mol %) of three independent samples were averaged and the SD is indicated. Plants were grown on soil for 4 weeks. PtdIns, phosphatidylinositol.

Values are significantly different from wild-type values based on Student’s t test (95% confidence interval).

This lipid sample also contains TGDG at <1 mol %.

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Figure 2. Ultrastructure of Chloroplasts in Leaves of the Wild Type and the tgd4-2 and tgd4-3 Mutants.

Representative transmission electron microscope images are shown. Plants were grown for 3 weeks on agar-solidified Murashige and Skoog (MS) medium, and leaves were harvested 4 h after onset of the light period. Arrows point to electron-dense particles outside the plastid present in the mutants, but absent in the wild type. Bars = 1 μm.
galactoglycerolipid biosynthesis (Kunst et al., 1988; Xu et al., 2006). The prediction was that concomitant disruption of the plastid and ER pathways of thylakoid lipid biosynthesis would lead to nonviable or severely impaired plants as had previously been shown for an ats-1-1 tgd-1-1 double mutant (Xu et al., 2005). When the T-DNA disruption alleles tgd-4-2 and tgd-4-3 were crossed into the ats-1-1 mutant, no viable homozygous seeds were obtained. Instead, aborted seeds were apparent in the siliques of plants homozygous for ats-1-1 and heterozygous for tgd-4-2 or tgd-4-3, presumably due to the presence of double homozygous embryos, as shown in Figure 4A. Crossing the point mutant allele tgd-4-1 into ats-1-1 gave rise to double homozygous ats-1-1 tgd-4-1 plants that were viable on agar-solidified medium containing sucrose but, unlike the tgd-4-1 homozygous mutant (cf. Figure 1A), were unable to grow photoautotrophically on soil (Figure 4B). Genotyping confirmed homozygosity at both loci, and like tgd-4-1, the ats-1-1 tgd-4-1 homozygous double mutant plants were infertile.

Sufficient material was available to conduct a careful lipid analysis of the double mutant, as shown in Figures 4C and 4D. The plastid-specific galactoglycerolipid MGDG was strongly reduced in the double mutant, while PtdCho was strongly increased to the same extent, suggesting a possible precursor (PtdCho) product (MGDG) relationship. The only phospholipid strongly reduced in the double mutant was PtdGro, which of all phospholipids is most prevalent in thylakoid membranes. While PtdGro is only mildly reduced in the ats-1-1 mutant (Kunst et al., 1988), introducing the tgd-4-1 mutation in addition had an additive effect on PtdGro synthesis. Possibly, this is an indication of an overall reduction in plastid membranes in the double mutant. As DGDG is mostly derived from the ER pathway, its relative amounts were not affected by the ats-1-1 mutation. Fatty acid analysis of the two galactoglycerolipids MGDG and DGDG in the double mutant revealed a nearly complete lack of 16 carbon fatty acids, particularly evident for 16:3, which is present in substantial

| Table 2. Fatty Acid Composition at the sn-2 Position of the Two Major Galactoglycerolipids in the Wild Type, tgd-4-1, tgd-4-2, and tgd-4-3 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| FAs             | 16:0 | 16:1 | 16:2 | 16:3 | 18:0 | 18:1 | 18:2 | 18:3 |
| MGDG            |      |      |      |      |      |      |      |      |
| WT              | 1.8  | 1.1  | NDb  | 57.3 | 1.0  | ND  | 2.7  | 35.9 |
| tgd-4-1         | 5.3  | 2.8  | ND   | 51.3 | 1.2  | ND  | 5.8  | 33.4 |
| tgd-4-2         | 18.3 | 7.3  | 1.5  | 59.7 | 2.4  | ND  | 1.3  | 9.2 |
| tgd-4-3         | 16.8 | 6.9  | 0.2  | 64.2 | 1.7  | ND  | 1.1  | 8.8 |
| DGDG            |      |      |      |      |      |      |      |      |
| WT              | 19.0 | 1.2  | 0.4  | 3.8  | 4.2  | 2.0 | 3.0  | 64.4 |
| tgd-4-1         | 29.2 | 1.8  | 0.4  | 1.2  | 7.4  | 4.5 | 5.4  | 49.3 |
| tgd-4-2         | 65.4 | 1.7  | 0.5  | 0.6  | 8.6  | 4.4 | 2.9  | 14.7 |
| tgd-4-3         | 74.0 | 1.1  | 0.6  | 3.6  | 3.7  | 2.7 | 1.9  | 11.9 |

aThree independent measurements were averaged for all samples, except for the tgd-4-2 DGDG samples, for which only two samples were available. In all cases, the SD was <10%. Plants were 4 weeks old and grown on soil. Fatty acids (FAs) are designated with number of carbons:number of double bonds.

bND, not detected at a limit of 0.05 mol %.
amounts in MGDG (cf. Table 2). This result is consistent with a complete block of the plastid pathway of galactoglycerolipid biosynthesis due to the presence of the \textit{ats1-1} mutation and a partial block of the ER pathway due to the presence of the leaky \textit{tgd4-1} allele.

During routine characterization of the \textit{ats1-1 tgd4-1} double mutant, we observed in electron micrographs that the double mutant cells contained only one, sometimes two or three, giant chloroplast(s), as shown in Figure 5. Apparently, chloroplast division in the double mutant was disrupted. Whether the wild-type TGD4 protein interacts with the chloroplast division machinery or whether the drastically altered lipid composition in the double mutant affects chloroplast division cannot be distinguished at this time.

Pulse-Chase Labeling Confirms an ER-to-Plastid Lipid Trafficking Defect in \textit{tgd4}

Lipid trafficking is a dynamic process, and one of the proven ways of detecting disruptions in ER-to-plastid lipid trafficking is pulse-chase labeling with radiolabeled oleic acid or acetate as previously discussed (Benning, 2008). A pulse of oleic acid applied to excised wild-type leaves was initially incorporated into PtdCho at the ER, as shown in Figure 6A. After switching to nonlabeled medium, label moved from the PtdCho pool to different lipids, including the exclusive thylakoid membrane lipid MGDG shown in Figures 6B and 6C. Comparing the previously characterized \textit{tgd1-1} lipid trafficking mutant and \textit{tgd4-2} (Figure 6C) and \textit{tgd4-3} alleles (Figures 6B and 6C), it was apparent that much less label moved from PtdCho to MGDG in the two \textit{tgd4} mutants during the chase phase of the experiment. This result is consistent with a defect in ER-to-plastid lipid trafficking as part of the ER pathway of thylakoid lipid biosynthesis.

A similar experiment was done from a different perspective using radiolabeled acetate. Acetate fed to excised leaves is taken up by chloroplasts where it is readily incorporated into fatty acids. As a result, lipid molecular species assembled in the plastid are labeled first. A large portion of MGDG is directly made in the plastid, and label in MGDG was substantial following a 10-min acetate pulse, as shown in Supplemental Figure 4 online. In the \textit{tgd1-1} and in the \textit{tgd4-3} mutants, the initial label in MGDG was much higher compared with the wild type (see Supplemental Figure 4 online). This result is consistent with a predominant synthesis of MGDG in the \textit{tgd4} mutants shown in Table 2.

ER-to-Outer Plastid Envelope Membrane Contact Sites Are Not Affected in \textit{tgd4}

Direct contact sites have been recently discussed as conduits for lipid transfer between the ER and the plastid (Kjellber et al.,...
To approach this question in a more quantitative way, we assayed the incorporation of labeled acyl groups from palmitoyl-CoA into PtdGro by isolated chloroplasts. This assay has been previously used to detect plastid-associated ER fragments (Kjellberg et al., 2000). The plastids of the tgd1-1 and tgd4-3 mutants showed higher incorporation of acyl groups from palmitoyl-CoA into PtdGro than the wild-type plastids (Figure 7E). Moreover, this activity was accessible to the large protease thermolysin, which cannot reach plastid-associated proteins inside of the outer envelope membrane. This result independently corroborates the cell biological findings described above that there is no decrease in the extent or number of ER-plastid contact sites in the tgd4-3 mutant.

It is interesting to note that in plastids of the tgd1-1 and the tgd4-3 mutants the incorporation of acyl groups from palmitoyl-CoA into PtdCho was also increased compared with wild-type plastids (Figure 7F). However, this activity was resistant to thermolysin treatment, suggesting that the acyltransferase or acyl-exchange protein responsible for this reaction is associated with the inner envelope of the plastid. The increased acyltransferase activities observed for the tgd1-1 and tgd4-3 mutants correlated well with the observed increase in PtdCho content of isolated plastids, as shown in Table 3. Other chloroplast lipid changes included a decrease in relative DGDG content (Table 3). In the wild type, this lipid is mostly derived from the ER pathway.

ER Prepared from tgd Mutants Is Less Efficient in PtdCho Transfer to Plastids

To directly assay lipid transfer from the ER to the plastid, ER preparations of etiolated Arabidopsis wild type and tgd1-1 and tgd4-1 mutants were incubated with lyso-PtdCho and labeled oleoyl-CoA. A thin layer chromatogram of lipids extracted from the ER preparations is shown in Figure 8A. Small but detectable amounts of galactoglycerolipids were present in the ER preparations and were indicative of low-level chloroplast envelope contamination that we could not eliminate even by using etiolated seedlings, which only contain etioplasts. The autoradiography of the thin layer chromatogram shown in Figure 8B confirms PtdCho as the predominantly labeled lipid in the labeled ER preparation. The transfer reaction was initiated by adding chloroplasts purified from pea (Pisum sativum) leaves, which can be readily prepared and are highly active. Transfer of labeled precursors from the ER to the plastid was measured as label incorporated into MGDG present in repurified chloroplasts following the incubation (Figure 8C). MGDG biosynthesis was stimulated by the addition of UDP-Gal when wild-type ER preparations were present, but less so for mutant ER. Equal amounts of ER were added based on fatty acid content in the preparations. Addition of soluble proteins prepared from pea leaves stimulated the transfer of label from PtdCho in the ER preparation to MGDG isolated from chloroplasts that were repurified from the reaction mixture. The soluble protein additions led to more pronounced differences between the wild-type and the mutant ER preparations. The effect of these soluble protein additions may reflect the need for additional cytosolic factors required for the transfer of PtdCho or the conversion of PtdCho into an intermediate that is ultimately transferred. Considering the small

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**Figure 5.** Disruption of Chloroplast Division in the ats1-1 tgd4-1 Double Mutant.

Ultrastructure of leaf cells of ats1-1 (A), tgd4-1 (B), and the ats1-1 tgd4-1 homozygous double mutant (C). Representative samples of 3-week-old plants grown on agar-solidified MS medium are shown. Note that the double mutant has a single large chloroplast. Bars = 5 μm.

2000; Andersson et al., 2007), and it seemed plausible that TGD4 might be involved in the formation of such contact sites. We generated wild-type and tgd4-1 mutant lines stably transformed with a construct expressing an ER-targeted GFP marker (Batoko et al., 2000) to test this hypothesis. Protoplasts isolated from leaves of the wild type (Figure 7A) or the tgd4-1 mutant (Figure 7B) showed no apparent difference in the extent or structure of the ER network. When chloroplasts from the transgenic plants were isolated, approximately the same fraction of plastids showed peripheral punctate GFP signals indicative of ER associated with plastids, as shown in Figures 7C and 7D. Of 991 plastids analyzed from the wild type, 129 (13%) showed a punctate GFP signal(s), while of 766 tgd4-1 plastids 111 (14.5%) had a GFP signal(s).
but statistically significant difference between the wild type and the mutants, one has to keep in mind that both mutants do not contain null alleles. Because of the sterility of the stronger tgd4-2 and tgd4-3 mutant alleles, only the leaky tgd4-1 mutant allele produced seeds that could be grown into etiolated seedlings needed for the preparation of the ER fraction.

DISCUSSION

ER Location Sets TGD4 Apart from Other TGD Proteins

Following the discovery of the two pathways of thylakoid lipid biosynthesis by Roughan et al. (1980), identifying the mechanisms of lipid transfer between the ER and the plastid became a cornerstone of the two-pathway hypothesis. Initially, lipid transfer proteins as defined by their ability to extract and transfer lipids from a donor membrane to an acceptor membrane in vitro were discussed as possible catalysts of ER-to-plastid lipid trafficking (Kader, 1996). However, over the years it has become evident that soluble lipid transfer proteins have functions unrelated to their observed in vitro activity (Thoma et al., 1993; Maldonado et al., 2002; Da Silva et al., 2005).

In mammalian and yeast cells, nonvesicular lipid transfer between the ER and mitochondria through direct contact sites has been recognized as an important mechanism of interorganellar lipid trafficking (Vance, 1990; Achleitner et al., 1999). Similarly, contact sites between the ER and the plastid envelope membrane were proposed by Sandelius and coworkers as conduits for lipid transfer in plants (Kjellberg et al., 2000; Andersson et al., 2007), but molecular components of such a lipid transfer system remained to be identified. The newly discovered TGD4 protein of Arabidopsis described here is a candidate for a protein critical for the transfer of lipid precursors from the ER to the plastid as part of the ER pathway of thylakoid lipid biosynthesis. Unlike the previously characterized TGD1,2,3 proteins, which are associated with the inner chloroplast envelope and presumably reach no further than the outer envelope (Xu et al., 2003, 2005; Awai et al., 2006; Lu et al., 2007), TGD4 appears to be associated with the ER (Figure 3). Although the presented data are based on transient overexpression of a TGD4cGFP fusion construct in tobacco cells, we did not observe an association with organelles other than the ER network visualized also by employing an ER-targeted GFP control protein in the transient expression system or in stably transformed Arabidopsis lines. Because the TGD4cGFP fusion construct also complemented the tgd4-1 mutation, it was assumed that the fusion protein was functional and correctly targeted in these experiments (see Supplemental Figure 1 online). Moreover, most bioinformatic evidence pointed toward ER location. Thus, while

Figure 6. In Vivo Pulse-Chase Labeling of Polar Lipids with [1-14C]-Oleate.

The wild type, the previously studied tgd1-1 mutant, and the tgd4-2 and tgd4-3 mutants were compared.

(A) Polar lipid labeling after 30 min in the presence of radiolabeled oleic acid (pulse). DGDG, SQDG, and phosphatidylinositol (PtdIns) could not be individually detected and were analyzed in a pool designated R.

(B) Polar lipid labeling following a 3-d chase.

(C) Representative autoradiograph of labeled lipids separated by thin layer chromatography following a 3-d chase.

For data shown in (A) and (B), three independent replicates were averaged, and SD is indicated.
TGD1,2,3 can only be indirectly involved in lipid transfer from the ER to the plastid, TGD4 is associated with the right membrane to play a more direct role in lipid transfer from the ER to the outer plastid envelope.

Multiple Lines of Evidence Point to an ER-to-Plastid Lipid Trafficking Defect in tgd4

The equally crucial function of all four known Arabidopsis TGD proteins in thylakoid lipid biosynthesis from ER-derived precursors is primarily supported by the fact that the corresponding tgd mutants share the same complex lipid phenotype. Although the accumulation of oligogalactoglycerolipids is diagnostic and led to the mutant name trigalactosyldiacylglycerol, this phenotype and the accumulation of triacylglycerols in the tgd mutants has been interpreted as a secondary effect (Xu et al., 2005). The accumulation of these two lipids is most likely a consequence of redirecting lipid intermediates into new end products due to the primary biochemical defect, the disruption of ER-to-plastid lipid trafficking.

Different criteria can be applied to determine whether ER-to-plastid lipid trafficking is indeed disrupted in the tgd4 mutant. Biochemical in vivo evidence derived from pulse-chase label experiments (Figure 6; see Supplemental Figure 4 online) showed a decreased galactoglycerolipid labeling over time from oleic acid but an initial increase in galactoglycerolipid labeling from acetate. The difference between the two labeling substrates is that oleic acid is first incorporated into PtdCho at the ER, while acetate enters the plastid and appears first in plastid-synthesized lipids such as MGDG. Thus, these observations are consistent with a slow conversion of ER-derived PtdCho into plastid-based MGDG and a relative higher activity of the plastid pathway of MGDG biosynthesis in the tgd4 mutant.

Fatty acid compositional analysis of the galactoglycerolipids led to the same conclusion (Table 2) because molecular species of the galactoglycerolipid MGDG in the tgd4 mutant are enriched in 16-carbon fatty acids in the sn-2 position of the glycerol backbone. The latter is the characteristic signature for plastid-derived molecular species of MGDG (Heinz and Roughan, 1983; Browse et al., 1986). Analysis of the ats1-1 tgd4-1 homozygous double mutant (Figure 4) provided genetic evidence consistent with a disruption of the plastid pathway by the ats1-1 mutation and the ER pathway by the tgd4-1 mutation. Disrupting both pathways was embryo-lethal as was previously shown for the ats1-1 tgd1-1 mutant (Xu et al., 2005).

<table>
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<th>tgd4-3</th>
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<td>MGDG</td>
<td>48.0 ± 3.5</td>
<td>46.4 ± 2.3</td>
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<tr>
<td>PtdGro</td>
<td>16.5 ± 2.7</td>
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<td>DGDD</td>
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<td>SQDG/PtdIns</td>
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</tr>
<tr>
<td>PtdCho</td>
<td>4.7 ± 0.5</td>
<td>7.2 ± 0.9</td>
<td>10.1 ± 1.7</td>
</tr>
</tbody>
</table>

Three to four independent samples were averaged, and the SD is indicated. PtdIns, phosphatidylinositol.

Figure 7. ER–Plastid Contact Sites in the Wild Type and the tgd4-1 Mutant.

(A) to (D) Visualization of the ER in the wild type (A) and the tgd4-1 mutant protoplasts (B) expressing an ER lumen–targeted GFP marker. ER adhesions (green) to plastids (red) isolated from wild-type (C) and tgd4-1 mutant seedlings (D) expressing an ER lumen–targeted GFP marker. Plants were 10 d old and grown on agar-solidified medium.

(E) and (F) Labeling of PtdGro (E) and PtdCho (F) using [14C]-palmitoyl-CoA as substrate. Thermolysin degrades accessible enzymes (e.g., on the surface of the plastids). PtdGro biosynthesis from palmitoyl-CoA is ER localized and thermolysin sensitive. PtdGro synthesis under the conditions used here is a biochemical marker for the presence of ER fragments. PtdCho synthesis from palmitoyl-CoA is not thermolysin sensitive and presumably happens at the inner plastid envelope. Plants were grown for 3 weeks on agar-solidified medium. Values are means of three measurements, and the SD is indicated.
Finally, a direct in vitro ER-to-plastid lipid trafficking assay was developed and demonstrated, with some caveats as pointed out below, a reduced ability of ER prepared from the tgd4-1 mutant to transfer label in PtdCho to MGDG in plastids (Figure 8). However, during interpretation of these assay results, a few potential issues have to be considered. First, all ER preparations, even those obtained from etiolated seedlings of Arabidopsis or other tissues, contained envelope detectable by the presence of MGDG (and DGDG) in the ER preparations (Figure 8A). One must assume that these contaminating envelopes contained galactolipid biosynthetic enzymes and could short-circuit the actual ER-to-plastid lipid transfer reactions in the ER preparation. This effect was visible when ER preparations of the tgd1-1 mutant were considered. These preparations also showed a reduced transfer of label from ER-based PtdCho to MGDG, despite the fact that TGD1 is apparently plastid localized (Xu et al., 2005) and wild-type TGD1 protein should be provided with the wild-type plastid preparation. Apparently, repurifying the chloroplasts from the reaction mixture did not solve the problem, as residual ER/envelope fractions might still have been present in the repurified plastids. However, the decrease in transfer was more severe when ER preparations from tgd4-1 were used (Figure 8C). The difference between ER preparations from tgd1-1 and tgd4-1 presumably reflected the net transfer activity from the ER preparation to the added wild-type plastids. Second, using pea chloroplasts instead of Arabidopsis wild-type chloroplasts provided more readily available material and gave more consistent results. However, the use of a heterologous system has to be kept in mind when interpreting the data. Third, because strong tgd4 mutant alleles were compromised in growth and fertility, sufficient amounts of material for ER preparations could only be obtained for the leaky tgd4-1 allele. Therefore, the observed moderate effects might reflect the leakiness of the tgd4-1 allele providing an attenuated picture of the true potential of the assay. However, differences were more pronounced when the reaction was stimulated by the addition of cytosolic fractions. Apparently, we are still missing components of the system that are present in cytosolic extracts. In principle, this system provides a biochemical assay for the purification of such cytosolic factors that could be exploited in the future.

**What Is the Molecular/Biochemical Function of TGD4?**

The bioinformatic analysis did not provide direct clues of the biochemical function of the TGD4 protein. Proteins with known biochemical function related to TGD4 were not present in the available genomic databases, and no distinct domains other than membrane-spanning domains were discernable. This makes TGD4 the founder of a family of proteins specific to plants, mosses, and the green algae. Because of its apparent role in ER-to-lipid trafficking as discussed above, it was considered that TGD4 plays a role in the formation of ER-to-plastid contact zones. However, biochemical and cell biological evidence (Figure 7) did not reveal a decrease in the number or the extent of potential ER-to-plastid contact sites in the tgd4-1 and the tgd4-3 mutants. Indeed, the incorporation of labeled palmitoyl-CoA into PtdGro was increased in chloroplast preparations from the tgd4-1 mutant and even more so from the more severe tgd4-3 mutant (Figure 7E). Possible causes are that there are more lysophospholipids present in the mutant chloroplast preparations or that the extent of ER-plastid contact is upregulated in the mutants to compensate for the deficiency in lipid transfer. However, at this stage of our analysis, we cannot rule out the

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**Figure 8.** Transfer of PtdCho from Prelabeled Microsomal Fractions of Arabidopsis Wild Type, tgd1-1, and tgd4-1 Mutants to Pea Chloroplasts.

(A) Microsomes were isolated from 7-d-old etiolated Arabidopsis seedlings. A chromatogram of polar lipids is shown. Lipids were stained by exposure to iodine. The presence of galactoglycerolipids gives the extent of plastid envelope contamination present in the ER microsomal preparations.

(B) Microsomal fractions shown in (A) were prelabeled with oleoyl-CoA in the presence of lyso-PtdCho. An autoradiograph of the thin layer chromatogram is shown.

(C) Equivalent amounts of prelabeled microsomal fractions were added to isolated pea chloroplasts, and the formation of monogalactosyldiacylglycerol in the plastids from PtdCho in the microsomal fractions was observed. The reaction was conducted in the absence or presence of UDP-Gal and unlabeled cytosol as indicated. Three independent measurements were averaged, and the SD is indicated. PtdIns, phosphatidylinositol.
possibility that the strength of ER–plastid contacts that was recently determined using optical tweezers (Andersson et al., 2007) is weakened in the leaky tgd4-1 mutant allele.

One striking phenotype of the homozygous ats1-1 tgd4-1 double mutant is the chloroplast division defect (Figure 5C). One possible cause is the strongly aberrant lipid class composition and fatty acid composition of individual lipid classes in the double mutant (Figures 4C and 4D). Local changes in the lipid environment of the plastid division machinery, as some of the components involved in organelle division have specific lipid binding sites (Osteryoung and Nunnari, 2003). Alternatively, if TGD4 is localized in ER–plastid contact sites it could interact with components of the organelle division machinery. While our current localization data suggest that TGD4 is not restricted to specific ER-to-plastid contact sites (Figure 3E), it also does not preclude its presence in those contact sites.

As pointed out, additional cytosolic factors are required for ER-to-plastid lipid trafficking evident in the stimulation of lipid transfer by cytosolic fractions in the in vitro transfer assay (Figure 8). These could include lipases that convert PtdCho produced in the ER into PtdOH, diacylglycerol, or lyso-PtdCho, which could be the ultimately transported lipids (Andersson et al., 2004). Lyso-PtdCho has been previously discussed as the lipid transported between the ER and the plastid (Mongrand et al., 1997, 2000), and PtdOH appears to be the substrate of the TGD1,2,3 lipid transfer complex of the inner plastid envelope membrane (Xu et al., 2005; Awai et al., 2006), while diacylglycerol is the direct precursor of MGDG biosynthesis. Although we have some knowledge about phospholipases involved in the remodeling of lipids following phosphate starvation (Andersson et al., 2005; Andersson et al., 2006b; Yamaryo et al., 2008), the identity of specific enzymes that would convert PtdCho into lyso-PtdCho, diacylglycerol, or PtdOH for the biosynthesis of ER-derived thylakoid lipids is not known. One hypothesis that remains to be tested is whether TGD4 could recruit phospholipases or other enzymes or factors required for ER-to-plastid lipid trafficking to ER-plastid contact sites.

METHODS

Plant Materials and Growth Conditions

All Arabidopsis thaliana plants used in this study were of the ecotypes Col-2 or Ler. The tgd4-1 mutant was isolated from a previously described, chemically induced mutant population in the Col-2 wild-type background (Dörmann et al., 1996) using a screening procedure as described by Xu et al. (2003). Plants were grown on soil or agar-solidified MS medium (Murashige and Skoog, 1962) with 1% sucrose under standard conditions as previously described (Xu et al., 2002).

The SAIL-T-DNA insertion lines were obtained from the ABRC at Ohio State University. Mutants were selected on growth medium containing 25 μg/mL of glufosinate (BASTA), and T-DNA insertions were verified using PCR primers specific for gene sequences (5’-ATTGTCGACGCGATTG-GAAAGG-3’ for SAIL_760_F05 and 5’-GACCAGACCTTCAGTTGCAC-GGAAAGGA-3’ for SAIL_133_H08) and the T-DNA left border primer (5’-GCTTCTAACATTTCTGGCAATC3AATACTACCA-3’). Insertion sites were confirmed by sequencing the PCR products using the left border primer.

The T-DNA line FLAG_410H05 was obtained from the Versailles Genetics and Plant Breeding Laboratory (http://dsgap.versailles.inra.fr/publiclines/). To verify the T-DNA insertion, the left border primer 5’-CGGTGGCAAGGTGCCCACCCGAAAGT-3’ and two gene-specific primers (5’-GATTAGCTCTGTAATGGA-3’ and 5’-GAATACATCCTGCGTATT-GGCA-3’) were used.

The double mutants were generated using Arabidopsis ats1-1 (Kunst et al., 1988; Xu et al., 2006) as the pollen donor and tgd4-1 or tgd4-3 as the pollen recipient. Following selfing of the F1 plants, the F2 seeds were selected for BASTA resistance. The BASTA-resistant plants were screened for the homozygous ats1-1 locus by gas chromatography detecting the diagnostic loss of 16:3 fatty acids. Screening the homozygous ats1-1 plants, the double mutant was identified by thin layer chromatography detecting the presence of TGD, diagnostic for plants carrying homozygous tgd4 mutant alleles.

Map-Based Cloning and Genetic Analysis

For mapping purposes, tgd4-1 plants were crossed to wild-type plants of the Ler ecotype. For fine mapping, the genetic linkage between tgd4-1 and molecular markers was determined by employing six simple sequence length polymorphism markers (Bell and Ecker, 1994) and four cleaved amplified polymorphism sequence (CAPS) markers (Konieczny and Ausubel, 1993), designing markers by taking advantage of the Monsanto Polymorphism and Ler Sequence Collection (www.arabidopsis.org/Cereon/index.jsp). Primers and restriction enzymes were as follows: F17A9, 5’-CCACCACATTGTTACTTAC-3’ and 5’-ATTGTCGACCT-GTCACA-3’, cut with MspI; F17A8d, 5’-ATTTTCCACCGCGAGAAC-3’ and 5’-GGAAAAAACTAGATCTGAAGA-3’, cut with BamBI; F17A9e, 5’-CGGATGCTCTTACAGTTCT-3’ and 5’-CAGAGAGTGTGCTTTAAAG-3’, cut with MspI; F3E22, 5’-CACAATGCTTCTGCAGA-3’ and 5’-GAAATAGTCTTGAGTCG-3’, cut with RsaI; F17A9a, 5’-GCATCACTCCCTCAAGA-3’ and 5’-GTATTCCATCAAGAGAT-3’; F17A9b, 5’-GCAGTGGCTCTCTAACT-3’ and 5’-TCTTTCCTACTCTACAC-3’, cut with NlaI; F17A9c, 5’-CATTCTTGCTGTTGA-3’ and 5’-GGCTTCTTCCGCTACT-3’, cut with NlaI; F17A9d, 5’-CTAGACTATGGTCTTTAAGC-3’ and 5’-GTCTTGAACATGTGAAC-3’, cut with NlaI and SaI.

Cosmids carrying TGD1 were isolated from an Arabidopsis cosmid library constructed in the binary T-DNA cosmid vector pBIC20 (Meyer et al., 1996). Mutant plants were transformed with various cosmid clones used in the floral dip method (Clough and Bent, 1998).

DNA Constructs, Complementation Analysis, and Genotyping

To construct the TGD4-GFP expression vector, the full-length coding sequence of TGD4 was amplified by RT-PCR from first-strand cDNA made from wild-type seedling mRNA, using the primers 5’-CATGGATC- CATGAAACGATGATGGGT-3’ and 5’-ACATGGACCTACTGTGCTTTAAGAAACGGACG-3’. Total RNA isolation and the first-strand cDNA synthesis were done as described (Xu et al., 2008). The PCR product was restricted with BamHI and SaI and inserted into the respective sites of a binary vector derived from pZPP211 carrying the GFP open reading frame, thereby creating the TGDc-GFP fusion (Hajdukiewicz et al., 1994). Stable transformation of Arabidopsis was achieved as described above. Transgenic plants were selected in the presence of gentamicin (100 μg/mL) on MS medium lacking sucrose. Genotyping at the tgd4-1 locus was performed using a CAPS marker (Konieczny and Ausubel, 1993) with the PCR primers 5’-CGGTGGCAAGGTGCCCACCCGAAAGT-3’ and 5’-AAAGCGGTG-GAAGAAACGATT-3’ (cut with HpaI).

For the construction of At2g46400 cDNA sense and antisense plasmids, the entire coding region of At2g46400 was amplified by RT-PCR using primers 5’-CTTGGTACATGGGCGAATCCTAATCGACG-3’ and 5’-CGGTGGCAAGGTGCCCACCCGAAAGT-3’.
Infiltration, tobacco leaves (Xu et al., 2005), which was predigested with the same enzyme.

Transient Expression of TGD4cGFP and Detection of TGD4-GFP in Cell Fractions

The transient expression of the TGD4cGFP construct in tobacco leaves was performed as previously described (Xu et al., 2005). Three days after infiltration, tobacco leaves (~3 g) were harvested, chilled to 4°C, and ground in 8 mL of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 330 mM sorbitol, 1 mM DTT, and 0.1 mM phenylmethanesulfonyl fluoride. The extract was centrifuged at 1500g for 5 min. The pellet was discarded, and the supernatant was then centrifuged at 8000g for 20 min. The pellet (P8) was resuspended in 2× loading buffer (Laemmli, 1970), and the supernatant (S8) was centrifuged at 100,000g for 1 h. The resulting supernatant and pellet were designated S100 and P100, respectively. The S8, P8, S100, and P100 fractions were analyzed by SDS-PAGE. Pretreatments of the P100 fraction for 1 h at 4°C with detergents, chaotropic agents, or salts were done as described in the legend of Figure 3. For GFP detection, a rabbit anti-GFP antibody (Molecular Probes) and anti-rabbit alkaline phosphatase-coupled antibody (Bio-Rad) were used at 1:1000 and 1:10,000 dilutions, respectively. The blots were developed using the Immun-Star AP detection system (Bio-Rad).

Microscopy

Confocal microscopy and transmission electron microscopy were performed as previously described (Xu et al., 2005).

Lipid Analysis

Lipids were extracted from leaves and analyzed by thin layer chromatography as previously described (Dörmann et al., 1995; Xu et al., 2005). For quantitative analysis, individual lipids were isolated from thin-layer plates and used to prepare fatty acyl methylesters. The methylesters were quantified by gas–liquid chromatography using myristic acid as internal standard. The fatty acid composition at the sn-2 position of the glycerol backbone was determined by Rhizopus arrhizus lipase digestion as described (Härtel et al., 2000).

Isolation of Chloroplasts, ER-Enriched Microsomes, Cytosol, and Protoplasts

Intact chloroplasts from Arabidopsis were isolated from 3-week-old plants according to Bruce et al. (1994) with some modifications. Briefly, leaves (5 g) were homogenized in 30 mL of grinding buffer containing 330 mM sorbitol, 50 mM HEPES-KOH, pH 7.9, 10 mM NaHCO3, 10 mM EDTA, 5 mM MgCl2, and 0.5% BSA. The homogenate was filtered and centrifuged at 1500g for 3 min. The pellet was suspended in grinding buffer without BSA and loaded onto a discontinuous Percoll gradient consisting of 5 mL Percoll 10, 3 mL Percoll 80, and 2 mL Percoll 80 (w/v) Cellulase onazuka R10 and 0.8% (w/v) Macerozyme R10 (Kinki Yakult) in a digestion buffer containing 400 mM sorbitol, 20 mM MES-KOH, pH 5.2, and 0.5 mM CaCl2 for 4 h. After digestion, the protoplasts were filtered through 200 µm of nylon mesh and spun down at 100g for 5 min. After washing with the digestion buffer twice, the protoplasts were ruptured by passing them through a 10-µm nylon mesh. Intact chloroplasts were then purified by discontinuous Percoll gradient as described above.

In Vivo Acetate and Oleate Labeling of Lipids

For oleate labeling experiments, [1-14C]oleic acid (~1.85 MBq:mmol; Moravek Biochemicals) was diluted to 0.5 MBq/mL with liquid paraffin and was applied to the upper surface of expanding leaves in 3-µL droplets as described (Roughan et al., 1987). At various times after application of label, samples of ~0.15 g (fresh weight) were harvested for lipid analysis. In some experiments, detached leaves were used and [1-14C]oleic acid was fed and traced as previously described (Xu et al., 2003). Very similar results were obtained by these two application methods.

For in vivo labeling experiments with acetate, detached leaves from 3-week-old plants were incubated with 1.85 MBq [1-14C]acetate sodium salt (2.22 GBq/mmol; Moravek Biochemicals) in 10 mL of MS medium in the presence of 0.01% Tween 20 for 10 min in the light (100 to 120 µmol m−2 s−1) at 22°C to 24°C. The reaction was stopped by immersion of samples in liquid nitrogen. Lipids were extracted and analyzed by thin layer chromatography as described above. Radiolabeled lipids were visualized by autoradiography, and radioactivity associated with the lipids was determined by liquid scintillation counting following isolation of the silica material containing the lipid.

Acyl-CoA Labeling of Chloroplasts

Intact chloroplasts purified from Arabidopsis leaves as described above equal to 30 µg of chlorophyll were incubated with 19 µM [1-14C]palmitoyl-CoA (53 KBq:mmol; Moravek Biochemicals) in buffer containing 330 mM sorbitol, 5 mM MgCl2, and 50 mM HEPES-KOH, pH 7.3, at 28°C for 30 min. The reaction was stopped by centrifugation through a 35% Percoll cushion. Labeled lipids were isolated from the resulting intact chloroplasts and analyzed as described above for in vivo–labeled lipids.

Protease pretreatment was done by incubating the intact chloroplasts with 0.8 mg/mL of thermolysin in the presence of 1 mM CaCl2 on ice for 30 min. The reaction was terminated by centrifugation through a 35% Percoll cushion.

In Vitro ER-to-Chloroplast Lipid Transfer Assay

Pea (Pisum sativum) chloroplasts used for lipid transfer assays were isolated from 8- to 10-d-old plants grown on soil as described above for Arabidopsis chloroplasts. Radiolabeled ER membranes were prepared by incubating ER (equal to 26 µmol of PtdCho as determined by quantification of fatty acid methylster in PtdCho isolated by thin layer chromatography of lipid extracts from the ER preparation) with 4.6 KBq of [1-14C]oleoyl-CoA (370 KBq:mmol; Moravek Biochemicals) and 5 µmol lyso-PtdCho (1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine; Avanti Polar Lipids) for 20 min at 28°C in 100 µL of incubation buffer containing 330 mM sorbitol, 5 mM MgCl2, and 50 mM HEPES-KOH, pH 7.3. The solution was then centrifuged at 100,000g for 10 min. The pellet was washed in the same buffer and submitted to a second centrifugation.
at 100,000g for 10 min. The pellet was resuspended in the same buffer and used immediately for lipid transfer assays.

To analyze lipid transfer between ER and pea chloroplasts, radiolabeled ER membranes (equal to 5.2 μmol PtdCho) were incubated with pea chloroplasts (30 μg chlorophyll) for 30 min at 28°C. After incubation, the intact chloroplasts were repurified by centrifugation through a 35% Percoll cushion for 5 min at 4000g. The chloroplasts were resuspended in incubation buffer and centrifuged again at 1500g. The resulting pellet was used for lipid analysis by thin layer chromatography of lipid extracts as described above. The radioactivity associated with the lipids was determined by liquid scintillation counting of lipids isolated from the thin layer chromatogram.

Accession Numbers

Arabidopsis Genome Initiative locus identifiers (www.Arabidopsis.org) used in this study are At3g06960 (TGD4) and At2g41640 (similar to TGD4). GenBank accession numbers for potential orthologs are as follows: Beta vulgaris, ABD83319; Oryza sativa, NP_001042598; Vitis vinifera, CAO70935; Physcomitrella patens, XP_001756157; Ostreococcus lucimarinus, XP_001418769; Ostreococcus tauri, CAL54660.

Supplemental Data

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

Supplemental Figure 2. Complementation Analysis Using a TGD4cGFP Fusion Construct.

Supplemental Figure 3. Relationships between Presumed TGD4 Orthologs. Unrooted Tree Showing the Sequence Relationships between Presumed TGD4 Orthologs.

Supplemental Figure 4. In Vivo Labeling of Polar Lipids with [14C]-Acetate.

Supplemental Table 1. Complete Fatty Acid Composition Data Sets for the Wild Type and tgd4-2 and tgd4-3 Mutants.

ACKNOWLEDGMENTS

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Lipid Trafficking between the Endoplasmic Reticulum and the Plastid in Arabidopsis Requires the Extraplastidic TGD4 Protein

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