A Small ATPase Protein of Arabidopsis, TGD3, Involved In Chloroplast Lipid Import*

Binbin Lu1,3, Changcheng Xu1, Koichiro Awai1, A. Daniel Jones1,2 and Christoph Benning1

From the Department of Biochemistry & Molecular Biology 1, the Department of Chemistry2, and the US Department of Energy Plant Research Laboratory3, Michigan State University, East Lansing, MI 48824-1312.

Running Title: A role for TGD3 in Arabidopsis Lipid Trafficking

Address all correspondence to: Christoph Benning, Dept. of Biochemistry & Molecular Biology, Michigan State University, Biochemistry 215, East Lansing, MI 48824-1319; Tel. 517-355-1609; Fax. 517-355-9334; E-Mail: benning@msu.edu

Current Address for K.A.: Graduate School of Science and Engineering, Saitama University, 255 Shimo-Okubo, Sakura-Ku, Saitama City, Saitama 338-8570, Japan

Polar lipid trafficking is essential in eukaryotic cells as membranes of lipid assembly are often distinct from final destination membranes. A striking example is the biogenesis of the photosynthetic membranes (thylakoids) in plastids of plants. Lipid biosynthetic enzymes at the endoplasmic reticulum (ER), and the inner and outer plastid envelope membranes are involved. This compartmentalization requires extensive lipid trafficking. Mutants of Arabidopsis are available that are disrupted in the incorporation of ER-derived lipid precursors into thylakoid lipids. Two proteins affected in two of these mutants, TGD1 and TGD2, encode the permease and substrate binding component, respectively, of a proposed lipid translocator at the inner chloroplast envelope membrane. Here we describe a third protein of Arabidopsis, TGD3, a small ATPase proposed to be part of this translocator. As in the tgd1 and tgd2 mutants, triacylglycerols and trigalactolipids accumulate in a tgd3 mutant carrying a T-DNA insertion just 5’ of the TGD3 coding region. The TGD3 protein shows basal ATPase activity and is localized inside the chloroplast beyond the inner chloroplast envelope membrane. Proteins orthologous to TGD1, 2, and 3 are predicted to be present in Gram-bacteria and the respective genes are organized in operons suggesting a common biochemical role for the gene products. Based on the current analysis, it is hypothesized that TGD3 is the missing ATPase component of a lipid transporter involving TGD1 and TGD2 required for the biosynthesis of ER-derived thylakoid lipids in Arabidopsis.

Interorganellar non-vesicular lipid trafficking in plant cells is required for the biogenesis of the characteristic plant cell organelle, the chloroplast (1). The predominant polar lipids found in the photosynthetic membranes (thylakoids) of chloroplasts are the galactoglycerolipids mono- (MGDG) and digalactosyldiacylglycerol (DGDG) (2). Their final assembly from diacylglycerol and UDP-Gal requires two lipid galactosyltransferases, which in the model
plant Arabidopsis are designated MGD1 and DGD1 (3). Of these two enzymes MGD1 is associated with the outside of the inner chloroplast envelope membrane (4) and is responsible for the bulk of MGDG formation (5), while DGD1 is located on the outside of the outer chloroplast envelope membrane (6) and is required for the bulk of DGDG biosynthesis (7). The diacylglycerol moiety of the galactoglycerolipids is either synthesized de novo in the plastid or it is assembled at the endoplasmic reticulum (ER) and needs to be imported into the plastid (8). Understanding this complexity of subcellular organization of glycerolipid biosynthesis in plants and the underlying lipid trafficking phenomena still poses some of the biggest challenges of modern plant biochemistry.

The recent isolation and characterization of genetic suppressors of the DGDG-deficient dgd1 mutant of Arabidopsis has provided the first components, TGD1 and TGD2, of a potential lipid transport complex associated with the inner chloroplast envelope membrane in Arabidopsis (4;9;10). The TGD1 protein is similar to the permease component of bacterial ABC transporters and the Arabidopsis tgd1 mutant shows a complex lipid phenotype: the accumulation of oligogalactolipids and triacylglycerols, a disruption of the assembly of galactolipids from ER-derived precursors, and the elevation of phosphatidic acid (PtdOH) levels (4;10). The name of the mutants, tgd (trigalactosyldiacylglycerol), refers to the characteristic accumulation of oligogalactolipids. Presumably, this is a secondary phenotype due to the activation of a processive galactosyl transferase of unknown function, for which the gene has not yet been identified. The accumulation of PtdOH and reduced incorporation of labeled PtdOH into galactoglycerolipids by isolated tgd1 chloroplasts, led to the suggestion that TGD1 is involved in the transport of PtdOH through the inner chloroplast envelope membrane (4). The TGD2 protein is similar to membrane-tethered substrate binding proteins associated with bacterial ABC transporters (9). Although not as extensively characterized as the tgd1 mutant, the Arabidopsis tgd1 and tgd2 mutants have a nearly identical lipid phenotype in all aspects tested. Moreover, the recombinant TGD2 protein lacking the single membrane-spanning domain specifically binds PtdOH (9). The nearly identical phenotypes of the two mutants and the organization of bacterial orthologs of TGD1 and TGD2 in operons (Fig. 1A) strongly suggest that both are involved in a common biochemical or cell biological process. Moreover, the specific binding of PtdOH to TGD2 corroborates the hypothesis that the two proteins are components of a PtdOH transporter at the inner chloroplast envelope.

Unlike other eukaryotic ATP binding cassette (ABC) transporters or the MsbA lipid transporter from E. coli (11), the TGD1 protein lacks an ATP binding domain. However, the Arabidopsis genome contains 26 genes encoding putative small ATPases with either one or two nucleotide binding domains (12). We hypothesized that one of these could encode a small ATPase potentially associated with the TGD1 protein. Here we describe the identification and biochemical characterization of TGD3, the small ATPase protein proposed to be associated with TGD1 and TGD2.

**EXPERIMENTAL PROCEDURES**

*Plant Materials and Growth Conditions—Arabidopsis thaliana* wild type and tgd1
and tgd2 mutant plants (9;10) were of the ecotype Columbia-2 (Col-2). The following T-DNA insertion lines were made available by the Salk Institute Genome Analysis Laboratory (SIGnAL, http://signal.salk.edu/) and were obtained from the Arabidopsis Biological Resource Center at Ohio State University (www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm): SALK_027270 At4g33460, SALK_129048 At5g60790, SALK_029696 At5g09930, SALK_113472 At5g64840, SALK_116866 At5g14100, SALK_055996 At1g32500, SALK_040335 At1g65410. Homozygous mutant lines were screened and used for all experiments. Seeds were surface-sterilized and grown on 0.8% (w/v) agar-solidified MS medium (13) supplemented with 1% (w/v) sucrose for 5 days. Wild-type and mutant seedlings of similar developmental stage were then transferred to a fresh MS agar plate with 1% sucrose and grown for three additional weeks prior to lipid analysis.

Genotyping and complementation—The TGD3 ORF was isolated by RT-PCR (standard conditions) from mRNA preparations using RNeasy and Omniscript kits (Qiagen, Valencia, CA), with the following primers: 5'-ACGGTACCATGGCTTCTGTATATATG CTC-3' and 5'-CTGGTACCCTAGCTCAGTGGTGC CAT-3'. The PCR products were ligated into pGEM-Teasy (Promega, Madison, WI) and sequenced. The resulting plasmids were digested with KpnI and inserted into pCAMBIA1300 (4) followed by transformation into Agrobacterium. Plants were transformed by the floral-dip method (14) and screened for resistance to hygromycin B (25 μg/ml) on agar-solidified MS medium. For an estimation of TGD3 transcript levels by semi-quantitative RT-PCR, RNA was isolated from Col-2 wild-type and tgd3 mutant plants and reverse transcription was done using oligo(dT)12-18 primers (Invitrogen, Carlsbad, CA). The TGD3 specific primers mentioned above were used for the PCR reaction. The abundance of ubiquitin (UBQ10) was tested for control purposes. The following UBQ10 specific primers were used: 5'-TCAATTCTCTCTACCGTGATCAAGA TGCA-3' and 5'-GTGTCAAGACTCTCCACCTCAAGAG TA-3'.

For genotyping during the complementation analysis DNA was prepared from 4-week-old plants and the following primers were used: T-DNA left border primer (LB), 5'-CGTGACCCGCTTGCTGCAAC-3'; P1, 5'-AATCTGATGGGGACGAGTATC-3'; P2, 5'-GCTATGCAACAGCAAGAGAC-3'. The PCR conditions were: 94°C 3min followed by 35 cycles at 94°C for 0.5 min, 54°C for 0.5 min, 72°C for 0.5 min followed by a final step of 5 min at 72°C.

Lipid Assays—Lipids were extracted from four-week-old Col-2 wild type and tgd3 mutant seedlings grown on MS medium with 1% sucrose. Fatty acid methylsters were prepared as previously described (15) and quantified by gas chromatography according to (16). Polar lipids were analyzed on activated ammonium sulfate-impregnated (15) silica gel TLC plates (Si250PA; Mallinckrodt, Baker, NJ) developed with chloroform/methanol/acetic acid/water (85/25/10/4, v/v). Neutral lipids were separated on untreated TLC plates developed with petroleum ether/ether/acetic acid (80/20/1, v/v).
Lipids were visualized by brief exposure to iodine vapor or staining with α-naphthol to detect glycolipids (17). The fatty acid compositions at the sn-2 position of individual lipids were determined using *Rhizopus* lipase (Sigma, St. Louis, MO) digestion according to (18), with modifications as described in (19). Fatty acid methyl esters were formed from lysolipids, and the fatty acid methylesters were quantified by gas chromatography.

**Mass Spectrometry**—Lipid extracts were characterized using liquid chromatography/mass spectrometry (LC/MS) on a Waters LCT Premier time-of-flight mass spectrometer equipped with Shimadzu LC-20AD pumps and SIL-5000 autosampler. Extracts were analyzed using V-mode operation and electrospray ionization in both positive and negative ion modes similar to described protocols (20) except that, in place of MS/MS spectra, mass spectra were acquired in alternating acquisition functions at low (15 V) and high (75 V) potentials on aperture one to generate spectra with, and without in-source collision-induced dissociation (CID). The latter conditions allow for production of characteristic fragment ions that support structure assignments. HPLC separations were performed using a Restek Allure C18 column (1 x 150 mm) using a ternary gradient based on: (a) 10 mM aqueous ammonium acetate, (b) methanol, and (c) 2-propanol.

**Subcellular Localization Assays**—For the generation of a GFP fusion construct, the entire coding region of At1g65410 was inserted into the *Kpn*I site of pCAMBIA1300GFP (4) using primers 5’-ACGGTACCATGCTTTCGTTATCATGCTC-3’ and 5’-CTGGTACCGTATCTGTGATGTTCCAT-3’. Transient expression of the construct in tobacco and microscopy of the resulting transgenic leaves was performed as previously described (4). For the *in vitro* import experiments using pea chloroplasts the same conditions and treatments were used as published for the localization of the MGD1 protein (4).

**Recombinant MBP-ΔTGD3 Protein Production and Purification**—The coding sequence for TGD3 leading to a truncated protein missing 46 N-terminal amino acids (designated as ΔTGD3) was PCR-amplified. As template the pGEM-Teasy plasmid derivative mentioned above was used in combination with primers 5’-GGATCCTGCATAGCTCCA-CCTCAGAAC-3’ and 5’-GTCGACTAGTATCTGATTGTTCCATCG-3’. The fragment was inserted into pGEM-Teasy (Promega) and sequenced. This plasmid served as template for F94A mutagenesis by using the forward/reverse primers and the QuickChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The forward and reverse primers for F94A are: 5’-GTAGAGATGTCTATAAATCGGCGGGAGAAACATATCTTG-3’ and 5’-CAAGATATGTTTCTCCCCCGCAGATTTATAGACATCTCTAC-3’. The underlined sequences are codons for the mutated residue (alanine). After digestion with *Bam*HI and *Sal*I, either the wild type ΔTGD3 or the mutant ΔTGD3F94A fragment was inserted 3’of the *E. coli* malE coding sequence into the pMalc2x vector (New England Biolabs, Ipswich, MA) to generate the maltose binding protein fusion proteins MBP-ΔTGD3 or MBP-ΔTGD3F94A. For recombinant protein production, a 500 ml culture was grown at 37°C, inoculated with 2 ml of an overnight culture. The protein was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside at an OD₆₀₀ of 0.4-
0.6 and incubated at 28°C for 4 hrs. The cells were then collected by centrifugation and lysed by sonication in the presence of 1 mg/ml lysozyme. Crude lysate was centrifuged at 18,000 g for 20 min and the soluble fraction was loaded onto an amylose column (New England Biolabs). Fusion proteins were purified according to manufacturer’s instructions. Protein concentration was determined according to Bradford (21), using bovine serum albumin as a standard. Protein purity was verified by SDS-PAGE. After purification, samples were stored at 4°C for a few weeks without significant loss of activity.

Assay for ATPase activity—Purified MBP-ΔTGD3 protein was assayed for ATPase activity at a protein concentration of ~0.5-8 μM in 50 μl reaction mixtures containing assay buffer (40 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM MgCl2). The MBP-ΔTGD3 protein was diluted in assay buffer to a final volume of 25 μl. To initiate the reaction, 25 μl of ATP in the assay buffer ranging from 0 to 2 mM (diluted from a 10 mM stock made in the same assay buffer) were added, giving a final ATP concentration of 0 to 1 mM. ATPase reactions were incubated at 37°C for the indicated time and stopped by the addition of 50 μl 12% sodium dodecylsulfate (SDS). Released P_i was quantified by a colorimetric method, as described by Chifflet et al. (22) using potassium phosphate as the standard. After the addition of SDS, 100 μl of a solution containing equal volumes of 12% ascorbic acid in 1M HCl and 2% ammonium molybdate were added, and the samples were incubated at room temperature for 5 min. This was followed by the addition of 150 μl of a solution composed of 2% sodium citrate, 2% sodium meta-arsenate, and 2% acetic acid. After 10 min incubation at 37°C, absorbance was measured at 850 nm. The assay was linear in the range of 0-25 nmol P_i. The activity was corrected for non-enzymatic release of P_i from ATP and for P_i contamination by subtracting absorbance readings obtained in the absence of protein and ATP, respectively. A vector expressing only MBP was used to control for endogenous ATPase activity from E. coli. The kinetic constants were determined by nonlinear least squares fitting to the equation 

\[ V_0 = \frac{V_{max}[S]}{K_m+[S]} \]

using Microcal Origin 7.0 (Microcal Software, Northampton, MA).

RESULTS

Identification of TGD3 by Genomics and Reverse Genetics—Bacterial genes involved in the same biochemical process are commonly linked in operons. This fact has been successfully exploited in genome annotation or the identification of individual genes (23). Accordingly, the location of TGD1 and TGD2 encoding homologs in bacterial operons (Fig. 1A) adjacent to putative ATP binding cassette proteins (ABC protein) suggests that these are the ABC components of the respective transport complex. Based on comparative genomics it seemed likely that Arabidopsis has an ABC protein associated with the TGD1/2 transporter.

An extensive and possibly complete inventory of transport ATPases and non-intrinsic ABC proteins potentially associated with membrane transporters in Arabidopsis has been published (12), listing 26 soluble nucleotide binding domain proteins. Because TGD1 and TGD2 are associated with the inner chloroplast membrane, which is homologous to the bacterial cell membrane, we expected that the respective ABC protein was localized on the inside of the inner chloroplast membrane requiring
an N-terminal chloroplast targeting sequence. Nine of the above mentioned ABC proteins were predicted to be targeted to the plastid using ChloroP (24): At1g32500, At1g65410, At3g10670, At4g04770, At4g33460, At5g09930, At5g14100, At5g60790, At5g64840. For seven (listed under Experimental Procedures) of these candidate genes T-DNA inactivation lines (25) were available at the time, and were selfed and selected for homozygosity. The homozygous mutant lines were tested for the presence of trigalactosyldiacylglycerol, which is diagnostic for mutants with impaired TGD1 or TGD2 function. Only one inactivation line (SALK_040335) carrying a T-DNA 5’ of the presumed ATG start codon of Arabidopsis gene At1g65410 (Fig. 2A) showed accumulation of the oligogalactolipid (Fig. 2 D, Fig. 3B). This mutant was clearly leaky as the abundance of the respective mRNA estimated by semi-quantitative RT-PCR was only partially reduced in the T-DNA line Fig. 2B. A completely disrupted allele was not available (see also below).

To confirm that the mutant phenotype was not due to secondary mutations, a wild-type cDNA was expressed in the homozygous T-DNA line disrupted in At1g65410. Two transgenic homozygous T-DNA lines lacking the accumulation of the oligogalactolipid are shown in Figure 2C, D. The restoration of the wild-type phenotype in the homozygous T-DNA lines by the corresponding wild-type cDNA confirmed that the accumulation of oligogalactolipids in the T-DNA lines was due to the disruption of the At1g65410 locus. From here on, the SALK_040335 line will be referred to as tgd3 mutant line (carrying the here characterized tgd3-1 mutant allele). The At1g65410 encoded protein was previously designated “Non-intrinsic ABC Protein 11” (NAP11) (12), and will be referred to here as TriGalactosylDiacylglycerol 3 (TGD3) fitting more closely with its function. Comparing the amino acid sequence of TGD3 with proteins encoded by bacterial genomes using BLAST (26), ABC protein-encoding ORFs adjacent to putative TGD1/2 homologs provided some of the best hits, with putative TGD3 homologs from cyanobacteria, e.g. Synechococcus (Fig. 1A), showing 52% identity and the E. coli homolog 32% identity.

The tgd3 Mutant Shares a Complex Lipid Phenotype with Other tgd Mutants—It is important to note that all three tgd mutant alleles (tgd1-1, tgd2-1, tgd3-1) analyzed thus far and discussed here are leaky leading to attenuated phenotypes. More severe impairment of the system as previously shown for TGD1 causes embryo-lethality (4) making fully gene-disrupted mutants currently inaccessible to analysis. If the TGD3 protein indeed is part of a lipid transport complex along with TGD1 and TGD2, mutant lipid phenotypes for all three loci should be similar. Therefore, leaf lipid extracts from the tgd1, tgd2 and the tgd3 lines were compared by thin-layer chromatography of neutral (Fig.3 A) and polar lipids (Fig. 3B). The tgd3 mutant extracts contained a lipid co-chromatographing with triacylglycerol (Fig. 3A), previously identified in the tgd1 mutant (4) and shown also to accumulate in tgd2 mutant extracts (9). In addition, polar lipid extracts of the tgd3 mutant contained a new lipid staining with α-naphthol, which is diagnostic for the presence of hexoses (Fig. 3B). This lipid co-chromatographed with authentic trigalactosyldiacylglycerol accumulating in tgd1 and tgd2.
Further confirmation of the identities of TGDG and TAG lipids was provided by LC/MS analyses of leaf extracts of Col-2 wild type and tgd3 mutants (Fig. 5A-C). Extracted ion chromatograms (XICs) generated in negative ion mode yielded a strong peak at m/z 1129.6 for the tgd3 mutant corresponding to [M+acetate]- for TGDG 34:6 which was the dominant TGDG lipid detected, but minimal amounts in the wild-type extracts. The in-source CID spectra supported this assignment in the form of a fragment ion at m/z 277 corresponding to the C18:3 fatty acid anion.

Triacylglycerols were characterized using positive ion electrospray ionization, displaying peaks at m/z 892.7 to 902.8 corresponding to [M+NH₄]⁺ of triacylglycerols with 54 carbons and a total number of double bonds ranging from 3 to 8 (Fig. 5F). Extracted ion chromatograms for signals in the range of m/z 890-905 showed about 5-fold greater signal in the tgd3 mutant than the wild type (Fig. 5D, E). Ion abundances for both TGDG and TAG lipids peaks were consistent with quantitative analysis of fatty acid methylesters by gas chromatography coupled to flame ionization detection.

The overall lipid composition of the tgd3 mutant differed from that of the wild type as shown in Figure 4A. Most notably, the relative amounts of the major chloroplast lipids mono and digalactosyldiacylglycerol were decreased, while the presumed precursor of galactolipids derived from the ER-pathway, phosphatidylcholine (8), was more abundant. Analysis of the fatty acid composition of mono- and digalactosyldiacylglycerol revealed distinct changes in the fatty acid profiles in tgd3 as compared to the wild type (Fig. 4B, C). Most notably, fatty acids were generally more saturated and 18:3 fatty acid content was reduced. Moreover, the tgd3 fatty acid profile changes were very similar to those observed for the tgd1 and tgd2 mutants (9).

In general, the tgd mutants impaired in the ER-pathway have an increased 16-carbon-to-18-carbon fatty acid ratio (9) in their galactolipids. This is particularly visible for digalactosyldiacylglycerol, which is to a large extent derived from the ER-pathway (27). Accordingly, the 16-carbon-to-18-carbon ratio for the digalactolipid increased from 0.29 to 0.76 in the tgd3 mutant (Fig. 4C). This phenomenon is due to the substrate specificities of the different acyltransferases in the plastid and the ER leading to 18-carbon fatty acids at the sn-1 position and 16-carbon fatty acids at the sn-2 position of the diacylglycerol backbone for plastid derived lipids. Those lipids derived from the ER-pathway carry 18-carbon fatty acids in both positions (27). Positional analysis using Rhizopus lipase (supplemental Fig. 1) confirmed an increase in 16-carbon fatty acids in the sn-2 position of monogalactosyldiacylglycerol and digalactosyldiacylglycerol of the tgd3 mutant.

Overall, tgd3 has a complex lipid phenotype very similar to tgd1 and tgd2 which is consistent with an impairment of the ER-pathway of galactolipid biosynthesis. This similarity in phenotypes also suggests that the TGD1, TGD2, and TGD3 proteins are involved in the same biochemical process.

The TGD3 Protein Is Imported into the Chloroplast—The TGD3 sequence has a predicted chloroplast transit peptide. To verify the localization of the protein, the cDNA was c-terminally fused with a green fluorescent protein (GFP) and transiently
expressed in tobacco. The result shown in Figure 6A-C shows the GFP fluorescence associated with chloroplasts, which are identified by their chlorophyll fluorescence.

To further narrow the location of TGD3, \textit{in vitro} translated labeled TGD3 protein was incubated with isolated pea chloroplasts. Two proteases were used to determine the sub chloroplast localization: Thermolysin, a large protease that can only digest cytosol exposed domains of outer envelope membrane proteins unless detergent such as Triton-X-100 is added (28), and Trypsin, which can penetrate the outer envelope membrane but not the inner (29). The result of this analysis is shown in Figure 6D. The TGD3 preprotein was shortened upon import consistent with the removal of a transit peptide. Moreover, the mature TGD3 protein was resistant to Thermolysin and Trypsin treatment, but was digested by Thermolysin in the presence of detergent. These results were consistent with import of TGD3 into the chloroplast beyond the inner chloroplast envelope membrane. Based on these results, TGD3 would be available for association with the TGD1/TGD2 complex at the stroma side of the inner envelope membrane.

\textit{Recombinant TGD3 Protein Has ATPase Activity}—The TGD3 protein contains motifs characteristic for other small ATPases associated with bacterial membrane transporters (30;31) designated Walker-A, Walker-B, A-loop and Q-loop (Fig. 1B). To test the biochemical function of TGD3, an N-terminally truncated cDNA missing the coding region for the first 46 amino acids representing the predicted transit peptide was fused to the C-terminus-encoding end of the \textit{E. coli} maltose-binding protein open reading frame and expressed in \textit{E. coli}. Of several expression systems tested, this was the only providing soluble TGD3 protein. Purification of the recombinant protein designated MBP-ATGD3 on an amylose column is shown in Figure 7A. The fusion protein was highly purified (estimated >90%) and migrated as 78 kD protein. The fusion protein in the E3 fraction was assayed for its ability to hydrolyze ATP. For control purposes, maltose binding protein by itself or a mutant cDNA giving rise to an F94A point mutant of TGD3 fused in the same manner as the truncated wild-type cDNA were included (MBP-ATGD3F94A). It should be noted that the F94A mutation did not affect protein expression or protein purification. Replacing the A-loop residue homologous to Phe94 in TGD3 in bacterial orthologs with a nucleophilic cysteine residue was previously shown to greatly decrease the ATP-dependent transport activities. (32). Under standard assay conditions as defined under EXPERIMENTAL PROCEDURES, the reaction was linear over 40 min (Fig. 7B). Furthermore, the reaction velocity increased linearly with MBP-ATGD3 protein concentration in the range tested (Fig. 7C). Varying the concentration of ATP, a classic Michaelis-Menten hyperbolic substrate velocity function was observed for the MBP-ATGD3 protein (Fig. 7D). Estimated kinetic constants based on this experiment were 151.7 \mu M for the \textit{K}_m and 0.026 \mu mol min^{-1}mg^{-1} protein for \textit{V}_\text{max}. These values are in the same range as previously reported for ATP hydrolysis by other ABC transporters (33). The MBP-ATGD3F94A mutant protein had less than 20% residual activity insufficient for accurate determination of the kinetic constants (Fig. 7D). The maltose binding protein had no activity.
DISCUSSION

The previous identification of TGD1 and TGD2 proteins and the characterization of the respective mutants (4;9;10) provided reasonable evidence suggesting that these two proteins are involved in PtdOH transfer possibly from the outer envelope through the inner envelope membrane of chloroplasts in Arabidopsis. Typically, ABC-type lipid transporters such as the biochemically well characterized MsbA protein from *E. coli* (34;35), consist of a dimer of a bifunctional protein, with a membrane-spanning permease domain and a second ATP-binding domain. In case of the TGD1/TGD2 transporter, TGD1 represents the permease domain only and TGD2 is similar to a membrane-tethered substrate binding protein like those associated with other bacterial ABC transporters. The TGD3 (NAP11) protein of Arabidopsis described here appears to be the missing ATP-binding component that would be required for a functional TGD1-TGD2-TGD3 complex as proposed in Figure 8.

Several lines of indirect evidence are consistent with the current hypothesis for the composition of the PtdOH transporter of the inner chloroplast envelope membrane and the involvement of the TGD3 (NAP11) protein: 1. Most Gram- bacteria and mycobacteria contain potential orthologs of the three Arabidopsis TGD proteins including the newly described TGD3 protein. Their respective genes are typically linked in operons consistent with their function in the same biochemical process (Fig. 1A). 2. Of the 26 genes of Arabidopsis encoding small ATP-binding proteins without membrane-spanning domains (12), nine were predicted to be targeted to the chloroplasts. Of the seven genes tested only the *tgd3* mutant corresponding to the gene encoding TGD3 (NAP11) showed an phenotype identical to the previously characterized *tgd1* and *tgd2* mutants. 3. The TGD3 (NAP11) protein was localized to the stroma of chloroplasts and showed basic ATPase activity *in vitro* when expressed as maltose-binding protein fusion.

With the presumed ATPase component of the TGD transport complex identified, reconstitution and direct proof of biochemical transport activity should - in principle - be possible. However, our current efforts have been hampered by the fact that the production of detergent-soluble, functional full-length TGD1 and TGD2 proteins in *E. coli* has not yet been feasible. Even the TGD3 protein described here could only be functionally produced fused to maltose binding protein. The difficulties in demonstrating lipid transport by integral membrane complexes are also evident from the fact that even for the well studied MsbA protein no direct evidence of lipid transfer across the membrane is available. Indirect evidence for lipid transport by MsbA is currently based on the respective mutant phenotype (34) and the observation that specific lipids stimulate the ATPase activity of MsbA *in vitro* (35). Testing the possible stimulation of ATPase activity of TGD3 was not considered a viable approach, because unlike the MsbA protein, the TGD3 protein lacks the permease domain that would directly interact with the lipid substrates. Like for the TGD complex of Arabidopsis, the precise function of MsbA still remains under debate, especially since a mutant of *Nisseria menengitis* lacking the MsbA ortholog remains capable of exporting phospholipid building blocks of the outer membrane (36). Unfortunately, technical barriers currently prevent us from directly and unambiguously demonstrating the lipid transfer activities
of MsbA-like or TGD complex-like lipid transporters.

Despite these difficulties, we are now reasonably certain about the localization of the TGD complex in the inner chloroplast membrane. Because the localization data for TGD1 or TGD2 left open questions, the clear result for TGD3 presented here provides crucial evidence. For example, TGD1 was partially Thermolysin-sensitive in chloroplast import experiments (4;10) and, therefore, it seemed possible that at least subpopulations of TGD1 might be localized in the inner and outer chloroplast envelope membranes. Alternatively, the TGD1 protein could be present in a fusion zone between the two membranes. Likewise, TGD2 wild-type protein was resistant to Trypsin, but the TGD2-GFP fusion protein was not (9). This result could be interpreted as the presence of TGD2 in localized domains between the inner and the outer envelope membrane, which might be inaccessible to Trypsin. On the other hand, the evidence for TGD3 presented here is unambiguous: TGD3 is imported into the chloroplast beyond the inner envelope membrane and processed during in vitro import experiments (Fig. 6B). The only way TGD3 could interact with TGD1 and TGD2 as suggested by the identical phenotypes for the respective mutants and the organization of predicted bacterial orthologs is, if TGD1 and TGD2 are intrinsic proteins of the inner chloroplast envelope membrane as shown in Figure 8, thereby reassuring previous conclusions drawn about TGD1 and TGD2 localization.

Demonstrating a direct interaction of the three TGD proteins in the inner chloroplast envelope has proven difficult in our hands using available in vivo techniques. It will require either the isolation of the native complex or its reconstitution from recombinant proteins, both of which have not yet been accomplished. However, our current data clearly suggest that TGD3 is the missing ATPase subunit of this transporter bringing us a step closer to the actual reconstitution of a core transporter possibly consisting of the TGD1 permease and the TGD3 ATP-binding protein.

ACKNOWLEDGMENTS

We would like to thank John Froehlich from the Michigan State University Department of Energy Plant Research Laboratory for his helpful advice on the chloroplast import experiments.
FOOTNOTES

*This work was supported in part by a grant from the US National Science Foundation, MCB 0453858, and a grant from the US Department of Energy (DE-FG02-98ER20305).

REFERENCES


FIGURE LEGENDS

FIG. 1. **Isolation of the TGD3 gene by reverse genetics.** (A) Linear representation of the Arabidopsis TGD homologs in different bacterial species. The number designations (1, 2, 3) correspond to ORFs presumed to encode homologs of Arabidopsis TGD1, TGD2, and TGD3 proteins respectively. Letters refer to the bacterial ORF designations in the respective operons. Species and GenBank protein accession numbers for TGD3 orthologs: *Escherichia coli* (ZP_00728201), *Pseudomonas putida* (ZP_01714441), *Xylella fastidiosa* (NP_779845), *Synechococcus* sp. JA-2-3B’a (YP_477326), *Arabidopsis thaliana* (NP_564850). (B) An alignment of the TGD sequence with various ABC-transporter associated ATPases for which crystal structures are available: HlyB (PDB: 1MT0), MalK (PDB: 1G29), MJ1267 (PDB: 1G9X), and HisP (PDB: 1B0U). Open boxes mark conserved residues, black boxes identical residues. The relative positions of the main functional sites are assigned and phenylalanine residue F94 of TGD3 which was mutated to alanine corresponds to the A-loop conserved aromatic residue as indicated.

FIG. 2. **Identification of the TGD3 locus (At1g65410).** (A) Structure for the tgd3 mutant allele corresponding to Salk_040335 line. The coding region of At1g65410 is shown as black boxes. T-DNA insertion position and primers (P1, P2 and left T-DNA border primer LB) used for PCR amplification are indicated. (B) Semi-quantitative RT-PCR analysis of TGD3 mRNA levels in wild-type (Col-2) and tgd3 plants. The expression of ubiquitin (UBQ10) in the same samples was used as a control. (C) Genotypic analysis at the TGD3 locus. Wild type (Col-2), tgd3 mutant, two tgd3 homozygous complementation lines expressing the TGD3 cDNA (cTGD3/tgd3) are shown. Primers were the same as in (A) and diagnostic DNA fragments are shown with their respective lengths in base pairs. (D) Lipid phenotype of the different plant lines in (C). A section of the thin-layer chromatogram stained for glycolipids is shown. DGDG, digalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol.

FIG. 3. **Lipid phenotype of the tgd3 mutant compared with the tgd1, tgd2 mutant and Col-2 wild type.** Total lipids were extracted from 4-week-old seedlings grown on MS agar plates containing 1% sucrose and separated by thin-layer chromatography. (A) Thin-layer chromatogram of neutral lipids. Lipids were visualized by exposure to iodine vapor. (B) Thin-layer chromatogram of polar lipids. Lipids were visualized by sugar-specific α-naphthol staining. DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; O, origin; PIG, pigments; TAG, triacylglycerol; TGDG, trigalactosyldiacylglycerol.
FIG. 4. Polar lipid composition and fatty acid content in the tgd3 mutant. (A) Four-week-old Col-2 wild type and tgd3 mutant seedlings grown on the same MS agar plate were analyzed. Five replicates were averaged and the standard errors were shown. (A) Polar lipid composition (relative mol%) determined by quantification of fatty acid methylesters derived from individual lipids. (B) Fatty acid composition of the two galactolipids MGDG and DGDG. Fatty acids are indicated with number of carbons: number of double bonds. DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol.

FIG. 5. LC/MS analyses of lipid extracts of tgd3 mutant and Col-2 wild type Arabidopsis leaves. Negative mode electrospray extracted ion chromatograms (XICs) for m/z 1129.6, the [M+acetate]⁺ ion for TGDG 34:6, for extracts of (A) tgd3 mutant and (B) Col-2 wild type leaves. The mass spectrum at retention time 21.32 min for the tgd3 mutant (C) shows additional peaks corresponding to [M+Cl]⁻ and [M+formate]⁻ (unlabeled). Positive mode electrospray XICs over the range of m/z 890-905 reflect the abundance of triacylglycerols with 54 carbons and various numbers of double bonds for (D) tgd3 mutant and (E) Col-2 wild type leaves. The summed mass spectrum over the peak eluting at 27.87 min shows [M+NH₄]⁺ peaks corresponding to triacylglycerols with 54 carbons and from 3-8 double bonds.

FIG. 6. Subcellular localization of TGD3. (A-C) Transient expression of the TGD3-encoding cDNA fused to the N-terminus of the green fluorescent protein coding sequence. Confocal images are shown with red chlorophyll fluorescence of chloroplasts (A), green fluorescence specific for the TGD3-GFP fusion protein (B) and an overlay of (A) and (B). The scale bar equals 8 µm. (D) Import of in vitro translated TGD3 protein into isolated pea chloroplasts. An autoradiogram of the protein gel is shown. The sensitivity of the imported protein to Thermolysin (TL), Trypsin (TR) and to Thermolysin in the presence of Triton-X-100 (TX) was tested. The type of plastid treatment is indicated by +/- . The translation product mixture (TP) containing the TGD3 preprotein (pP) is shown as well. The mature protein (mP) following removal of the transit peptide is indicated.

FIG. 7. Purification and ATPase activity of recombinant TGD3 protein. The TGD3 protein is N-terminally truncated lacking the transit peptide and fused to the C-terminus of maltose binding protein designated as MBP-ΔTGD3. (A) Purification of MBP-ΔTGD3 in E. coli. Analysis of 10 µl of total protein from flow-through (F), washing fraction (W) and a series of different elution fractions (1-7) by SDS-PAGE is shown. Proteins were visualized by Comassie Blue staining. The E3-fraction contained approximately 5 µg protein. (B) Time-course of ATPase activity. The assay was conducted at a protein concentration of ~8 µM and final ATP concentration of 1 mM at 37°C. Buffer solutions containing no protein were included as background control. Maltose biding protein treated identical to MBP-ΔTGD3 was included to test for endogenous ATPase activity. Relative activity was shown as absorbance readings at 850 nm. (C) Production of inorganic phosphate as a function of protein concentration at 1 mM ATP. Reactions were...
performed at 37°C with an incubation time of 30 min. Absorbance readings were corrected for ATP spontaneous hydrolysis. Three replicates were averaged and the standard errors are shown. (D) ATPase activity of MBP-ΔTGD3 and MBP-ΔTGD3F94A. The phenylalanine at position 94 (A-loop) was mutated to an alanine residue by site-directed mutagenesis. ATPase assays were performed at various ATP concentrations as indicated. Three replicates were averaged and the standard errors are shown.

FIG. 8. Model of the proposed TGD lipid transporter complex. A dimeric composition is shown with numbers corresponding to the TGD1, TGD2, and TGD3 proteins. Outer (oE) and inner chloroplast envelope membranes (iE) are indicated.
Fig. 1, Lu et al. 2007
Fig. 2, Lu et al., 2007
Fig. 3, Lu et al., 2007
Fig. 4, Lu et al., 2007
Fig. 5, Lu et al., 2007
Fig. 7, Lu et al. 2007
Fig. 8, Lu et al. 2007
FIG. S1. Fatty acid composition of lyso-MGDG (A) and lyso-DGDG (B) in the tgd3 mutant and Col-2 wild type lines. Relative abundance (mol%) of 16 and 18-carbon fatty acids at the glycerol sn-2 position of MGDG and DGDG were determined from the lyso derivatives after digestion of the lipids with Rhizopus sp. lipase. Values represent the means of three measurements and the standard error is shown.
A small ATPase protein of Arabidopsis, TGD3, involved in chloroplast lipid import
Binbin Lu, Changcheng Xu, Koichiro Awai, A. Daniel Jones and Christoph Benning

*J. Biol. Chem.* published online October 15, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M704063200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2007/10/17/M704063200.DC1