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FUNCTIONAL ANALYSIS OF A PHOSPHATIDIC ACID TRANSPORT SYSTEM IN *ARABIDOPSIS THALIANA*

presented by

Binbin Lu

has been accepted towards fulfillment
of the requirements for the

Ph.D degree in Biochemistry and Molecular Biology

Major Professor's Signature

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Date

FUNCTIONAL ANALYSIS OF A PHOSPHATIDIC ACID TRANSPORT SYSTEM IN *ARABIDOPSIS THALIANA*

By

Binbin Lu

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ABSTRACT

FUNCTIONAL ANALYSIS OF A PHOSPHATIDIC ACID TRANSPORT SYSTEM IN *ARABIDOPSIS THALIANA*

By

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Membranes consisting of lipid bilayers are indispensable structures of all organisms. Membranes of lipid assembly are often distinct from final destination membranes, which require substantial polar lipid trafficking and lipid transport. A striking example is the biogenesis of the photosynthetic membranes (thylakoids) in plastids of plants. Many lipid biosynthetic enzymes at the endoplasmic reticulum (ER) and the inner and outer plastid envelope membranes are involved in this process. *Arabidopsis thaliana* mutants that are disrupted in the incorporation of ER-derived lipid precursors into thylakoid lipids are available due to systematic genetic screening and bioinformatics studies. Three proteins identified in three of these mutants, TRIGALACTOSYLDIACYLGLYCEROL 1 (TGD1), TGD2, and TGD3, are postulated to be the permease, substrate binding component, and ATPase, respectively, of a proposed lipid translocator at the inner chloroplast envelope membrane. In chapter 2, I describe the identification and characterization of TGD3, a small ATPase proposed to be part of this translocator. The TGD3 protein shows basal ATPase activity when expressed as a maltose-binding protein fusion and is localized inside the chloroplast beyond the inner envelope membrane. Similar to the *tgd1* and *tgd2* mutants, a *tgd3* mutant carrying a T-DNA insertion just 5' of the *TGD3* coding region (*tgd3-1*) also accumulates triacylglycerols and trigalactolipids. However, the *TGD3* gene expression level is only slightly reduced in this *tgd3-1* mutant. Mutants fully disrupted in *TGD3* were unavailable

to analysis at the time of the study. By utilizing RNA silencing, Arabidopsis mutant plants with more severely disrupted TGD3 function were generated and analyzed, as described in chapter 3. The phenotype of these RNAi mutant plants are nearly identical to *tgd3-1*, and do not show the expected severe growth defect and embryo lethality seen in other *tgd* mutants, raising the possibility of an additional ATPase associated with the proposed TGD complex. Chapter 4 focuses on the in-depth biochemical studies of the TGD2 protein, whose C-terminal domain (TGD2C) was previously shown to specifically bind phosphatidic acid (PtdOH). The PtdOH binding site was further narrowed down to a 25 amino acid segment, which by itself is sufficient to mediate specific binding of TGD2 to PtdOH. However, binding characteristics of this 25-mer were different from that of wild-type TGD2C suggesting that additional sequences of TGD2 are needed for this 25-mer to provide the proper context for wild-type like PtdOH binding.

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Chapter 1

Galactoglycerolipid biosynthesis and lipid trafficking between the endoplasmic reticulum and the plastid in plants

1.1. Introduction

The chloroplast is critical for plants because it is where photosynthesis takes place. It harbors a unique and also the most extensive membrane system in nature-the thylakoid membrane. Photosynthetic protein complexes are embedded into the thylakoid membrane and carry out photosynthesis to provide oxygen in the atmosphere and chemical energy to heterotrophic organisms in the form of reduced carbon.

The chloroplast has two boundary envelope membranes to separate the interior from the extraplastidic compartments of the cell. Both membranes consist of lipid bilayers. However, different from many organisms, such as fungi, animals and bacteria, whose predominant lipid classes are phosphoglycerolipids, the most abundant lipids in photosynthetic organisms are non-phosphorous glycoglycerolipids. Among those glycoglycerolipids, mono- and digalactosyldiacylglycerol (MGDG and DGDG) are predominant in chloroplasts (Dörmann and Benning 2002).

Glycoglycerolipids are derived from fatty acid precursors, whose synthesis is conducted in the chloroplasts (Ohlrogge *et al.* 1979). Fatty acids can either stay in chloroplasts and be directly used for glycerolipid synthesis, or they can be exported and assembled into lipids in the endoplasmic reticulum (ER). Glycerolipids assembled in the ER serve as building blocks for extraplastidic membranes. Alternatively, lipids could also return to chloroplasts, where their diacylglycerol backbones are incorporated into thylakoid lipids (Roughan and Slack 1982).

Apparently, the assembly of thylakoid membranes requires substantial lipid exchange between the ER and the chloroplasts, however, most of the lipid trafficking phenomena still remain poorly understood. In this chapter, I will review studies of

chloroplast lipid biosynthesis, with a focus on the role of lipid trafficking between the ER and the plastid relevant to chloroplast biogenesis. In addition, I will introduce our current working model suggesting that the TGD complex is a critical ABC (ATP-binding cassette) transporter that is involved in phosphatidic acid transfer from the outer chloroplast to inner chloroplast envelope.

1.2. Galactoglycerolipids are the most abundant lipids in plants

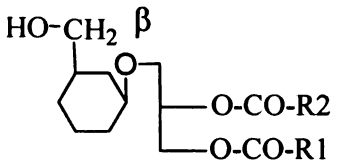
Membranes consisting of lipid bilayers are indispensable structures of all organisms. In fungi, animals and bacteria, the predominant lipid classes are glycerolipids that carry a phosphate head group. Likewise, those lipid classes are the major components of plasma membranes and ER, peroxisomal and mitochondrial membranes in seed plants. However, in plastids, non-phosphorous glycerolipids carrying one (monogalactosyldiacylglycerol, MGDG) or two (digalactosyldiacylglycerol, DGDG) galactose head moieties are the main constituents of the membranes (Dörmann and Benning 2002). MGDG and DGDG account for approximately 50% and 25% of the thylakoid membrane lipids, respectively. Together, they represent the bulk of the photosynthetic membranes in plant chloroplasts. In addition, sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PtdGro) represent about 15% of the thylakoid lipid species (Dörmann and Benning 2002).

Normally, in MGDG, the galactosyl head group is linked to the diacylglycerol backbone in β-configuration (Gal [β1′→3]-diacylglycerol), whereas the second galactose of DGDG is bound to the first one in α-configuration (Gal [α1″→6′]- Gal[β1′→3]-diacylglycerol) (Figure 1.1 A) (Carter *et al.* 1956). Unusual oligogalactoglycerolipids

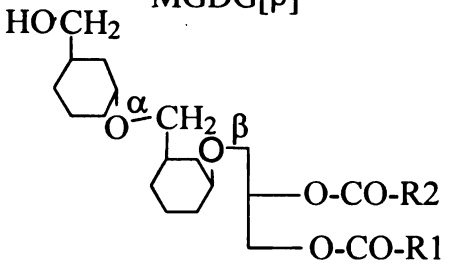
such as trigalactosyldiacylglycerol (TGDG) and tetragalactosyldiacylglycerol (TeGDG) are also found in some plant species, including *Chlorella*, pumpkin, rice bran and Adzuki bean (Benson *et al.* 1958, Fujino and Miyazawa 1979, Galliard 1969, Ito and Fujino 1975, Kojima *et al.* 1990). These lipids have three or four galactosyl head moieties attached to a diacylglycerol backbone and can be divided into at least three categories based on their anomeric configuration, as shown in Figure1.1. They can be derived either from the normal isomer β,α-DGDG or isomer β,β-DGDG by galactosylation. It should be noticed that TGDG detected in the Arabidopsis *tgd* mutants, which will be described in detail later, have all β configuration (TGDG[β, β, β], Figure1.1 C) (Xu *et al.* 2003).

Figure1.1. Chemical structures of plant galactoglycerolipids, adapted from Dörmann and Benning 2002. *A*, isomers of MGDG and DGDG. *B-D*, three categories of TGDG and TeGDG isomers. The anomeric linkage (α or β) of the glycosidic C1 atoms are indicated in parentheses and the structures. The open hexagons represent the $C_5H_8O_4$ units derived from galactose. R1-CO- and R2-CO- are acyl groups esterified to the sn-1 or sn-2 position of glycerol backbone, respectively.

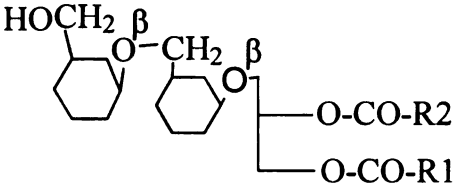
A



MGDG[β]

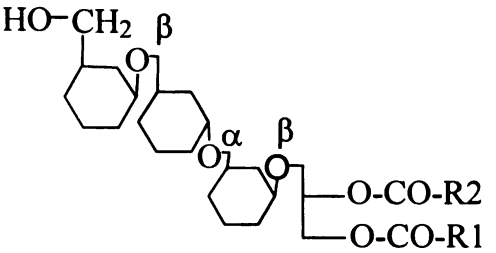


DGDG[β,α]

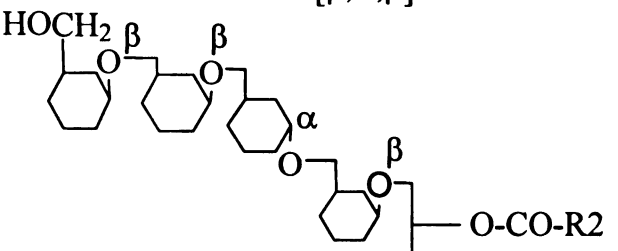


DGDG[β,β]

B

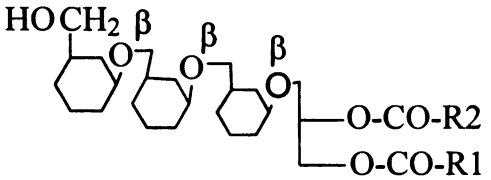


TGDG[β,α,β]

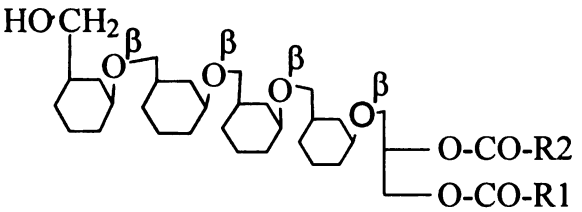


TeGDG[β,α,β,β]

C

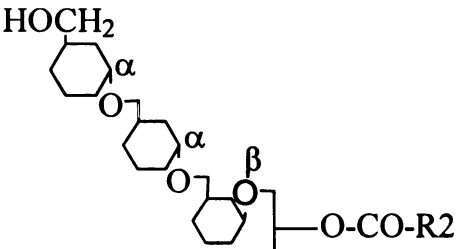


TGDG[β,β,β]

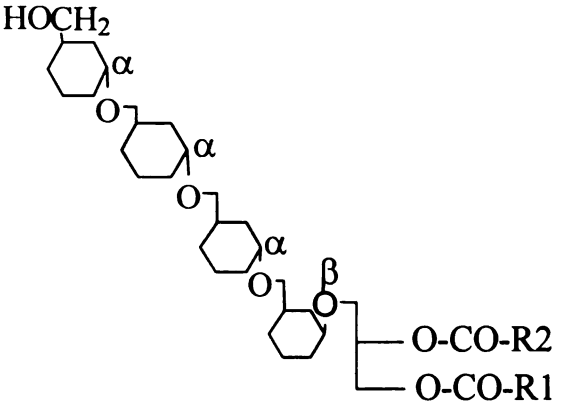


TeGDG[β,β,β,β]

D



TGDG[β,α,α]



TeGDG[β,α,α,α]

1.3. Three enzyme systems are involved in galactoglycerolipid biosynthesis in plants

Three classes of galactosyltransferases are associated with plastid envelopes and are responsible for galactoglycerolipid biosynthesis, namely monogalactosyldiacylglycerol synthase (MGD1, MGD2/3), digalactosyldiacylglycerol synthase (DGD1, DGD2), and processive galactolipid:galactolipid galactosyl transferase (GGGT) (Figure 1.2) (Benning and Ohta 2005).

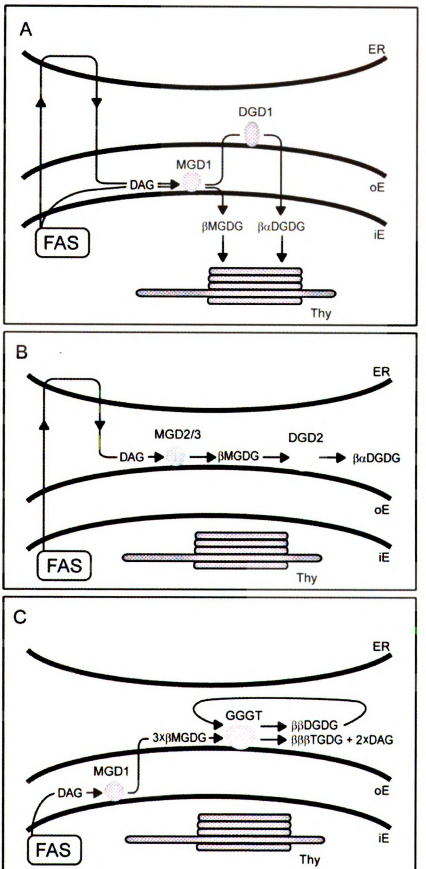
The genes that encode the enzymes of MGDG and DGDG synthesis have been isolated from different plants, including Arabidopsis (Dörmann and Benning 2002, Jorasch and Heinz 1999, Joyard *et al.* 1998). MGDG is synthesized in plants by MGDG synthase (EC 2.4.1.46), which catalyzes the transfer of one galactosyl residue from UDP-galactose to the sn-3 position of diacylglycerol (DAG) (Benning and Ohta 2005). The Arabidopsis genome contains three genes encoding functional MGDG synthases, which could be grouped into the A-type (MGD1) (Figure 1.2 A) and the B-type (MGD2 and MGD3) (Figure 1.2 B) (Awai *et al.* 2001, Miege *et al.* 1999). These different enzymes have different subcellular localizations, functions, as well as substrate specificities with regard to the DAG precursor. Though mutant analysis, MGD1 was shown to be the major isoform responsible for the bulk of MGDG synthesis in Arabidopsis. Even partial inactivation of the gene in the mutant led to a dramatic reduction in MGDG synthase activity and MGDG level (Awai *et al.* 2001, Jarvis *et al.* 2000). MGD1 was associated with the inner chloroplast envelope whereas MGD2 and MGD3 were localized to the outer chloroplast envelope (see Figure1.2 A and B)(Awai *et al.* 2001, Miege *et al.* 1999). In terms of substrate specificity, MGD1 utilizes both 18:2/18:2-DAG and 18:1/16:0-DAG

equally, while MGD2 and MGD3 prefer 18:2/18:2 DAG over 18:1/16:0-DAG (Awai *et al.* 2001).

DGDG synthase catalyzes galactosylation of MGDG to form DGDG. Isolation of the *dgd1* mutant in Arabidopsis led to the identification of the gene encoding the enzyme responsible for the bulk of DGDG synthesis, *DGD1* (Dörmann *et al.* 1995) (Figure 1.2 A). Disruption of the function of DGD1 in the *dgd1* mutant results in 90% reduction in DGDG content (Dörmann *et al.* 1995). Arabidopsis also has a second DGDG synthase, DGD2 (Figure 1.2 B), identified by sequence similarity to DGD1 (Kelly and Dörmann 2002). Both DGD1 and DGD2 enzymes are associated with the outer chloroplast envelope and catalyze the formation of β,α-DGDG from UDP-galactose and MGDG (Kelly *et al.* 2003, Kelly and Dörmann 2002).

A third class of galactosyltransferase, processive galactolipid:galactolipid galactosyltransferase (GGGT), is capable of synthesizing unusual oligogalactoglycerolipids such as TGDG and TeGDG (Figure 1.2 C). Its activity has been observed in chloroplast, however, its function and molecular identity are not yet known (Heemskerk *et al.* 1990, Heemskerk *et al.* 1991).

Figure1.2. Three enzyme systems involved in galactoglycerolipid biosynthesis in plants, adapted from Benning and Ohta 2005. *A*, MGD1 and DGD1 are the major isoforms of galactosyltransferases to synthesize the bulk of chloroplast galactoglycerolipids. *B*, MGD2/3 and DGD2 synthesize galactoglycerolipids in non-photosynthetic tissues and under phosphate deprivation conditions. *C*, GGGT is responsible for the synthesis of oliogalactoglycerlipids. *FAS*, fatty acid synthase complex; *iE*, inner chloroplast envelope; *oE*, outer chloroplast envelope; *Thy*, thylakoids.


A
ER
DGD1
MGD1
DAG
oE
βMGDG
βαDGDG
iE
FAS
Thy
B
ER
MGD2/3
DGD2
DAG
βMGDG
βαDGDG
oE
iE
FAS
Thy
C
ER
GGGT
ββDGDG
3xβMGDG
βββTGDG + 2xDAG
MGD1
DAG
oE
iE
FAS
Thy

Current evidence suggests that galactoglycerolipids are exclusively synthesized by these three sets of enzymes associated with the two chloroplast envelopes in plants. However, galactoglycerolipids could also be present in extraplastidic membranes in non-photosynthetic tissues or under phosphate-limiting growth conditions (Härtel *et al.* 2000). This fact requires substantial transfer of lipid precursors as well as galactoglycerolipids themselves between different membranes, including ER, chloroplast, thylakoid etc. However, most of the lipid trafficking processes remain unknown.

1.4. The two-pathway hypothesis of galactoglycerolipids biosynthesis

Galactoglycerolipids could be assembled either *de novo* in the plastids or from imported precursors assembled at the ER. This two-parallel-pathway theory was first proposed by Roughan and his colleagues (Roughan *et al.* 1980, Roughan and Slack 1982). The current hypothesis is shown in Figure 1.3. In the plastid (also called prokaryotic) pathway, fatty acids synthesized in chloroplasts are directly incorporated into chloroplast lipid species catalyzed by plastid acyl-acyl carrier protein (ACP):glycerol 3-phosphate acyltransferase encoded by *ATS1* (Kunst *et al.* 1988, Xu *et al.* 2006) and plastid acyl-ACP:lyso-phosphatidic acid (PtdOH) acyltransferase encoded by *ATS2* (Kim and Huang 2004, Yu and Benning 2003). The PtdOH generated is then dephosphorylated by a phosphatase to diacylglycerol (DAG). A candidate PtdOH phosphatase (PAP) was recently reported (Nakamura *et al.* 2007), however, the definitive proof that this enzyme is involved is still lacking. The produced DAG together with UDP-galactose serves as substrate for MGD1 to form MGDG (Awai *et al.* 2001, Jarvis *et al.* 2000). DGD1 then

catalyzes the next step of adding one more galactose to MGDG to form DGDG (Dörmann *et al.* 1999, Kelly *et al.* 2003).

In the ER (also called eukaryotic) pathway, those plastid-derived fatty acids are transported to the ER where they serve as building blocks for extraplastidic membrane lipids by ER-specific acyltransferases. Recent pulse-chase labeling data suggested that newly exported fatty acids first appear in phosphatidylcholine (PtdCho) by a very active acyl editing mechanism (Bates *et al.* 2007, Williams *et al.* 2000). Alternatively, DAG moieties of PtdCho can be transported back to the plastid and then enter the galactoglycerolipids biosynthesis as mentioned above. Therefore, only DAG origins for galactolipid biosynthesis differ in the two pathways, the final assembly into galactoglycerolipids by MGD1 and DGD1 are the same in either pathway.

Two-pathways of galactolipid biosynthesis are present in many plant species, including the well-studied genetic model plant Arabidopsis. In Arabidopsis, galactolipid species coming from both pathways are approximately equal in proportion (Browse *et al.* 1986). Lipid species derived from different pathways can be distinguished because they have different fatty acids in the diacylglycerol backbone, a fact due to distinct substrate specificities of the acyltransferases or acyl exchange enzymes in the ER or the plastid outer envelope. Lipids derived from the plastid pathway often have a 16-carbon acyl group at the sn-2 position of the DAG backbone whereas lipids derived from the ER pathway often have an 18-carbon acyl group at this position (Heinz and Roughan 1983). Plants that harbor the prokaryotic pathway such as Arabidopsis and spinach are referred to as '16:3 plants'; plant species that lost the prokaryotic pathway and thus depend solely

on the eukaryotic pathway for glycolipid biosynthesis such as Pea are called '18:3 plants'(Heinz and Roughan 1983).

Two types of Arabidopsis mutants that affect lipid trafficking in either galactolipid biosynthesis pathway are available. The *act1 (ats1)* mutant is deficient in the plastid glycerol 3-phosphate acyltransferase, and thus most of the galactoglycerolipids in this mutant are of ER origin (Bates *et al.* 2007, Kunst *et al.* 1988, Xu *et al.* 2006). (Figure 1.3). In other words, the *ats1* mutation turned Arabidopsis from a '16:3 plant' into an '18:3 plant'. Another type of mutants deficient in the ER pathway was obtained through a suppressor screen in the *dgd1* mutant background (Xu *et al.* 2003). Galactoglycerolipids in these so called *tgd* mutants are derived primarily from the plastid pathway (Awai *et al.* 2006, Lu *et al.* 2007, Xu *et al.* 2003, Xu *et al.* 2005) (Figure 1.3). The identification and characterization of these *tgd* mutants and resulting protein products are the main subject of this dissertation and will be described in detail below (see chapter 1.6).

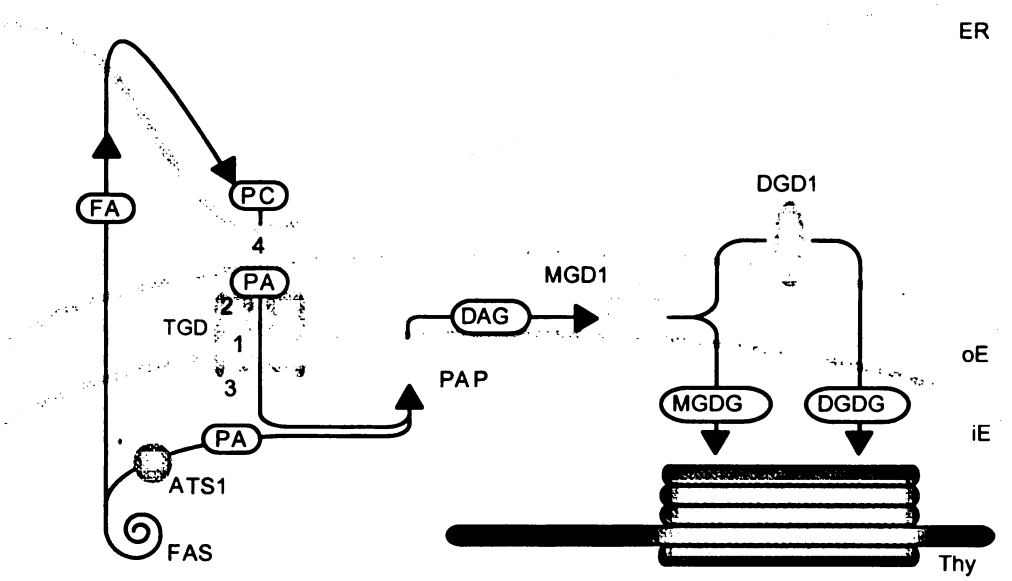


Figure 1.3. Two-pathway hypothesis of galactoglycerolipid biosynthesis in plants, adapted from Benning 2008. *ER*, endoplasmic reticulum; *oE*, outer chloroplast envelope; *iE*, inner chloroplast envelope; *Thy*, thylakoids; *FAS*, fatty acid synthase complex; *ATS1*, plastid acyl-acyl carrier protein (ACP): glycerol 3-phosphate acyltransferase; *PAP*, plastid phosphatidic acid phosphatase; *TGD1,2,3,* components of the proposed ATP-binding cassette (ABC) transporter for phosphatidic acid import through chloroplast inner envelope; *TGD4*, a TGD protein associated with the ER; *FA*, fatty acid; *PA*, phosphatidic acid; *PC*, phosphatidylcholine; *DAG*, diacylglycerol. Other designations are as in Figure 1.2. Arrows indicate movement of lipids.

1.5. Basic polar lipid transfer mechanisms involving plastids

Glycerolipid biosynthesis occurs mainly in plastid envelope and ER membranes. Since most lipids are not confined in the membranes where they are synthesized, extensive glycerolipid trafficking within plant cells is required to accomplish membrane specific lipid distribution. Among all possible aspects of lipid trafficking in plant cells, those lipid trafficking phenomena that involve the plastid are of our interest and will be discussed in this chapter.

Plastids not only export newly synthesized fatty acids, but also import ER-derived glycerolipids whose diacylglycerol backbones are incorporated into thylakoid lipids. Since plastids originate from ancestral endosymbiotic Gram-negative photosynthetic bacteria (Reyes-Prieto *et al.* 2007), similar bacterial lipid transfer mechanisms might still be present in plastids. In fact, recent studies support this hypothesis by showing that many bacterial and cyanobacterial components of cell membranes have orthologs in plastids (Reumann *et al.* 2005, Vothknecht and Soll 2007). Therefore, the well characterized lipid trafficking in bacteria and yeast provides us new insights into the less well studied and more complex plant phenomena. Polar lipid transfer in plants can be divided into three basic mechanisms: (1) flip-flop movements, (2) intermembrane transport and transfer by membrane contact sites and (3) vesicular lipid transfer mechanisms (see Figure 1.4. and below).

1.5.1. Flip-flop movement

Bilayer structures of biological membranes generally consist of two leaflets of polar glycerolipids. The polar head groups normally face the aqueous environment and

the acyl chain groups face the center. Galactoglycerolipids synthesized by DGD1, an enzyme associated with the cytosolic face of the outer chloroplast envelope membrane (Figure 1.2) have to be exchanged with the leaflet facing the intermembrane space (Figure 1.4, process 1). Similarly, the bulk synthesis of MGDG on the outside of the inner chloroplast envelope membrane by MGD1 (Figure 1.2) requires lipid exchange with the stroma side of the inner envelope membrane (Figure 1.4, process 2).

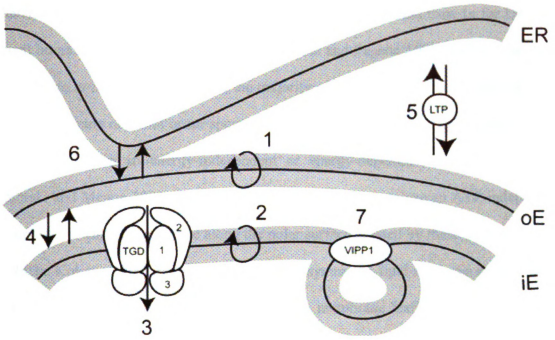


Figure 1.4. Postulated lipid transfer processes involved in ER-to-plastid lipid trafficking in plants, adapted from Benning 2008. Membrane designations are the same as in Figure 1.3. Bilayer membranes with their two leaflets are drawn. Arrows indicate different lipid transfer processes: (1) polar lipid flipping across the outer envelope membrane; (2) polar lipid flipping across the inner envelope membrane; (3) transmembrane movement of polar lipids through the proposed TGD123 complex; (4) transfer of lipids between the two envelope membranes; (5) lipid transfer mediated by lipid transfer proteins (LTPs); (6) lipid transfer through ER-outer envelope membrane contact zones (PLAMs); and (7) vesicle formation at the inner envelope membrane mediated by a vesicle inducing protein in plastids (VIPP1).

The spontaneous movement across the bilayer is dependent on the physical and chemical properties of lipids and membranes and usually is very slow. For example, the spontaneous flip-flop movement through a lipid bilayer takes less than 1s for DAG but several hours for lipids with larger polar heads (Bai and Pagano 1997, Jouhet *et al.* 2007, Reyes-Prieto *et al.* 2007). Therefore, the majority of the flipping of polar lipids across a bilayer membrane does not occur spontaneously, but is catalyzed by lipid transporters or flippases, either in an energy-independent or energy-dependent way (Daleke 2007, Pomorski and Menon 2006). Energy-independent flippases catalyze an ATP-independent movement, in which lipids move along the polar surface created by these proteins (Kol *et al.* 2002a, Kol *et al.* 2002b). They play a role in asymmetric lipid synthesis by facilitating the equilibration of polar lipids between the two membrane leaflets. Energy-dependent translocases can assist the movement of specific lipid classes against equilibrated gradients and thus are involved in the maintenance of membrane asymmetry (Seigneuret and Devaux 1984).

To date, no energy-independent flippase has been characterized in plants cells although some gene sequences reveal similarities to flippase sequences from other organisms. For instance, the first flippase studied in eukaryotic cells are the RFT1 protein, a yeast reticulum pyrophosphoryloligosaccharide-dolichol flippase (Helenius *et al.* 2002) and the scramblase, a red blood cell plasma membrane calcium-dependent flippase (Zhou *et al.* 1997). RFT1 transfers dolichol from the reticulum cytosolic leaflet to the luminal leaftlet (Helenius *et al.* 2002). Scramblase is activated by increasing calcium influx and catalyzes bidirectional transfer of phospholipids between the two plasma membrane leaflets (Zhou *et al.* 1997). In Arabidopsis, one RFT1 (At5g07630) and one scramblase

(At2g04940) homologue are identified based on sequence similarity, but functional characterizations are still pending (Jouhet *et al.* 2007).

ATP-requiring lipid transporters or flippases can be divided into two families: (a) aminophospholipid translocases that belong to the P-type ATPase superfamily and (b) ATP-binding cassette (ABC) transporters that comprise transporters for a variety of organic and inorganic compounds. In Arabidopsis, 11 genes belong to the P-type ATPase superfamily (ALA1 to ALA11, see Jouhet *et al.* 2007). Among them, ALA1 is found to be involved in cold tolerance and might affect plasma membrane lipid asymmetry (Gomès *et al.* 2000). However, ALA1 localization and function remain unknown.

ABC transporters, which are found in all organisms, are one of the largest and most conserved protein families (Moody *et al.* 2002, Pohl *et al.* 2005). These proteins transport a wide range of compounds including lipids, peptides and sugars through biological membranes. ABC transporters are often multicomponent protein complexes that typically consist of two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). In most eukaryotes, all four domains are encoded by one gene to generate a single polypeptide chain, while the TMDs and NBDs of prokaryotes are encoded by separate genes (Davidson and Chen 2004, Moody *et al.* 2002, Pohl *et al.* 2005). Many prokaryotic ABC transporters require a fifth subunit that functions as a substrate binding protein (SBP). Nucleotide binding proteins of ABC transporters are highly conserved among all organisms and share three well conserved motifs required for nucleotide binding and hydrolysis: the Walker A site ($GX_4GK(S/T)$, where X=any residue), the Walker B site ($RX_{6-8}\Phi_4D$, where Φ=hydrophobic residue), and the LSGGQ signature sequence (Moody *et al.* 2002).

ABC transporters have been extensively studied. In humans, mutations of members of the ABC protein families cause defects in lipid transport and metabolic disorders, such as Tangier disease. Fibroblasts from patients with Tangier disease have a defect in the export of cellular cholesterol and phospholipids from the plasma membrane to high density lipoproteins (Francis *et al.* 1995, Remaley *et al.* 1997). Additionally, the role of ABC proteins in lipid transport is being studied in non-human eukaryotes such as Arabidopsis and yeast, as well as in prokaryotes such as *Escherichia coli* and *Lactococcus lactis* (Pohl *et al.* 2005). A good example is the MsbA protein of *E. coli*, which has been implicated in lipid transfer from the inner cell membrane to the outer membrane; its crystal structure has already been solved and is very similar to other reported ABC transporters (Chang and Roth 2001, Doerrler *et al.* 2001).

The Arabidopsis genome contains 129 open reading frames (ORFs) belonging to ABC protein families (Sanchez-Fernandez *et al.* 2001). They play a key role in a variety of processes including chlorophyll biosynthesis, stomata movement, and ionic fluxes (Martinoia *et al.* 2002). Some ABC transporters involved in the export of precursors for cuticle or wax biosynthesis in the plant epidermis are also reported (Bird *et al.* 2007, Luo *et al.* 2007, Pighin *et al.* 2004, Ukitsu *et al.* 2007). A potential ABC transporter identified as At1g54350 in the Arabidopsis genome is proposed to mediate free fatty acids transfer from the inner plastid envelope to the outer plastid envelope, where fatty acids are reactivated to acyl-CoA and enter numerous metabolic pathways (Koo *et al.* 2004). A well studied ABC transporter in Arabidopsis, COMATOSE (CTS), PEROXISOMAL ABC TRANSPORTER1(PXA1) or PEROXISOME DEFECTIVE3 (PED3), is localized in peroxisomes to import fatty acids or acyl-CoAs for β-oxidation (Footitt *et al.* 2002,

Hayashi *et al.* 2002, Zolman *et al.* 2001). Recent labeling experiments on *pxa1/cts/ped3* mutant seedlings suggested that this ABC transporter could also mediate the import of acetate into glyoxysomes, specialized peroxisomes that play a role in seed germination, when fatty acids are converted to carbohydrates (Hooks *et al.* 2007). All the above mentioned ABC transporters are homodimers encoded by a single peptide with a multi-membrane-spanning permease domain and an ATP-binding cassette (ABC) domain. To date, the only Arabidopsis ABC transporter involved in ER-to-plastid lipid exchange identified is the TGD1, 2, 3 complex. These three proteins constitute a bacterial-type multipartite ABC transporter (Awai *et al.* 2006, Lu *et al.* 2007, Xu *et al.* 2003, Xu *et al.* 2005). The transporter is localized to the inner chloroplast envelope and proposed to function in PtdOH import through this membrane as will be discussed in detail below (Figure 1.4, process 3).

1.5.2. Intermembrane transport and transfer by membrane contact sites

As mentioned above, most plant species have two sources of thylakoid lipid precursors, the ER and plastids. This fact requires substantial lipid exchange between these two organelles (Figure1.4, process5). In addition, lipids delivered to the outer chloroplast envelope have to be exchanged with lipids synthesized in the inner chloroplast envelope to keep the specific lipid distribution and lipid homeostasis (Figure 1.4, process 4). These intermembrane lipid transport events are often carried out by lipid transfer proteins (LTPs) or membrane contact sites.

LTPs have to target both donor and acceptor membranes to mediate trafficking of particular lipids between organelles (Holthuis and Levine 2005, Kader 1996). Some LTPs

are localized at membrane contact sites where two membranes are in close proximity, usually less than 10nm (Holthuis and Levine 2005, Loewen *et al.* 2003a, Loewen *et al.* 2003b). Some LTPs have two targeting domains for both the lipid donor membrane and the receptor membranes thus they can either move from one membrane to another or bind to both membranes simultaneously. This simultaneous binding to both membranes could potentially stabilize membrane contact sites and promote lipid transport (Holthuis and Levine 2005). There are many LTPs identified in plants, however, most of the LTPs characterized so far are generally found to be involved in a variety of cellular processes such as cell death (Brodersen *et al.* 2002). Moreover, there is no information concerning their possible localizations to specific membranes or membrane contact sites. This questions the actual role of LTPs in ER-to-plastid lipid transfer.

Another type of lipid transfer involves membrane contact between two organelles (Voelker 2000). MAMs (mitochondria associated membranes) are present in the contact sites between ER and mitochondria. They are well studied in mammals and yeast (Achleitner *et al.* 1999, Vance 1990). MAMs are proposed to be involved in mitochondrial phosphatidylethanolamine (PtdEtn) biosynthesis, which occurs by decarboxylation of phosphatidylserine (PtdSer) catalyzed by an isoform of PtdSer decarboxylase localized to the mitochondria (Rontein *et al.* 2003). PtdEtn biosynthesis is dependent on PtdSer supply from ER due to spatial localization of PtdSer synthase in the ER membrane (Voelker 2000). PtdSer is transported to the mitochondrial inner membrane by an unknown mechanism, maybe going through contact sites between the inner and outer membranes of mitochondria (Reichert and Neupert 2002). PtdSer is subsequently decarboxylated in the mitochondria inner membrane to form PtdEtn before

a retro-transfer of PtdEtn to ER probably going also through the MAM (Voelker 2000). Genetic screens using mutant strains selectively inactivated in the PtdSer decarboxylase of the mitochondria led to the isolation of enthanolamine auxotrophs that are impaired in the regulation of aminoglycerophospholipid biosynthesis (Storey *et al.* 2001, Trotter *et al.* 1998, Wu *et al.* 2000). In addition, MAMs are also possibly involved in the transfer of other mitochondrial phospholipids synthesized in ER such as PtdCho (Voelker 2000).

Based on yeast studies, it was proposed that PtdEtn is transferred by a similar mechanism involving plasma membrane associated membrane (PAM) from ER to plasma membranes (Holthuis and Levine 2005). Membrane contact sites are also observed between ER and vacuoles. These contact sites possibly participate in the transfer of PtdEtn, PtdCho and PtdIns with the involvement of previously described LTPs (Staehelin 1997).

Finally, lipid transfer by membrane contact sites between plastid envelope and other organelles was also reported. Sandelius and coworkers were the first to discover direct contact sites between the ER and the outer chloroplast envelope membrane (Figure 1.4, process 6) that can be isolated in the form of PLAMs (plastid associated membranes). During pea chloroplast isolation, some ER-fractions were found to remain associated with the plastid envelopes (Kjellberg *et al.* 2000). Moreover, *in vivo* optical manipulation reveals strong attracting forces at membrane contact sites between the ER and the plastids (Andersson *et al.* 2007). It should be noted that the existence of PLAMs by itself does not provide a mechanism of ER-to-plastid lipid exchange. It is still not clear what the physical arrangements of polar lipids in such contact sites are and whether or not proteins are required to form these contact sites. However, the well studied MAM in yeast system

suggests the possibilities of also applying genetic approaches to address lipid trafficking in plants. Recently, a genetic screen carried out in Arabidopsis has led to the isolation of mutants disrupted in the transfer of lipids from the ER to the plastids. Mapping of the mutant genes might provide information about components participating in the formation of PLAMs in the near future. For instance, the possibility that the TRIGALACTOSYLGLYCEROL 4 (TGD4) protein functions in PLAMs and lipid trafficking between the ER and the plastid is emerging (Xu *et al.* 2008b).

1.5.3 Vesicular lipid transfer

All the lipid transfer mechanisms discussed so far are non-vesicular. On the other hand, lipid transfer could be realized through a vesicular mechanism, in which vesicles bud from a donor membrane and fuse with an acceptor membrane. The general mechanism of vesicular transfer includes several steps with a series of protein factors (Jürgens 2004, Nebenfuhr 2002). Briefly, protein complexes are recruited and anchored to the donor membrane by small type Ras GTPases, coupled to GTP exchange factors. Then, vesicles bud from the donor membrane through a not-yet-clear mechanism and move away using the actin cytoskeleton and myosin. A large GTPase called dynamin is involved in the scission of nascent vesicles from the donor membrane. Finally, SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) proteins, located both on the vesicles (v-SNARE) and on the target membrane (t-SNARE), together with small GTPases help anchoring and fusion to the acceptor membrane.

Vesicular transfer mechanisms have been extensively studied and a lot of protein factors have been identified. The process was suggested to mediate numerous transfer

events such as ER and Golgi exchanges (Brandizzi *et al.* 2002, Hawes 2005), lipid transfer to the plasma membrane (Ikonen 2001, Keller *et al.* 2001), and lipid transfer to the vacuoles (Matsuoka and Bednarek 1998). No evidence for the involvement of vesicular transfer mechanism in ER-to-plastid lipid trafficking has appeared yet. However, some studies suggest a possibility that a vesicular lipid transfer system might exist in the transfer of galactoglycerolipids from the inner chloroplast envelope to the thylakoid membrane, contributing to the biogenesis of the thylakoids (Figure 1.4, process 7). First, vesicle budding from the inner chloroplast envelope membrane was directly observed by electron microscopy (Carde *et al.* 1982, Morré *et al.* 1991). Later, an Arabidopsis chloroplast-localized THYLAKOID FORMATION 1 (THF1) protein was shown to control a step required for the organization of vesicles derived from the inner envelope membrane into mature thylakoid stacks. Deletion of THF1 in Arabidopsis leads to deficient thylakoid formation and variegated leaves (Wang *et al.* 2004).

The plastid vesicular pathway is dependent on ATP and stromal proteins, among which a NSF (for *N*-ethylmaleimide sensitive factor) homolog protein (Hugueney *et al.* 1995), a dynamin-like protein (Park *et al.* 1998) and a vesicle inducing protein in plastids (VIPP1) (Aseeva *et al.* 2007, Kroll *et al.* 2001) are identified. VIPP1 is essential for basic thylakoid membrane formation so that thylakoid membrane formation and chloroplast vesicle transport are abolished in Arabidopsis VIPP1 depleted *vipp1* mutant (Aseeva *et al.* 2007, Kroll *et al.* 2001). Some data also demonstrate that VIPP1 forms a high molecular weight complex closely associated with the inner chloroplast envelope membrane and that the C-terminus of the protein protrudes from the complex into the stroma of chloroplasts possibly for interaction with other proteins (Aseeva *et al.* 2004).

Accordingly, interactions between VIPP1 and the chaperon proteins HSP70B-CDJ2-CGE1 are observed in the green alga Chlamydomonas. This tight association might play a role in assembly and disassembly of VIPP1 oligomers to recycle the system for another round of vesicle formation (Liu *et al.* 2005, Liu *et al.* 2007). Taken together, current evidence demonstrates that VIPP1 is a crucial component of a complex potentially required for vesicular lipid trafficking between the inner plastid envelope and the thylakoids. Bioinformatics studies suggest that some other vesicular pathway components are present in plastids (Andersson and Sandelius 2004), yet further characterization by genetic and biochemical approaches is still pending.

1.6. Current progress in the study of lipid trafficking in Arabidopsis

Arabidopsis is a well developed genetic model that has been used to study lipid transfer phenomena. Two types of Arabidopsis mutants that affect lipid trafficking between the ER and the plastid have been identified. The *act1 (ats1)* mutant is deficient in the plastid glycerol 3-phosphate acyltransferase, and thus most of the galactoglycerolipids in this mutant are derived from the ER pathway (Bates *et al.* 2007, Kunst *et al.* 1988, Xu *et al.* 2006). All known mutant alleles of *ats1* are leaky and further repression of the activity of the mutant gene leads to a severe growth phenotype. Therefore, the leaky *ats1-1* allele has become an important tool in double-mutant studies focusing on lipid trafficking in plants, as mutations in genes encoding components of the eukaryotic pathway in an *ats1-1* background severely impair chloroplast biogenesis and cause embryo lethality (Dörmann *et al.* 1999, Xu *et al.* 2003, Xu *et al.* 2005, Xu *et al.* 2008b).

Previous studies in the Benning laboratory have identified another type of mutants deficient in the ER-derived galactolipid biosynthesis pathway (Awai *et al.* 2006, Lu *et al.* 2007, Xu *et al.* 2003, Xu *et al.* 2005). Galactoglycerolipids in these mutants are derived primarily from the plastid pathway. These mutants were given the name *trigalactosyldiacylglycerol (tgd)* for the diagnostic accumulation of oligogalactoglycerolipids (TGDG), which is typically absent in wild-type Arabidopsis plants.

The *tgd* mutants were isolated during a suppressor screen in the *dgd1* mutant background (Xu *et al.* 2003), which is deficient in DGD1 activity and thus lacks 90% of DGDG content (Dörmann *et al.* 1995, Dörmann *et al.* 1999). The primary goal of the screen was to identify mutants that bypass the major galactoglycerolipid biosynthetic pathway involving MGD1 and DGD1 (Awai *et al.* 2001, Härtel *et al.* 2000, Kelly and Dörmann 2002). Two group of mutants were isolated, with one meeting the original criteria for the disruption of a regulator of the alternative galactolipid biosynthesis pathway, which was affected in a mitochondrial outer membrane protein (Xu *et al.* 2008b). The second group of mutants was the *tgd* mutants that accumulated novel oligogalactoglycerolipids TGDG and restored the wild-type level of DGDG content compared to *dgd1* parent plants. TGDG accumulated in the *tgd* mutants have all β glycosidic linkages of their head group galactoses, indicating that their production is independent of MGD1 and DGD1 galactosyltransferases (See Figure1.1C, Figure 1.2C). Probably, a processive galactolipid:galactolipid galactosyltransferase (GGGT) is involved in the synthesis of TGDG in the mutants.

The *tgd1* mutant was the first and best analyzed *tgd* mutant so far (Xu *et al.* 2003, Xu *et al.* 2005). It accumulates both TGDG and triacylglycerol (TAG) in leaves. The PtdOH level in the mutant is greatly increased. Pulse-chase labeling experiment with acetate and oleic acid indicates that the transfer of lipid molecular species from the ER to the plastid is impaired in the *tgd1* mutant. Isolated chloroplasts from *tgd1* plants exhibit a decreased rate of conversion of labeled PtdOH into galactoglycerolipids. Loss of function of TGD1 is embryo lethal. The TGD1 protein is localized in the inner chloroplast envelope membrane and is similar to the permease component of bacterial ABC transporters.

Detailed studies of the other two *tgd* mutants, *tgd2* and *tgd3*, are the major components of this dissertation and will be introduced chapter by chapter. Chapter 2 and chapter 4 will be focused on the identification and biochemical study of the TGD3 protein and TGD2 protein, respectively. Characterization of Arabidopsis mutants disrupted in TGD3 function will be described in chapter 3.

Briefly, both *tgd2* and *tgd3* mutants have the same phenotype as *tgd1*. The TGD2 protein is similar to the substrate binding subunit of bacterial ABC transporters and specifically binds to PtdOH *in vitro* (Awai *et al.* 2001). Recently, I identified a 25-amino-acid minimal domain that is sufficient for specific PtdOH binding when fused with *Discosoma sp.* red fluorescent protein (DsRed) (Lu and Benning 2009). Presumably, TGD2 is anchored with its N-terminus into the inner chloroplast envelope membrane. With its C-terminus facing the intermembrane space, it could extract PtdOH from the outer envelope membrane (Awai *et al.* 2006, Lu and Benning 2009). The TGD3 protein was identified through a reverse genetics approach, and contains sequence similarity with

non-intrinsic ATPase found adjacent in operons to bacterial orthologs of TGD1 and TGD2 (Lu *et al.* 2007). Recombinant TGD3 protein fused with maltose binding protein shows basal ATPase activity and is localized inside the chloroplast beyond the inner chloroplast envelope membrane (Lu *et al.* 2007). It could potentially associate with TGD1 on the inside of the inner envelope membrane.

Taken together, it was proposed that TGD1, TGD2 and TGD3 form a bacterial type ABC transporter complex that transports PtdOH through the inner chloroplast envelope for synthesis of galactoglycerolipids (See Figure 1.3 and Figure 1.4, process 3) (Benning 2008). The *tgd1,2,3* mutants are disrupted in this transporter and thus most of the galactoglycerolipids in these mutants are derived from the plastid pathway (Awai *et al.* 2006, Lu *et al.* 2007, Xu *et al.* 2003, Xu *et al.* 2005). At this stage, how PtdOH would be formed at the outer envelope remains unclear. However, the recent identification of the TGD4 protein involved in ER-to-plastid lipid trafficking, which is the first TGD protein associated with the ER (See Figure 1.3), provides new avenues towards an understanding of PtdOH formation from ER-derived precursors in predicted contact zones between the ER and the outer plastid envelope membrane (Xu *et al.* 2008a).

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Chapter 2

Identification and characterization of TGD3[1]

[1]This work has been published: **Lu, B., Xu, C., Awai, K., Jones, A. D., and Benning, C.** (2007) A small ATPase protein of Arabidopsis, TGD3, involved in chloroplast lipid import. *J. Biol.Chem.* **282,** 35945-35953. I contributed to all the figures except Figure 2.4, Figure 2.6 and Figure 2.8.

2.1. Abstract

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 endoplasmic reticulum-derived lipid precursors into thylakoid lipids. Two genes affected in two of these mutants, *trigalactosyldiacylglycerol 1 (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 gene of Arabidopsis, *TGD3*, encodes 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 when expressed as a maltose-binding protein fusion 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*.

2.2. Introduction

Interorganellar non-vesicular lipid trafficking in plant cells is required for the biogenesis of the characteristic plant cell organelle, the chloroplast (Benning *et al.* 2006). The predominant polar lipids found in the photosynthetic membranes (thylakoids) of chloroplasts are the galactoglycerolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG)(Dörmann and Benning 2002). Their final assembly from diacylglycerol (DAG) and UDP-galactose requires two major lipid galactosyltransferases, which in the model plant *Arabidopsis* are designated MGD1 and DGD1(Benning and Ohta 2005). Of these two enzymes MGD1 is associated with the outside of the inner chloroplast envelope membrane (Xu *et al.* 2005) and is responsible for the bulk of MGDG formation (Jarvis *et al.* 2000), whereas DGD1 is located on the outside of the outer chloroplast envelope membrane (Froehlich *et al.* 2001) and is required for the bulk of DGDG biosynthesis (Dörmann *et al.* 1999). 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 (Roughan and Slack 1982).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* (Awai *et al.* 2006, Xu *et al.* 2003, Xu *et al.* 2005). The TGD1 protein is similar to the permease component of bacterial ATP binding cassette (ABC) transporters,

and the *Arabidopsis tgd1* mutant shows a complex lipid phenotype; that is, 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(Xu *et al.* 2003, Xu *et al.* 2005). 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 galactolipid:galactolipid galactosyl transferase (GGGT) 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 (Xu *et al.* 2005).The TGD2 protein is similar to membrane-tethered substrate-binding proteins associated with bacterial ABC transporters (Awai *et al.* 2006). Although *tgd2* was 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 (Awai *et al.* 2006). The nearly identical phenotypes of the two mutants and the organization of bacterial orthologs of *TGD1* and *TGD2* in operons (Figure 2.1A) 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 ABC transporters or the MsbA lipid transporter from *Escherichia coli* (Doerrler 2006), 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 (Sanchez-Fernandez *et al.* 2001). 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.

2.3. Experimental procedures

2.3.1 Plant materials and growth conditions

Arabidopsis thaliana wild type and *tgd1* and *tgd2* mutant plants (Awai *et al.* 2006, Xu *et al.* 2003) 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) and were obtained from the *Arabidopsis* Biological Resource Center at Ohio State University: SALK_027270 At4g33460, SALK_129048 At5g60790, SALK_029696 At5g09930, SALK_113472 At5g64840, SALK_116866 At5g14100, SALK_055996 At1g32500, and 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 Murashige-Skoog medium (Murashige and Skoog 1962) supplemented with 1% (w/v) sucrose for 5 days. Wild-type and mutant seedlings of similar developmental stage were then transferred to a fresh Murashige-Skoog agar plate with 1% sucrose and grown for three additional weeks before lipid analysis.

2.3.2 Genotyping and complementation

The *TGD3* open reading frame was isolated by reverse transcriptase-PCR (standard conditions) from mRNA preparations using RNeasy and Omniscript kits (Qiagen, Valencia, CA) with the primers 5'-ACGGTACCATGCTTTCGTTATCATGC-TC-3' and 5'-CTGGTACCCTAGTATCTGATTGGTCCAT-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 (Xu *et al.* 2005) followed by transformation into *Agrobacterium*. Plants were transformed by the floral dip method (Clough and Bent 1998) and screened for resistance to hygromycin B (25µg/ml) on agar-solidified Murashige-Skoog medium. For an estimation of *TGD3* transcript levels by semi-quantitative reverse transcriptase-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). 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'-TCAATTCTCTCTACCGTGATCAA-GATGCA-3' and 5'-GTGTCAGAACTCTCCACCTCAAGAGTA-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'-GCGTGGACCGC-TTGCTGCAAC-3'; P1, 5'-AACTGATTGGGACGAGTATC-3'; P2, 5'-GCTATGCAA-CAGCAAGAGAC-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, and 72 °C for 0.5 min followed by a final step of 5 min at 72 °C.

2.3.3 Lipid assays

Lipids were extracted from 4-week-old Col-2 wild-type and *tgd3* mutant seedlings grown on Murashige-Skoog medium with 1% sucrose. Fatty acid methyl esters were prepared as previously described (Dörmann *et al.* 1995) and quantified by gas chromatography according to Rossak *et al. (Rossak et al. 1997)*. Polar lipids were analyzed on activated ammonium sulfate-impregnated (Dörmann *et al.* 1995) silica gel TLC plates (Si250PA; Mallinckrodt Baker) 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 (Benning *et al.* 1995). The fatty acid compositions at the *sn*-2 position of individual lipids were determined using *Rhizopus* lipase (Sigma) digestion according to Siebertz and Heinz (Siebertz and Heinz 1977) with modifications as described in Miquel *et al. (Miquel et al. 1998)*. Fatty acid methyl esters were formed from lyso-lipids, and the fatty acid methyl esters were quantified by gas chromatography.

2.3.4 Mass spectrometry

Lipid extracts were characterized using liquid chromatography/mass spectrometry (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 (Houjou *et al.* 2005) 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. The latter conditions allow for production of characteristic fragment ions that support structure assignments. High performance liquid chromatography separations were performed using a Restek Allure C18 column (1×150 mm) and a ternary gradient based on (*a*) 10 mM aqueous ammonium acetate, (*b*) methanol, and (*c*) 2-propanol.

2.3.5 Subcellular localization assays

For the generation of a green fluorescent protein fusion construct, the entire coding region of At1g65410 was inserted into the KpnI site of pCAMBIA1300GFP (Xu *et al.* 2005) using primers 5'-ACGGTACCATGCTTTCGTTATCATGCTC-3' and 5'-CTGGTACCGTATCTGATTGGTCCAT-3'. Transient expression of the construct in tobacco and microscopy of the resulting transgenic leaves was performed as previously described (Xu *et al.* 2005). 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 (Xu *et al.* 2005).

2.3.6 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'-GGATCCTGCATAGCTCCACCTCAGAAC-3' and 5'-GTCGACTAGTATCTGAT-TGGTCCATCG-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 QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The forward and reverse primers for F94A are 5'-GTAGAGA-TGTCTATAAATCGGCGGGGGAGAAACATATCTTG-3' and 5'-CAAGATATGTTT-CTCCCCCGCCGATTTATAGACATCTCTAC-3'. The underlined sequences are codons for the mutated residue (alanine). After digestion with BamHI and SalI, 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 A_{600} of 0.4–0.6 and incubated at 28 °C for 4 h. The cells were then collected by centrifugation and lysed by sonication in the presence of 1mg/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 (Bradford 1976) 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.

2.3.7 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

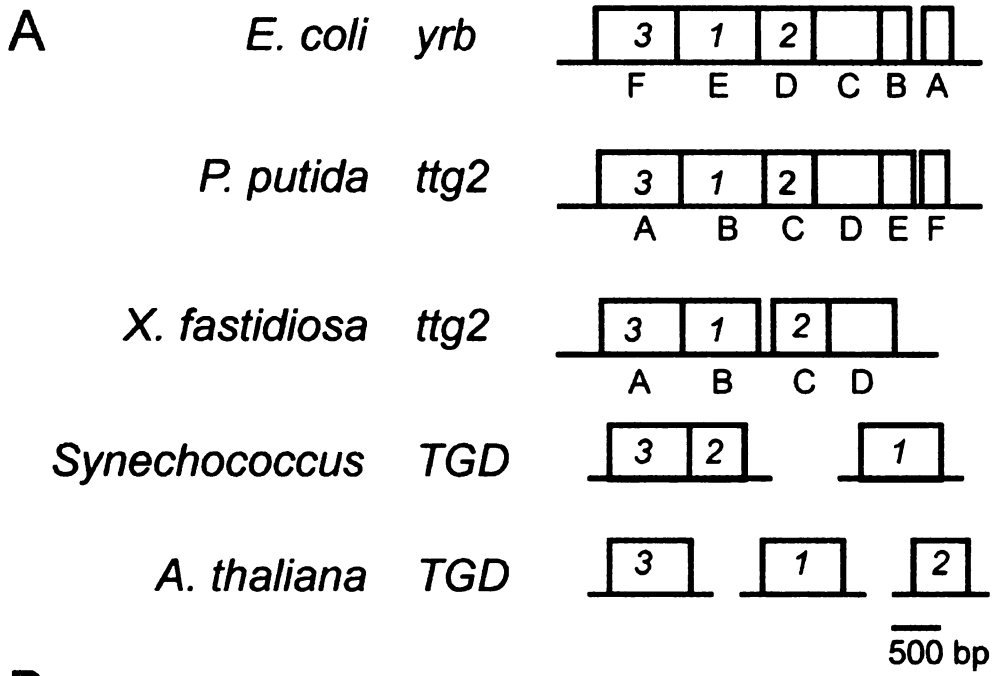
Tris-HCl, pH 7.6,150 mM NaCl, 2 mM $MgCl_2$). The MBP-ΔTGD3 protein was diluted in assay buffer to a final volume of 25 µl. To initiate the reaction, 25 µl of ATP in the assay buffer ranging from 0 to 2 mM (diluted from a 10 mM stock made in the same assay buffer) were added, giving a final ATP concentration of 0 to 1mM. ATPase reactions were incubated at 37 °C for the indicated time and stopped by the addition of 50µl 12% SDS. Released Pi was quantified by a colorimetric method, as described by Chifflet *et al.* (Chifflet *et al.* 1988) using potassium phosphate as the standard. After the addition of SDS, 100 µl of a solution containing equal volumes of 12% ascorbic acid in 1 M 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*-arsenite, and 2% acetic acid. After 10 min of incubation at 37 °C, absorbance was measured at 850 nm. The assay was linear in the range of 0–25 nmol of Pi. The activity was corrected for nonenzymatic release of Pi from ATP and for Pi 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 $V_o = V_{max}[S]/(K_m + [S])$ using Microcal Origin 7.0 (Microcal Software, Northampton, MA).

2.4. Results

2.4.1 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 (Osterman and Overbeek 2003). Accordingly, the location of TGD1 and TGD2 encoding homologs in bacterial operons (Figure 2.1A) adjacent to putative ABC proteins 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.

Figure 2.1. Isolation of the *TGD3* gene by reverse genetics. *A*, linear representation of the *Arabidopsis TGD* homologs in different bacterial species. The number designations (*1–3*) correspond to open reading frames presumed to encode homologs of *Arabidopsis* TGD1, TGD2, and TGD3 proteins, respectively. *Letters* refer to the bacterial open reading frame designations in the respective operons. Species and GenBankTM protein accession numbers for TGD3 orthologs: *E. 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 code 1MT0), MalK (PDB code 1G29), MJ1267 (PDB code 1G9X), and HisP (PDB code 1B0U). *Open boxes* mark conserved residues, and *black boxes* indicate identical residues. The relative positions of the main functional sites are assigned, and phenylalanine residue Phe94 of TGD3, which was mutated to alanine, corresponds to the A-loop conserved aromatic residue as indicated.



B

```
                    60        70        80        90       100
TGD3   51 PQNLDNDATKFDSLTKSGGGMCKERGLENDSDVLIECRDVYKSFGEKHIL
HlyB    1 ..............................DITFRNIRFRYKPDSPVIL
MalK    1 ..............................MAGVRLVDVWKVFGEVTAV
MJ1267  1 ............................MRDTMEILRTENIVKYFGEFKAL
HisP    1 ............................MMSENKLHVIDLHKRYGGHEVL
                                                 A-loop

                  110       120       130       140
TGD3       KGVSFKIRHGEAVGVIGPSGTGKSTILKIMAGLLAPDKGEVYIRGKKRA.
HlyB       DNINLSIKQGEVIGIVGRSGSGKSTLTKLIQRFYIPENGQVLIDGHDLAL
MalK       REMSLEVKDGEFMILLGPSGCGKTTTLRMIAGLEEPSRGQIYIGDKLVA.
MJ1267     DGVSISVCKGDVTLIIGPNGSGKSTLINVITGFLKADEGRVYFENKDIT.
HisP       KGVSLQARAGDVISIIGSSGSGKSTFLRCINFLEKPSEGAIIVNGQNINL
                          Walker A

           150       160       170       180
TGD3       ..GLISDEEI.........SGLRIGLVFQSAALFDSLSVRENVGF......
HlyB       ..........ADPNWLR...RQVGVVLQD.NVLLNRSIIDNISLA..NPG
MalK       ..DPEKGIFVPP......KDRDIAMVFQSYALYPHMTVYDNIAF......
MJ1267     ..NK.EPAEL.........YHYGIVRTFQTPQPLKEMTVLENLLIGEINPG
HisP       VRDKDGQLKVADKNQLRLLRTRLTMVFQHFNLWSHMTVLENVME......
                                Q-loop
```

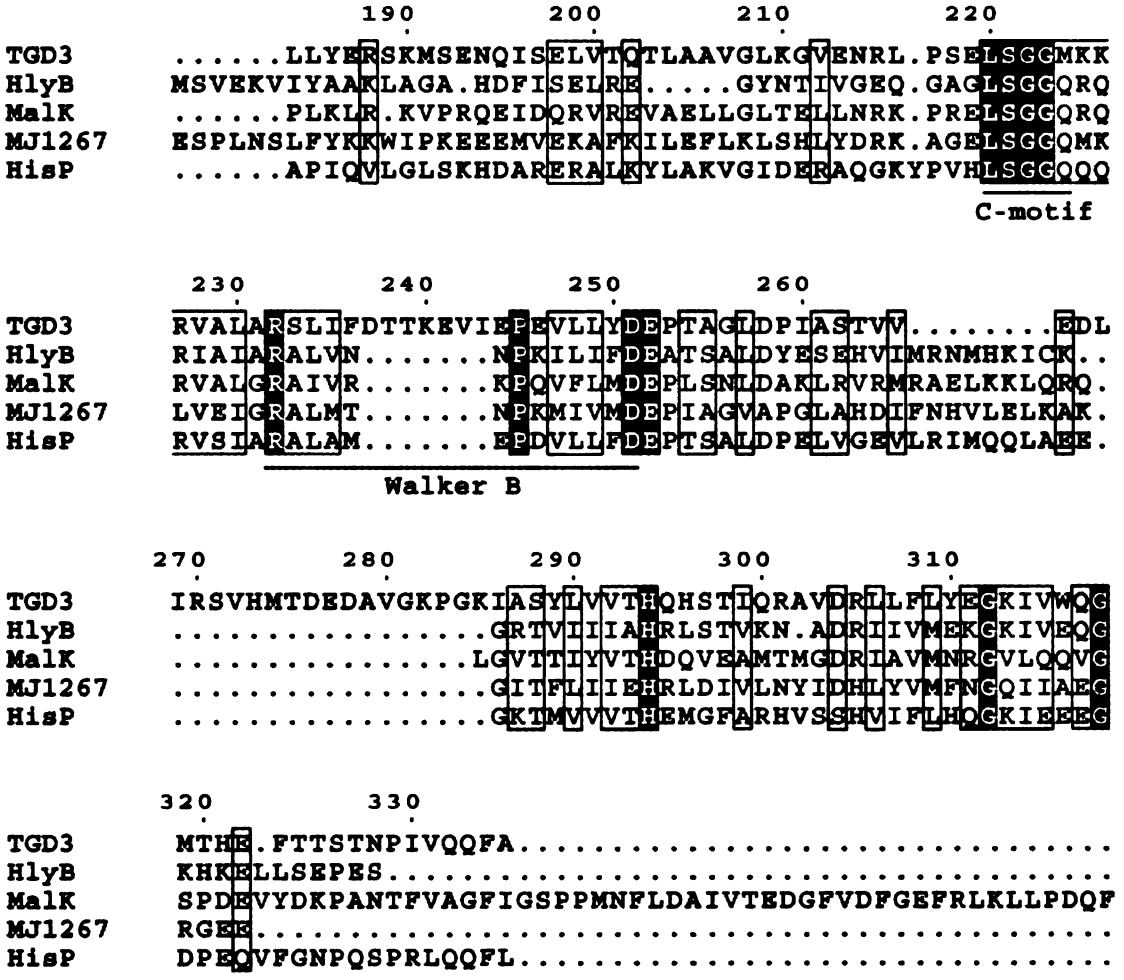
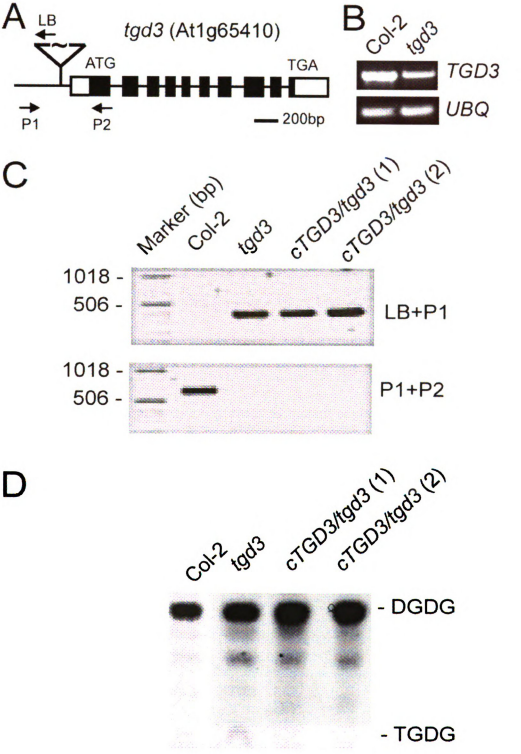


Figure 2.1. (cont'd)

An extensive and possibly complete inventory of transport ATPases and non-intrinsic ABC proteins potentially associated with membrane transporters in *Arabidopsis* has been published (Sanchez-Fernandez *et al.* 2001) 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 (Emanuelsson *et al.* 1999): At1g32500, At1g65410, At3g10670, At4g04770, At4g33460, At5g09930, At5g14100, At5g60790, At5g64840. For seven (listed under "Experimental Procedures") of these candidate genes, T-DNA inactivation lines (Alonso *et al.* 2003) 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 (Figure 2.2A) showed accumulation of the oligogalactolipid (Figures 2.2D and 2.3B). This mutant was clearly leaky as the abundance of the respective mRNA estimated by semi-quantitative reverse transcriptase-PCR was only partially reduced in the T-DNA line (Figure 2.2B). 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 2.2C and 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) (Sanchez-Fernandez *et al.* 2001) 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 (Altschul *et al.* 1997), ABC protein-encoding open reading frames adjacent to putative *TGD1/2* homologs provided some of the best hits, with putative TGD3 homologs from Cyanobacteria, *e.g. Synechococcus* (Figure 2.1A), showing 52% identity and the *E. coli* homolog 32% identity.

Figure 2.2. 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*, semiquantitative reverse transcriptase-PCR analysis of *TGD3*mRNAlevels 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, and 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.


A
LB
tgd3 (At1g65410)
ATG
TGA
P1
P2
200bp
B
Col-2
tgd3
TGD3
UBQ
C
Marker (bp)
Col-2
tgd3
cTGD3/tgd3 (1)
cTGD3/tgd3 (2)
1018 -
506 -
LB+P1
1018 -
506 -
P1+P2
D
Col-2
tgd3
cTGD3/tgd3 (1)
cTGD3/tgd3 (2)
- DGDG
- TGDG

2.4.2 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 (Xu *et al.* 2005) 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 (Figure 2.3A) and polar lipids (Figure 2.3B). The *tgd3* mutant extracts contained a lipid co-migrating with triacylglycerol (Figure 2.3A), previously identified in the *tgd1* mutant (Xu *et al.* 2005) and shown also to accumulate in *tgd2* mutant extracts (Awai *et al.* 2006). 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 (Figure 2.3B). This lipid co-chromatographed with authentic trigalactosyldiacylglycerol (TGDG) accumulating in *tgd1* and *tgd2*.

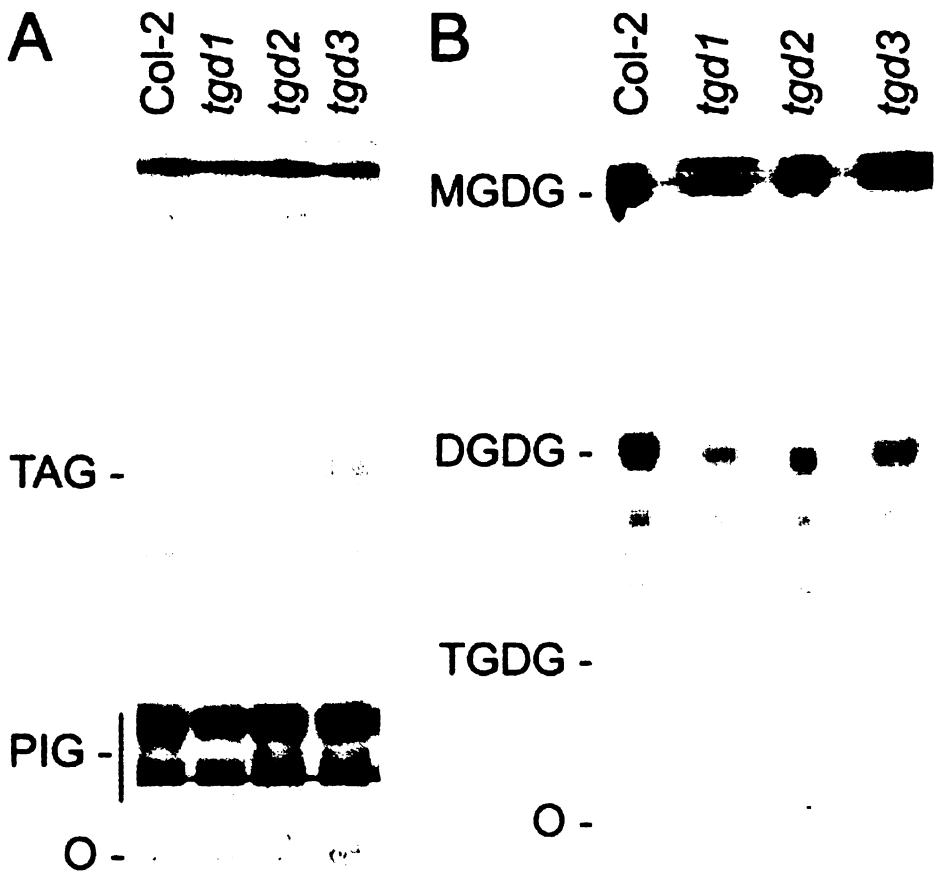
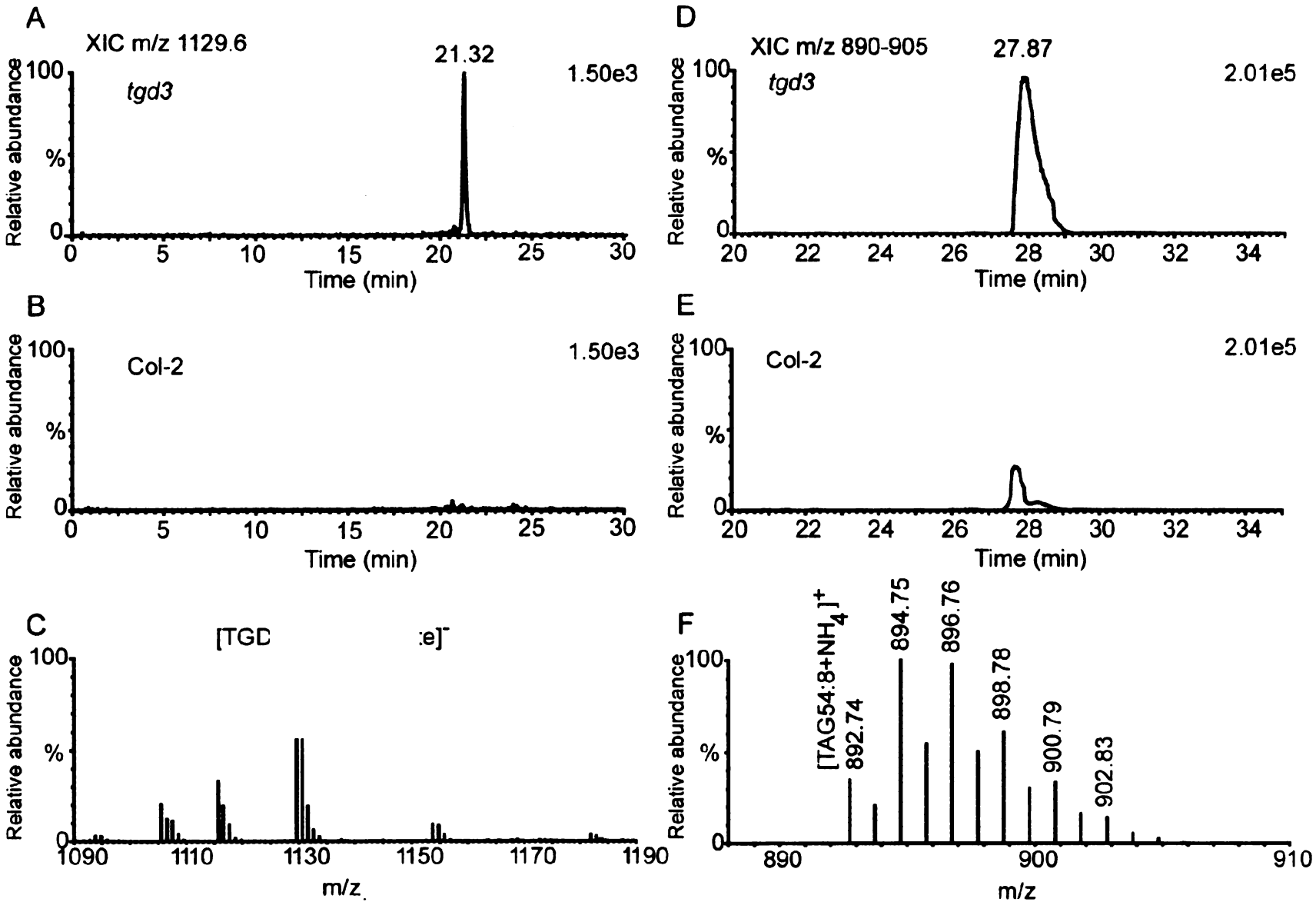


Figure 2.3. Lipid phenotype of the *tgd3* mutant compared with the *tgd1*, *tgd2* mutant, and Col-2 wild type. Total lipids were extracted from 4-weekold seedlings grown on Murashige-Skoog agar plates containing 1% sucrose and separated by thin-layer chromatography. *A*, thin-layer chromatogram of neutral 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. *O*, origin; *PIG*, pigments; *TAG*, triacylglycerol.

Further confirmation of the identities of TGDG and triacylglycerol lipids was provided by liquid chromatography/MS analyses of leaf extracts of Col-2 wild type and *tgd3* mutants (Figure 2.4, A–C). Extracted ion chromatograms 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 collision-induced dissociation 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 $[M+NH_4]^+$ of triacylglycerols with 54 carbons and a total number of double bonds ranging from 3 to 8 (Figure 2.4F). 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 (Figure 2.4, D and E). Ion abundances for both TGDG and triacylglycerol lipid peaks were consistent with quantitative analysis of fatty acid methyl esters by gas chromatography coupled to flame ionization detection.

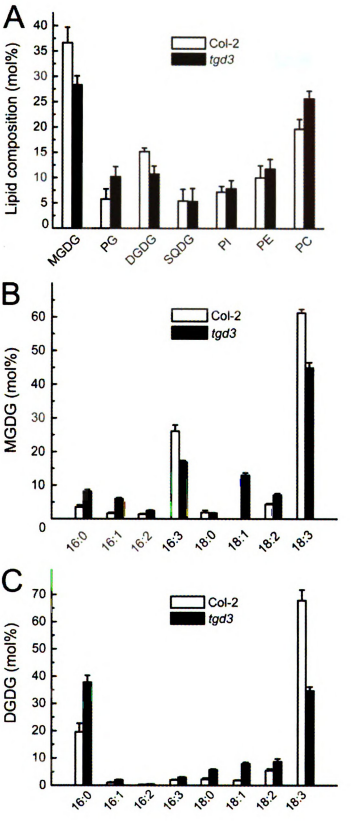
Figure 2.4. Liquid chromatography/MS analyses of lipid extracts of tgd3 mutant and Col-2 wild type Arabidopsis leaves. Negative mode electrospray extracted ion chromatograms (*XIC*) for *m/z* 1129.6, the $[M+acetate]^-$ ion for TGDG 34:6, for extracts of *tgd3* mutant (*A*) and Col-2 wild type (*B*) 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 extracted ion chromatograms over the range of *m/z* 890–905 reflect the abundance of triacylglycerols with 54 carbons and various numbers of double bonds for *tgd3* mutant (*D*) and Col-2 wild type (*E*) leaves. The summed mass spectrum over the peak eluting at 27.87 min (*F*) shows $[M+NH_4]^+$ peaks corresponding to triacylglycerols with 54 carbons and from 3–8 double bonds.

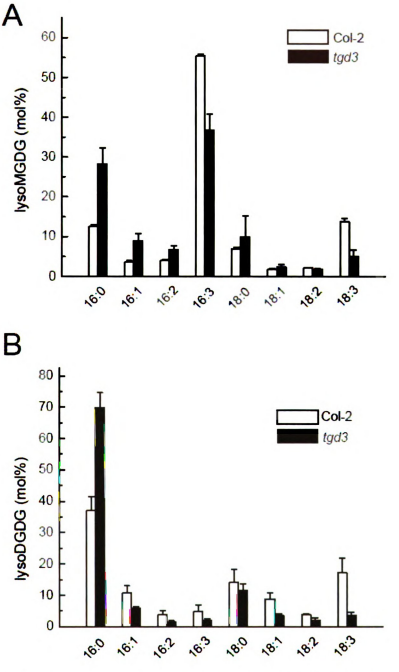

A
XIC m/z 1129.6
21.32
1.50e3
tgd3
Relative abundance
100
%
0
0 5 10 15 20 25 30
Time (min)
B
Col-2
1.50e3
Relative abundance
100
%
0
0 5 10 15 20 25 30
Time (min)
C
[TGD :e]-
Relative abundance
100
%
0
1090 1110 1130 1150 1170 1190
m/z
D
XIC m/z 890-905
27.87
2.01e5
tgd3
Relative abundance
100
%
0
20 22 24 26 28 30 32 34
Time (min)
E
Col-2
2.01e5
Relative abundance
100
%
0
20 22 24 26 28 30 32 34
Time (min)
F
[TAG54:8+NH4]+
892.74
894.75
896.76
898.78
900.79
902.83
Relative abundance
100
%
0
890 900 910
m/z

The overall lipid composition of the *tgd3* mutant differed from that of the wild type as shown in Figure 2.5A. Most notably, the relative amounts of the major chloroplast lipids MGDG and DGDG were decreased, whereas the presumed precursor of galactolipids derived from the ER pathway, phosphatidylcholine (Roughan and Slack 1982), was more abundant. Analysis of the fatty acid composition of MGDG and DGDG revealed distinct changes in the fatty acid profiles in *tgd3* as compared with the wild type (Figure 2.5, B and 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 (Awai *et al.* 2006). In general, the *tgd* mutants impaired in the ER pathway have an increased 16-carbon-to-18-carbon fatty acid ratio (Awai *et al.* 2006) in their galactolipids. This is particularly visible for DGDG, which is to a large extent derived from the ER pathway (Heinz and Roughan 1983). Accordingly, the 16-carbon-to-18-carbon ratio for the digalactolipid increased from 0.29 to 0.76 in the *tgd3* mutant (Figure 2.5C). 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 (Heinz and Roughan 1983). Positional analysis using *Rhizopus* lipase (Supplemental figure 2.1) confirmed an increase in 16-carbon fatty acids in the *sn*-2 position of MGDG and DGDG of the *tgd3* mutant. Overall, *tgd3* has a complex lipid phenotype very similar to *tgd1* and *tgd2* that 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.

Figure 2.5. Polar lipid composition and fatty acid content in the *tgd3* mutant. *A*, 4-week-old Col-2 wild type and *tgd3* mutant seedlings grown on the same Murashige-Skoog agar plate were analyzed. Five replicates were averaged, and the S.E. are shown. *A*, polar lipid composition (relative mol%) determined by quantification of fatty acid methyl esters derived from individual lipids. Fatty acid composition of MGDG (*B*) and DGDG (*C*). Fatty acids are indicated with the number of carbons:number of double bonds. *PE*, phosphatidylethanolamine; *PG*, phosphatidylglycerol; *PI*, phosphatidylinositol; *SQDG*, sulfoquinovosyldiacylglycerol.


A
Lipid composition (mol%)
Col-2
tgd3
MGDG
PG
DGDG
SQDG
PI
PE
PC
B
MGDG (mol%)
16:0
16:1
16:2
16:3
18:0
18:1
18:2
18:3
C
DGDG (mol%)

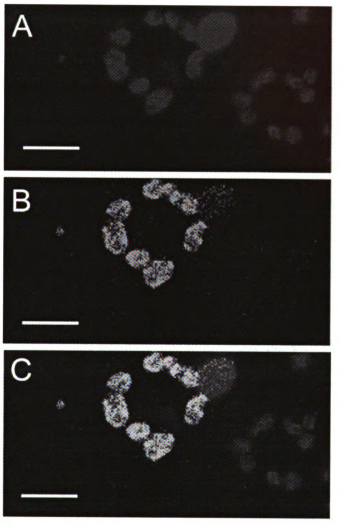


Supplemental Figure 2.1. Fatty acid composition of lyso-MGDG (A) and lyso-DGDG (B) in the *tgd3* mutant and Col 2 wild type lines. Relative abundance (mol%) of 16 and 18-carbon fatty acids at the glycerol sn-2 position of MGDG and DGDG were determined from the lyso derivatives after digestion of the lipids with *Rhizopus sp.* lipase. Values represent the means of three measurements and the standard error is shown.

2.4.3 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 and transiently expressed in tobacco. The result shown in Figure 2.6, A–C, shows the green fluorescent protein fluorescence associated with chloroplasts, which are identified by their chlorophyll fluorescence. To further narrow the location of TGD3, *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 (Cline *et al.* 1984), and trypsin, which can penetrate the outer envelope membrane but not the inner (Jackson *et al.* 1998). The result of this analysis is shown in Figure 2.6D. 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.

Figure 2.6. 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-green fluorescent protein 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 theTGD3preprotein (*pP*) is shown as well. The mature protein (*mP*) after removal of the transit peptide is indicated.

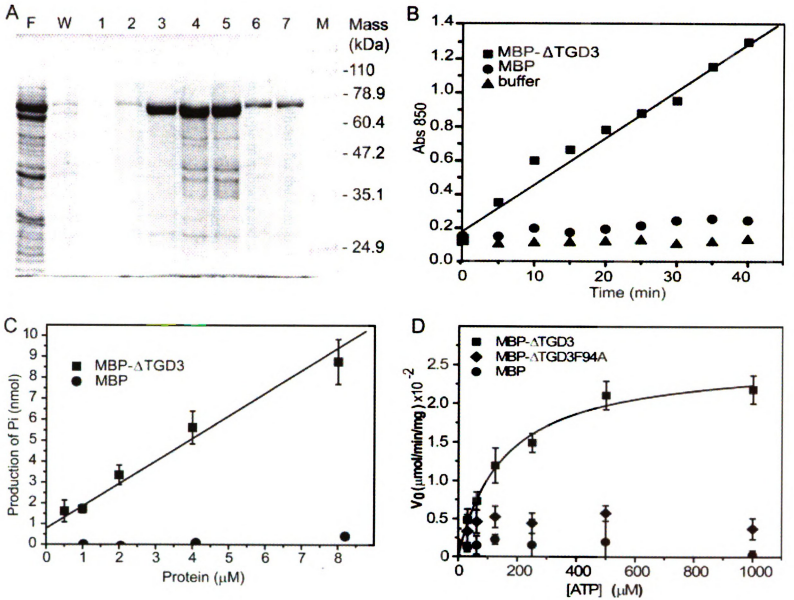

A
B
C
D
- + + - TL
+ - - - TR
TP - - + - TX
- pP
- mP

2.4.4 Recombinant TGD3 protein has ATPase activity

The TGD3 protein contains motifs characteristic for other small ATPases associated with bacterial membrane transporters (Ambudkar *et al.* 2006, Davidson and Chen 2004) designated Walker-A, Walker-B, A-loop, and Q-loop (Figure 2.1B). To test the biochemical function of TGD3, an N-terminal 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 *E. coli* maltose binding protein open reading frame and expressed in *E. coli*. Of several expression systems tested, this was the only providing soluble TGD3 protein. Purification of the recombinant protein designated MBP-ΔTGD3 on an amylose column is shown in Figure 2.7A. The fusion protein was highly purified (estimated ~90%) and migrated as a 78-kDa 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-ΔTGD3F94A). 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 (Zhao and Chang 2004). Under standard assay conditions as defined under "Experimental Procedures," the reaction was linear for more than 40 min (Figure 2.7B). Furthermore, the reaction velocity increased linearly with MBP-ΔTGD3 protein concentration in the range tested (Figure 2.7C). Varying the concentration of ATP, a classic Michaelis-Menten hyperbolic substrate velocity function was observed for the MBP-ΔTGD3 protein (Figure 2.7D). Estimated

kinetic constants based on this experiment were 151.7 μM for the K_m and 0.026 μmol $min^{-1}mg^{-1}$ for V_{max}. These values are in the same range as previously reported for ATP hydrolysis by other ABC transporters (Schneider and Hunke 1998). The MBP-ΔTGD3F94A mutant protein had less than 20% residual activity insufficient for accurate determination of the kinetic constants (Figure 2.7D). The maltose-binding protein had no activity.

Figure 2.7. Purification and ATPase activity of recombinant TGD3 protein. The TGD3 protein is N terminal 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 Coomassie Blue staining. The E3-fraction contained ~5 µg of protein. *B*, time-course of ATPase activity. The assay was conducted at a protein concentration of ~8 µM and final ATP concentration of 1mM at 37 °C. Buffer solutions containing no protein were included as background control. Maltose-binding protein treated identical to MBP-_TGD3 was included to test for endogenous ATPase activity. Relative activity is shown as absorbance readings at 850 nm. *C*, production of inorganic phosphate as a function of protein concentration at 1mM 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 S.E. 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 S.E. are shown.


A
F W 1 2 3 4 5 6 7 M
Mass (kDa)
-110
-78.9
-60.4
-47.2
-35.1
-24.9
B
MBP-ΔTGD3
MBP
buffer
Abs 850
Time (min)
C
MBP-ΔTGD3
MBP
Production of Pi (nmol)
Protein (μM)
D
MBP-ΔTGD3
MBP-ΔTGD3F94A
MBP
V0 (μmol/min/mg) x10-2
[ATP] (μM)

2.5. Discussion

The previous identification of TGD1 and TGD2 proteins and the characterization of the respective mutants (Awai *et al.* 2006, Xu *et al.* 2003, Xu *et al.* 2005) 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* (Doerrler *et al.* 2001, Doerrler and Raetz 2002) consist of a dimer of a bifunctional protein, with a membrane-spanning permease domain and a second ATP binding domain. In the 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 2.8. 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 (Figure 2.1A). 2) Of the 26 genes of *Arabidopsis* encoding small ATP-binding proteins without membrane-spanning domains (Sanchez-Fernandez *et al.* 2001), 9 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 a 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 a 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 (Doerrler *et al.* 2001) and the observation that specific lipids stimulate the ATPase activity of MsbA *in vitro* (Doerrler and Raetz 2002). 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. As for the TGD complex of *Arabidopsis*, the precise function of MsbA still remains under debate, especially since a mutant of *Neisseria meningitis* lacking the MsbA ortholog remains capable of exporting phospholipid building blocks of the outer membrane (Tefsen *et al.* 2005). 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 (Xu *et al.* 2003, Xu *et al.* 2005), 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-green fluorescent protein fusion protein was not (Awai *et al.* 2006). 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 (Figure 2.6B). 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 2.8, thereby confirming 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.

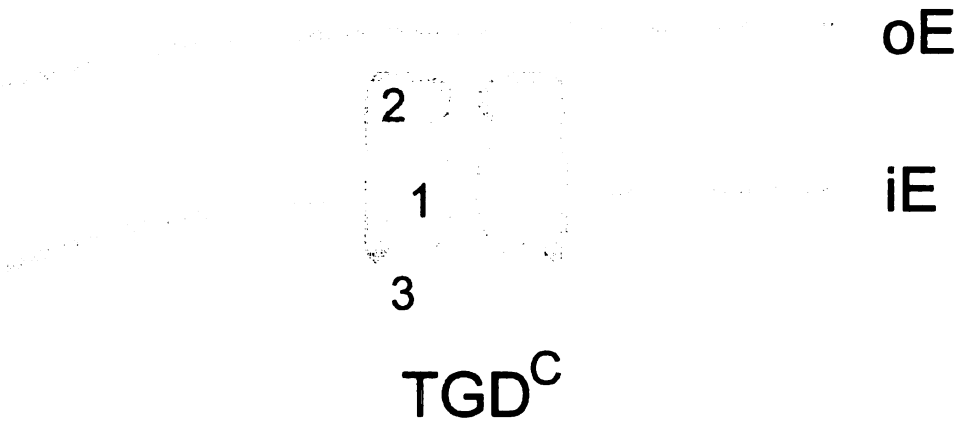


Figure 2.8. Model of the proposed TGD lipid transporter complex. A dimeric composition is shown with numbers corresponding to the TGD1, TGD2, and TGD3 proteins. Outer (*oE*) and inner chloroplast envelope membranes (*iE*) are indicated.

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Chapter 3

Germination, growth and lipid analysis of Arabidopsis plants disrupted in *TGD3*

3.1. Abstract

Galactoglycerolipids are predominant in the chloroplasts of seed plants. They can either be synthesized *de novo* in the plastids or transported to endoplasmic reticulum and later return to the plastids and enter the galactolipid biosynthesis pathway. Arabidopsis mutants that are disrupted in the incorporation of endoplasmic reticulum-derived lipid precursors into thylakoid lipids are available. Three genes affected in these mutants, *trigalactosyldiacylglycerol 1 (TGD1), TGD2*, and *TGD3*, encode the permease, substrate binding component and ATP binding domain, respectively, of a proposed phosphatidic acid (PtdOH) translocator at the inner chloroplast envelope membrane. The TGD3 protein shows basal ATPase activity when expressed as a maltose-binding protein fusion and is localized inside the chloroplast beyond the inner chloroplast envelope membrane. Similar to the *tgd1* and *tgd2* mutants, a *tgd3* mutant carrying a T-DNA insertion just 5' of the *TGD3* coding region (*tgd3-1*) also accumulates triacylglycerols and trigalactolipids. However, the *TGD3* gene expression level is only slightly reduced in this *tgd3-1* mutant. Mutants fully disrupted in *TGD3* were unavailable to analysis at the time of the study. By utilizing RNA silencing technique, we generated Arabidopsis mutant plants with more severely disrupted TGD3 function. The phenotype of these RNAi mutant plants are nearly identical to *tgd3-1*, and do not show the expected severe growth defect and embryo lethality seen in other *tgd* mutants, raising the possibility of another ATPase associated with the proposed TGD complex.

3.2. Introduction

The chloroplast harbors the most extensive membrane system in nature- the thylakoid. Different from most organisms and organelles, the major lipid classes in the chloroplasts are galactoglycerolipids with one (monogalactosyldiacylglycerol, MGDG) or two (digalactosyldiacylglycerol, DGDG) galactose moieties (Dörmann and Benning 2002). MGD1 and DGD1 are the two major galactosyltransferases required for galactoglycerol lipids final assembly from diacylglycerol (DAG) and UDP-galactose (Benning and Ohta 2005). MGD1 is located at the outside of the inner chloroplast envelope membrane (Xu *et al.* 2005) and responsible for the bulk of MGDG synthesis (Jarvis *et al.* 2000), whereas DGD1 is located at the outside of the outer chloroplast envelope membrane (Froehlich *et al.* 2001) and plays a major role in the bulk of DGDG synthesis (Dörmann *et al.* 1999).

The original location of lipid biosynthesis and final destinations of the lipids are often different, a fact which requires substantial interorganellar lipid trafficking within the plant cells (Benning *et al.* 2006). For example, the diacylglycerol moiety of the galactoglycerolipids is either synthesized *de novo* in the plastid or is assembled at the endoplasmic reticulum (ER) and imported into the plastid (Roughan and Slack 1982). Previous studies identified a group of *tgd* mutants in Arabidopsis that are disrupted in the ER-pathway so that most of the galactolipids in these mutants are of plastid origin (Awai *et al.* 2006, Lu *et al.* 2007, Xu *et al.* 2003, Xu *et al.* 2005). These mutants have nearly identical lipid phenotypes in all aspects tested such as the accumulation of oligogalactolipids and triacylglycerols (Awai *et al.* 2006, Lu *et al.* 2007, Xu *et al.* 2003, Xu *et al.* 2005). Four *TGD* genes have been identified so far. Their bacterial orthologs are

often organized in operons (Lu *et al.* 2007). Taken together, these facts strongly suggest that the *TGD* gene products are involved in a common biochemical or cell biological process. Our current hypothesis is that the *TGD1, TGD2* and *TGD3* gene encodes a protein similar to the permease component, membrane-tethered substrate-binding protein and the ATP binding domain of a bacterial ATP binding cassette (ABC) transporter (Lu *et al.* 2007), respectively. The specific binding of phosphatidic acid (PtdOH) to TGD2 suggests that PtdOH is the substrate transported by this TGD1-3 transporter complex located at the inner chloroplast envelope (Awai *et al.* 2006).

In chapter 2, I detailed the identification and biochemical characterization of TGD3, the small ATPase protein proposed to be associated with TGD1 and TGD2 (Lu *et al.* 2007). The TGD3 protein shows basal ATPase activity when expressed as a maltose-binding protein fusion and is localized inside the chloroplast beyond the inner chloroplast envelope membrane (Lu *et al.* 2007). It is important to notice that all three *tgd* mutant alleles (*tgd1-1*, *tgd2-1*, *tgd3-1*) analyzed thus far are leaky, leading to attenuated phenotypes. More severe impairment of the system as previously shown for *TGD1* causes embryo-lethality (Xu *et al.* 2005), making fully gene-disrupted mutants currently unavailable to analysis. An Arabidopsis mutant carrying a T-DNA insertion in the promoter region of *TGD3* (*tgd3-1*) does not have obvious growth defects as compared to wild-type Col-2, possibly due to the leakiness of *TGD3* gene expression. Therefore, I utilized RNA interference (RNAi) technique to silence *TGD3* expression and generate transgenic Arabidopsis plants disrupted more severely in the TGD3 function. Here, I performed experiments to systematically characterize germination, root growth and lipid phenotypes of these *tgd3* mutants.

3.3. Experimental Procedures

3.3.1. Plant materials and growth conditions

Arabidopsis thaliana wild type plants were of the ecotype Columbia-2 (Col-2). The *tgd3-1* T-DNA insertion lines (SALK_040335, At1g65410) were obtained from the Arabidopsis Biological Resource Center at Ohio State University. The *TGD3* transgenic lines were generated as described below. Homozygous mutant lines were screened and used for all experiments. All seeds were surface-sterilized with 20% bleach, 0.05% TritonX-100 for 10 min and were rinsed 5 times in sterile water before grown on 0.8% (w/v) agar-solidified Murashige-Skoog medium (Murashige and Skoog 1962) supplemented with 0 or 1% (w/v) sucrose when appropriate.

For germination and seedling growth assay, wild-type and mutant seeds were first stratified at 4°C in the dark for 3 days prior to being transferred into an incubator. The incubator has a photon flux density of 60–80 μmol m^{-2} sec^{-1}, a light period of 16 h (22°C), and a dark period of 8 h (18°C). After 10 days, seedlings were either transferred to soil or to fresh agar plates. Soil grown plants were put into a 16h photoperiod with a day temperature of 22°C and a night temperature of 20°C at a photon flux density of 100–120 μmol m^{-2} sec^{-1}. Wild-type and mutant seedlings of similar developmental stage were used for lipid analysis and growth measurement.

3.3.2. Lipid and fatty acid analysis

Total lipids were extracted by vigorously shaking leaf tissue for 5 min in 300 μL of methanol/chloroform/formic acid (2:1:0.1, v/v). Then 150 μL of 1 M KCl, 0.2 M

H_3PO_4 was added and the tubes were vortexed before centrifuged at 16,000 g for 5 min. The organic phase (upper phase) was loaded onto a treated (soaked in 0.15 M $(NH_4)_2SO_4$ and dried, then baked at 120°C for 2 hrs) silica-60 TLC plate (Si250PA; Mallinckrodt Baker, Phillipsburg, NJ). For lipid quantification, a solvent system of acetone/toluene/water (91:30:7, v/v) was used and lipids were visualized with iodine vapor. Distinct bands were then scraped off the plate and lipid composition was determined by fatty acid methyl ester (FAME) analysis and gas chromatography as described previously (Dörmann *et al.* 1995, Rossak *et al.* 1997)

3.3.3. Root elongation assays

All seeds used were produced from plants grown in identical conditions and were of the same age. When appropriate, 0 or 1% sucrose was added to the medium prior to being autoclaved. Seeds were sown in a straight line and the agar plates were arranged vertically. Plant growth were recorded by a SPOT CCD microscope digital camera (Diagnostic instruments, Sterling Heights, MI), and root length was measured by software Spot Advanced (version 4.0.2) every 24 h. The same set of agar plates was used throughout the experiment. Ten individual plants were measured and values were averaged.

3.3.4. Generation and analysis of TGD3 RNAi plants

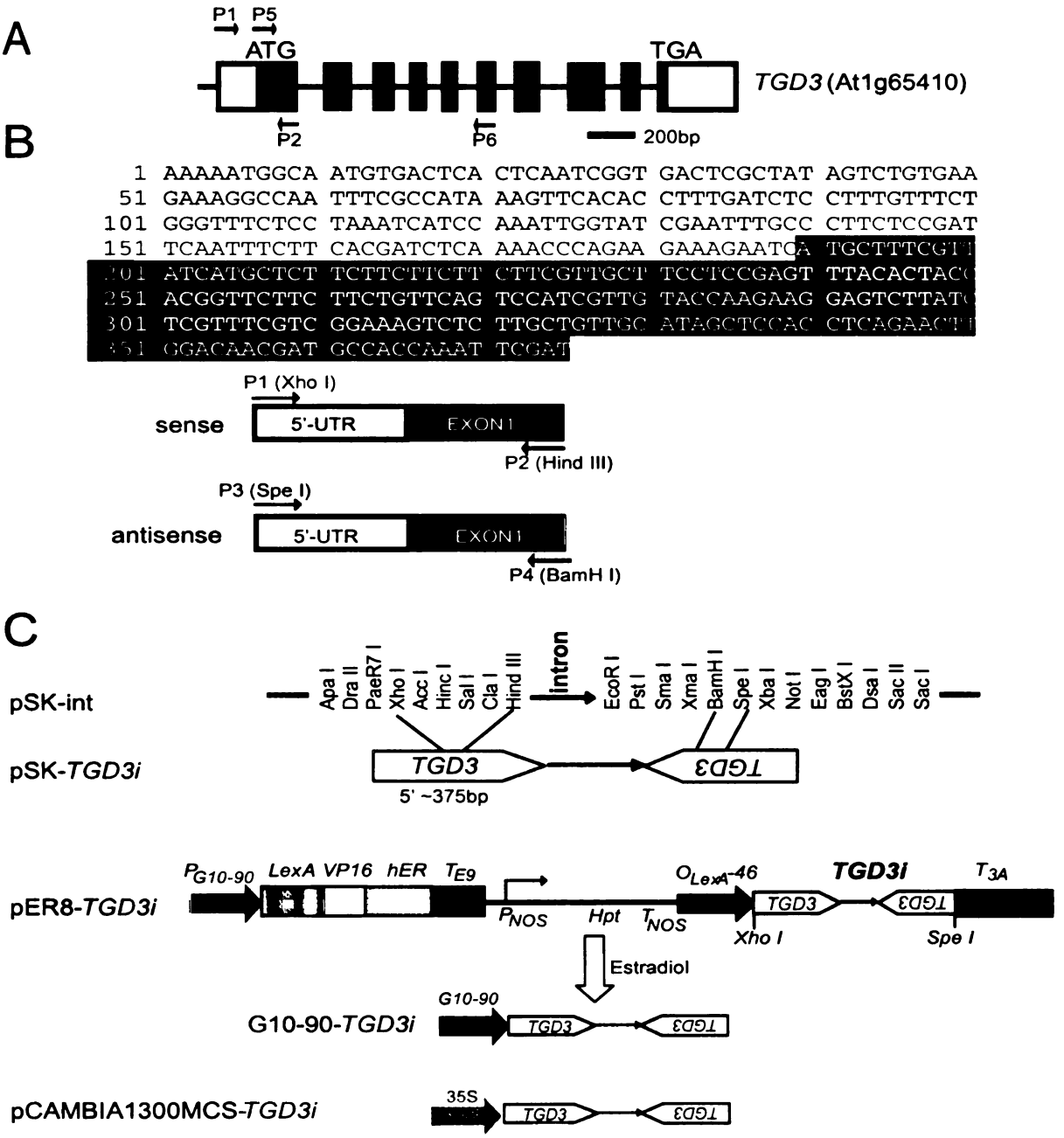
For the generation of a TGD3 RNA interference (RNAi) line, 5'UTR and the first exon region of TGD3 gene sequence (~375bp) were amplified by PCR using the primers 5'- CCGCTCGAGAAAAATGGCAATGTGAC -3' and 5'-CGCAAGCTTATCGAATT-

TGGTGGCATC-3' (XhoI and HindIII; for amplification of sense fragment) and 5'-CG-GACTAGTAAAAATGGCAATGTGACTCAC-3' and 5'-ATTGGATCCATCGAATTT-GGTGGCATC-3'(SpeI and BamHI; for amplification of antisense fragment). The underlined sequences indicate the restriction enzyme sites. The resulting fragment was cloned into the intermediate vector pSK-int (Guo *et al.* 2003) in sense and antisense orientations separated by an actin intron using the corresponding restriction enzyme sites on the vector. Subsequently, the entire sense-intron-antisense cassette was cloned into the hormone-inducible expression vector pER8 (Zuo *et al.* 2000). Alternatively, the pSK-int vector harboring TGD3 RNAi cassette (pSK-TGD3i) was cut by XhoI and SacI and inserted into the SalI and SacI sites of pCambia1300MCS (Xu *et al.* 2005) followed by transformation into *Agrobacterium*. The details of construction of these plasmids are illustrated in Figure 3.1. Plant transformation was achieved by the floral dip method (Clough and Bent 1998). Transgenic plants were selected by MS medium containing 25 μg/ml of Hygromycin B. For an estimation of *TGD3* transcript levels by semi-quantitative reverse transcriptase-PCR, RNA was isolated from Col-2 wild-type and *TGD3 RNAi* mutant plants, and reverse transcription was done using oligo$(dT)_{12-18}$ primers (Invitrogen). The *TGD3* specific primers used for the PCR reaction were: 5'-AC-GGTACCATGCTTTCGTTATCATGCTC-3' and 5'-AGGTTTGTGTCACAAGCTCA-3'. Meanwhile, the abundance of actin (*ACT1*) was tested for control purposes. The following *ACT1* specific primers were used: 5'-CCTCTCGATTTCAGGTAGAAGAAA-ATG-3' and 5'-CCATCTCCAGAGTCGAGCACAATA-3'.

To evaluate the function of the inducible *TGD3i*-pER8 system, T2 seeds from independent transgenic lines were germinated on the regular MS medium containing

Hygromycin B. After two weeks, resistant plants were transferred into a liquid MS solution containing inducer β-estradiol. Samples were collected and subject to TLC analysis after 6 days induction.

Figure 3.1. Generation of *TGD3i* constructs. *A,* linear representation of the *TGD3* gene (At1g65410). The coding region is shown as black boxes. *B*, nucleic tide sequences from the *TGD3* gene 5'-UTR and the first exon. Exon was shown as black box.PCR primers with restriction enzyme sites used to amplify and clone sense and antisense fragments are indicated. *C*, schematic diagrams of the inducible and constitutive *TGD3i* constructs. pSK-int, an intermediate vector contains actin 11 intron (ATU27981, nt 1957-2111) with multiple cloning sites on both arms of the intron. DNA fragments encoding sense and antisense RNA (from *B*) were cloned into pSK-int at 5' and 3' arms of the intron in appropriate restriction sites indicated by solid lines, resulting pSK-*TGD3i*. pER8-*TGD3i*, the inducible pER8-*TGD3i* construct contains a chimeric transactivator with the regulator domain of an estrogen receptor (see Zuo *et al.*, 2000 for details of pER8 vector). *TGD3i*, *TGD3* DNA sequences encoding the intron-containing inverted-repeats. pCAMBIA1300MCS-*TGD3i*, a constitutive *TGD3i* construct with a fragment of *TGD3i* cloned into the binary vector pCAMBIA1300MCS.


A
P1 P5
ATG
TGA
TGD3 (At1g65410)
P2
P6
200bp
B
1 AAAAATGGCA ATGTGACTCA CTCAATCGGT GACTCGCTAT AGTCTGTGAA
51 GAAAGGCCAA TTTCGCCATA AAGTTCACAC CTTTGATCTC CTTTGTTTCT
101 GGGTTTCTCC TAAATCATCC AAATTGGTAT CGAATTTGCC CTTCTCCGAT
151 TCAATTTCTT CACGATCTCA AAACCCAGAA GAAAGAATCA TGCTTTCGTT
201 ATCATGCTCT TCTTCTTCTT CTTCGTTGCT TCCTCCGAGT TTACACTACC
251 ACGGTTCTTC TTCTGTTCAG TCCATCGTTG TACCAAGAAG GAGTCTTATC
301 TCGTTTCGTC GGAAAGTCTC TTGCTGTTGC ATAGCTCCAC CTCAGAACTT
351 GGACAACGAT GCCACCAAAT TCGAT
P1 (Xho I)
sense
5'-UTR
EXON1
P2 (Hind III)
P3 (Spe I)
antisense
5'-UTR
EXON1
P4 (BamH I)
C
pSK-int
Apa I
Dra II
PaeR7 I
Xho I
Acc I
Hinc I
Sal I
Cla I
Hind III
Intron
EcoR I
Pst I
Sma I
Xma I
BamH I
Spe I
Xba I
Not I
Eag I
BstX I
Dsa I
Sac II
Sac I
pSK-TGD3i
TGD3
TGD3
5' ~375bp
pER8-TGD3i
P G10-90
LexA VP16 hER
T E9
P NOS
Hpt
T NOS
O LexA-46
TGD3i
T 3A
TGD3
TGD3
Xho I
Spe I
Estradiol
G10-90-TGD3i
G10-90
TGD3
TGD3
pCAMBIA1300MCS-TGD3i
35S
TGD3
TGD3

3.3.5. Dominant-negative mutation analysis

Coding region of *TGD3* was amplified by reverse transcriptase-PCR (standard conditions) from mRNA preparations using Plant RNeasy kit (Qiagen, Valencia, CA) with the primers 5'-ACGGTACCATGCTTTCGTTATCATGCTC-3' and 5'-CTGG-TACCCTAGTATCTGATTGGTCCAT-3'. The PCR products were ligated into pGEM-Teasy (Promega, Madison, WI) and sequenced. The resulting plasmids served as template to create F94A mutation by site-directed mutagenesis strategy. The following mutagenesis primers were used: 5'-GTAGAGATGTCTATAAATCGGCGGGGGAGA-AACATATCTTG-3' and 5'-CAAGATATGTTTCTCCCCCGCCGATTTATAGACAT-CTCTAC-3'. The underlined sequences are codons for the mutated residue (Alanine). After digestion with SalI, the mutant *TGD3F94A* fragment was inserted into pCambia1300MCS (Xu *et al.* 2005) followed by transformation into *Agrobacterium.* Plant transformation was achieved by the floral dip method (Clough and Bent 1998). Transgenic plants were selected by MS medium containing 25 μg/ml of Hygromycin B and analyzed for lipid phenotype as described above.

For semi-quantitative PCR of *TGD3* and *TGD3F94A (tgd3)* transcripts, the following primers were used: *TGD3*, P1: 5'-CCGCTCGAGAAAAATGGCAATGTGA-C-3' and P2: 5'-CGCAAGCTTATCGAATTTGGTGGCATC-3'; *TGD3* and *tgd3*, P5: 5'-CCGGTCGACATGCTTTCGTTATCATG- 3' and P6: 5'-AGGTTTGTGTCACAAGCT-CA-3'; *ACT1,* 5'-CCTCTCGATTTCAGGTAGAAGAAAATG-3' and 5'-CCATCTCC-AGAGTCGAGCACAATA-3'. Primers are shown by arrows in Figure 3.1. Isolation of RNA and reverse transcription were done as described above. Standard PCR conditions were used.

3.3.6. Chlorophyll quantification

Chlorophyll was extracted from leaves and seedlings using 100 volumes (μL/mg) of 80% acetone and was quantified as previously described (Lichtenthaler 1987).

3.4. Results

3.4.1. Oligogalactolipids accumulate in TGD3i leaves

The *TGD3* gene was identified via a reverse-genetics approach. Previously, a T-DNA insertion line (SALK_040335, *tgd3-1*) carrying a T-DNA 5' of the presumed ATG start codon of the *TGD3* gene showed accumulation of oligogalactolipids (Lu *et al.* 2007). The growth of the *tgd3-1* mutant line was only slightly reduced, possibly due to leakiness of the allele.

Because no knockout allele of *TGD3* was readily available, we used an RNA interference (RNAi) approach to enhance the mutant phenotype of *tgd3-1* and thereby demonstrate the leakiness of the *tgd3-1* allele. Two representative RNAi lines are shown in Figure 3.2. The abundance of the *TGD3* mRNA was greatly reduced in these lines (Figure 3.2C), resulting in slightly diminished growth (Figure 3.2A) and obvious accumulation of the oligogalactolipids-TGDG (Figure 3.2B).

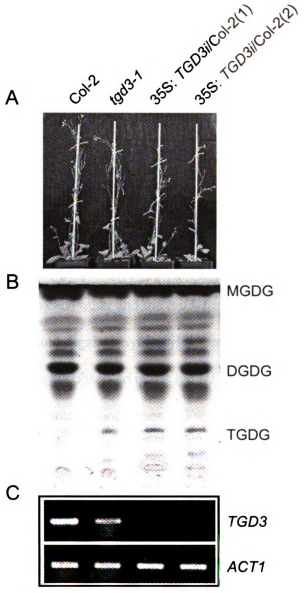


Figure 3.2. Oligogalactolipids accumulate in *TGD3-RNAi* plants. *A*, morphology of 6-week-old plants grown on soil. Wild-type (Col-2), *tgd3-1* T-DNA mutant, and two constitutively expressed *TGD3-RNAi* homozygous lines (35S:*TGD3i*/Col-2) are shown. *B*, lipid phenotype of the different plant lines in *A*. A section of the thin-layer chromatogram stained for glycolipids is shown. *C*, semi-quantitative reverse transcriptase-PCR analysis of *TGD3* mRNAlevels in wild-type (Col-2) and *TGD3-RNAi* plants. The expression of actin (*ACT1*) in the same samples was used as a control.

3.4.2. The phenotype of TGD3i and tgd3-1 are very similar

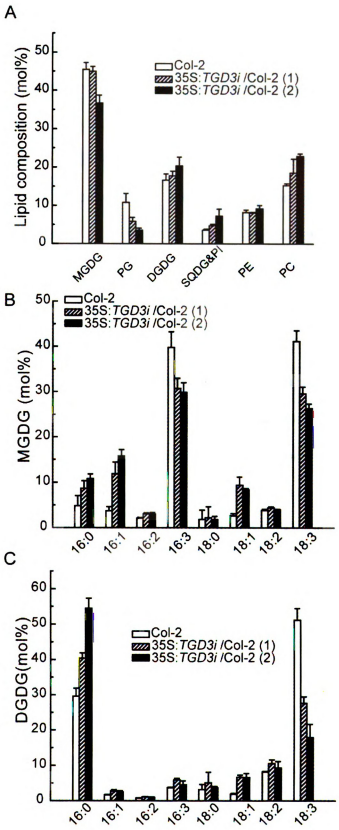
The overall lipid composition of the *tgd3-1* mutant differed from that of the wild type (Lu *et al.* 2007). Most notably, the relative amounts of the major chloroplast lipids MGDG and DGDG were decreased, whereas the presumed precursor of galactolipids derived from the ER pathway (Roughan and Slack 1982), phosphatidylcholine, was more abundant.

Two independent homozygous 35S: *TGD3i*/Col-2 lines were screened and analyzed for their lipid phenotype (Figure 3.3A). The overall lipid compositions of these two lines are very similar to each other and the *tgd3-1* mutant, with the exception of phosphatidylglycerol (PG) and digalactosyldiacylglycerol (DGDG). A decrease in DGDG and increase in PG were observed in the *tgd3-1* mutant, however, an opposite trend was found in *TGD3i* plants. In both lines, PG was decreased about 50-60% while DGDG was increased 10-20%.

Analysis of the fatty acid composition of MGDG and DGDG also revealed distinct changes in the fatty acid profiles in 35S: *TGD3i*/Col-2 plants as compared with the wild type (Figure 3.3 B and C). Similar to *tgd3-1* plants, fatty acids were generally more saturated, and 18:3 fatty acid content was reduced. In general, the *tgd* mutants impaired in the ER pathway have an increased 16-carbon-to-18-carbon fatty acid ratio in their galactolipids (Awai *et al.* 2006). This is particularly visible for DGDG, which is to a large extent derived from the ER pathway (Browse *et al.* 1986). Accordingly, the 16-carbon-to-18-carbon ratio for the digalactolipid increased 2.6 fold in the *tgd3-1* mutant and 1.8 and 3 fold for the two 35S: *TGD3i*/Col-2 lines, respectively (Figure 3.3 C). 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 (Heinz and Roughan 1983). Overall, 35S: *TGD3i*/Col-2 plants have a complex fatty acid composition very similar to *tgd3-1* that is consistent with an impairment of the ER pathway of galactolipid biosynthesis.

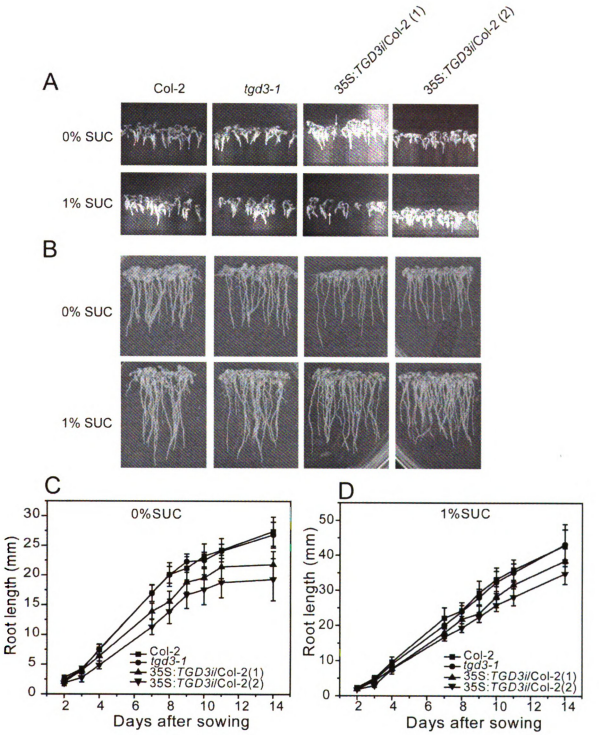
Figure 3.3. Polar lipid composition and fatty acid content in the *tgd3* mutant. 4-week-old seedlings grown on the Murashige-Skoog agar plates from Col-2 wild type, *tgd3-1* T-DNA mutant and two independent homozygous 35S:*TGD3i*/Col-2 lines were analyzed. Five replicates were averaged, and the S.E. was shown. *A*, polar lipid composition (relative mol%) determined by quantification of fatty acid methyl esters derived from individual lipids. *B*, fatty acid composition of MGDG. *C*, fatty acid composition of DGDG. Fatty acids are indicated with the number of carbons:number of double bonds. *MGDG*, monogalactosyldiacylglycerol; *PG*, phosphatidylglycerol; *DGDG*, digalactosyldiacylglycerol; *SQDG*, sulfoquinovosyldiacylglycerol; *PE*, phosphatidylethanolamine; *PI*, phosphatidylinositol; *PC*, phosphatidylcholine.


A
Lipid composition (mol%)
Col-2
35S:TGD3i /Col-2 (1)
35S:TGD3i /Col-2 (2)
MGDG
PG
DGDG
SQDG&PI
PE
PC
B
MGDG (mol%)
16:0
16:1
16:2
16:3
18:0
18:1
18:2
18:3
C
DGDG(mol%)

3.4.3. Impaired growth and development of TGD3i

Chapter 2 describes identification of the *tgd3-1* T-DNA mutant allele and includes some data about lipid phenotype and fatty acid composition. It must be pointed out that during routine growth of the *tgd3-1* plants, no obvious growth defect was observed except that their leaves are slightly pale-green and growth is slightly retarded both in leaves and roots compared to the wild type. After obtaining homozygous 35S: *TGD3i* plants, systematic analyses of the growth phenotype of these transgenic plants defective in TGD3 function were conducted, as described in this chapter. Root elongation assays were performed. As shown in Figure 3.4, *tgd3-1* plants grow almost the same as wild type Col-2, but 35S: *TGD3i* plants grow slower than wild type. In general, *TGD3i* plants grow more slowly on MS medium supplemented with 1% (w/v) sucrose compared to Col-2, and this growth delay is more obvious when plants are grown on MS medium containing no sucrose. The result also indicated that the initiation of root and shoot growth maybe delayed in *TGD3i* mutants as at 2 days after sowing (DAS) mutants are notably smaller and pale with shorter roots compared with Col-2. One possibility is that the germination itself is inhibited in these seedlings.

Figure 3.4. Delayed root growth in *tgd3* mutants. Morphology of 2-day-old (*A*) or 8-day-old (*B*) Col-2 wild type and *tgd3* mutant seedlings gown on MS medium contains no sucrose or 1% (w/v) sucrose. White arrows indicate un-germinated seeds. Root length were measured and shown as a function of days-after-sowing (DAS) for Col-2 wild type, and *tgd3* mutants grown on MS medium contains no sucrose (*C*) or 1% sucrose (*D*). 10 seedlings from each line were measured, values were averaged and SE was shown.


A
Col-2
tgd3-1
35S:TGD3i/Col-2 (1)
35S:TGD3i/Col-2 (2)
0% SUC
1% SUC
B
0% SUC
1% SUC
C
0%SUC
Root length (mm)
Days after sowing
Col-2
tgd3-1
35S:TGD3i/Col-2(1)
35S:TGD3i/Col-2(2)
D
1%SUC
Root length (mm)
Days after sowing
Col-2
tgd3-1
35S:TGD3i/Col-2(1)
35S:TGD3i/Col-2(2)

Germination tests were subsequently conducted to explore the possibility that the *tgd3* mutants germinate later than wild type plants. Figure 3.5 shows the germination rates of wild type, *tgd3-1*, and two RNAi lines using seeds of the same age. As early as 1 DAS, wild type seeds have completely germinated (defined by radicle emergence from the seed coat) and the roots are already elongating on either 0% or 1% sucrose (Figure 3.5). However, there are only about 90% of the seeds germinated for *tgd3-1* and 35S:*TGD3i* line1 sown on MS medium containing no or 1% sucrose and they reach a maximum germination of ~98% after 4DAS. Moreover, only 80% of the 35S: *TGD3i* line 2 seeds germinated and reached its maximum of 95% at 8 DAS, suggesting that this line carries a stronger allele of *TGD3* mutation.

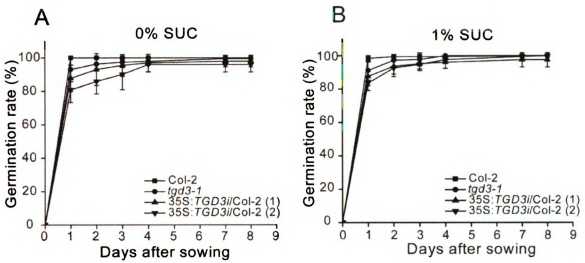


Figure 3.5. Delayed germination in *tgd3* mutants. Col-2 wild type and *tgd3* mutant seeds of the same age were first stratified at 4°C in the dark for 3 days prior to being transferred into an incubator. Germination rates were recorded for seeds sown on 0% (*A*) or 1% (*B*) sucrose containing MS medium.

Another noticeable morphology of *tgd3* mutants is slight chlorosis, namely, pale-green leaves, and early flowering (Figure 3.6, A and B). When grown on agar plates, total chlorophyll content is reduced by 15-30% (Figure 3.6D). Shoot fresh weight was also reduced by ~30% in *tgd3* mutants compared to the Col-2 wild type (Figure 3.6C).

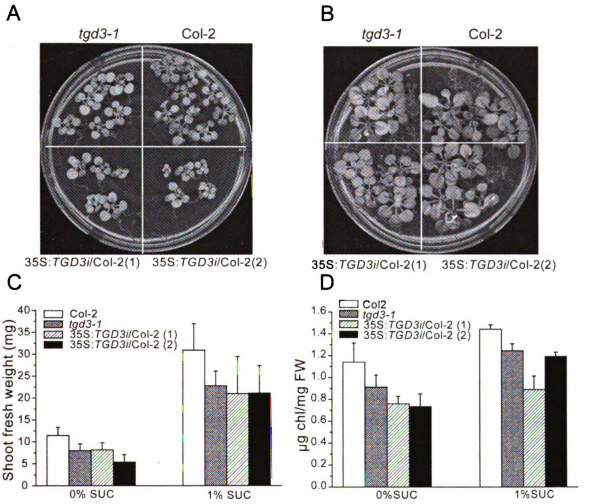


Figure 3.6. Impaired growth and slight chlorosis phenotype of *tgd3* mutants. Pictures were taken for 4-weeks-old seedlings grown on 0% (*A*) or 1% (*B*) sucrose containing MS medium. Shoot fresh weight and chlorophyll content were measured. Values are represented as Mean ± SE from 10 seedlings.

3.4.4. Overexpression of TGD3F94A cDNA in wild type causes a dominant negative effect

The similarities of *tgd1*, *tgd2*, *tgd3* mutant phenotypes and the fact that bacterial orthologs of these Arabidopsis genes are organized in operons suggested that these proteins might form a large complex and act together in the same cellular process. However, due to technical difficulties, we still lack definitive evidence to support this hypothesis. Previous studies on TGD2 demonstrated that expression of the *tgd2-1* cDNA in the wild type recapitulates the *tgd2-1* phenotype (Awai *et al.* 2006).

There are no *tgd3* point mutant alleles available except the T-DNA insertion line at the time of the study, so we set out an experiment to generate a mutant version by site-directed mutagenesis. In Chapter 2, we have reported that mutation of Phenylalanine 94 to Alanine greatly decreased TGD3 ATPase activity *in vitro* when expressed as a MBP-fusion protein in *E.coli* (Lu *et al.* 2007). We introduced this TGD3F94A mutant protein in wild type plants by expressing the mutant cDNA under the control of the 35S-CMV promoter in the wild type background.

Initially, two independent T1 transgenic lines were obtained and further analyzed (Figure 3.7). It is interesting that growth of *35S: TGD3F94A*/Col-2 line 2 was obviously different than wild type and the other *35S: TGD3F94A*/Col-2 line (Figure 3.7A). One possible explanation is that overexpression of the mutant *TGD3F94A* cDNA also randomly inactivates another gene involved in flowering so that a late flowering phenotype was observed. This should be an exception. Further isolation of other independent transgenic lines proved that most of the overexpression lines behave similar to wild-type and *tgd3-1* in growth (data not shown).

Constitutive overexpression of mutant *TGD3F94A* cDNA led to the accumulation of a lipid cochromatographing with the trigalactolipid accumulating in the *tgd3-1* mutant, namely TGDG (Figure 3.7B). Semi-quantitative RT-PCR confirmed that this dominant negative effect was not due to cosuppression of the genomic wild type *TGD3* gene and the *tgd3* (*TGD3F94A*) cDNA expression construct, since mRNA derived from both genes was abundant in the transgenic lines (Figure 3.7C). One explanation of this dominant negative effect is that the TGDF94A mutant protein is impaired in its ATPase activity but can still physically be part of its native protein complex, thereby disrupting overall function of the process involving the complex. This result provided indirect proof that TGD3 is part of a large lipid transfer complex that involves TGD1 and TGD2. An antibody against native TGD3 protein would eventually be required to explore the expression level and function of TGD3 proteins *in vivo*.

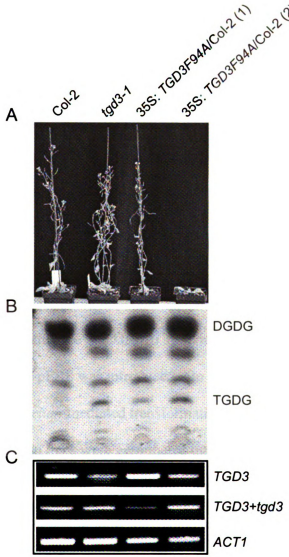


Figure 3.7. Dominant negative effect caused by expression of *TGD3F94A* mutant cDNA in Col-2 wild type. *A*, morphology of 6-week-old plants grown on soil. Wild-type (Col-2), *tgd3-1* T-DNA mutant, and two constitutively expressed *TGD3F94A* T1 lines (35S:*TGD3F94A*/Col-2) are shown. *B*, lipid phenotype of the different plant lines in *A*. A section of the thin-layer chromatogram stained for glycolipids is shown. *C*, semi-quantitative RT-PCR analysis of both *TGD3* and *TGD3F94A (tgd3)* mRNA levels in wild-type (Col-2) and *TGD3F94A* plants. The expression of actin (*ACT 1*) in the same samples was used as a control.

3.4.5. Inducible silencing of TGD3 in Arabidopsis

Potentially, the most effective silencing mediated by intron-containing double-strand RNAi could produce phenotypes resembling those of the null alleles of the target genes. However, if the target gene is required for basic cell function or development, constitutive silencing of the gene may produce detrimental effects or even cause plant lethality resulting in no recovery of transgenic plants for further investigation. To circumvent this problem, an inducible gene silencing approach was recently developed and considered as a effective substitute for constitutive silencing (Guo *et al.* 2003).

In the study of *TGD3* function, parallel to the constitutive overexpression of *TGD3RNAi,* a chemical inducible *TGD3RNAi* system was also constructed and evaluated. The construct *TGD3RNAi*-pER8 was introduced into Arabidopsis wild-type Col-2 background by *Agrobacterium*-mediated transformation (Xu *et al.* 2005). T2 seeds from independent transformation lines were germinated on the regular MS medium containing Hygromycin B. After two weeks, resistant plants were transferred into a liquid MS solution containing β-estradiol. Samples were collected and subject to TLC analysis after 6 days. As shown in Figure 3.8A, 3 out of the 6 lines tested have accumulated TGDG after β-estradiol induction for 6 days at a concentration as low as 2μM. Similarly, 2 out of the 12 lines tested showed accumulation of triacylglyerols (TAG) (see Figure 3.8B). However, in the control wild-type Col-2 plant treated the same way, a faint band migrated at the same position as TGDG was also observed, suggesting that the presence of oligogalalctolipids might be due to stress condition, .e.g. hypoxia (seedlings immersed under liquid solution for 6 days). Other ways of applying the inducer such as spraying the surface of leaves or direct germination of seeds on inducer-containing MS medium did

not lead to positive results. Therefore, the actual effectiveness of this inducible RNAi system is still under question. Compared with 35S promoter driven constitutive *TGD3i*, which leads to clear and unambiguous results, I conclude that in the case of *TGD3*, constitutive RNAi works better.

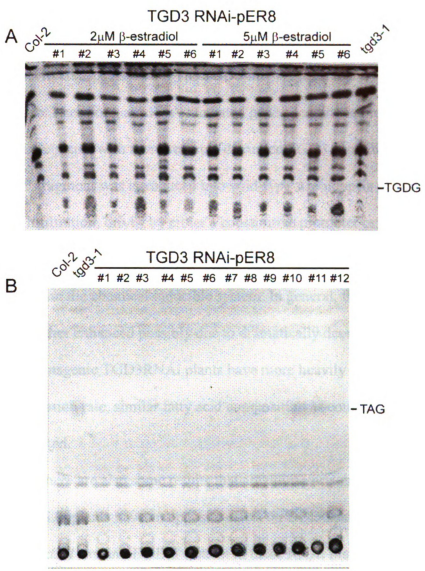


Figure 3.8. Inducible silencing of *TGD3* in Arabidopsis. The construct *TGD3RNAi*-pER8 was introduced into Arabidopsis wild-type Col-2 background by *Agrobacterium*-mediated transformation. T2 seeds from independent transgenic lines were germinated on the regular MS medium containing Hygromycin B. After two weeks, resistant plants were transferred into a liquid MS solution containing 2 or 5μM β-estradiol. *A*, TGDG phenotype of the seedlings after induction. *B*, TAG phenotype of the seedlings after induction with 2μM β-estradiol.

3.5. Discussion

The previously identified *tgd3-1* T-DNA mutant is a much weaker allele than all other *tgd* mutants in all aspects tested. The *TGD3* mRNA level is only partially reduced in the T-DNA line. Therefore, a dsRNA mediated gene silencing approach was used to generate transgenic plants with more severely disrupted *TGD3* gene function. Inverted repeats of *TGD3* fragment was introduced into wild-type Arabidopsis plants mediated by *Agrobacterium* infiltration, driven by either a constitutive promoter or a chemical inducible promoter. The results demonstrated that constitutive expression system works more effectively than the chemical inducible system. In general, the *tgd3-1* mutant phenotype was further enhanced possibly due to dramatically decreased mRNA expression. The transgenic TGD3RNAi plants have more heavily impaired root growth and slower germination rate, similar fatty acid composition as compared to *tgd3-1*, all of which are as expected.

However, there are also some results that are uninterpretable at this stage. For example, TGD3RNAi plants have an opposite effect on DGDG and PG levels as compared to the *tgd3-1* mutant. DGDG content in these RNAi plants is slightly increased whereas the PG content is greatly decreased (Figure 3.3). This might be due to low efficiency or specificity of RNA silencing mediated by dsRNA that possibly affects other genes (see below).

Moreover, the growth phenotype of *tgd3* plants also differs from *tgd1* and *tgd2*. Previous studies showed that ~50% of the seeds from the homozygous *tgd1-1* allele are aborted during seed development. This abortion ratio can be further increased to ~90% in the *tgd1-1*/*TGD1*-RNAi lines with the reduced *tgd1-1* mRNA level, suggesting that

TGD1 has an essential role in seed development (Xu *et al.* 2005). Similar to the *tgd1-1* mutant, the *tgd2-1* mutant also produced ~ 43% of aborted seeds (Awai *et al.* 2006). However, both *tgd3-1* and TGD3RNAi seeds are developed normally. No abortion of seeds was observed.

Taken together, there are two possible explanations:

1) The mRNA level was still only partially reduced by dsRNA-mediated silencing. Although semi-quantitative RT-PCR revealed almost complete disrupt of *TGD3* (Figure 3.2C), it can only give an estimate. More accurate quantitative assays such as real-time quantitative PCR or northern blot are required to demonstrate the actual abundance of TGD3 mRNA. Therefore, it is possible that *TGD3* expression is still leaky in *TGD3i* transgenic plants and thus lead to weak phenotypes.

2) There is probably an additional ATPase associated with TGD1. The *TGD3* gene was identified via a reverse genetics approach (Lu *et al.* 2007). The Arabidopsis genome has 26 genes encoding small ATPases. Among those 26 genes, only nine have chloroplast transit peptide as predicted by ChloroP (Emanuelsson *et al.* 1999). We analyzed seven T-DNA insertion lines (Alonso *et al.* 2003) available at the time for those nine genes and identified Non-intrinsic ABC Protein 11 (NAP11) as the missing ATPase associated with the TGD1/2 transporter. Later, NAP 11 was renamed *TGD3* because the mutant disrupted in *NAP 11* accumulates TGDG.

However, recently STRING (Search Tool for the Retrieval of Interacting Genes/Proteins) database is being developed for known and predicted protein interactions (Jensen *et al.* 2009). In addition to TGD3, another ATPase NAP3 (At1g67940) was found to be associated with TGD1. The predicted interaction of NAP3 with TGD1 has a bit

score of 0.671, which follows that of TGD3 (NAP11) (a score of 0.868), indicating strong confidence of this prediction.

NAP3 and NAP11 proteins share a sequence similarity of ~34%. NAP3 does not have a chloroplast targeting peptide, as predicted by ChloroP, a primary reason why it was ruled out of initial consideration. However, based on the information from SUBA(the Arabidopsis Subcellular Database), several other programs predict its targeting to cytosol or Golgi apparatus whereas MS/MS data shows its localization to plasma membrane or vacuole (Heazlewood *et al.* 2007). This indicates that no definite localization of the NAP3 gene product is known so that the possibility of its association with TGD1, which is a chloroplast targeted protein, is emerging. Unfortunately, no T-DNA insertion lines are available which are disrupted in the NAP3 gene. In order to demonstrate whether or not NAP3 could be another ATPase associated with TGD1/2 transporter, either antisense or RNA silencing approach would be required to generate mutant alleles disrupted in the target genes and used for testing the TGDG lipid phenotype. Hopefully, the study of this NAP3 protein would help us explain some of the currently uninterpretable results for the 35S:TGD3RNAi mutant plants.

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Chapter 4

Biochemical analysis of the phosphatidic acid binding site in Arabidopsis TGD2 protein[2]

[2]This work has been published: **Lu, B. and Benning, C.** (2009) A 25-amino acid sequence of the Arabidopsis TGD2 protein is sufficient for specific binding of phosphatidic acid. *J. Biol. Chem.* 284(26): 17420-17427. I performed all the experiments and wrote the first draft of the paper.

4.1. Abstract

Genetic analysis suggests that the TGD2 protein of Arabidopsis is required for the biosynthesis of endoplasmic reticulum (ER) derived thylakoid lipids. TGD2 is proposed to be the substrate binding protein of a presumed lipid transporter consisting of the TGD1 (permease) and TGD3 (ATPase) proteins. The TGD1, 2, and 3 proteins are localized in the inner chloroplast envelope membrane. TGD2 appears to be anchored with an N-terminal membrane-spanning domain into the inner envelope membrane while the C-terminal domain faces the inter-membrane space. It was previously shown that the C-terminal domain of TGD2 binds phosphatidic acid (PtdOH). To investigate the PtdOH binding site of TGD2 in detail, the C-terminal domain of the TGD2 sequence lacking the transit peptide and trans-membrane sequences was fused to the C-terminus of the *Discosoma sp.* red fluorescent protein (DR). This greatly improved the solubility of the resulting DR-TGD2C fusion protein following production in *E. coli*. The DR-TGD2C protein bound PtdOH with high specificity as demonstrated by membrane lipid-protein overlay and liposome association assays. Internal deletion and truncation mutagenesis identified a previously undescribed minimal 25 amino acid fragment in the C-terminal domain of TGD2 that is sufficient for PtdOH binding. Binding characteristics of this 25-mer were different from that of TGD2C suggesting that additional sequences of TGD2 providing the proper context for this 25-mer are needed for wild-type like PtdOH binding.

4.2. Introduction

Many plants including Arabidopsis have two parallel pathways of thylakoid lipid biosynthesis involving enzymes at the inner plastid envelope or the endoplasmic reticulum (ER), respectively (Roughan *et al.* 1980, Roughan and Slack 1982). In Arabidopsis the two pathways contribute nearly equally to the bulk of galactoglycerolipids (Browse *et al.* 1986), which are the predominant lipids in thylakoid membranes. Synthesis by the ER pathway involves the assembly of phosphatidic acid (PtdOH) and phosphatidylcholine (PtdCho) at the ER from fatty acids synthesized in the chloroplast. The current hypothesis suggests that PtdCho returns to the outer plastid envelope, where it is converted to PtdOH by a phospolipase D (Benning 2008). The TGD1, 2, and 3 proteins are postulated to be involved in the transfer of PtdOH from the outer envelope membrane to the inside of the inner envelope membrane (Awai *et al.* 2006, Lu *et al.* 2007, Xu *et al.* 2003, Xu *et al.* 2005), where PtdOH is dephosphorylated by a PtdOH phosphatase to diacylglycerol, which is the precursor of galactoglycerolipid biosynthesis at the inner chloroplast envelope membrane. Alternatively, nascent fatty acids are assembled *de novo* at the chloroplast inner envelope into PtdOH, which then enters galactoglycerolipid biosynthesis as described above. In either pathway, PtdOH is a key intermediate for thylakoid lipid biosynthesis.

The TGD1, 2, and 3 proteins resemble the permease, substrate binding, and ATPase subunits of a multipartite ABC transporter which is localized in the inner chloroplast envelope (Awai *et al.* 2006, Lu *et al.* 2007, Xu *et al.* 2003, Xu *et al.* 2005) . Mutants with reduced function of these proteins have complex lipid phenotypes. They accumulate diagnostic oligogalactolipids such as trigalactosyldiacylglycerol giving rise to

their name, and triacylglycerols in their leaves. Moreover, galactolipid molecular species derived from the ER-pathway are underrepresented and pulse-chase-labeling experiments indicate a reduced flux through the ER-pathway in the mutants.

TGD2 appears anchored with its N-terminal domain into the inner envelope membrane such that the C-terminus faces the inter-membrane space (Awai *et al.* 2006) . The C-terminus of TGD2 was shown to specifically bind PtdOH. The TGD2 protein does not share sequence similarity with known PtdOH-binding proteins or domains. However, it does contain a mycobactrial cell entry domain (MCE) required for mycobacterial entry into host cells (Chitale *et al.* 2001).

For the few cellular protein targets of PtdOH described thus far, no uniform PtdOH binding motif has emerged making the reliable prediction of protein-PtdOH interactions or PtdOH binding domains difficult. In mammalian cells, protein kinase Raf-1 (Ghosh *et al.* 1996, Ghosh *et al.* 2003), protein phosphatases SHP-1 (Frank *et al.* 1999) and PP1 (Jones and Hannun 2002), and protein kinase Cε (Lopez-Andreo *et al.* 2003) are among the best studied PtdOH binding proteins. In yeast, the SNARE protein Spo20p (Nakanishi *et al.* 2004) and the inositol-regulated transcriptional repressor Opi1p (Loewen *et al.* 2004) are recognized as PtdOH binding proteins.

At this time, even fewer PtdOH-binding proteins have been identified in plants. The PtdOH binding proteins ABI1 (ABA insensitive 1) (Zhang *et al.* 2004) and PDK1 (phosphoinositide-dependent kinase 1) (Anthony *et al.* 2004) were only recently reported. ABI1 is a protein phosphatase 2C that negatively regulates signaling of the growth regulator abscisic acid. Arabidopsis PDK1 is a protein kinase that binds both PtdOH and phosphoinositides (Deak *et al.* 1999), but its kinase is activated only by PtdOH (Anthony

et al. 2004). Additional PtdOH targets were isolated from Arabidopsis cell lysates employing a PtdOH-analogue-affinity matrix followed by mass spectrometry analysis (Testerink *et al.* 2004). Phosphoenolpyruvate carboxylase was identified and shown to bind preferentially PtdOH over other phospholipids (Testerink *et al.* 2004). The TGD2 protein contains a new PtdOH binding site which we characterized and delineated as described below.

4.3. Experimental procedures

4.3.1. Expression and purification of DR-TGD2 fusion proteins

All TGD2 truncated proteins used in this study were derived from the TGD2-dTMD-pQE31 plasmid (also known as TGD2C-pQE31) using a PCR based strategy(Awai *et al.* 2006). Following digestion with *Nco* I and *Xho* I, the PCR fragment was ligated into the DsRed-plw01-His plasmid (provided by Dr. Michael Garavito, Michigan State University, East Lansing) to produce *Discosoma sp.* red fluorescent protein (DR)-TGD2C fusion proteins. Internal deletion mutants and/or point mutants were generated by site-directed mutagenesis applied to TGD2C-DsRed-plw01-His using a PCR-based protocol, with the primers and mutation sites listed in Supplemental table 4.1. All fusion proteins were expressed in the *Escherichia coli* strain, BL21 (DE3) (Novagen, Madison, WI). An overnight pre-culture of LB medium (5 mL) was used to start a 200 mL culture in LB medium. The protein was induced with 50 μM IPTG (isopropyl-β-D-thiogalactopyranoside) at OD_{600} 0.6-0.8, 16°C and growth was continued overnight. Cultures were cooled to 4°C, washed twice and resuspended in lysis buffer (50

mM Tris-HCl, pH7.5, 300 mM NaCl, 10 mM imidazole). The cells were lysed by sonication, followed by centrifugation at 18,000×g. The resulting supernatant was applied to a Ni-NTA agarose column (Qiagen, Valencia, CA). Proteins binding non-specifically were washed off the column using lysis buffer containing 20 mM imidazole. The His-tagged protein was then eluted with lysis buffer containing 250 mM imidazole. Samples were concentrated and dialyzed into assay buffer (10 mM KH_2PO4, pH7.4), using Amicon centrifugal filter devices (Millipore, Billerica, MA). Protein concentration was determined according to Bradford (Bradford 1976) using bovine serum albumin as a standard. The fusion proteins were analyzed for purity by SDS-PAGE followed by Coomassie Brilliant Blue staining (Laemmli 1970) and stored at $4^{o}C$ for several weeks without significant degradation or loss of lipid binding activity. The fusion proteins were soluble and, typically, their purity was greater than 90%. The different DR-TGD2 fusion proteins used in this study are listed in Supplemental table 4.1.

Supplemental table 4.1 PCR primers used for generation of DR-TGD2 fusion proteins

DsRed fusion protein	mutation	5' PCR primer sequence	3' PCR primer sequence
TGD2C WT	119-381	CCGGAGCTCGGTTTTCAAATGCGGTC	CGGCTCGAGTAGTAGCCTGCTTAGGG
TGD2C T1(119-250)	119-250	CCGGAGCTCGGTTTTCAAATGCGGTC	GCGCTCGAGAATACGAGTGAAAATTCC
TGD2C T2(171-300)	171-300	CCGGAGCTCGCTGAGATAGAAGATG	CGACTCGAGGCTATCACGAAACTCAG
TGD2C T3(221-350)	221-350	CAGGAGCTCAAGGAAGGTCTGATCG	CGGCTCGAGGACGTTCTTCAAAGTAT
TGD2C T4(201-381)	201-381	CCGGAGCTCATTATGCCTAGGAATCCG	CGGCTCGAGTAGTAGCCTGCTTAGGG
TGD2C T5 (119-300)	119-300	CCGGAGCTCGGTTTTCAAATGCGGTC	CGACTCGAGGCTATCACGAAACTCAG
TGD2C T6(119-225)	119-225	CCGGAGCTCGGTTTTCAAATGCGGTC	CGGCTCGAGGATCAGACCTTCCTTAC
TGD2C T7(171-225)	171-225	CCGGAGCTCGCTGAGATAGAAGATG	CGGCTCGAGGATCAGACCTTCCTTAC
TGD2C T8(201-225)	201-225	CCGGAGCTCATTATGCCTAGGAATCCG	CGGCTCGAGGATCAGACCTTCCTTAC
TGD2C T9(221-250)	221-250	CAGGAGCTCAAGGAAGGTCTGATCG	GCGCTCGAGAATACGAGTGAAAATTCC
TGD2C T10(225-381)	225-381	CCGGAGCTCATCGTTTGTGATAGGCAG	CGGCTCGAGTAGTAGCCTGCTTAGGG
TGD2C T11(225-350)	225-350	CCGGAGCTCATCGTTTGTGATAGGCAG	CGGCTCGAGGACGTTCTTCAAAGTAT
TGD2C T12(225-300)	225-300	CCGGAGCTCATCGTTTGTGATAGGCAG	CGACTCGAGGCTATCACGAAACTCAG
TGD2C T13(225-250)	225-250	CCGGAGCTCATCGTTTGTGATAGGCAG	GCGCTCGAGAATACGAGTGAAAATTCC
TGD2C T14(119-204)	119-204	CCGGAGCTCGGTTTTCAAATGCGGTC	CGGCTCGAGCCTAGGCATAATGTCG
TGD2C T15(119-160)	119-160	CCGGAGCTCGGTTTTCAAATGCGGTC	CGGCTCGAGAACACGGATAATCGTACC
TGD2C T16(291-381)	291-381	CCGGAGCTCCAACCTTTGCTCTCTG	CGGCTCGAGTAGTAGCCTGCTTAGGG
TGD2C T17(341-381)	341-381	CCGGAGCTCTACACTCTGGTTTATAC	CGGCTCGAGTAGTAGCCTGCTTAGGG
TGD2C D1(Δ221-250)	221-250 deleted	CTGCATCCTGAATGTGGTGGACGCGAA GTTGAGGCC	GGCCTCAACTTCGCGTCCACCACATTCA GGATGCAG
TGD2C D2 (Δ221-225)	221-225 deleted	CTGCATCCTGAATGTGGTGTTTGTGAT AGGCAGACA	TGTCTGCCTATCACAAACACCACATTCA GGATGCAG

Supplemental table 4.1 (Cont'd)

TGD2C D3 (Δ201-225)	201-225 deleted	ATGGAAACTATGATCGACGTTTGTGAT AGGCAGACA	TGTCTGCCTATCACAAACGTCGATCATA GTTTCCAT
TGD2C P1(201-225)	R204A	GAATTCGAGCTCATTATGCCTGCGAAT CCGATACCAGAACCTTC	GAAGGTTCTGGTATCGGATTCGCAGGC ATAATGAGCTCGAATTC
TGD2C P2(201-225)	N205A	CGAGCTCATTATGCCTAGGGCGCCGAT ACCAGAACCTTCAG	CTGAAGGTTCTGGTATCGGCGCCCTAG GCATAATGAGCTCG
TGD2C P3(201-225)	E209A	CCTAGGAATCCGATACCAGCGCCTTCA GTAGGACCTCTG	GCAGAGGTCCTACTGAAGGCGCTGGTA TCGGATTCCTAGG
TGD2C P4(201-225)	H216A	CCTTCAGTAGGACCTCTGGCGCCTGAA TGTGGTAAGGAAG	CTTCCTTACCACATTCAGGCGCCAGAG GTCCTACTGAAGG
TGD2C P5(201-225)	E218A	CAGTAGGACCTCTGCATCCTGCGTGTG GTAAGGAAGGTCTGATC	GATCAGACCTTCCTTACCACACGCAGG ATGCAGAGGTCCTACTG
TGD2C P6(201-225)	C219A	GGACCTCTGCATCCTGAAGCGGGTAAG GAAGGTCTGATCC	GGATCAGACCTTCCTTACCCGCTTCAGG ATGCAGAGGTCC
TGD2C P7(201-225)	K221A	CTCTGCATCCTGAATGTGGTGCGGAAG GTCTGATCCTCGAG	CTCGAGGATCAGACCTTCCGCACCACA TTCAGGATGCAGAG
TGD2C P8(201-225)	E222A	CATCCTGAATGTGGTAAGGCGGGTCTG ATCCTCGAGCACC	GGTGCTCGAGGATCAGACCCGCCTTAC CACATTCAGGATG

4.3.2. Phylogenetic analysis of TGD2

Full-length TGD2 amino acid sequences were searched against the National Center for Biological Information (NCBI) non-redundant protein database using the BLAST tool (Altschul *et al.* 1997) and the resulting sequences with high similarities and identities were aligned using ClustalX software (version 2.0.10) (Larkin *et al.* 2007). Generation of the bootstrapped phylogenetic tree was performed using the PHYLIP software package as previously described (Mamedov *et al.* 2005).

4.3.3. Protein-lipid overlay assay

Membrane lipid strips were purchased from Echelon Biosciences (Salt Lake City, UT). The strips were first blocked with 3% bovine serum albumin in TBST (10mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.25% Tween-20) for two hours and incubated in 0.5 μg/mL DR-TGD2 fusion protein solution in the blocking buffer at 4°C overnight. The strips were washed 3 times, 10 min each with TBST and soaked in 3% bovine serum albumin in TBST with a penta-His mouse monoclonal antibody (Sigma-Aldrich, St. Louis, MO) at 1:2,000 dilution at 4°C overnight. The strips were washed twice with TBST and soaked in 3% bovine serum albumin in TBST with horse radish peroxidase-conjugated anti mouse antibody (Bio-Rad, Hercules, CA) at 1:20,000 dilution for an hour at room temperature. Following washing with TBST for 1 hour, the protein was detected by using a chemiluminescence detection system (Sigma-Aldrich, St. Louis, MO).

4.3.4. Liposome-association assay

The liposome association assay was performed according to (Sano *et al.* 1998). Lipids (dioleoyl-phosphatidylcholine, or dioleoyl-phosphatidic acid) were dried under a stream of nitrogen and incubated in TBS (50 mM Tris-HCl, pH 7, 0.1 M NaCl) at 37°C for an hour followed by vigorous vortexing for 5 min. The liposomes were precipitated at 20,000×g and washed twice with ice-cold TBS. Liposomes (250 µg) were mixed with purified DR-TGD2 fusion protein and TBS to make a final 100 µL solution. The mixture was incubated at 30°C for 30 min and washed twice with ice-cold TBS by centrifugation at 20,000×g at 4°C. The liposome pellet mixed with sample buffer was analyzed by SDS-PAGE (Laemmli 1970). Immuno-detection of the His-tagged protein was accomplished using the above mentioned penta-His antibody at 1:10,000 and the anti mouse antibody at 1:50,000 dilution. For visualization and quantification a chemiluminescence detection kit from Sigma was used.

4.3.5. Quantitative analysis of immunoblots

Quantification and detection of signals on protein immunoblots were done by two methods: 1) by film-based autoradiography followed by densitometry scanning. Distinct protein bands were quantified using the computer software Multi Gauge V3.0 (Fujifilm USA, Valhalla, NY), and 2) using a camera-based gel documentation system (a LAS-3000, Fuji Film Life Science USA, Stamford, CT). The linear range for detection extended over 4 orders of magnitude for the camera based system as compared to 1.5 orders of magnitude for the film-based system. Linearity of the response of the respective detection system was established using a serial dilution of a test protein. Under the

conditions used, both detection systems were in the linear range of detection and gave similar results during quantification of signals and were used interchangeably as indicated. The data obtained by both methods were fit to a Hill equation modified for ligand binding data (Ghosh *et al.* 1996, Orr and Newton 1992) using OriginPro8 (Origin lab corporation, Northampton, MA). Relevant variables calculated by this method were k indicating the PtdOH lipid fraction at half maximal binding and the Hill coefficient n indicating cooperativity.

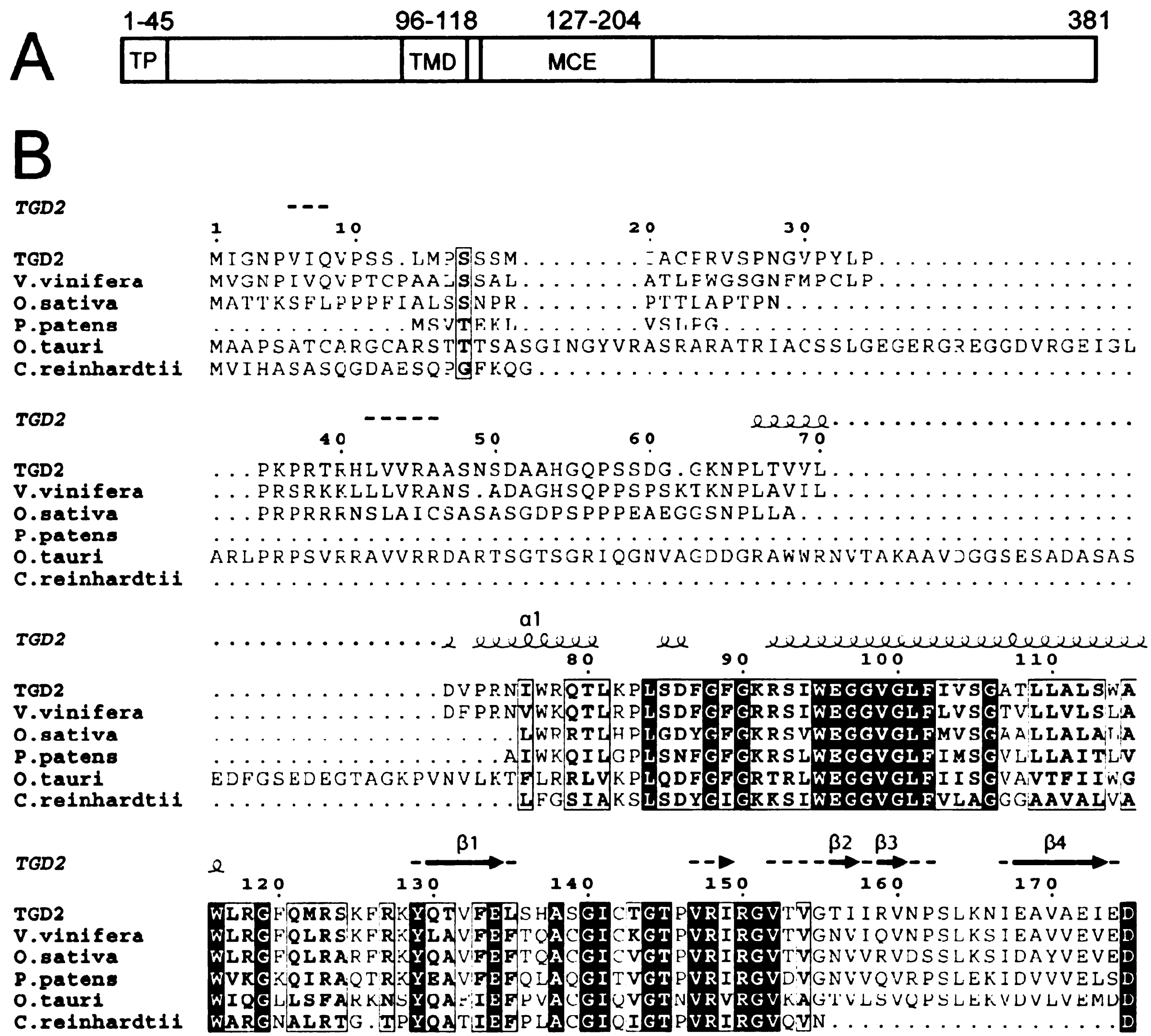
4.4. Results

4.4.1. TGD2 orthologs in plants and Cyanobacteria

The *TGD2* gene encodes a 381 amino acid protein with a calculated molecular mass of 41.6 kDa as summarized for At3g20320 at The Arabidopsis Information Resource (http://www.arabidopsis.org). The TGD2 protein contains a mycobacterial cell entry domain (MCE, amino acids 127-204), which is the only known conserved domain in TGD2 and is found in surface proteins of pathogenic mycobacteria. These proteins are essential virulence factors proposed to facilitate the bacterial entry into mammalian host cells (Chitale *et al.* 2001). The molecular or biochemical functions of this domain, which was strictly defined by conservation in different proteins, are not known. At the outset of this study it was hypothesized that the MCE domain might play a role in mediating PtdOH binding to TGD2. A trans-membrane-spanning domain (amino acids 96-118) and a chloroplast targeting peptide (amino acids 1-45) in TGD2 were also predicted as shown in Figure 4.1A. Putative TGD2 orthologs in plants, green algae and cyanobacteria (Figure

4.1, B and C) have conserved regions beyond the MCE domain (Figure 4.1B). None of the conserved amino acid residues in the primary sequence resembled previously described PtdOH binding motifs. Therefore, a systematic mutagenesis approach was necessary to identify amino acids in TGD2 relevant to PtdOH binding.

Figure 4.1. Sequence analysis of TGD2. *A*, primary structure of TGD2 indicating a predicted transit peptide (TP), transmembrane domain (TMD) and a conservative mycobacterial cell entry (MCE) domain. *B*, an alignment of the TGD2 amino acid sequence with its orthologs in plants and green algae is shown. The predicted TGD2 secondary structure is indicated above the sequence. Open boxes mark conserved residues, and black boxes indicate identical residues. *C*, an unrooted tree showing the relatedness of predicted TGD2 orthologs in plants, green algae and Cyanobacteria is shown. Boot strapping values >950 are marked by +, those between 500 and 950 are marked with a solid circle, and those under 500 are marked by open square. The gene bank accession numbers for the included sequences in *B* and *C* are: *Arabidopsis thaliana*, NP_566659.1; *Vitis vinifera*, CAN71395.1; *Oryza sativa*, EAY77419.1; *Physcomitrella patens*, XP_001778862.1; *Ostreococcus tauri*, CAL53419.1; *Chlamydomonas reinhardtii*, XP_001699315.1; *Prochlorococcus marinus str. NATL2A*, YP_292846.1; *Prochlorococcus marinus str. MIT 9301*, YP_001090537.1; *Synechococcus sp. WH 5701*, ZP_01083418.1; *Synechococcus sp. CC9902*, YP_376253.1; *Synechococcus sp. JA-2-3B'a(2-13)*, YP_477327.1; *Anabaena variabilis*, YP_323182.1; *Nodularia spumigena*, ZP_01630545.1; *Crocosphaera watsonii*, ZP_00516249.1; *Cyanothece sp. PCC 8801*, ZP_02940544.1; *Microcystis aeruginosa*, CAO90615.1; *Acaryochloris marina*, YP_001516641.1; *Thermosynechococcus elongatus*, NP_683197.1.


A
1-45
96-118
127-204
381
TP
TMD
MCE
B
TGD2
1 10 20 30
TGD2 MIGNPVIQVPSS.LMPSSSM.........ACFRVSPNGVPYLP
V.vinifera MVGNPIVQVPTCPAALSSAL........ATLFWGSGNFMPCLP
O.sativa MATTKSFLPPPFIALSSNPR........PTTLAPTPN
P.patens ....MSVTEKL........VSLFG
O.tauri MAAPSATCARGCARSTTTSASGINGYVRASRARATRIACSSLGEGERGREGGDVRGEIGL
C.reinhardtii MVIHASASQGDAESQPGFKQG
TGD2
40 50 60 70
TGD2 ...PKPRTRHLVVRAASNSDAAHGQPSSDG.GKNPLTVVL
V.vinifera ...PRSRKKLLLVRANS.ADAGHSQPPSPSKTKNPLAVIL
O.sativa ...PRPRRRNSLAICSASASGDPSPPPEAEGGSNPLLA
P.patens
O.tauri ARLPRPSVRRAVVRRDARTSGTSGRIQGNVAGDDGRAWWRNVTAKAAVDGGSESADASAS
C.reinhardtii
α1
TGD2
80 90 100 110
TGD2 ................DVPRNIWRQTLKPLSDFGFGKRSIWEGGVGLFIVSGATLLALSWA
V.vinifera ................DFPRNVWKQTLRPLSDFGFGRRSIWEGGVGLFLVSGTVLLVLSLA
O.sativa ....................LWRRTLHPLGDYGFGKRSVWEGGVGLFMVSGAALLALALA
P.patens ...................AIWKQILGPLSNFGFGKRSLWEGGVGLFIMSGVLLLAITLV
O.tauri EDFGSEDEGTAGKPVNVLKTFLRRLVKPLQDFGFGRTRLWEGGVGLFIISGVAVTFIIWG
C.reinhardtii ....................LFGSIAKSLSDYGIGKKSIWEGGVGLFVLAGGGAAVALVA
β1
β2
β3
β4
TGD2
120 130 140 150 160 170
TGD2 WLRGFQMRSKFRKYQTVFELSHASGICTGTPVRIRGVTVGTIIRVNPSLKNIEAVAEIED
V.vinifera WLRGFQLRSKFRKYLAVFEFTQACGICKGTPVRIRGVTVGNVIQVNPSLKSIEAVVEVED
O.sativa WLRGFQLRARFRKYQAVFEFTQACGICVGTPVRIRGVTVGNVVRVDSSLKSIDAYVEVED
P.patens WVKGKQIRAQTRKYEAVFEFQLAQGITVGTPVRIRGVDVGNVVQVRPSLEKIDVVVELSD
O.tauri WIQGLLSFARKNSYQA-IEFPVACGIQVGTNVRVRGVKAGTVLSVQPSLEKVDVLVEMDD
C.reinhardtii WARGNALRTG.TPYQATIEFPLACGIQIGTPVRIRGVQVN..................D

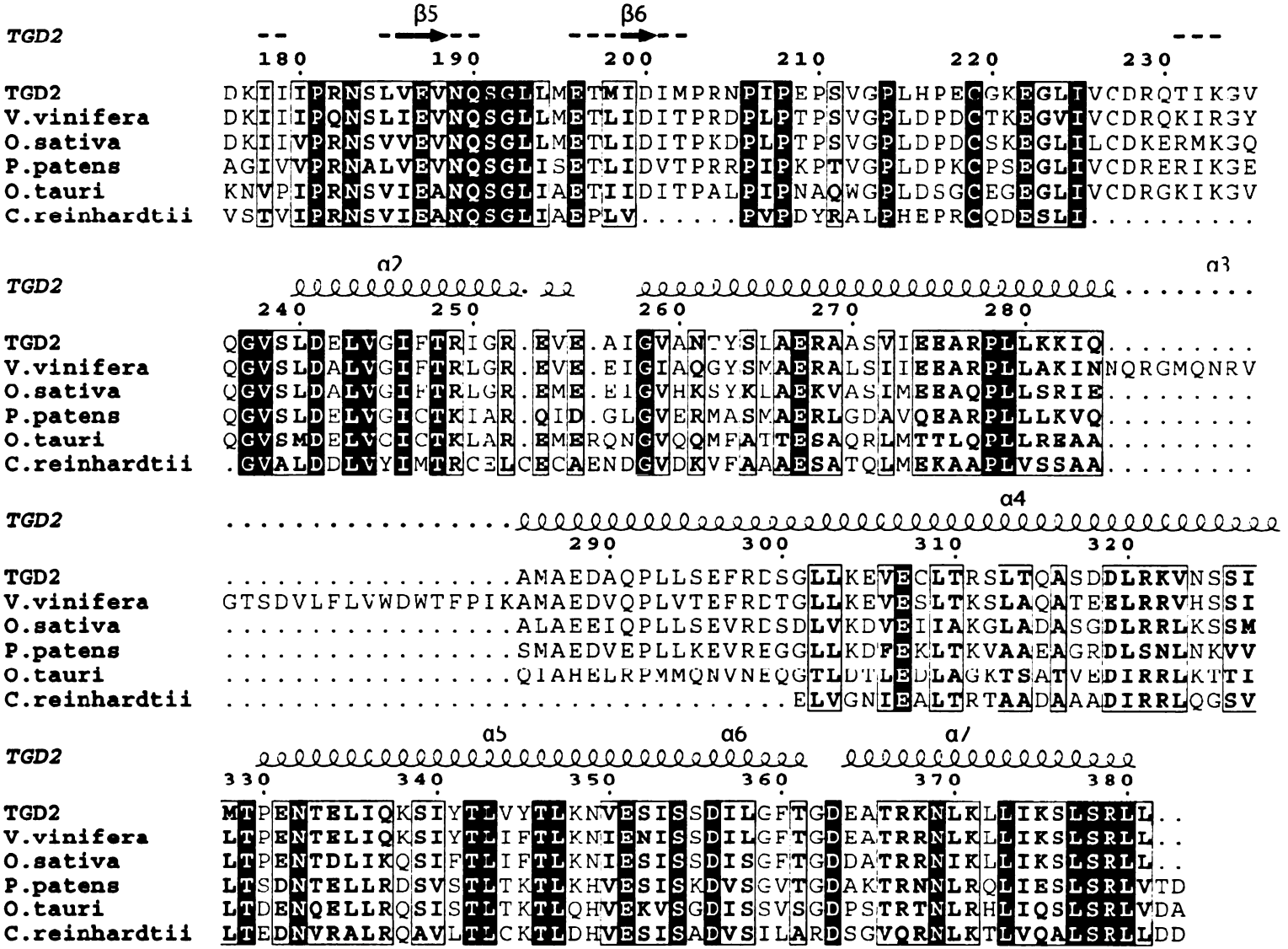


Figure 4.1 (Cont'd)

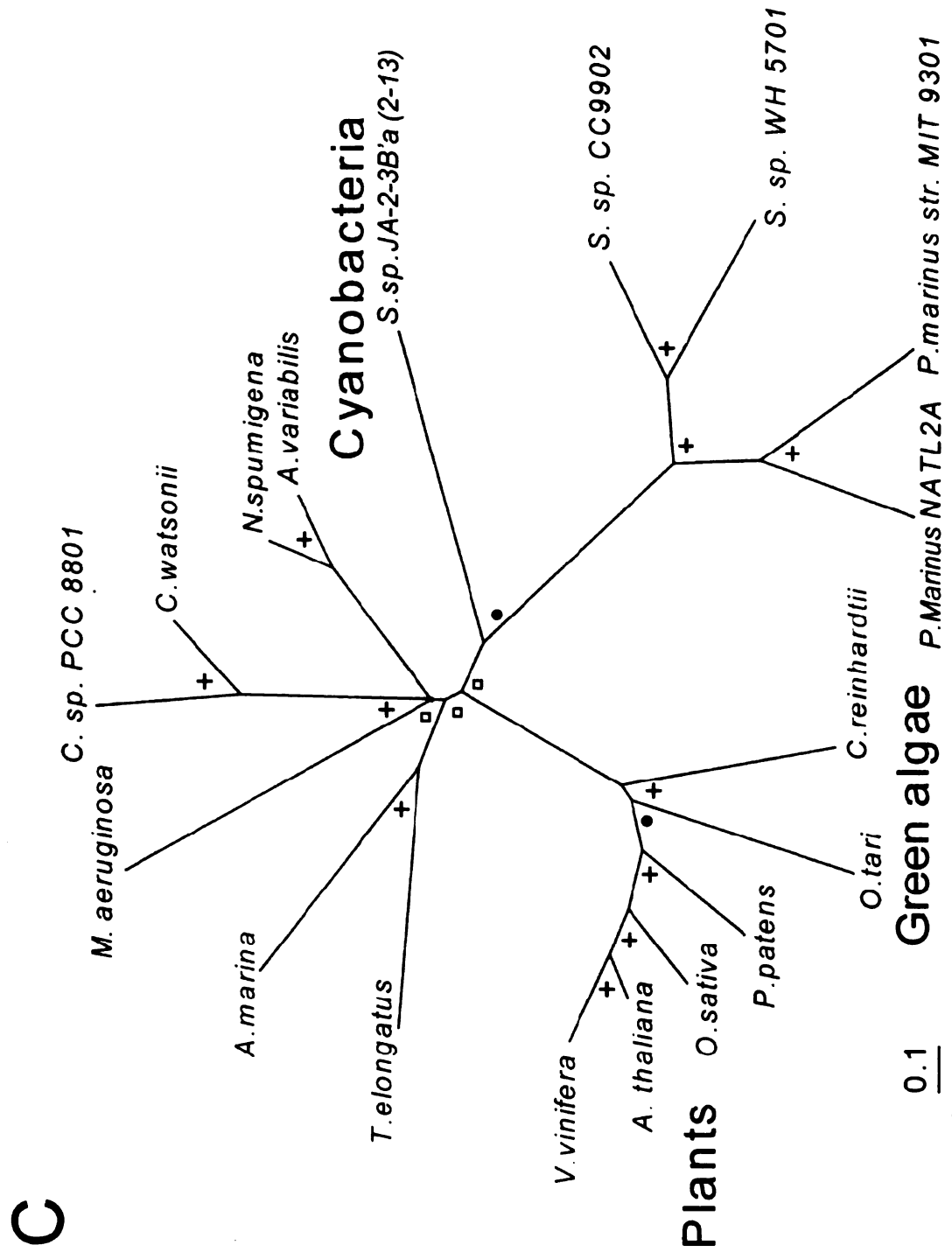
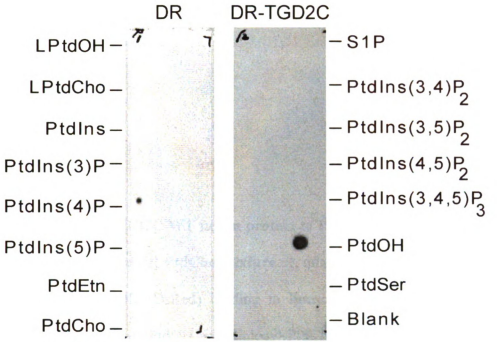


Figure 4.1 (Cont'd)

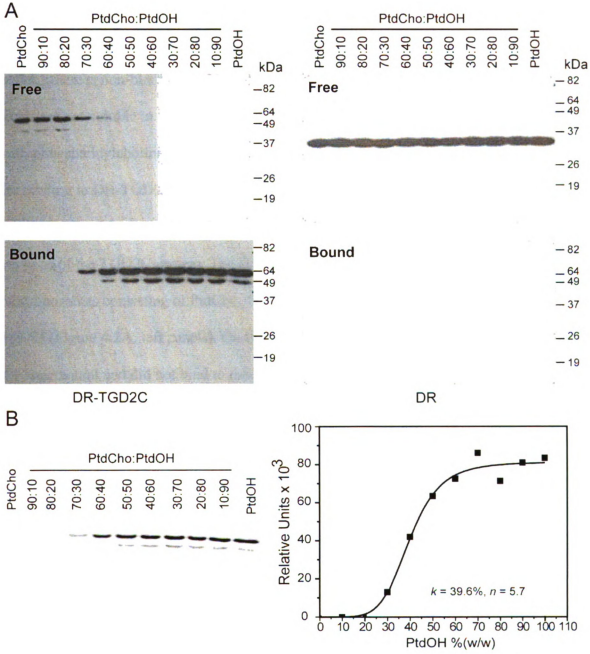
4.4.2. Specific binding of the DR-TGD2C WT fusion protein to PtdOH

A His-tagged version of the C-terminal domain of TGD2, TGD2C-His, was found to specifically bind PtdOH(Awai *et al.* 2006). This truncated TGD2 protein was difficult to solubilize and mutant forms were completely insoluble when the respective constructs were expressed in *E. coli*. To overcome this problem, we fused the C-terminal TGD2 domain to the C-terminus of an engineered monomeric *Discosoma sp.* red fluorescent protein (DR) (Bevis and Glick 2002). Contrary to maltose binding protein and glutathione-*S*-transferase that were initially considered as fusion partners for TGD2C, DR did not show lipid binding by itself in the protein-lipid overlay or liposome lipid binding assays that were employed in this study (see supplemental Figure 4.1 and Figure 4.2). However, the soluble DR-TGD2C fusion protein specifically bound to PtdOH as demonstrated in the protein-lipid overlay assay shown in Supplemental Figure 4.1, confirming previous results on the TGD2C-His-tagged protein(Awai *et al.* 2006). Moreover, deletion and point mutant variants of the DR-TGD2C protein encoding constructs generally expressed well in *E. coli* and the resulting proteins were soluble.



Supplemental figure 4.1. Binding of DsRed-TGD2C WT fusion protein to PtdOH. The TGD2 protein is N-terminally truncated lacking the TMD and C-terminally fused to the *Discosoma sp.* red fluorescent protein (DsRed, DR) open reading frame. Fusion protein was expressed and protein-lipid overlay assay was conducted with commercial phospholipid-containing membrane strip. Lipids: LPtdOH, lysophosphatidic acid; LPtdCho, lysophosphatidylcholine; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate;PtdIns(5)P, phosphatidylinositol 5-phosphate; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; S1P, sphingosine 1-phosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-bisphosphate; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine.

Figure 4.2. Binding of the DR-TGD2C WT fusion protein to PtdOH as a function of weight percent of PtdOH in a PtdOH/PtdCho mixture. *A*, qualitative analysis of DR-TGD2C wild-type protein and DR (DsRed) binding to liposomes. Liposomes were prepared from a mixture of dioleoyl-PtdOH and dioleoyl-PtdCho in which the weight percent of PtdOH was varied from 0-100% (w/w), maintaining total lipid at 250 μg and total protein at 1μg. Western-blots detected by chemiluminescent kit are shown. *B*, quantitative analysis of the association of DR-TGD2C wild-type protein to PtdOH/PtdCho liposomes determined by direct fluorometry using a digital camera based system (left). The values are plotted as a function of PtdOH concentration in the liposomes (right). The data were fit to a modified Hill equation for receptor-ligand binding.


A
PtdCho:PtdOH
PtdCho
90:10
80:20
70:30
60:40
50:50
40:60
30:70
20:80
10:90
PtdOH
kDa
Free
Bound
82
64
49
37
26
19
DR-TGD2C
DR
B
PtdCho:PtdOH
Relative Units x 10^3
k = 39.6%, n = 5.7
PtdOH %(w/w)

To obtain corroborating and more quantitative evidence for PtdOH binding, a liposome binding assay was used. Purified recombinant proteins were incubated as described under Experimental Procedures with liposomes of different lipid composition. Following centrifugation, proteins bound to the liposomes were associated with the lipid pellet, whereas non-binding proteins remained in the supernatant (Figure 4.2A). Because lipids are not-soluble in water, PtdOH was mixed at different weight-per-weight ratios with phosphatidylcholine (PtdCho) to determine the weight% fraction of PtdOH required for binding to DR-TGD2C. This fraction provided an indication for the affinity of the DR-TGD2C wild-type protein to PtdOH and allowed a quantitative comparison to mutant versions of the TGD2C protein. The DR-TGD2C wild-type fusion protein was found to bind liposomes consisting of PtdCho, PtdOH lipid mixtures depending on the fraction of PtdOH (Figure 4.2A, left panels). On the contrary, DR alone was exclusively present in the supernatant and did not bind to the liposomes (Figure 4.2A, right panels). At the protein concentration tested (1μg total protein), a significant increase in binding between 30-40% PtdOH was observed. In Figure 4.2B, the blot was analyzed using a digital camera system which shows linear responses over four orders of magnitude. The individual signals were quantified, and the resulting data were plotted and fit to the Hill equation modified for receptor-ligand binding (Ghosh *et al.* 1996, Orr and Newton 1992). Because we established that the detection system itself was not saturated under the conditions used (see Experimental Procedures), we interpreted the observed saturation as true saturation of binding of the protein to the liposomes. When the data were fit to the modified Hill plot, half maximal binding at 39.6% of PtdOH was apparent. Using the film-based detection methods, a similar value was obtained (see below). This value is

comparable to the results obtained for RafC-PtdOH association (20 mol% PtdOH)(Ghosh *et al.* 1996). From the binding plot, a Hill number of 5.7 was calculated, suggesting positive cooperativity (Figure 4.2B). Again, this value is similar to that obtained for RafC-PtdOH interaction (Hill number between 3.3 and 6.2) (Ghosh *et al.* 1996). It seems likely that TGD2 is forming a homo-multimer showing cooperativity of PtdOH binding among the involved C-terminal TGD2 domains.

4.4.3. Identification of TGD2C amino acid sequences necessary for PtdOH binding

Because most previously described amino acid sequences involved in PtdOH-binding lack recognizable similarities at the primary sequence level (Munnik and Testerink 2005), a deletion and truncation approach was used to systematically identify sequences necessary for PtdOH binding by TGD2C. The described liposome binding assay was sufficiently quantitative to compare PtdOH binding to different versions of DR-TGD2C. Pure PtdOH liposomes were used which gave maximal binding of DR-TGD2C (ref. Figure 4.2B). In parallel, a negative control was included using pure PtdCho liposomes. To test for specificity of binding, liposomes containing 50% (w/w) PtdOH and PtdCho were included as well. All DR-TGD2C mutant proteins described here were soluble and more than 90% pure based on Coomassie Brilliant Blue protein gel staining (see Supplemental figure 4.2). Schematic representations of the mutant proteins and the respective binding data are shown in Figure 4.3. In an initial set, five truncated mutant versions ranging in lengths from 130-to-180 amino acids across the length of the TGD2C sequence (Figure 4.3, B and C) were tested for binding. All of the truncated proteins (DR-TGD2C T1-T5, Figure 4.3, B and C) showed significant PtdOH binding to pure

PtdOH liposomes, suggesting that a fragment from residues 221-to-250 common to these fragments might be involved in PtdOH binding.

In a second set of experiments, two internal deletion mutants probing the 221-to-250 sequence were generated (DR-TGD2C D1 and D2; Figure 4.3C) and tested for PtdOH binding. Surprisingly, deleting the entire segment (residues 221-250, DR-TGD2C D1; Figure 4.3C) did not affect binding, while deleting a smaller fragment within this segment (residues 221-225, DR-TGD2C D2; Figure 4.3C) diminished binding of the fusion protein to PtdOH. It seems possible that DR-TGD2C D1 with the larger deletion folds just right to still reconstitute a nearby site, whereas the smaller deletion in DR-TGD2C D2 does not allow reconstitution.

Supplemental figure 4.2. SDS-PAGE of the proteins used for liposome binding assay. 1μg of total proteins were loaded each lane and stained with Coomassie Blue R-250. *A*, same proteins as in Figure 4.3B. *B*, same proteins as included in Figure 4.3C. *C*, same proteins as in Figure 4.4A. *D*, same proteins as in Figure 4.4C. *E*, same proteins as in Figure 4.6B. Protein molecular weight markers are indicated where appropriate. All the proteins including DsRed control show an additional weaker band underneath the band which is identical to calculated mass, most likely due to alternative translation starting site or N-terminal truncation, since both bands can be detected by anti-His antibody.

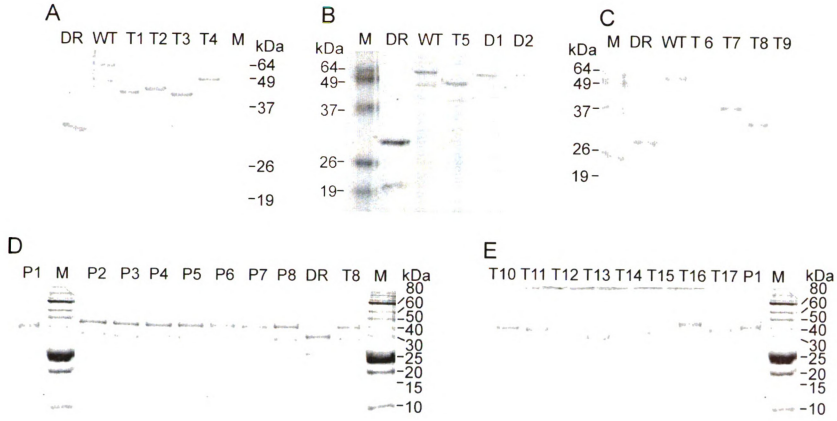
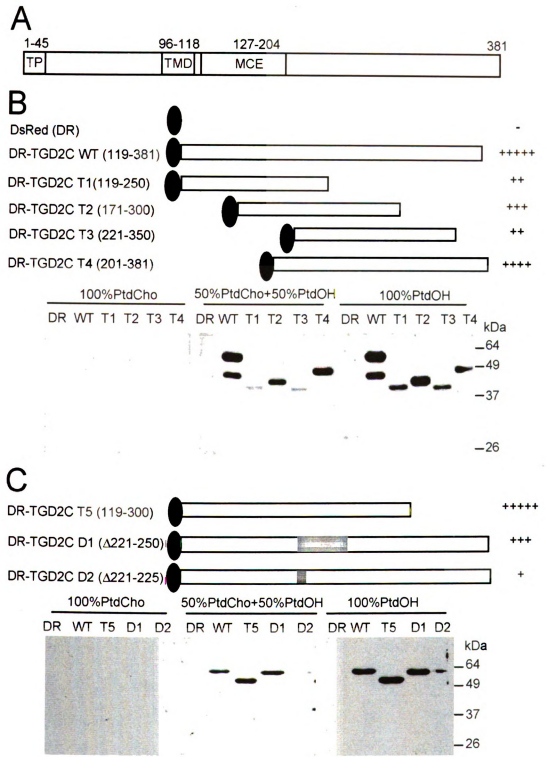

A
DR WT T1 T2 T3 T4 M kDa
-64
-49
-37
-26
-19
B
kDa M DR WT T5 D1 D2
64-
49-
37-
26-
19-
C
kDa M DR WT T 6 T7 T8 T9
64-
49-
37-
26-
19-
D
P1 M P2 P3 P4 P5 P6 P7 P8 DR T8 M kDa
80
60
50
40
30
25
20
15
10
E
T10 T11 T12 T13 T14 T15 T16 T17 P1 M kDa
80
60
50
40
30
25
20
15
10

Figure 4.3. Identification of a PtdOH binding domain in TGD2C. *A*, primary structure of TGD2 indicating a predicted transit peptide (TP), transmembrane domain (TMD) and a conservative mycobacterial cell entry (MCE) domain. *B,C* series of deletion and truncation mutants were generated for TGD2C and fused to the C-terminus of the DR open reading frame. The black oval represents the DR (DsRed) carrier protein, grey bars represent deletions. Liposome-association assays were performed to assess binding of various mutants to PtdCho, PtdOH/PtdCho mixtures or PtdOH liposomes. Western-blots detected by chemiluminescent kit are shown. PtdOH-specific binding data are summarized on the right based on visually examined signal intensity (+++++, ++++, +++, ++, +, indicate decreasing signal intensity, – no binding).

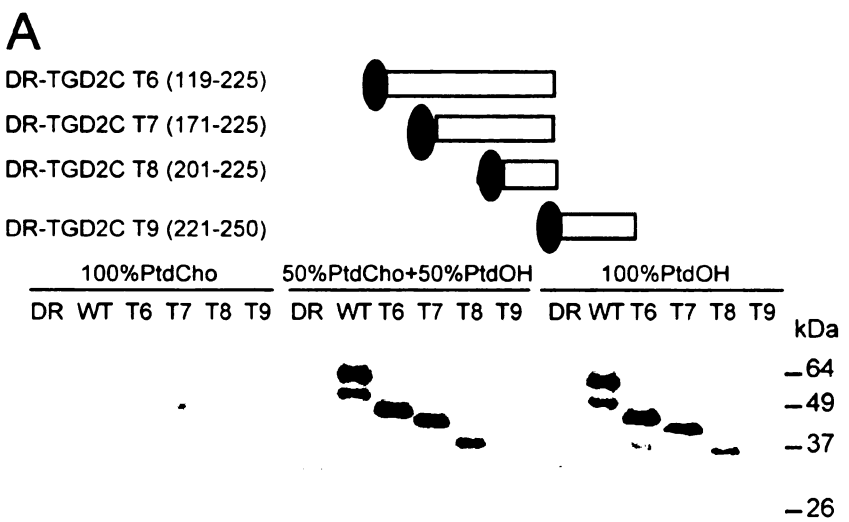

A
1-45
96-118
127-204
381
TP
TMD
MCE
B
DsRed (DR)
DR-TGD2C WT (119-381)
DR-TGD2C T1(119-250)
DR-TGD2C T2 (171-300)
DR-TGD2C T3 (221-350)
DR-TGD2C T4 (201-381)
-
+++++
++
+++
++
++++
100%PtdCho
50%PtdCho+50%PtdOH
100%PtdOH
DR WT T1 T2 T3 T4 DR WT T1 T2 T3 T4 DR WT T1 T2 T3 T4
kDa
64
49
37
26
C
DR-TGD2C T5 (119-300)
DR-TGD2C D1 (Δ221-250)
DR-TGD2C D2 (Δ221-225)
+++++
+++
+
100%PtdCho
50%PtdCho+50%PtdOH
100%PtdOH
DR WT T5 D1 D2 DR WT T5 D1 D2 DR WT T5 D1 D2
kDa
64
49
37
26

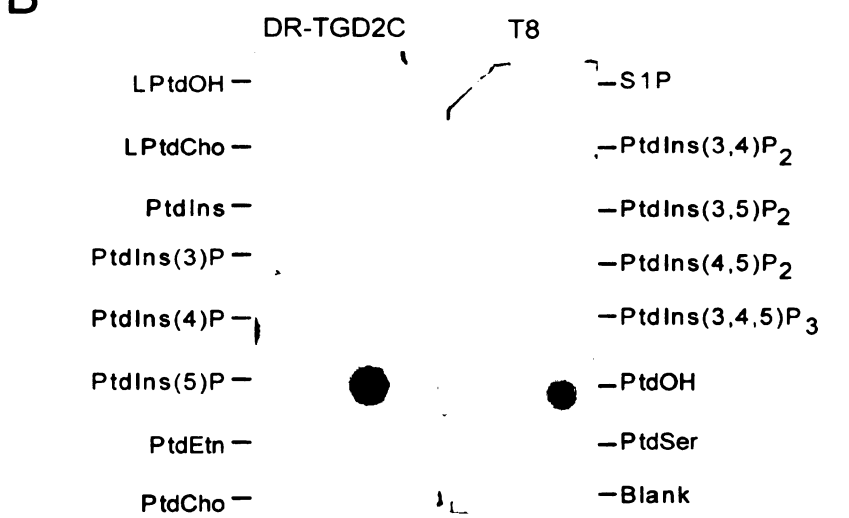
4.4.4. A TGD2 minimal domain sufficient for PtdOH binding

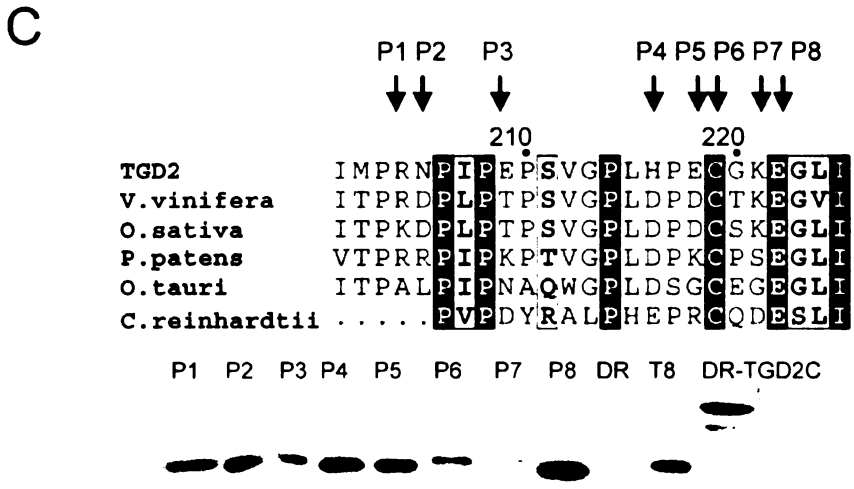
Coarse mapping described above suggested peptide sequences overlapping or surrounding residues 221-225 as a major contributing factor to PtdOH binding by TGD2. Fine mapping was initiated from residue 225 in both directions starting with the fragment containing residues 119-225 which fused to DR (DR-TGD2C T6; Figure 4.4A). This fragment tested positive for PtdOH binding with liposomes. Truncations were made from the N-terminus of DR-TGD2C T6 to narrow down this PtdOH binding site in the TGD2 protein. The two truncation clones DR-TGD2C T7 and T8 still showed binding to PtdOH (Figure 4.4A). Apparently, the clones DR-TGD2C T6-T8 have a 25 amino acid peptide (residues 201-225) in common that is sufficient to mediate binding to PtdOH. Specificity of PtdOH binding by the minimal fragment DR-TGD2C T8 was confirmed using the protein-lipid overlay assay applied to a membrane containing multiple lipids (Figure 4.4B).

Probing from TGD2 amino acid 221 towards the C-terminus, a fragment consisting of residues 221-250 (DR-TGD2C T9; Figure 4.4A) did not show binding of PtdOH when fused to DR, indicating that this sequence might not play a direct role in PtdOH binding. This result was consistent with the previous observation that DR-TGD2C D1 lacking this specific fragment still binds PtdOH (Figure 4.3C).

Figure 4.4. Fine mapping of a TGD2 minimal PtdOH binding sequence. *A*, truncation mutants fused to DR (DsRed) focusing on the PtdOH binding domain. PtdOH binding activity was assessed by liposome-association assay. *B*, specificity of the minimal PtdOH binding domain fused to DR was verified by protein-lipid overlay assay using a phospholipid-containing membrane strip. Lipids: LPtdOH, lysophosphatidic acid; LPtdCho, lysophosphatidylcholine; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate;Ptdlns(5)P, phosphatidylinositol 5-phosphate; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; S1P, sphingosine 1-phosphate; PtdIns(3,4)P2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,5)P2, phosphatidylinositol 3,5-bisphosphate; PtdIns(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-bisphosphate; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine. *C*, PtdOH binding of point mutations of the minimal PtdOH binding domain fused to DR shown by liposome-association assay with 100% PtdOH liposomes. A section (residues 201-225) of the same sequence alignment as in Figure 4.1 was shown. Point mutations in the sequence are indicated by arrows. P1, DR-TGD2C T8 R204A; P2, DR-TGD2C T8 N205A; P3, DR-TGD2C T8 E209A; P4, DR-TGD2C T8 H216A; P5, DR-TGD2C T8 E218A; P6, DR-TGD2C T8 C219A; P7, DR-TGD2C T8 K221A; P8, DR-TGD2C T8 E222A.


A
DR-TGD2C T6 (119-225)
DR-TGD2C T7 (171-225)
DR-TGD2C T8 (201-225)
DR-TGD2C T9 (221-250)
100%PtdCho
50%PtdCho+50%PtdOH
100%PtdOH
DR WT T6 T7 T8 T9
DR WT T6 T7 T8 T9
DR WT T6 T7 T8 T9
kDa
64
49
37
26


B
DR-TGD2C
T8
LPtdOH
LPtdCho
PtdIns
PtdIns(3)P
PtdIns(4)P
PtdIns(5)P
PtdEtn
PtdCho
S1P
PtdIns(3,4)P2
PtdIns(3,5)P2
PtdIns(4,5)P2
PtdIns(3,4,5)P3
PtdOH
PtdSer
Blank


C
P1 P2 P3 P4 P5 P6 P7 P8
210
220
TGD2 IMPRNPIPEPSVGPLHPECGKEGLI
V.vinifera ITPRDPLPTPSVGPLDPDCTKEGVI
O.sativa ITPKDPLPTPSVGPLDPDCSKEGLI
P.patens VTPRRPIPKPTVGPLDPKCPSEGLI
O.tauri ITPALPIPNAQWGPLDSGCEGEGLI
C.reinhardtii .....PVPDYRALPHEPRCQDESLI
P1 P2 P3 P4 P5 P6 P7 P8 DR T8 DR-TGD2C

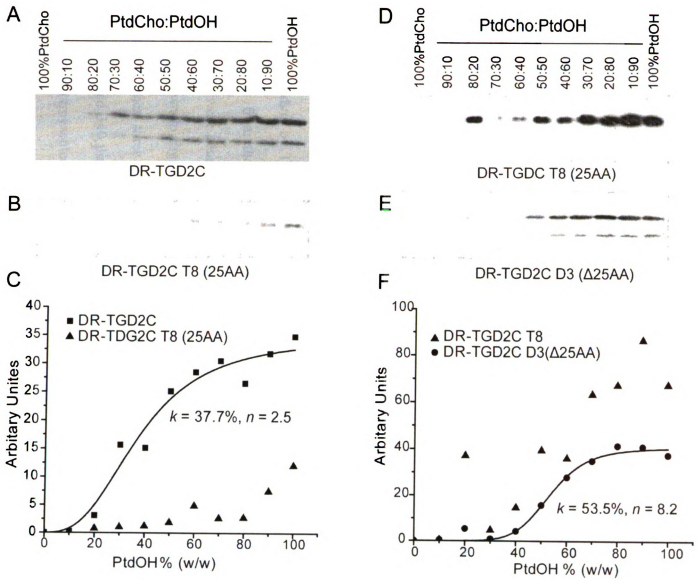
In the few cases in which PtdOH binding sites have been analyzed in detail, basic amino acids or tryptophan were found to be critical for PtdOH binding (Munnik and Testerink 2005). In particular, a recent study suggested that electrostatic interactions of PtdOH with basic amino acids (*i.e.* lysine and arginine), along with hydrogen bond interactions, form the basis of the specific binding of PtdOH to its cognate targets (Kooijman *et al.* 2007). Similarly, we set out to map residues critical to PtdOH binding within the identified minimal PtdOH binding region of TGD2C (residues 201-225). Based on sequence similarity of TGD2 to its most closely related orthologs in plants and green algae, several charged or conserved amino acids were targeted for mutational analysis. In every case these amino acids were changed to alanine to simplify the analysis. Point mutant versions of DR-TGD2C T8 are shown in Figure 4.4C and were tested for PtdOH binding using pure PtdOH liposomes. Of all the point mutants analyzed only the substitution in DR-TGD2C T8 K221A drastically reduced PtdOH binding. No detectable binding to pure PtdCho liposomes was observed for any of the point mutants (data not shown). Taken together, these data suggest that TGD2 residues 201-225 represent a not previously described PtdOH binding domain, which is localized near but not in the MCE domain. Lysine 221, a positively charge amino acid at physiological conditions seems to be a critical residue within this PtdOH binding domain that might be required for electrostatic interactions with the negatively charged phosphoryl group of PtdOH.

4.4.5. PtdOH binding properties of the minimal PtdOH binding domain

Positive cooperativity of ligand binding to its target as observed for PtdOH binding by DR-TGD2C (Figure 4.2B) usually requires the interaction of multiple protein

subunits or protein domains. On the other hand, the minimal 25-amino acid PtdOH binding domain represented by DR-TGD2C T8 (residues 201-225) was hardly expected to show this cooperativity. To test this hypothesis, we measured PtdOH binding to DR-TGD2C T8 (see Figure 4.5, A and C) using PtdCho/PtdOH mixed liposomes and pure lipid liposomes. For consistency, we analyzed DR-TGD2C in parallel as well (Figure 4.5B). In this experiment we used film-based detection and quantification of the Western signal as described under Experimental Procedures and obtained very similar results for DR-TGD2C (Figure 4.5, A and C; k=37.7%, n =2.5) to results observed for camera-based detection (Figure 4.2B; k =39.6%, n =5.7). Binding of DR-TGD2C T8 to PtdOH was much weaker compared to DR-TGD2C to the extent that interpretations regarding cooperativity were inconclusive (Figure 4.5C) and a fit to a Hill plot was not attempted.

Figure 4.5. Reduced affinity of the minimal PtdOH binding domain for PtdOH. *A*, PtdOH binding for DR-TGD2C (wild type). *B*, PtdOH binding for DR-TGD2C T8 carrying the 201-to-225 residue minimal domain. *C*, quantification of relative binding of PtdOH for DR-TGD2C and DR-TGD2C T8. *D*, PtdOH binding for DR-TGD2C T8. *E*, PtdOH binding for DR-TGD2C D3 (DR-TGD2C with deletion of minimal PtdOH binding domain residues 201-225). *F*, quantification of relative binding of PtdOH for DR-TGD2C T8 and DR-TGD2C D3. Liposome association assays were performed. Data shown in A, B, and C were obtain in the same representative experiment, likewise data obtained in D, E, and F were obtained in the same representative experiment.


A
PtdCho:PtdOH
100%PtdCho
90:10
80:20
70:30
60:40
50:50
40:60
30:70
20:80
10:90
100%PtdOH
DR-TGD2C
B
DR-TGD2C T8 (25AA)
C
DR-TGD2C
DR-TDG2C T8 (25AA)
k = 37.7%, n = 2.5
Arbitary Unites
PtdOH% (w/w)
D
PtdCho:PtdOH
DR-TGDC T8 (25AA)
E
DR-TGD2C D3 (Δ25AA)
F
DR-TGD2C T8
DR-TGD2C D3(Δ25AA)
k = 53.5%, n = 8.2
Arbitary Units
PtdOH % (w/w)

We also constructed an additional DR-TGD2C D3 deletion mutant lacking only residues 201-225 and compared it directly to DR-TGD2C T8 carrying only residues 201-225 (Figure 4.5, D-F). The deletion mutant DR-TGD2C D3 still had residual binding activity but less than observed for DR-TGD2C T8 carrying the identified 25 amino acid minimal PtdOH binding domain. When the data were fit to a Hill plot, positive cooperativity was indicated (Figure 4.5F; k = 53%, n = 8.2). Again, the analysis of DR-TGD2C T8 gave data too noisy to draw conclusion regarding cooperativity (Figure 4.5F). Taken together, these data suggest that TGD2C residues 201-225 are important to PtdOH binding and sufficient by themselves. They might represent the core of a primary PtdOH binding site, but in its absence a secondary weaker PtdOH binding site may be present, or possibly additional residues participate in the formation of a genuine PtdOH binding site in the native TGD2 protein.

To test the possibility for secondary PtdOH binding sites or residues participating in PtdOH binding by TGD2, an additional series of mutants with truncated sequences starting either from the C-terminus (residue 381) or from the center of the protein (residue 204) were generated, fused to DR and tested in pure PtdOH liposome binding assays as shown in Figure 4.6. Comparing the PtdOH binding activity of the different truncation clones, three regions were identified between residues 161 and 204, 251 and 300, or 291 and 340 that could contribute to binding of PtdOH to TGD2 outside of the core segment represented by residues 201-225.

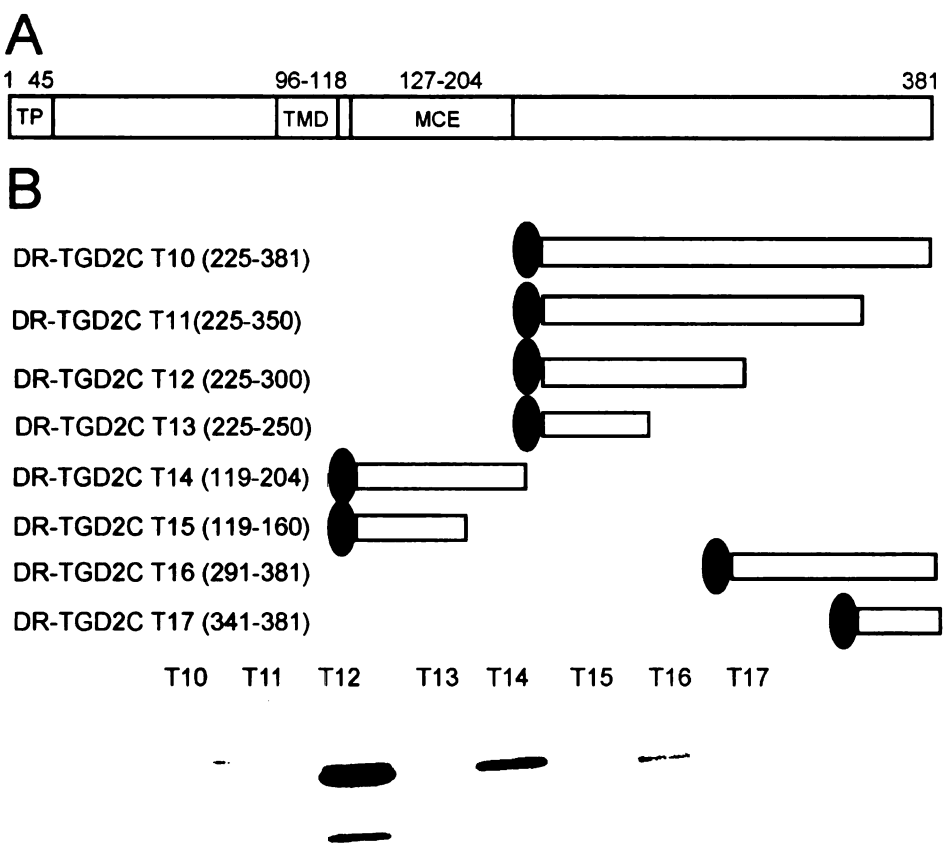


Figure 4.6. Additional TGD2 sequences contributing to PtdOH binding. *A*, primary structure of TGD2 indicating a predicted transit peptide (TP), transmembrane domain (TMD) and a conserved mycobacterial cell entry (MCE) domain. *B*, internal deletion and truncation mutants of TGD2C fused to the C-terminus of the DsRed protein (black oval). Liposome-association assays were performed to assess binding of various mutants to PtdOH liposomes.

4.5. Discussion

A crystal structure for TGD2 binding to its ligand PtdOH will ultimately be required to map the three-dimensional relationships between TGD2 amino acid residues and the ligand. In the absence of such a structure, systematic functional dissection of the primary amino acid sequence offers a valuable opportunity and the next logical step to learn more about this interaction. Previous genetic evidence has provided a solid in vivo framework for the function of TGD2 as the possible substrate binding protein of an ABC-type transporter hypothesized to be involved in ER-to-plastid lipid trafficking and required for chloroplast development(Awai *et al.* 2006). Other proteins involved in this process constitute the permease (TGD1) and ATPase (TGD3) of this presumed lipid ABC-type transporter in the inner plastid envelope (Benning 2008). The evidence to date that this system transports PtdOH is still indirect and is based on the observations that the *tgd1* mutant of Arabidopsis accumulates PtdOH and that isolated *tgd1* plastids show reduced incorporation of PtdOH into plastid lipids (Xu *et al.* 2005), and most convincingly that TGD2 specifically binds PtdOH (Awai *et al.* 2006). TGD2 appears anchored with a transmembrane domain into the inner envelope membrane, but a major C-terminal domain presumably faces the intermembrane space (Awai *et al.* 2006). The fact that the TGD2 wild-type protein is resistant to the protease trypsin, which penetrates the outer envelope but not the inner envelope membrane of chloroplasts, but that a mutant TGD2 protein and a green fluorescent protein fused to TGD2 are sensitive to the protease, suggests that the TGD2 protein is in a trypsin-inaccessible location, possibly in a junction between the inner and outer envelope membrane. Taken together, these observations let us hypothesize that TGD2 might provide the substrate PtdOH to the TGD1-TGD3

complex in the inner envelope membrane by extracting PtdOH from the intermembrane leaflet of the outer plastid envelope membrane. While this hypothesis is difficult to prove at this time, one step towards a better understanding of TGD2 function is a delineation of its actual PtdOH binding site and its mode of PtdOH binding. Important questions are whether the PtdOH binding site of TGD2 is localized in the intermembrane space, whether it is close to the predicted transmembrane domain and, therefore, close to the inner plastid envelope membrane, or whether it is synonymous to the MCE domain, for which a molecular function is not yet known, but which appears to be involved in membrane penetration by mycobactrial pathogens (Chitale *et al.* 2001).

The initially encountered protein solubility issue was solved by fusing the TGD2C protein lacking the predicted N-terminal membrane-spanning domain to an engineered highly soluble monomeric DR carrier protein (Bevis and Glick 2002). It should be noted that neither glutathione-*S*-transferase, nor maltose-binding protein were useful for this purpose, because in our hands both carrier proteins showed significant lipid binding above background on their own. However, DR does not bind lipids by itself. We generally tested binding of PtdOH to the fusion proteins using two assays, a protein-lipid overlay assay providing a measure for the specificity of binding and a liposome binding assay providing a semi-quantitative assessment of the affinity of TGD2 or its mutant derivatives for PtdOH. To avoid misinterpretation of the binding data, care was taken to stay within the linear range of the assays. Baring the technical limitations of the available approaches in mind, we were able to delineate a 25 amino acid stretch in the TGD2 protein that was sufficient for PtdOH binding, even though with reduced affinity. Upon generation of a point mutant (K221A) within the minimal domain, PtdOH binding

was diminished, identifying lysine 221 as a critical residue involved in this process. This finding is consistent with other work proposing basic amino acids and/or tryptophan as critical residues for PtdOH binding (Kooijman *et al.* 2007, Munnik and Testerink 2005).

In the reverse experiment, deleting residues 201-225 from TGD2C did not fully abolish PtdOH binding activity (Figure 4.5, E and F) suggesting the presence of other, secondary amino acids involved in PtdOH binding. The presence of six proline residues in the identified primary binding site represented by TGD2 residues 201-225 suggests that this minimal region forms a loop-strand fold lacking helical or β-strand structure as indicated by secondary structure prediction (Figure 4.1B). Additional elements are clearly required to hold this loop in place and enable the TGD2 protein to bind PtdOH in a cooperative manner as suggested by the DR-TGD2C binding data.

In general, PtdOH binding sites of different proteins lack conservation in their primary structures (Munnik and Testerink 2005). Likewise, the observed TGD2 PtdOH binding site does not follow an established pattern. Presumed orthologs of TGD2 (Figure 4.1) show more similarity in sequences outside the identified minimal PtdOH binding site than within it. N-terminally adjacent to the minimal PtdOH binding is the MCE domain, a highly conserved feature initially found in mycobacterial cell surface proteins required for entry of the pathogen into the host cell (Chitale *et al.* 2001). This study clearly shows that the MCE domain is not required for PtdOH binding and must have other functionalities that nevertheless could lead to cell membrane fusion or penetration as would be necessary for mycobacterial entry into mammalian host cells. Whether the close proximity of the two sequence features in TGD2 is necessary for the function of the protein can only be speculated at this time. The central location of the identified binding

domain approximately ~100 residues C-terminal of the predicted membrane-spanning domain does not rule out the possibility that TGD2 could extract PtdOH out of the outer envelope membrane assuming that it is anchored with its N-terminus into the inner envelope membrane. How PtdOH would be formed at the outer envelope remains unclear. However, the recent identification of the TGD4 protein involved in ER-to-plastid lipid trafficking, which is the first TGD protein associated with the ER(Xu *et al.* 2008), provides new avenues towards an understanding of PtdOH formation from ER-derived precursors in predicted contact zones between the ER and the outer plastid envelope membrane.

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Chapter 5

Conclusions and perspectives

5.1. Major Conclusions

This dissertation focused on the study of TGD3 and TGD2 protein, the ATPase component and the substrate binding protein of a proposed bacterial type ABC transporter, respectively. The *TGD3* gene was isolated by a reverse genetics approach. Arabidopsis plants with a T-DNA inserted in the 5'-UTR region have reduced *TGD3* mRNA expression levels and phenotypes similar to that of *tgd1-1* (Xu *et al.* 2003, Xu *et al.* 2005), including retarded growth, accumulation of trigalactosyldiacylglycerol (TGDG) and triacylglycerol (TAG) (Lu *et al.* 2007). By using a double stranded RNA mediated RNA silencing technique, the *TGD3* mRNA level was further reduced and the phenotype was enhanced (see chapter 3). However, the fact that the resulting *TGD3i* plants still have normal seed development in combination with bioinformatics data led to the speculation that there might be a second ATPase associated with TGD1 that could partially substitute for TGD3. Further experiments to test this hypothesis are still pending.

When fused with MBP, the TGD3 protein has basal ATPase activity *in vitro*. Upon mutating the conserved phenylalanine to alanine, the ATPase activity was almost abolished. Overexpression of this F94A mutant cDNA caused a dominant-negative effect in the Col-2 wild-type background, supporting the hypothesis that TGD3 is involved in a complex. One explanation is that the TGD3F94A mutant protein is nonfunctional but could still be part of its native complex thereby disrupting the overall function of the complex.

The TGD2 protein binds specifically to phosphatidic acid (PtdOH) (Awai *et al.* 2006). In this study, the binding domain of TGD2 was narrowed down to a 25 amino acid segment, which by itself is sufficient to mediate specific PtdOH binding of TGD2. The

binding property of this 25-mer is different from that of WT, suggesting that there might be other sequences involved to fulfill complete and complex WT-like binding. A crystal structure of TGD2 is ultimately required to demonstrate the three dimensional relationship between TGD2 and its ligand.

5.2. Remaining questions and future directions

Lipid transfer into and out of plastids is extensive as tissues expand. The discovery of the TGD proteins provides new insights into lipid-trafficking phenomena involving plastids, particularly the import of lipid precursors from the ER during chloroplast biogenesis. However, at this time there are still some important issues that need to be addressed. For example, no direct interaction among the three TGD proteins has been demonstrated, the transporter has not been reconstituted *in vitro*, and its substrate specificity has not yet been determined.

Meanwhile, additional components involved in the ER that facilitate the ER-chloroplast outer envelope membrane contact sites and lipid import into the plastid remain to be discovered, as some evidence suggests that such proteins must exist (Xu *et al.* 2008). Hopefully, the availability of the extensive genomic resources for plants and photosynthetic bacteria will accelerate the discovery process in the near future.

I will discuss below remaining questions in the study of TGD proteins and propose some future directions as well.

5.2.1 Demonstration of direct interaction of the three TGD proteins

The TGD proteins are hypothesized to form a bacterial type ABC transporter. This is based on the nearly identical lipid phenotypes of the respective Arabidopsis mutants with disrupted TGD protein function and the fact that their bacterial orthologs are often organized in an operon. However, direct proof that the proteins interact with each other is lacking.

Polyclonal antibodies against individual TGD proteins were generated. Further purification and characterization of the antibodies are needed to test their efficiency and specificity. Upon availability of specific antibodies, co-immunoprecipitation experiments should be conducted according to the methods described in ref (Pandey and Assmann 2004). Total proteins will have to be extracted from HA-tagged TGD1 plants. The TGD1 protein will be pulled down by anti-HA antibodies and protein A-Sepharose. TGD1 and/or the putative complex will be extracted and solubilized by nonionic detergents such as Triton X-100, Nonidet P-40, and CHAPS. Protein immunoprecipitated with anti-HA antibodies will be probed with anti-TGD2 (Awai *et al.* 2006) or anti-TGD3 antibodies (Lu *et al.*, unpublished) by immunoblotting. TGD2 and/or TGD3 proteins are expected in the complex, confirming that TGD1 is indeed associated with the TGD3 and/or TGD2 proteins.

Additionally, the purified complex could be submitted to the Mass spectrometry facility of MSU and explore all the components of the complex. This will help unveil additional components involved in lipid transport between the ER and the chloroplasts that might be associated with the currently known TGD proteins. The potential sites of protein-protein interactions between TGD1 protein and other proteins can be explored by

using homobifunctional amino group-specific chemical cross-linkers such as dimethyladipimidate (DMA), dimethylsuberimidate (DMS) and bis (sulfosuccinimidyl) suberate (BS). The reactive residues will be identified by matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) of tryptic peptides. Such an approach has been successfully used to map protein-protein interactions between MutL and MutH (components of an *E.coli* DNA repair system) (Giron-Monzon *et al.* 2004) and interactions between UreD, UreF and Urease from *Klebsiella aerogenes* (Chang *et al.* 2004).

5.2.2. Investigation of the function of MCE domain in TGD2

The amino acid sequence of the TGD2 protein between residues 127-204 has similarity to the MCE (mammalian cell entry) related proteins (Marchler-Bauer *et al.* 2009). These proteins are from *Mycobacterium tuberculosis* and found to be necessary for colonization of, and survival within macrophages (Arruda *et al.* 1993, Flesselles *et al.* 1999)

Previously, the MCE gene in the *Mycobacterium tuberculosis* genome was cloned and the protein product promoted uptake into Hela cells of polystyrene latex microspheres coated with the protein (Chitale *et al.* 2001). Although the exact function of the MCE protein is still unknown, it might serve as a membrane interacting domain on the surface of *Mycobacterium tuberculosis* that is capable of eliciting plasma membrane perturbations in non-phagocytic mammalian cells.

In-depth biochemical analysis revealed that the MCE domain in TGD2 appears to be not critical for PtdOH binding (Lu and Benning 2009). However, the identified

minimal PtdOH binding domain (residue 201-225) of TGD2 is just outside of the MCE domain and even overlaps partially with it (see Chapter 4, Figure 4.4). Furthermore, residues161-204, which are part of the predicted MCE domain, still might be involved in PtdOH binding (see Chapter 4, Figure 4.6). Therefore, PtdOH binding by the MCE domain itself needs to be tested. For that purpose, the MCE domain of TGD2 should be cloned and expressed as a DsRed fusion protein and tested for specific PtdOH binding similar as described in Chapter 4.

In addition, even if the MCE domain turns out to be not directly involved PtdOH binding, there are still possibilities that the MCE domain has other functions such as membrane fusion or penetration as that would be required by bacterial cell entry into mammalian host cells. To test that, an uptake and internalization experiment using HeLa cells and latex beads coated with DsRed-TGD2MCE domains according to previously reported methods (El-Shazly *et al.* 2007) should be conducted.

Briefly, latex beads coated with purified recombinant DsRed-TGD2MCE protein will be incubated with HeLa cell monolayer for varying lengths of time at 37°C. Cell suspension will be collected and observed under fluorescence microscope with an excitation at 556 nm and emission at 586 nm for DsRed-monomer red fluorescence. Transmission electron microscopy will also be used as a second method to confirm the uptake of beads coated with DsRed-TGD2MCE protein by HeLa cells. I expect that the DsRed-TGD2MCE protein could also facilitate the uptake and internalization of latex beads by HeLa cells, similar to Mce1A, Mce4A, and LprM/Mce3E (Chitale *et al.* 2001, El-Shazly *et al.* 2007, Saini *et al.* 2008). The expected result would suggest a possible function of TGD2 MCE domain in membrane perturbations and invaginations, which

might be required for the transport of lipids or other substrates across chloroplast envelopes in plants.

5.2.3 Reconstitution of TGD transporter and in vitro uptake assay

Previously, we hypothesized that TGD2 binds a lipid and contributes to the substrate specificity of the transporter. This is based on the facts that TGD2 is similar to the substrate binding protein of bacterial ABC transporters and that the Arabidopsis *tgd2* mutant phenotype is consistent with an impairment in ER-to-plastid lipid trafficking. Later, TGD2 was shown to bind PtdOH specifically, and the incorporation of PtdOH into galactoglycerolipids was reduced by isolated *tgd1* chloroplasts, which is consistent with PtdOH as the substrate for the proposed transporter complex (Awai *et al.* 2006, Xu *et al.* 2005).

However, PtdOH is usually maintained at very low levels and often serves as precursor. It is also rapidly converted into other lipids in plants under normal conditions. Therefore, we cannot rule out the possibilities that there are other lipids that are transported by the proposed TGD complex. For example, recent rapid pulse-chase labeling experiments with pea or rape seed leaves have shown that newly exported fatty acids are first incorporated into PtdCho instead of PtdOH, presumably due to a very active acyl editing mechanism (Bates *et al.* 2007, Williams *et al.* 2000). These results suggest that the first glycerolipid formed outside the plastid from newly synthesized fatty acids is PtdCho.

The recent discovery of TGD4, a novel Arabidopsis TGD protein, suggests that TGD4 protein is possibly associated with the ER. It has been speculated that TGD4 is

directly involved in the formation of plastid associated microsomes (PLAMs) and is required for the transfer of PtdCho to the outer plastid envelope (Xu *et al.* 2008). Recombinant TGD4 protein has some PtdCho binding activity when expressed as a maltose binding protein fusion (Xu et al., unpublished). Therefore, it is reasonable to hypothesize that the TGD transporter system might be required for the transfer of PtdOH formed at the outer chloroplast envelope from PtdCho to the inside of the inner envelope membrane where the PtdOH phosphatase resides (Malherbe *et al.* 1992). This process could produce diacylglycerol for subsequent galactoglycerolipid biosynthesis (See Chapter 1, Figure 1.3). For this hypothesis, one would expect a phospholipase D involved in the conversion of PtdCho to PtdOH at the outside of the outer chloroplast envelope membrane, for which the gene has not been identified.

It is extremely difficult to determine the substrates and substrate specificity of lipid transporters because no reliable *in vitro* assays are available. Also, a functional reconstitution of these membrane proteins involved is generally very challenging. The latter issue poses an even greater hurdle for the analysis of multi-component transporters assembled from different peptides, including the proposed TGD transporter. Recently, we successfully generated a recombinant maltose binding protein-TGD1-TGD3 fusion protein and purified it to homogeneity, which provides hope for functional reconstitution of the transporter *in vitro*. Preliminary data suggested that this single MBP-TGD1-TGD3 fusion protein potentially has basal ATP hydrolysis activity (Z.Wang, B.Lu, C.Benning, unpublished work), similar to the TGD3 protein alone. Experiments need to be conducted in the near future to confirm the result and test whether the ATPase activity could be

stimulated in the presence of PtdOH, the proposed substrate of the transporter, or inhibited by vanadate and other compounds.

In addition, purified fusion protein could be functionally reconstituted into proteoliposomes and tested for its ability to translocate or transport fluorescent lipids. This could be done by using a dithionite reduction assay in the presence or absence of DsRed-TGD2 protein. Such a system has been successfully used to demonstrate the function of the *E.coli* MsbA protein in a reconstituted system *in vitro* (Eckford and Sharom 2008). MsbA functions as an ATP-dependent lipid translocase that transports lipid A from the inner to the outer leaflet of the cytoplasmic membrane. By using a fluorescence quenching assay, MsbA was shown to have ATP-dependent flippase activity for several fluorescent NBD-lipids (Eckford and Sharom 2008)

5.3. Summary

Only a few PtdOH targets have so far been identified in plants. Moreover, most of the PtdOH targets studied are involved in PtdOH signaling, including the first PtdOH target ABI1(ABA insensitive 1) (Zhang *et al.* 2004) and PDK1 (phosphoinositide-dependent kinase 1) (Anthony *et al.* 2004). However, PtdOH can also serve as an important metabolite involved in phospholipids biosynthesis and membrane remodeling. For instance, in the model plant *Arabidopsis thaliana*, there are two parallel pathways that contribute to the bulk synthesis of the predominant galactolipids in thylakoid membrane (Roughan *et al.* 1980, Roughan and Slack 1982). In either pathway, PtdOH is an important intermediate for the bulk of thylakoid lipid biosynthesis.

The TGD2 protein of Arabidopsis has been identified as a novel PtdOH target in this described dissertation (See Chapter 4). However, together with TGD1 and TGD3, these Arabidopsis proteins, which are involved in lipid transfer from the outer to the inner chloroplast envelope membrane, need to be reconstituted and tested for transport activity as well as substrate specificity.

How the TGD transport complex works still remains unclear. Our current speculation is that the TGD2 protein seems critical to trigger the first step of import by binding to the lipid substrates and thus modulates the activity of the TGD1, 3 core transporter. With its N-terminus tethered into the inner chloroplast envelope, the TGD2 C-terminus could face the intermembrane space and even touch the outer envelope. Therefore, TGD2 could extract PtdOH formed at the outer envelope and deliver it to TGD1, which then acts as a flippase to mediate transport of PtdOH through the membrane with the energy supplied by ATP hydrolysis catalyzed by TGD3. Two pieces of evidence supported TGD2 localization in two-membrane contact zones: 1) TGD2 contains an MCE domain typically found in cell surface proteins that are needed by mycobacteria cell entry into mammalian host cells (Chitale *et al.* 2001). Functional study of the TGD2 MCE domain is currently under way. 2). It was observed that both the TGD2-GFP fusion and the *tgd2-1* mutant protein are sensitive to Trypsin treatment, whereas the wild type TGD2 protein by itself was resistant (Awai *et al.* 2006). One possible interpretation is that TGD2 is in a complex or a membrane contact domain inaccessible to Trypsin.

In summary, whether or not this TGD complex is located in membrane contact zones between the outer and inner envelopes and whether or not this complex transports

PtdOH from the outer to the inner envelope still remains an interesting and challenging hypothesis, which will require substantial amount of work to test. If transport of PtdOH by the TGD complex can be confirmed by future efforts, this will be the first PtdOH transporter identified and will greatly advance our understanding of lipid transport and chloroplast biogenesis.

On the other hand, the proposed TGD complex belongs to ABC transporters, one of the largest and best conserved protein families found in all organisms (Moody *et al.* 2002, Pohl *et al.* 2005). Some ABC transporters involved in the export of precursors for cuticle or wax biosynthesis in the plant epidermis are reported (Bird *et al.* 2007, Luo *et al.* 2007, Pighin *et al.* 2004, Ukitsu *et al.* 2007). Another well studied ABC transporter in Arabidopsis, COMATOSE (CTS), PEROXISOMAL ABC TRANSPORTER1(PXA1) or PEROXISOME DEFECTIVE3 (PED3), is localized in peroxisomes to import fatty acids or acyl-CoAs for β-oxidation (Footitt *et al.* 2002, Hayashi *et al.* 2002, Zolman *et al.* 2001). However, all the currently identified ABC transporters in plants are a homodimer encoded by a single peptide with a multi-membrane-spanning permease domain and an ATP-binding cassette (ABC) domain. To date, the TGD1, 2, 3 complex is the only Arabidopsis ABC transporter involved in ER-to-plastid lipid exchange that resembles a bacterial-type multipartite ABC transporter (Awai *et al.* 2006, Lu *et al.* 2007, Xu *et al.* 2003, Xu *et al.* 2005).

Many bacteria have orthologs of the Arabidopsis *TGD1, 2, 3* genes organized in operons. For example, the TGD orthologs in *Pseudomonas putida* were proposed to form a toluene efflux pump and thus required for toluene tolerance (Kieboom *et al.* 1998b, Kieboom *et al.* 1998a, Kim *et al.* 1998). However, toluene has not yet been confirmed as

a substrate. It is possible that the bacterial orthologs are involved in lipid remodeling of the cell membrane in response to solvent stress. Hopefully, in-depth functional studies of the TGD transporter will lead to a better overall understanding of ABC lipid transporters and provide new insights into their bacterial orthologs.

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