FUNCTIONAL ANALYSIS OF A PROTEIN INVOLVED IN THE ENDOPLASMIC RETICULUM-TO-CHLOROPLAST LIPID TRAFFICKING PATHWAY IN PLANTS

By

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ABSTRACT

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Photosynthesis is arguably the most important trait of plants for improving human conditions. Chloroplast is the dedicated cellular organelle where photosynthesis takes place. Chloroplast is made up of intricate membrane systems, where inner and outer envelopes enclose the photosynthetic thylakoid membranes. Chloroplast membranes mainly consist of non-phosphorous glycolipids that are synthesized through two pathways: the prokaryotic and the eukaryotic pathway.

In the prokaryotic pathway, fatty acids synthesized within the chloroplast are incorporated into glycerol-3-phosphate for glycolipids and phospholipids synthesis. All reactions take place inside the chloroplast. In the eukaryotic pathway, fatty acid residues are first exported from the chloroplast to the endoplasmic reticulum (ER) for the synthesis of extraplastidic membrane lipids. A certain portion of these lipids is transferred back from the ER to the chloroplast for chloroplast lipid synthesis.

Forward genetic screening has revealed a few Arabidopsis mutants impaired in the eukaryotic pathway. They accumulate an abnormal galactolipid trigalactosyldiacylglycerol (TGDG), and the mutants are, therefore, designated as *tgd* mutants. *tgd* mutants exhibit various phenotypes including dwarfed growth, pale-colored leaves, and partially aborted seeds. Four genes have been found responsible for the phenotypes: *TGD1, 2, 3, 4*. The *TGD1, 2, 3* gene

products, TGD1, 2, 3 proteins, form a putative ATP-binding cassette (ABC) transporter on the inner envelope of the chloroplast, however the molecular function of TGD4 protein cannot be inferred from previous knowledge.

In this work, TGD4 is found to be a transmembrane protein embedded in the chloroplast outer envelope. It is predicted to be a β-barrel protein reminiscent of gram-negative bacteria outer membrane β-barrel transporters. The recombinant TGD4 protein binds phosphatidic acid (PtdOH) specifically and two regions, amino acids 1-80 and 110-145 are responsible for PtdOH binding. *tgd4* mutants accumulate PtdOH outside of the chloroplast suggesting TGD4 is a PtdOH transporter. TGD4 is indeed able to transfer PtdOH from one membrane bilayer to the other *in vitro*. TGD4 does not interact strongly with any other proteins but dose interact with itself and forms a homodimer *in vivo*. In conclusion, TGD4 is a putative chloroplast outer envelope PtdOH transporter that transfers PtdOH from the ER to the chloroplast intermembrane space.

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As I am writing the last part of this dissertation, fall leaves are turning for the last time of my stay in East Lansing. I have had the most productive and pleasant five years so far in my life and have met some of the most important people to me here in Michigan.

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My committee members, Dr. Federica Brandizzi, Dr. Michael Garavito, Dr. Eric Hegg and Dr. John Ohlrogge, are the source of wisdom and continues support. I learned a lot about con-focal microscopy from Dr. Brandizzi and her lab members. Dr. Garavito gave me valuable advices about membrane protein biochemistry and is now carrying out a project for TGD4 crystallization. I appreciate Dr. Hegg's willingness to be my committee chair who has to do most of the paper work. I enjoyed discussing specific questions regarding plant lipid metabolism with Dr. Ohlrogge who always gave me a comprehensive view of the field.

Lastly, I thank my parents, my boyfriend, and my friends for years of support. There are still many people that I want to acknowledge but the space is limited. I want to end this section by quoting Steve Jobs, the founder of Apple Inc.:

"The people who are crazy enough to think they can change the world are the ones who do."

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CHAPTER 1

Chloroplast lipid synthesis and the lipid trafficking through endoplasmic reticulum-to-plastid membrane contact sites in *Arabidopsis thaliana*[1]

[1]This work has been published: **Wang Z., Benning C.** (2012) Chloroplast lipid synthesis and lipid trafficking through ER-plastid membrane contact sites. *Biochem. Soc. Trans.* **40**, 456-463. I designed all the figures and wrote the first draft of the review.

1.1. Abstract

Plant chloroplasts contain an intricate photosynthetic membrane system, the thylakoids, and are surrounded by two envelope membranes at which thylakoid lipids are assembled. The glycoglycerolipids mono- and digalactosyldiacylglycerol, and sulfoquinovosyldiacylglycerol as well as phosphatidylglycerol are present in thylakoid membranes giving them a unique composition. Fatty acids are synthesized in the chloroplast and are either directly assembled into thylakoid lipids at the envelope membranes or exported to the endoplasmic reticulum (ER) for extraplastidic lipid assembly. A fraction of lipid precursors is re-imported into the chloroplast for the synthesis of thylakoid lipids. Thus, polar lipid assembly in plants requires tight coordination between the chloroplast and the ER and necessitates inter-organelle lipid trafficking. Here we discuss our current knowledge of the export of fatty acids from the chloroplast and the import of chloroplast lipid precursors assembled at the ER. Direct membrane contact sites between the ER and the chloroplast outer envelopes are discussed as possible conduits for lipid transfer.

1.2. Introduction

Photosynthesis is a ubiquitous biochemical process of plants and other photosynthetic organisms converting light into metabolic energy. It is essential for life on earth. The organelle in plant cells responsible for photosynthesis, the chloroplast, is enclosed by two envelopes and contains the inner thylakoid membranes harboring the photosynthesis apparatus. These intricate membrane systems constitute about 70% of total plant cell membranes of which ~80% are galactoglycerolipids (Dörmann and Benning, 2002). In fact, the chloroplast glycoglycerolipids monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and the sulfur-containing lipid sulfoquinovosyldiacylglycerol (SQDG) predominate over the phosphoglycerolipid, phosphatidylglycerol (PtdGro), present in thylakoids (Jouhet *et al.*, 2007). Chloroplast lipids directly interact with photosynthetic complexes. For example, galactoglycerolipids have been found in the crystal structure of photosystem I and II (Guskov et al., 2009; Jordan et al., 2001). Indeed, Arabidopsis mutants deficient in either MGDG, DGDG, SQDG or PtdGro synthesis, *mgd1*, *dgd1, sqd2 or pgp1,* respectively, are impaired in photosynthesis apparent in the decrease in chlorophyll content, defects in chloroplast ultrastructure, and reduced photosynthetic activity (Dörmann *et al.*, 1995; Jarvis *et al.*, 2000; Xu *et al.*, 2002; Yu *et al.*, 2002). The assembly of chloroplast lipids primarily takes place at the chloroplast envelope membranes, but is complicated by the fact that lipid precursors originate at different locations/membranes within the cell. Aside from the chloroplast envelope membranes, the ER is also involved in the biosynthesis of chloroplast lipids. Therefore, extensive lipid trafficking takes place between the ER and chloroplast envelope membranes.

1.3. Structural and functional properties of chloroplast lipids

In leaves of Arabidopsis grown under normal conditions, the ratios of MGDG, DGDG, SQDG, and PtdGro relative to total glycerolipids are about 50%, 20%, 2%, and 10%, respectively (Dörmann and Benning, 2002). In an MGDG molecule, a galactose is linked by a β-glycosidic bond in the *sn*-3 position of the glyceryl residue of the diacylglyceryl (DAG) moiety (Figure 1.1.). The predominant fatty acids in MGDG are α-linolenic acid ($C_{18:3}^{\Delta 9,12,15}$; 18 carbon acyl chain with *cis*-double bonds at carbons 9, 12, 15 counting from the carboxyl group) and hexadecatrienoic acid ($C_{16:3}^{\Delta 7,10,13}$) esterified at the *sn-2* position of the glyceryl backbone. Because of the relatively small galactosyl head group of MGDG comparred to DGDG and the high degree of unsaturation of the respective fatty acids, the MGDG molecule adapts a conical shape (with the head group at its tip) which is thought to accommodate the curvature of envelopes and thylakoid membranes (Webb and GREEN, 1991). In fact, MGDG is a non-bilayer forming lipid when mixed with water in the absence of other lipids. MGDG is synthesized by MGDG synthase 1, or MGD1 in Arabidopsis, which is an UDP-Gal: diacylglycerol galactosyl transferase localized at the inner envelope membrane of the chloroplast facing the intermembrane space (Miège *et al.*, 1999; Xu *et al.*, 2005). In spinach leaves, MGDG constitutes 17% of total lipids on the outer envelope, 55% on the inner envelope, and 58% on the thylakoid membranes (Jouhet *et al.*, 2007).

The head group of DGDG consists of MGDG with a second galactosyl residue connected in an α1-6 linkage (Figure 1.1.). The major fatty acids in DGDG are α-linolenic acid ($C_{18:3}^{\Delta 9,12,15}$) and palmitic acid ($C_{16:0}$). Since DGDG has two galactosyl moieties in its head group, it is more cylindrically shaped than MGDG and, hence, a bilayer-forming lipid (Webb

and GREEN, 1991). In Arabidopsis, the major DGDG synthase, DGD1, is localized at the outer envelope membrane facing the cytosol (Froehlich *et al.*, 2001) and catalyzes the transfer of a galactosyl residue from UDP-Gal to MGDG (Kelly *et al.*, 2003). In spinach, DGDG makes up 30% of total lipids in both envelope membranes and 27% in the thylakoid membrane (Jouhet *et al.*, 2007).

The sulfolipid SQDG and phospholipid PtdGro are anionic lipids essential for photosynthesis (Yu and Benning, 2003). In SQDG, the sulfoquinovosyl head group is linked to DAG with an α-glycosidic bond (Figure 1.1.). Similar to DGDG, the most abundant fatty acids in SQDG are α-linolenic acid ($C_{18:3}^{\Delta 9,12,15}$) and palmitic acid ($C_{16:0}$). SQDG synthase, SQD2, is associated with the inner chloroplast envelope membrane and transfers a sulfoquinovose from UDP-sulfoquinovose to diacylglycerol forming SQDG (Yu *et al.*, 2002), which accounts for about 6% of the lipids in both envelope and thylakoids membranes in spinach (Jouhet *et al.*, 2007). In Arabidopsis, PtdGro is synthesized from CDP-diacylglycerol and glycerol-3-phosphate by PtdGro-phosphate synthases PGP1 and PGP2 and subsequently dephosphorylated by PtdGro-phosphate phosphatase associated with the inner envelope membrane of chloroplasts (Xu et al., 2002; Müller and Frentzen, 2001). Aside from α-linolenic acid, PtdGro also possesses a unique $16{:}1^{\Delta 3}$ fatty acid with its double bond in *trans*-configuration (Figure 1.1.) (Gao *et al.*, 2009). PtdGro represents 10% of polar lipids in both envelope membranes and 7% in thylakoids of spinach (Jouhet *et al.*, 2007).

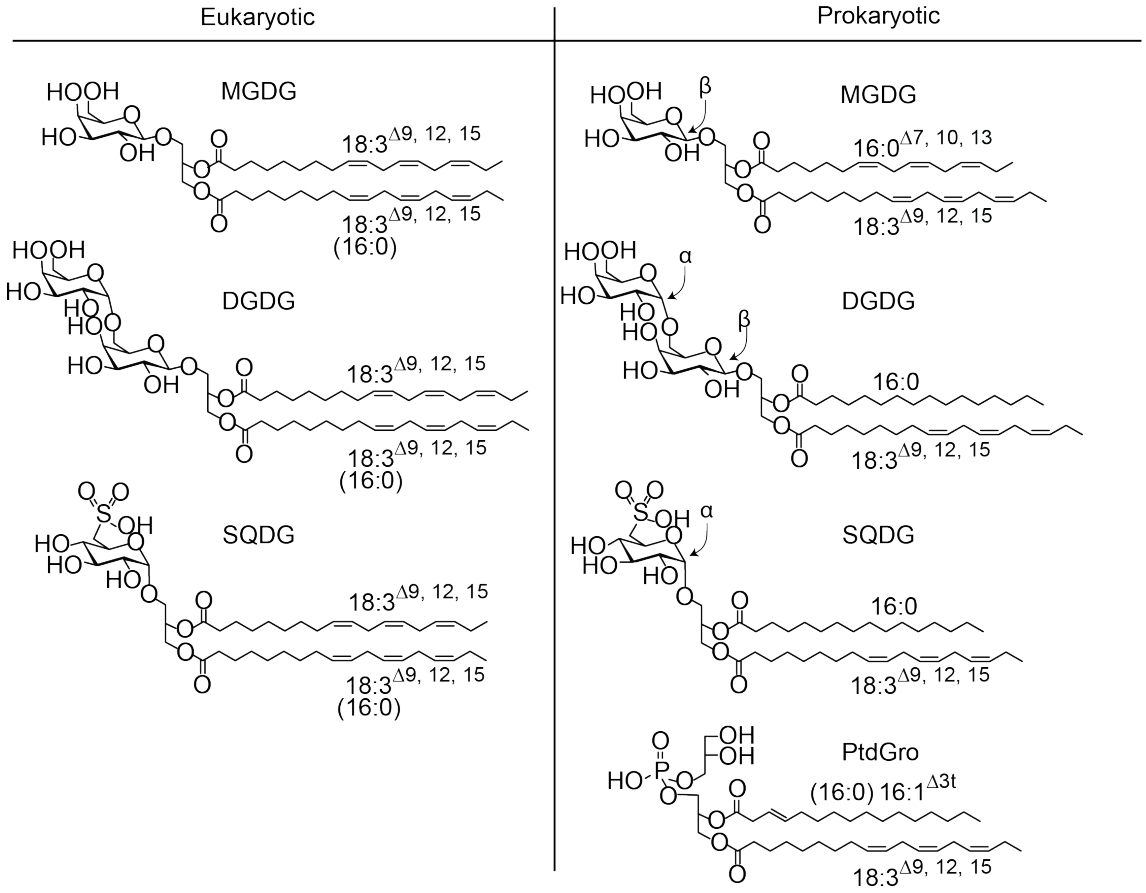

Figure 1.1. Structures of predominant eukaryotic and prokaryotic chloroplast lipids in Arabidopsis. The predominant membrane lipids in Arabidopsis chloroplasts are monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), sulfoquinovosyldiacylglycerol (SQGD), and phosphatidylglycerol (PtdGro). MGDG, DGDG, and SQDG can be assembled *de novo* in the plastid by the prokaryotic pathway leading to molecular species with an 16-carbon acyl chain in the *sn*-2 position of the glyceryl backbone, or at the ER by the eukaryotic pathway giving rise to molecular species with a 18-carbon acyl chain in the same position. However, plastid PtdGro is synthesized exclusively from the prokaryotic

Figure 1.1. (cont'd)

pathway. In MGDG and DGDG, a β-glycosidic bond joins the galactosyl and diacylglyceryl groups, while in SQDG the anomeric carbon of the sulfoquinovosyl residue adopts the α-configuration. In DGDG, the anomeric carbon of the second galactosyl residue is in the α-configuration. Fatty acids common to chloroplast lipids are: α -linolenic acid ($18:3^{\Delta 9,12,15}$), palmitic acid (16:0), and hexadecatrienoic acid ($16:3^{\Delta 7,10,13}$) as shown. An unusual $16:1^{trans\text{-}\Delta 3}$ fatty acid is found exclusively in the *sn*-2 position of PtdGro.

1.4. Two pathways for chloroplast lipid synthesis

During the biogenesis of complex lipids, fatty acids synthesized *de novo* in the chloroplast can have two fates: either they remain in the plastid where they are assembled into complex lipids by the prokaryotic pathway, or they are exported to the ER where they enter the eukaryotic pathway of lipid assembly (Figure 1.2.) (Benning, 2009). The two pathway hypothesis was first proposed by Roughan and coworkers (P G Roughan, 1980). *In vivo* pulse-chase experiments with intact spinach leaves incubated with [^{14}C]-labeled acetate showed a biphasic kinetics, an initial labeling of MGDG followed by a decrease and a subsequent increase. The initial rapid incorporation of fatty acids into MGDG is interpreted as *de novo* biosynthesis by the prokaryotic pathway. The subsequent decrease is due to lipid turnover, e.g. the conversion of MGDG to DGDG. The second increase reflects the import of DAG moieties assembled at the ER into the chloroplast where they serve as the substrate for MGDG synthesis (Benning, 2008). Glycerolipids originating from the prokaryotic pathway carry a 16-carbon acyl chain at the *sn-2*

position of the glyceryl backbone while glycerolipids assembled by the eukaryotic pathway contain an 18-carbon acyl chain at the same position (Figure 1.1.) (Frentzen *et al.*, 1983) . In Arabidopsis MGDG, DGDG, and SQDG can be synthesized from either pathway while plastid PtdGro is synthesized exclusively by the prokaryotic pathway (Xu *et al.*, 2006). Many seed plants, such as pea, only use the eukaryotic pathway for chloroplast glycoglycerolipid assembly. These plants have a high proportion of α-linolenic acid (18:3) in chloroplast lipids, giving rise to their designation "18:3 plants". In other plants such as Arabidopsis and spinach, both pathways are involved in the biosynthesis of chloroplast lipids and they are named "16:3 plants" for the preponderance of hexadecatrienoic acid (16:3) in their chloroplast lipid (Heinz and Roughan, 1983). In Arabidopsis, both pathways contribute approximately equal to galactolipids synthesis (Warwick *et al.*, 1986). The two-pathway hypothesis was confirmed by the discovery of Arabidopsis mutants impaired in either of the pathways. In the *ats1* mutant, the prokaryotic pathway was disrupted converting Arabidopsis essentially into an 18:3 plant without any significant growth phenotype (Kunst *et al.*, 1988). However, mutants impaired in the eukaryotic pathway such as *tgd1* are severely affected in growth and strong alleles cause embryo lethality (Xu *et al.*, 2003; Xu *et al.*, 2005). Pulse-chase labeling experiments have shown that *ats1* plants lack the rapid phase of MGDG synthesis while the *tgd1* mutant lacks the second phase increase (Xu *et al.*, 2003). The eukaryotic pathway involving both the ER and the chloroplast necessitates extensive lipid trafficking between the three membranes, especially during the export of fatty acids from the chloroplast to the ER and the import of DAG moieties from the ER to the chloroplast. The mechanisms of these processes will be discussed below in detail.

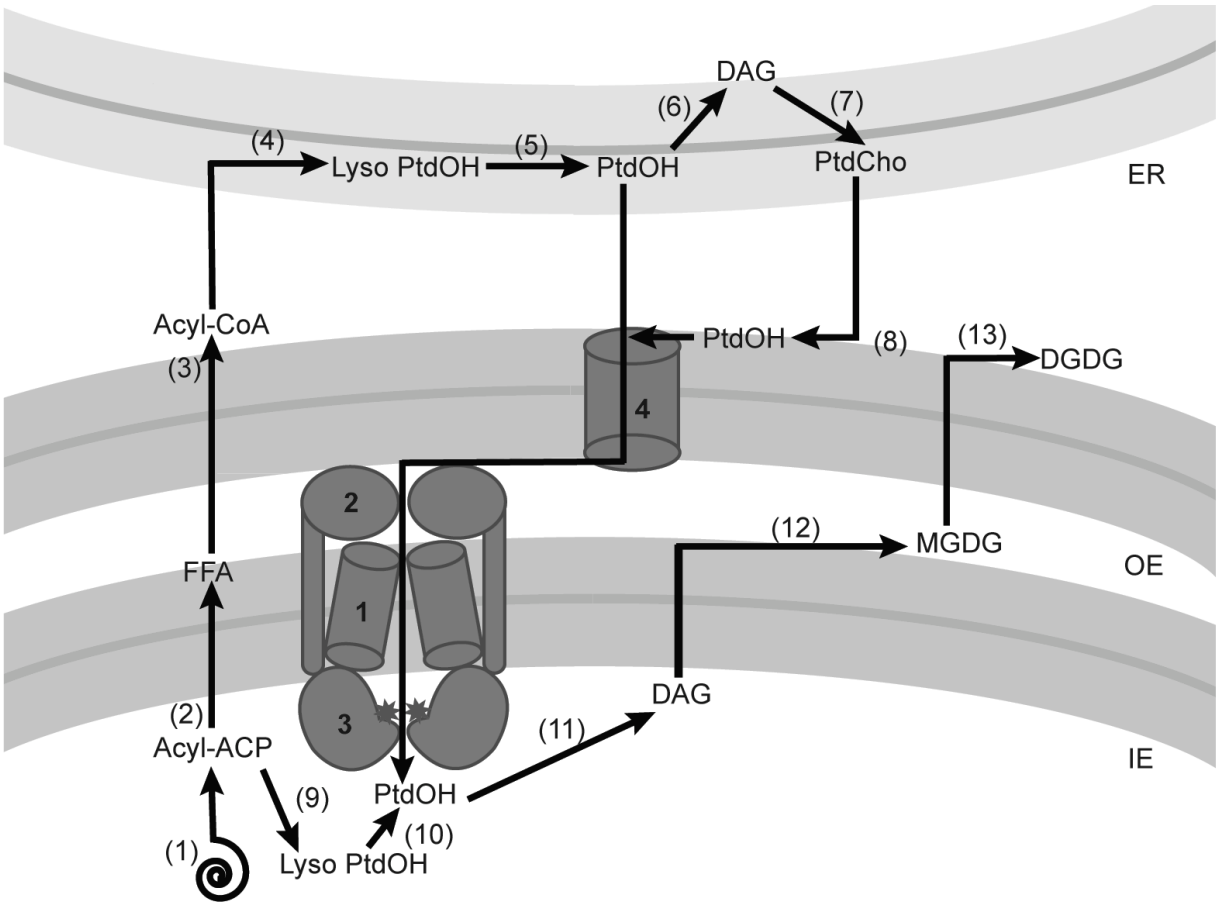

Figure 1.2. Schematic representation of the ER-to-chloroplast lipid trafficking and the biosynthesis of galactolipids through the prokaryotic and eukaryotic pathways. TGD1, 2, 3, 4 form two distinctive protein complexes on the outer (OE) and inner (IE) chloroplast envelopes responsible for the transfer of lipid precursors from the ER to the chloroplast. This process requires ATP (star) hydrolysis to provide energy. Abbreviations: Acyl-ACP: Acyl-acyl carrier protein; FFA: free fatty acids; Acyl-CoA: Acyl-Coenzyme A; Lyso PtdOH: lysophosphatidic acid; DAG: diacylglycerol; PtdCho: phosphatidic choline. Numbers in brackets refer to the enzymes involved in each step: 1. Fatty acid synthase complex; 2. Acyl-ACP thioesterase; 3. Acyl-CoA synthetase; 4. ER glycerol-3-phosphate: acyl-CoA acyltransferase; 5. ER

Figure 1.2. (cont'd)

lysophosphatidic acid: acyl-CoA acyltransferase; 6. ER phosphatidic acid phosphatase; 7. Diacylglycerol: CDP-choline phosphotransferase; 8. Putative phospholipase D; 9. Plastid glycerol-3-phosphate: acyl-ACP acyltransferase or ATS1; 10. Plastid lysophosphatidic acid: acyl-ACP acyltransferase or ATS2; 11. Plastid phosphatidic acid phosphotase; 12. DAG: UDP-Galactose galactosyltransferase or MGD1; 13. MGDG: UDP-Galactose galactosyltransferase or DGD1.

1.5. Fatty acid export from chloroplast

In seed plants, essentially all fatty acids are synthesized inside the chloroplast attached to acyl carrier protein (ACP) and become available for complex lipid assembly primarily in the form of oleoyl- and palmitoyl-ACP (Ohlrogge *et al.*, 1979). Fatty acids must be exported from the chloroplast to the ER where they are assembled into various glycerolipids. The molecular mechanism of fatty acid export is still under debate. Rapid label experiments suggested that phosphatidylcholine is the first acyl-incorporating product following fatty acid export from the plastid and not acyl-CoA as previously assumed (Bates *et al.*, 2007). At least two enzymes are expected to be involved in this process: acyl-ACP thioesterases and long chain fatty acyl-CoA synthetases (LACS). Acyl-ACP thioesterases catalyze the hydrolysis of acyl-ACP to free fatty acids that are later activated to be acyl-CoA in the presence of ATP by LACS. Chloroplast fractionation experiments have shown that acyl-ACP thioesterases are localized at the inner envelope membrane while LACS are associated with the outer envelope, consistent with a mechanism of acyl-ACPs hydrolysis and reactivation of the acyl groups to acyl-CoAs on the

respective sides of the membrane (Andrews and Keegstra, 1983). Indeed, experiments following [^{18}O]-labeled acetate incorporation into plastid versus extraplastidic lipids were consistent with the export of a free carboxylate anion intermediate produced by intermittent hydrolysis of acyl-ACP (Pollard and Ohlrogge, 1999). Furthermore, [^{14}C]-labeling revealed fast turnover of a free fatty acid pool with a half-life of less than 1s (Koo *et al.*, 2004). Although long chain fatty acids can cross the membrane bilayer by simple diffusion, as their membrane permeability is several orders of magnitude higher than hydrophilic metabolites such as glucose or amino acids (Hamilton, 2007; Kamp and Hamilton, 2006), this process may be facilitated and regulated by the involvement of proteins. It has been shown that in the presence of bovine serum albumin, which removes free fatty acids outside of the chloroplast, LACS activity was not affected, suggesting that fatty acid transport is facilitated by a vectorial mechanism coupling transport and reactivation of the acyl group (Koo *et al.*, 2004). This mechanism has been postulated also for fatty acid import systems in *E. coli* and yeast (Black and DiRusso, 2003). However, little is known about how free fatty acids cross the inter-membrane space.

In Arabidopsis, two acyl-ACP thioesterases, FatA and FatB, have been described. While FatA is specific to oleoyl-ACP, FatB has a broader substrate spectrum, but prefers palmitoyl-ACP (Salas and Ohlrogge, 2002). There are nine genes encoding LACSs in Arabidopsis, but LACS9 is responsible for 90% of the fatty acyl-CoA synthetase activity (Shockey *et al.*, 2002). However, neither altered growth nor impaired fatty acid export from chloroplasts was observed in the LACS9 T-DNA knockout mutant, consistent with functional overlap between different LACS in Arabidopsis (Schnurr et al., 2002; Zhao et al., 2010).

1.6. The ER-chloroplast contact site

Scattered throughout the literature are reports of observations of the ER in close vicinity to plastid envelope membranes. In different groups of algae, the chloroplast was surrounded by periplastidic ER (GIBBS, 1962; GIBBS, 1979). In specific cells of *Acer pseudoplatanus* and the resin canal cells from *Pinus pinea*, immature plastids were completely sheathed by an ER membrane (Wooding and Northcote, 1965). In resin canal cells, presumed precursors for resin synthesis were observed between the outer envelope membrane of plastids and the sheathing ER. ER-to-chloroplast contact sites were also reported in embryonic pea leaves where lipid droplets were associated with the developing chloroplast (Kaneko and Keegstra, 1996). In the mature chloroplast, the complete sheathing ER disappeared and instead, localized ER-to-chloroplast attachment was observed with Green Fluorescent Protein (GFP) targeted to the ER or Yellow Fluorescent Protein (YFP) fused to reticulon-like proteins in Arabidopsis (Hanson and Köhler, 2001; Nziengui et al., 2007). The chloroplast associated ER adopted a reticulum structure rather than cisterna. Chloroplasts isolated from pea or Arabidopsis leaves had ER membrane fragments attached (Hawes et al., 2010). This attachment was sufficiently strong to withstand a force of 400 pN applied with optical tweezers, suggesting strong protein-protein interactions. Using rapid freeze-fracture, continuity of the ER and the outer envelope of chloroplasts in the green alga *Chara globularis var. capillacea* and the non-seed vascular plant *Equisetum telmateia* were observed (McLean *et al.*, 1988), but this has not been reported for seed plants. In addition, osmiophilic particles, most likely lipid droplets, were enriched at the ER-to-chloroplast contact sites, suggesting a possible role of these contact sites in lipid metabolism.

Labeling of cellular compartments with specific fluorescent markers enabled the dynamic imaging of the ER-chloroplast interaction. Following laser stimulation at ER-to-chloroplast

contact sites, GFP labeled ER grew on the surface of the chloroplast forming a denser network (Griffing, 2011). Myosin might be involved in this movement as it was localized at the chloroplast-associated ER tubules (Liebe and Menzel, 1995). In addition, chloroplast stromules, which are extensions of chloroplast suggested to be involved in inter-plastidic communication and exchange of substances, have been shown to be surrounded by ER tubules and the movement of stromules coincided with the movement of the surrounding ER (Schattat, Barton, Baudisch, *et al.*, 2011; Schattat, Barton and Mathur, 2011).

While there is compelling evidence for the existence of ER-to-chloroplast membrane contact sites, the functional significance of this structure remains to be determined. The ribosomes associated with the ER surrounding the plastids observed in early studies with algae led to an early hypothesis that the ER-to-chloroplast contact sites may serve as a conduit for plastid protein import (GIBBS, 1979). However, for seed plants it is well established that proteins are imported directly through the Toc/Tic complex of the envelope membranes of the chloroplasts. That is, with notable exceptions as some, if not all, N-glycosylated proteins, such as nucleotide pyrophosphatase/phosphodiesterase (NPP) in rice and α-carbonic anhydrase (CAH1) in Arabidopsis, depend on the secretory pathway to reach the chloroplast (Nanjo et al., 2006; Villarejo et al., 2005). One can speculate that during early stages of plastid symbiosis as represented by algae, plastid proteins encoded by the nucleus genome may not have had chloroplast targeting sequences and, thus, reached the chloroplast via the secretary pathway. In seed plants, although targeting of nuclear encoded plastid proteins is mostly independent of the secretary pathway, those plastid proteins requiring post-translational modification in the ER may still utilize this mechanism. It is unclear how N-glycosylated proteins transfer from the ER to the

chloroplast, but ER-to-chloroplast contact sites may represent a possible structure involved in this process.

Membrane contact sites as lipid conduits have been reported in yeast microsomal fractions associated with mitochondria (MAM) (Achleitner et al., 1999; Gaigg et al., 1995). Phosphatidylserine synthesized at the ER is imported through MAMs into mitochondria where it is utilized for the synthesis of phosphatidylethanolamine. It is possible that similar lipid transfer also takes place at the ER-to-chloroplast contact sites, as the eukaryotic pathway for chloroplast lipid synthesis requires the participation of the ER. The TGD proteins involved in this process were discovered during a forward genetic screen in Arabidopsis (Xu et al., 2003). The *tgd* mutants accumulated abnormal oligogalactolipids, most prominently **t**ri**g**alactosyl**d**iacylglycerol, after which they were named. The amount of chloroplast lipids derived from the eukaryotic pathway was decreased in these mutants, and the above mentioned pulse-chase labeling experiment with [^{14}C]-acetate showed that the *tgd1-1* mutant was impaired in the eukaryotic pathway for chloroplast lipid biosynthesis. The accumulation of trigalactosyldiacylglycerol resulted from the activation of a processive galactosyltransferase named SENSITIVE TO FREEZING 2 (SFR2) (Moellering et al., 2010). Genes responsible for the *tgd* mutants have been identified and the proteins have been designated TGD1, 2, 3, 4 (Figure 1.2.). The TGD1, 2 and 3 proteins form a bacterial-type ATP Binding Cassette (ABC) transporter in the inner envelope of chloroplasts touching the outer envelope (Benning, 2009). TGD1 is a permease and TGD3 is an ATPase providing the energy for lipid transport (Xu *et al.*, 2005; Xu *et al.*, 2003; Lu *et al.*, 2007) likely making the lipid transfer from the ER to the chloroplast unidirectional (Xu et al., 2010). TGD2 has an N-terminal transmembrane domain and a C-terminal soluble domain facing the intermembrane space (Awai et al., 2006). The C-terminal domain specifically binds to PtdOH

and disturbs adjacent membranes (Lu and Benning, 2009; Roston et al., 2011). TGD4 lacks known functional domains but its C-terminus may fold into a β-barrel (Haarmann et al., 2010). A GFP-TGD4 fusion protein transiently overproduced in tobacco was localized to the ER membrane (Xu et al., 2008). In addition, the TGD4 protein was found in chloroplast envelope fractions during proteomics studies (Zybailov et al., 2008). These conflicting results may indicate that TGD4 is localized at ER-to-chloroplast contact sites, through which lipid precursors might transfer from the ER to the chloroplast. Besides TGD4, a cytochrome P450 monooxygenase, CYP86B1, which is required for suberin biosynthesis in roots and seeds, has also been localized to both ER and the chloroplast using two different approaches. CYP86B1 has an N-terminal sequence rich in Ser/Thr interpreted to be a chloroplast targeting peptide, and the protein was localized to the outer envelope of the chloroplast facing the cytosol using a chloroplast protein import assay (Compagnon et al., 2009). However, transiently expressing CYP86B1-YFP in tobacco localized the fusion protein to the ER membrane. With prolonged inspection, however, some of the fluorescence started to associate with chloroplasts (Compagnon et al., 2009). Either overproduction of fluorescent fusion proteins generally leads to mistargeting to the ER, or this protein is also localized at the ER chloroplast contact sites thereby explaining the ambiguous results.

1.7. Conclusions

The biosynthesis of chloroplast membrane lipids in seed plants involves at least two organelles and three membrane bilayers: the ER and the chloroplast inner and outer envelope

membranes. Using Arabidopsis as a model system, the development of forward genetic screening and the completion of the genome sequence enabled the identification of nearly all of the enzymes involved in the chloroplast lipid synthesis in the past two decades. Fluorescent protein fusions, cell fractionation, and proteomics revealed the fine localization of these enzymes. However, our understanding of how the lipid intermediates are handed from one enzyme to the next, and subsequently transferred from one organelle to another has just begun to emerge. Direct membrane contacts between the ER and the chloroplast are suggested as the sites for the transfer of lipids.

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CHAPTER 2

Glycerolipid analysis using Thin Layer Chromatography (TLC) coupled with Gas-Liquid Chromatography (GLC)[2]

[2]This work has been published: **Wang, Z., Benning, C.** (2011) Arabidopsis thaliana polar glycerolipid profiling by thin layer chromatography (TLC) coupled with gas-liquid chromatography (GLC). *J. Vis. Exp.* **49**, e2518. I performed all the experiments and wrote the first draft of the paper.

2.1. Abstract

Biological membranes separate cells from the environment. From a single cell to multicellular plants and animals, glycerolipids, such as phosphatidylcholine or phosphatidylethanolamine, form bilayer membranes which act as both boundaries and interfaces for chemical exchange between cells and their surroundings. Unlike animals, plant cells have a special organelle for photosynthesis, the chloroplast. The intricate membrane system of the chloroplast contains unique glycerolipids, namely glycolipids lacking phosphorus: monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG) (Benning, 2009). The roles of these glycolipids are beyond simply structural. MGDG was found in the crystal structure of photosystem I suggesting the involvement of glycolipids in photosynthesis (Guskov *et al.*, 2009; Jordan *et al.*, 2001). During phosphate starvation, DGDG is transferred to extraplastidic membranes to compensate for the loss of phospholipids (Härtel *et al.*, 2000; Kobayashi *et al.*, 2009).

Much of our knowledge of the biosynthesis and function of these lipids has been derived from a combination of genetic and biochemical studies with *Arabidopsis thaliana* (Ohlrogge and Browse, 1995). During these studies, a simple procedure for the analysis of polar lipids has been essential for the screening and analysis of lipid mutants and will be outlined in detail. A leaf lipid extract is first separated by thin layer chromatography (TLC) and glycerolipids are stained reversibly with iodine vapor. The individual lipids are scraped from the TLC plate and converted to fatty acyl methylesters (FAMEs), which are analyzed by gas-liquid chromatography coupled with flame ionization detection (FID-GLC) (Figure 2.1.). This method has proven to be a reliable tool for mutant screening. For example, the *tgd1,2,3,4* endoplasmic reticulum-to-plastid lipid trafficking mutants were discovered based on the accumulation of an abnormal

galactoglycerolipid, trigalactosyldiacylglycerol (TGDG), and a decrease in the relative amount of 18:3 (carbons : double bonds) fatty acyl groups in membrane lipids (Xu *et al.*, 2003; Xu *et al.*, 2005; Awai *et al.*, 2006; Lu *et al.*, 2007). This method is also applicable for determining enzymatic activities of proteins using lipids as substrate (Kaneda and Tominaga, 1975).

2.2. Video Link

The video component of this protocol can be found at http://www.jove.com/details.php?id=2518

2.3. Experimental procedures

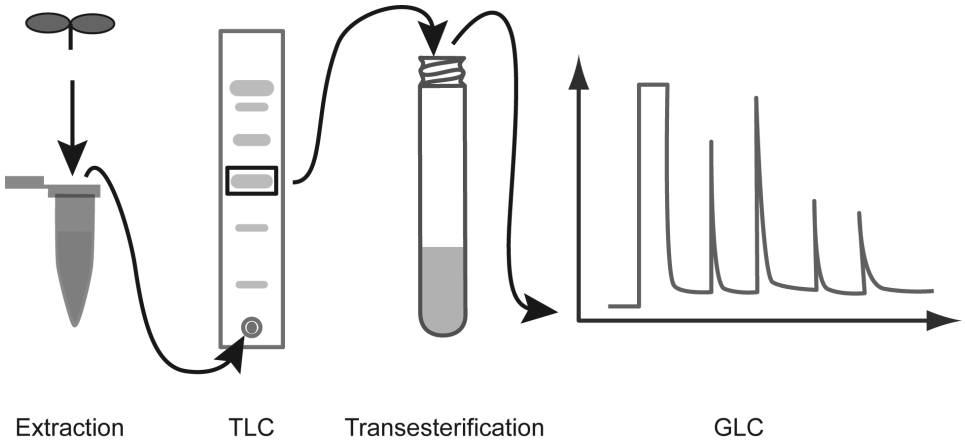

Figure 2.1. Flow chart of polar lipid analysis using Arabidopsis seedlings. Total lipids are extracted from 4-week-old Arabidopsis seedlings and separated by TLC. Lipids are stained either irreversibly with sulfuric acid or α-naphthol or reversibly with iodine. The iodine-stained lipids can be scraped from TLC plate for FAME generation followed by GLC analysis.

2.3.1. Lipid extraction

i. Harvest 30 mg 4-week-old Arabidopsis leaves from plants grown on agar solidified medium or soil and transfer them into 1.5 ml polypropylene reaction tubes. Fresh leaves can be flash frozen in liquid nitrogen and stored at -80 °C.

ii. Add 300 µl of extraction solvent composed of methanol, chloroform and formic acid (20:10:1, v/v/v) to each sample. Shake vigorously (using a paint shaker or similar) for 5 minutes.

iii. Add 150 µl of 0.2 M phosphoric acid (H_3PO_4), 1 M potassium chloride (KCl) and vortex briefly.

iv. Centrifuge at 13,000×g at room temperature for 1 minute. Lipids dissolved in the lower chloroform phase will be spotted onto TLC plates.

2.3.2. Thin Layer Chromatography (TLC) (STAHL, 1956)

i. To prepare TLC plates, submerge a 20cm×20cm Si250-PA TLC plate for 30 sec into 0.15 M ammonium sulfate ($(NH_4)_2SO_4$) solution, dry for at least 2 days in a covered container. The sublimation of ammonium leaves behind sulfuric acid during activation which protonates phosphatidylglycerol necessary for its separation from other glycerolipids.

ii. On the day of experiment, activate TLC plates by baking in an oven at 120 °C for 2.5 hours.

iii. After cooling down the activated plates to room temperature, use a pencil to draw a straight line (1.5 cm from the edge of the plate) across the plate at the origin of the chromatogram.

iv. In a fume hood, slowly deliver 60 µl of lipid extract in the lower chloroform phase using a 20 µl pipette with 200 µl yellow plastic tips under a slow stream of N_2. For this purpose, a Pasteur pipette is connected with Tygon Tubing to the regulator of the N_2 tank. Keep the spot smaller than 1 cm in diameter. Each plate can hold up to 10 samples (when subsequent GLC analysis is planned).

v. While allowing for complete drying of the lipid spots in the fume hood, prepare the developing solvent composed of acetone, toluene, water (91: 30: 7.5, v/v/v). If the ambient relative air humidity is high, separation could be affected. In this case water should be reduced to give (91: 30: 7.0. v/v/v) to achieve the desired separation.

vi. Pour 80 ml of developing solvent into a sealable TLC developing chamber (L: H: W=27.0: 26.5: 7.0, cm/cm/cm) and place the plate into the tank with the sample end facing down. Seal the tank using the clamp. The solvent will ascend the plate and lipids will be separated. The development time is ~50 minutes at room temperature.

vii. When the solvent front has reached 1 cm from the top of the plate, carefully remove the plate from the tank and dry completely in the fume hood for ~10 minutes.

viii. Lipids separated by TLC can be either reversibly stained briefly with iodine for quantitative analysis or irreversibly stained with sulfuric acid or α-naphthol (Figure 2.2.).

viii.1. Sulfuric acid charring: spray the plate with 50% sulfuric acid in water in a glass spray bottle in the fume hood and bake at 120 °C for 15 minutes (Figure 2.2.A).

viii.2. α-naphthol staining for glycolipids: spray the plate with 2.4% (w/v) α-naphthol in 10% (v/v) sulfuric acid, 80% (v/v) ethanol and bake at 120 °C for 3-5 minutes until glycolipid bands

are stained pink or purple (Figure 2.2.B). Overtreatment will lead to charring due to presence of sulfuric acid in the reagent.

viii.3. Iodine staining: in a fume hood, place the plate into a closed TLC tank with iodine crystals (in a tray on the bottom leading to saturation of the atmosphere with iodine vapor until lipids are visible). Don't expose the plate to iodine too long as iodine may covalently modify polyunsaturated fatty acids (Figure 2.2.C). Alternatively, to avoid any oxidation of lipids, only standard lanes interspersed with sample lanes should be stained using a glass wool plugged Pasteur pipette with iodine crystals through which N_2 is blown over individual standard lanes.

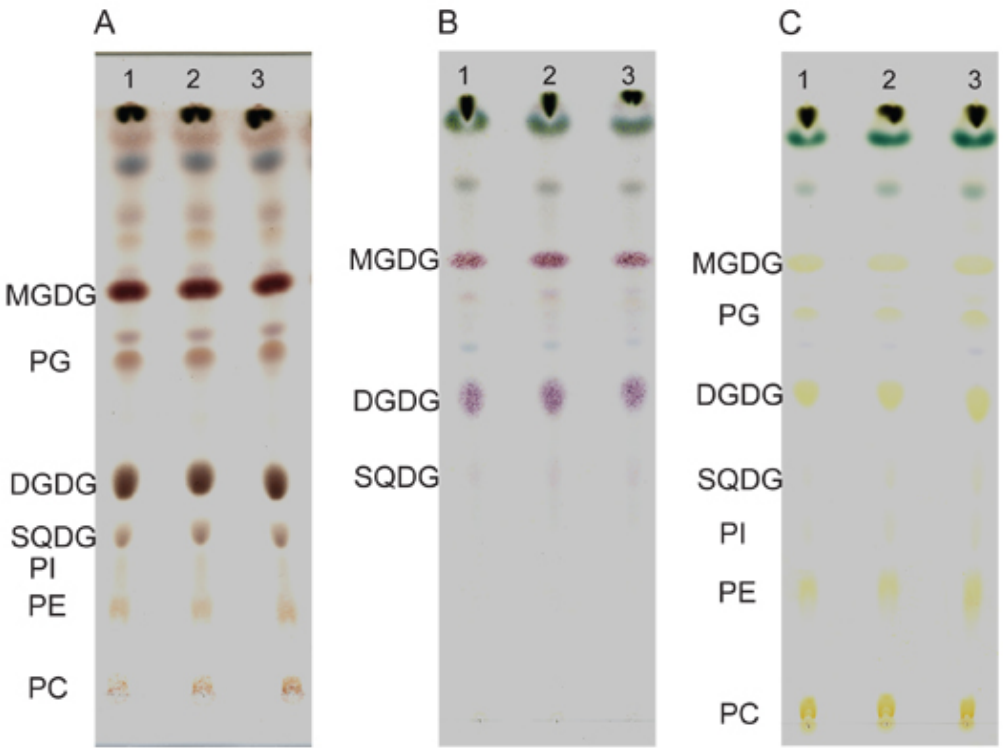

Figure 2.2. Staining of lipids on TLC plates. Lipid extracts of 35 mg (fresh weight) wild-type seedlings are separated by TLC and stained with sulfuric acid **(A)**, α-naphthol **(B)** or iodine vapor **(C)**. Three repeats are shown in each staining method. DGDG, digalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; SQDG, sulfoquinovosyldiacylglycerol. (For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this dissertation.)

2.3.3. Fatty Acyl Methylester (FAME) reaction (STOFFEL *et al.*, 1958)

i. Remove silica surrounding identified lipid bands with a razor blade from the TLC plate. Scrape the lipid containing silica and transfer the silica powder using a funnel into a glass tube with Teflon (PTFE)-lined screw cap.

ii. Add 1 ml 1 N hydrochloric acid (HCl) in anhydrous methanol to each sample by glass pipette.

iii. Add 100 µl 50 µg ml^{-1} pentadecenoic acid (15:0) to each sample as internal standard using a pipette with 200 µl yellow plastic tip. Keep a tube with only pentadecenoic acid in methanolic HCl as a control. Close glass tubes tightly with Teflon-lined caps.

iv. Incubate glass tubes in an 80 °C water bath for 25 minutes. Tubes need to be sealed so that the solvent does not evaporate.

v. After tubes have cooled down, add 1 ml 0.9% sodium chloride followed by 1 ml hexane and vortex vigorously. Centrifuge samples at 1000×g for 3 minutes.

vi. In the fume hood, remove the hexane/upper layer of the sample with Pasteur pipette and place it into a new 13×100 mm glass tube.

vii. Evaporate hexane under a slow stream of N_2 without drying completely.

viii. Dissolve the resulting FAMEs in 60 µl hexane and transfer samples into autosampler vials and cap tightly. Samples can be stored at 4 °C for short term and -20 °C for a few days.

2.3.4. Gas-Liquid Chromatography (GLC) (James, 1952)

i. Ensure that the helium, hydrogen and air bottles are filled.

ii. Sufficient hexane must be added to the solvent reservoir and the waste container must be empty.

iii. Place vials into the autosampler. Start the Chemstation software for GLC on the system computer.

iv. Set the inlet temperature at 250 °C with helium flow rate at 48.6 ml min^{-1} and the pressure at 21.93 psi. The split ratio is 30.0: 1.

v. The oven temperature is set initially at 140 °C for 2 min and raise to 160 °C at a rate of 25 °C min^{-1}. Then set the temperature to increase from 160 °C to 250 °C at a rate of 8 °C and hold at 250 °C for 4 min followed by a decrease to 140 °C at a rate of 38 °C min^{-1}. One run takes ~21 minutes.

vi. The temperature of the flame ionization detector is 270 °C with a hydrogen flow rate of 30.0 ml min^{-1}, air flow rate at 400 ml min^{-1}, and helium flow rate at 30.0 ml min^{-1}.

vii. Enter the number of vials and sample names in the run sequence table. Set the 10 µl injector to inject 2 µl sample per vial.

viii. When the instrument is ready, initiate the run sequence.

2.4. Representative results

Examples of irreversible staining of TLC-separated lipids from 4-week-old Arabidopsis seedlings are shown in Figure 2.2. The sulfuric acid stained lipids (Figure 2.2.A) are charred and appear as brown spots. α-naphthol is preferred to stain glycolipids such as MGDG, DGDG, SQDG etc. Glycolipids stained with α-naphthol carry a pink-purple color while other polar lipids stain yellow (Figure 2.2.B). The iodine staining is reversible and gives lipids a yellowish color that will disappear over a short time as the iodine evaporates (Figure 2.2.C). Briefly iodine stained lipids can be subjected to GLC analysis although unstained lipids are preferable to reduce break down of lipids.

If done successfully, distinctive signals representing different FAMEs will be observed after GLC (Figure 2.3.). FAMEs with shorter carbon chain and fewer double bonds have shorter retention time using the DB-23 column as described above. FAME profiling is a sensitive tool to identify mutants with altered lipid composition. In Figure 2.4., the MGDG18:3 fatty acid molar ratio is decreased in mutant *tgd4-1* compared to the wild type (Xu *et al.*, 2008). By dividing the moles of FAMEs for one lipid class with the moles of FAMEs of all lipid classes, the molar ratio of each lipid are calculated. For example, to calculate the molar ratio of MGDG:

$$(\text{MGDG})\ \text{mol\%} = \sum [\text{FAMEs}_{(\text{MGDG})}] / \sum [\text{FAMEs}_{(\text{total})}] \times 100\%$$

The resulting molar ratios of each lipid class from both the wild type and the mutant can be compared. For instance, the *tgd4-1* mutant has increased relative amounts of MGDG and PG but decreased amounts of DGDG and PE (Figure 2.5.) (Xu *et al.*, 2008).

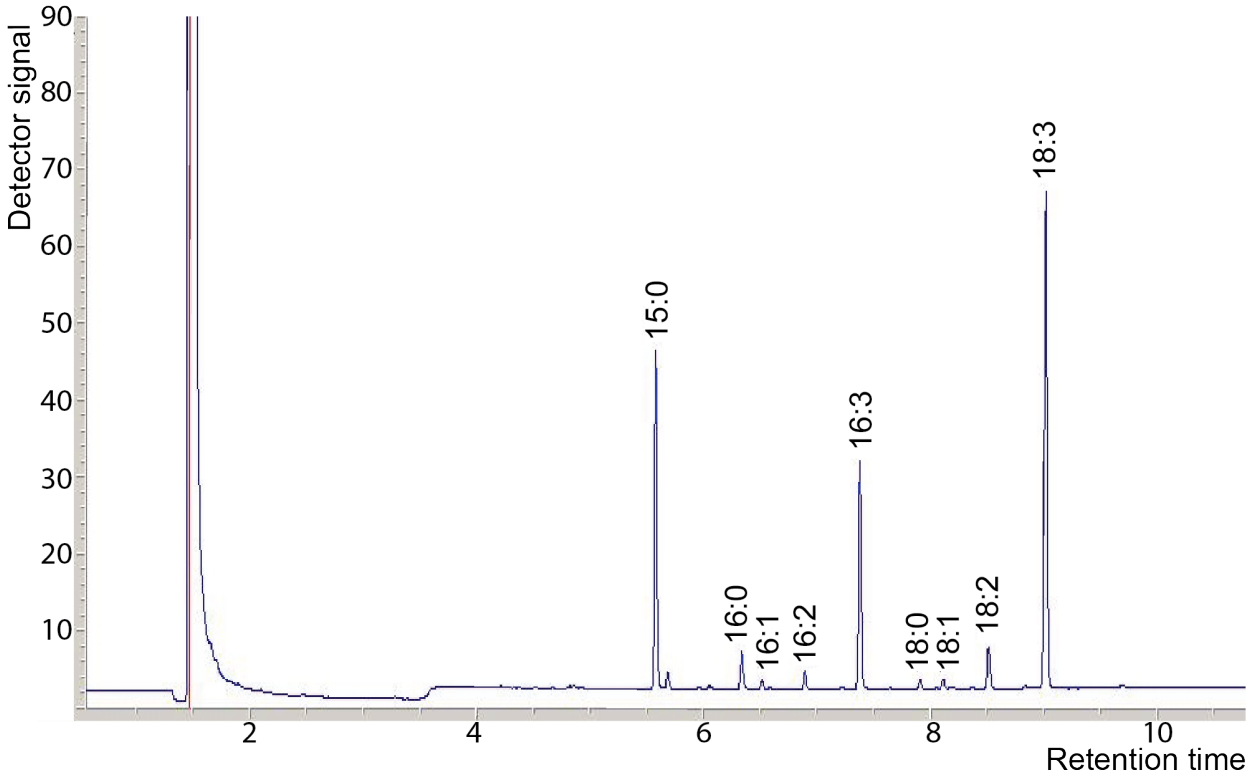

Figure 2.3. GLC analysis of Fatty Acid Methyl Esters (FAMEs) derived from MGDG of the wild type. FAMEs are separated on a 30 m capillary column and detected by flame ionization. Pentadecenoic acid (15:0) is used as an internal standard.

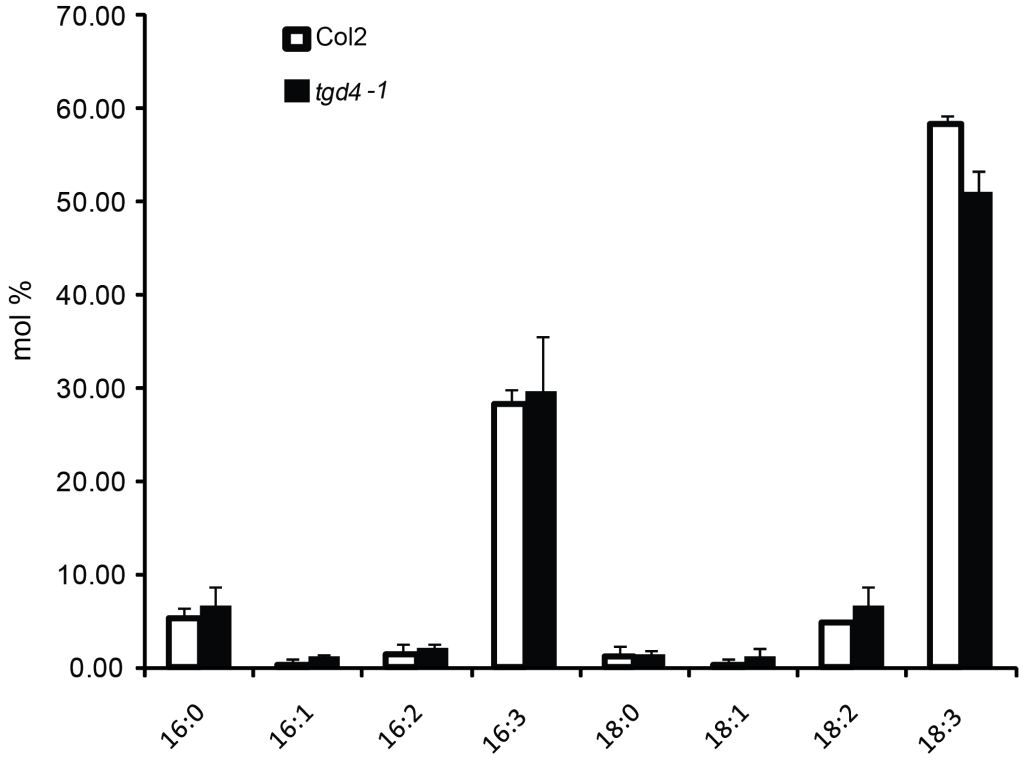

Figure 2.4. Fatty acid profile of MGDG in the wild type Col2 (white columns) and the mutant *tgd4-1* (black columns). Fatty acids are presented as the number of carbons followed by the number of double bonds. Three repeats are averaged and standard deviations are denoted by three repeats.

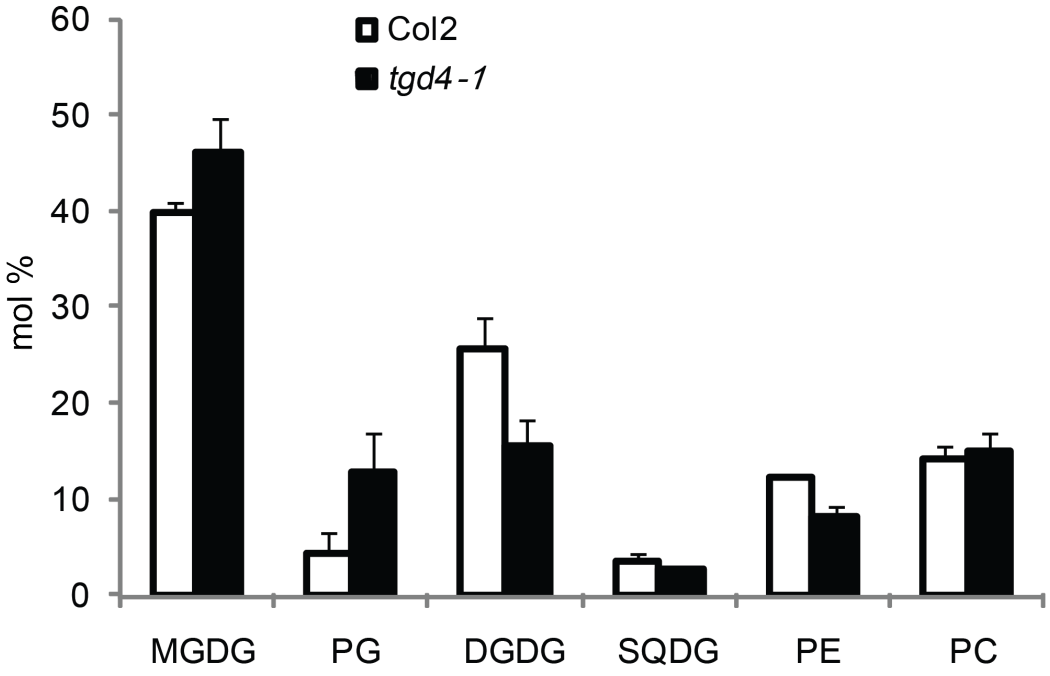

Figure 2.5. Polar lipids composition of the wild type Col2 (white columns) and the mutant *tgd4-1* (black columns). Three repeats are averaged and standard deviations are denoted by error bars.

2.5. Discussion

TLC coupled with GLC provides a robust and rapid tool for quantitative analysis of polar lipids in plants. Small changes in lipid composition can be identified; therefore, this method has been used for large scale screening of mutants impaired in polar lipid metabolic pathways (Ajjawi *et al.*, 2010; Xu *et al.*, 2003). This method is also widely used for monitoring activities of enzymes utilizing polar lipids as a substrate (Andersson *et al.*, 2004; Gaude *et al.*, 2008; Kaneda and Tominaga, 1975).

Besides leaves, the lipid composition of other plant tissues such as roots and seeds or subcellular fractions such as chloroplasts and mitochondria can also be determined using this method.

The solvent system (acetone, toluene, water) used here is optimized for the separation of glycolipids and phospholipids in plants. However, in *tgd1,2,3,4* mutants and isolated chloroplasts, TGDG runs together with PE while tetragalactosyldiacylglycerol runs with PC. In this case, a solvent system with chloroform, methanol, acetic acid, and water (85: 20: 10: 4, v/v/v/v) is used (Lu *et al.*, 2007). Sometimes two-dimensional TLC using two different solvent systems is performed to further separate glycolipids and phospholipids (Xu *et al.*, 2005). In addition, plant tissues can be directly subjected to FAME production followed by GLC to determine the total fatty acid profile without initial separation on TLC (Browse *et al.*, 1986). Beside the demonstrated TLC-GLC system, another method used for lipid profiling is based on direct electrospray ionization tandem mass spectrometry (Welti *et al.*, 2003). In this method the initial chromatographic separation of lipids in the extract is omitted. However, this method

requires expensive equipment and experienced personnel, which makes it less useful for routine analysis in the lab or for mutant screening.

Table 2.1. List of reagent used in this protocol.

Name of the reagent	Company	Catalogue number	Comments (optional)
α-naphthol	Sigma-Aldrich	N1000	
Methanolic HCL 3N	Sigma-Aldrich	33050-U	Dilute to 1N by methanol
Si250-PA TLC plates	J.T.Baker	7003-04	With pre-absorbent
TLC chamber	Sigma-Aldrich	Z266000	
Screw cap tubes	VWR	53283-800	
Scew caps	Sun Sri	13-425	
PTFE disk	Sun Sri	200 608	
GLC system	Hewlett Packard	HP6890	
DB-23 column	J&W Scientific	122-2332	
GLC vials	Sun Sri	500 132	
Caps of GLC vials	Sun Sri	201 828	
Chemstation software	Hewlett Packard	G2070AA	

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CHAPTER 3

Biochemical and cellular biological characterization of Arabidopsis TGD4 protein[3]

[3]This work has been published: **Wang Z., Xu C., Benning C.** (2012) TGD4 involved in endoplasmic reticulum-to-chloroplast lipid trafficking is a phosphatidic acid binding protein. *Plant J.* **70,** 614-623. I performed all the experiments except making the transgenic line in Figure 3.5, and wrote the first draft of the paper.

3.1. Abstract

The synthesis of galactoglycerolipids, which are prevalent in photosynthetic membranes, involves enzymes at the Endoplasmic Reticulum (ER) and the chloroplast envelope membranes. Genetic analysis of TGD proteins in Arabidopsis has demonstrated their role in polar lipid transfer from the ER to the chloroplast. The TGD1, 2, and 3 proteins resemble components of a bacterial-type ATP-Binding Cassette (ABC) transporter, with TGD1 representing the permease, TGD2 the substrate binding protein, and TGD3 the ATPase. However, the function of the TGD4 protein in this process is less clear, and its location in plant cells remains to be firmly determined. With its predicted C-terminal *β*-barrel structure, TGD4 is weakly similar to proteins of the outer cell membrane of Gram-negative bacteria. Here, we show that like TGD2, the TGD4 protein fused to *Ds*RED specifically binds phosphatidic acid (PtdOH). As previously shown for *tgd1* mutants, *tgd4* mutants have elevated PtdOH content, likely in extraplastidic membranes. Using highly purified and specific antibodies to probe different cell fractions, the TGD4 protein was present in the outer envelope membrane of chloroplasts, where it appeared to be deeply buried within the membrane except for the N-terminus, which was found to be exposed to the cytosol. It is proposed that TGD4 is either directly involved in the transfer of polar lipids, possibly PtdOH, from the ER to the outer chloroplast envelope membrane or in the transfer of PtdOH through the outer envelope membrane.

3.2. Introduction

Plant chloroplasts, the unique organelles of plant cells, harness solar energy and convert it to chemical energy by conducting photosynthesis, thereby providing food and oxygen for most of the living organisms on earth. The thylakoid membranes in the mature chloroplast represent

an extensive and intricate membrane system harboring the photosynthetic apparatus. The thylakoid lipids provide the structural matrix for the photosynthetic membrane into which the electron transport chain components are embedded. Thylakoid lipids have been observed in the crystal structures of both photosystem I and II (Guskov *et al.*, 2009, Jordan *et al.*, 2001) consistent with their possible roles in the proper assembly or function of photosynthetic complexes.

Unlike extraplastidic membranes, such as the endoplasmic reticulum (ER) or the plasma membrane, in which phosphoglycerolipids predominate, chloroplast membranes contain primarily galactoglycerolipids, which can account for approximately 70% of the total lipids in leaf tissue (Dörmann and Benning, 2002). Of the galactoglycerolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) represent the two most abundant classes. The amount of DGDG increases further during phosphate deprivation in leaves to substitute for phospholipids in extraplastidic membranes (Härtel *et al.*, 2000).

Galactolipids are synthesized at the chloroplast envelope membranes (Benning and Ohta, 2005). The MGDG synthase encoded by *MGD1* in Arabidopsis transfers a galactosyl residue from UDP-Gal to diacylglycerol (DAG) generating an MGDG (Awai *et al.*, 2001, Jarvis *et al.*, 2000). MGD1 is localized at the inner envelope facing the intermembrane space (Xu *et al.*, 2005). Transfer of a second galactosyl residue from UDP-Gal to MGDG is catalyzed by the DGDG synthase encoded by *DGD1* (Dörmann *et al.*, 1999), which is localized at the outer envelope of the chloroplast facing the cytosol (Froehlich *et al.*, 2001). There are two pathways contributing to the DAG precursor pool for galactoglycerolipid synthesis (Benning, 2009). In the "prokaryotic pathway", DAG assembly from *de novo* synthesized fatty acids takes place within

the chloroplast. In the "eukaryotic pathway", acyl groups are exported from the plastid to be available for polar lipid assembly at the ER where most of the extraplastidic phosphoglycerolipids are synthesized. DAG moieties transferred from the ER to the chloroplast serve as precursors in the synthesis of galactoglycerolipids. Thylakoid lipids derived from the prokaryotic pathway carry a 16-carbon acyl chain at the *sn-2* position of the glycerol backbone, while the lipids derived from the eukaryotic pathway an 18-carbon acyl chain at the same position (Heinz and Roughan, 1983).

Four genes, *TGD1, 2, 3,* and *4*, identified in a genetic mutant screen, encode proteins involved in ER-to-chloroplast lipid transfer in Arabidopsis (Awai *et al.*, 2006, Lu *et al.*, 2007, Xu *et al.*, 2003, Xu *et al.*, 2008). The respective *tgd* mutants accumulate abnormal oligogalactolipids, most prominently trigalactosyldiacylglycerol (TGDG) giving rise to the mutant and protein designation, *tgd* and TGD, respectively. The *tgd* mutants also have fewer thylakoid lipids derived from the eukaryotic pathway. The accumulation of oligogalactolipids in these mutants results from the activation of a processive galactosyltransferase, likely SFR2 (Moellering *et al.*, 2010). The TGD1, 2, and 3 proteins resemble the components of a bacterial-type ATP Binding Cassette (ABC) transporter complex and are likely associated with the inner envelope membrane (Benning, 2009). TGD1 contains multiple transmembrane domains and is proposed to be the permease of the complex (Xu *et al.*, 2003). TGD2 is similar to the substrate binding protein and binds specifically phosphatidic acid (PtdOH) (Awai *et al.*, 2006, Lu and Benning, 2009, Roston *et al.*, 2011). TGD3 is an ATPase localized in the chloroplast stroma (Lu *et al.*, 2007).

The TGD4 protein encoded by At3g06960.1 does not contain any known functional domains but is conserved from green algae to higher plants (Xu *et al.*, 2008). It is also distantly

related to the bacterial LptD protein which is an outer membrane *β*-barrel protein in *E. coli* and is involved in Lipid A transport (Haarmann *et al.*, 2010). Conflicting evidence has arisen with regard to the cellular localization of TGD4. Overexpression of functional TGD4 with the N-terminus fused to Green Fluorescent Protein (GFP) was consistent with TGD4 localization at the ER. However, chloroplast proteomic studies suggested chloroplast localization of TGD4 (Ferro *et al.*, 2003, Zybailov *et al.*, 2008). The goals of this study were to determine the molecular function of TGD4 and to resolve conflicting data regarding the cellular localization of the TGD4 protein.

3.3. Experimental procedures

3.3.1. Plant materials and growth conditions

Arabidopsis thaliana ecotype Col 2 and *tgd4* mutant plants were grown as previously described (Xu *et al.*, 2005). Surface-sterilized seeds were germinated on 0.5% (w/v) agar-solidified MS medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and transferred to soil after 10 days for propagation. Aerial parts of 4-week-old plants grown on agar-solidified MS medium were harvested for chloroplast isolation and lipid analysis.

3.3.2. Construction of transgenic lines

The HA-TGD4 producing transgenic line was generated in the *tgd4-1* mutant background. Full-length TGD4 was amplified by PCR from wild-type derived cDNA using a forward primer encoding the HA tag (Supplemental Table 1). The PCR product was cloned into a binary vector

derived from pPZP211 (Hajdukiewicz *et al.*, 1994) using restriction sites *Bam* HI and *Sal* I. The construct was introduced into the *tgd4-1* mutant using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on the MS medium supplemented with 100 μg/ml Gentamycin. Genotyping of the *tgd4-1* allele was performed using CAPs markers as previously described (Xu *et al.*, 2008).

3.3.3. Expression and purification of *Ds*RED-TGD4 fusion proteins

The TGD4 cDNA was initially cloned into the pMalc2x vector (New England Biolabs, Ipswich, MA). The pMalc2x/TGD4 construct was modified to give rise to pMalc2x/ΔTGD4 by deleting the 859-924nt (referring to coding sequence NM_111576) fragment encoding the hydrophobic region using site-directed mutagenesis. pMalc2x/TGD4 and pMalc2x/ΔTGD4 were used as PCR templates for the amplification of TGD4 (*Sac*I, *Not*I), TGD4N (*Nco*I) and ΔTGD4 (*Sac*I, *Not*I), TGD4C (*Sac*I, *Not*I) respectively. The restriction sites were included in the primers (Table 3.1). Following restriction digestion, the PCR fragments were ligated into the pLW01/*Ds*RED-His vector (Lu and Benning, 2009). Sequence identities were confirmed by sequencing at the MSU Research Technology Support Facility. To express *Ds*RED-TGD4-His proteins, constructs pLW01/*Ds*RED-TGD4-His, pLW01/*Ds*RED-ΔTGD4-His, pLW01/TGD4N-*Ds*RED-His and pLW01/*Ds*RED-TGD4C-His were transformed into *E.coli* strain BL21 (DE3) (Novagen, Madison, WI). A 5 ml overnight culture was used to inoculate a 200 ml culture. When the cell density reached A_{600}=0.6~0.8, isopropyl-β-D-thiogalactopyranoside (IPTG) was added at a final concentration of 0.1 mM to induce protein expression at 16°C overnight. The cells were centrifuged at 5,000×g for 10 minutes, and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0, 1% (w/v) foscholine-12 and protease inhibitor cocktail

(Roche, Indianapolis, IN)) with 0.2 mg/ml lysozyme (Sigma, St. Louis, Mo). After incubating on ice for 30 minutes, cells were lysed by sonication followed by centrifugation at 10,000×g for 20 minutes. The supernatant was filtered through a 0.45 μm filter and was loaded onto a Ni-NTA column (Qiagen, Valencia, CA). Protein purification was carried out according to manufacturer's instructions except of the addition of 0.1 % foscholine-12 to the wash and elution buffers. The purified proteins were concentrated with an Amicon centrifugal filter device (Millipore, Billerica, MA) and the buffer was changed to Tris-buffered saline (TBS; 10mM Tris-HCl, pH 8.0, 150 mM NaCl,) with 2 M choline chloride, which stabilizes *Ds*RED-TGD4-His proteins (Figure 3.1). Protein concentration was determined by the Bradford assay and protein purity was assessed by SDS-PAGE. The fusion proteins were then frozen in 10 μl aliquots at -80 °C.

Table 3.1. Primers for producing the pLW01/*Ds*RED-TGD4-His protein construct series and the HA-TGD4 transgenic line.

Primer name	Primer sequence
pLW01/*ds*RED-TGD4-His Fw	CGAGCTCATGAACAGAATGAGATGGTC
pLW01/*ds*RED-TGD4-His Rw	ATAGTTTAGCGGCCGCTGTCTCAAAGAAACGAAGCTC
pLW01/*ds*RED-ΔTGD4-His Fw	CGAGCTCATGAACAGAATGAGATGGTC
pLW01/*ds*RED-ΔTGD4-His Rw	ATAGTTTAGCGGCCGCTGTCTCAAAGAAACGAAGCTC
pLW01/TGD4N-*ds*RED-His Fw	CATGCCATGGATATGAACAGAATGAGATGGGTC
pLW01/TGD4N-*ds*RED-His Rw	CATGCCATGGTATAGGGCTTGCAAGTTTCG
pLW01/*ds*RED-TGD4C-His Fw	CGAGCTCGGTGAAAATTCAATCAGATCAAA
pLW01/*ds*RED-TGD4C-His Rw	ATAGTTTAGCGGCCGCTGTCTCAAAGAAACGAAGCTC
	CATGGATCCATGTACCCTTACGACGTCCCAGACTACG
pCHF1/HA-TGD4 Fw	CTAACAGAATGAGATGGGT
pCHF1/HA-TGD4 Rw	CACAGTCGACCTATGTCTCAAAGAAACGAAGC

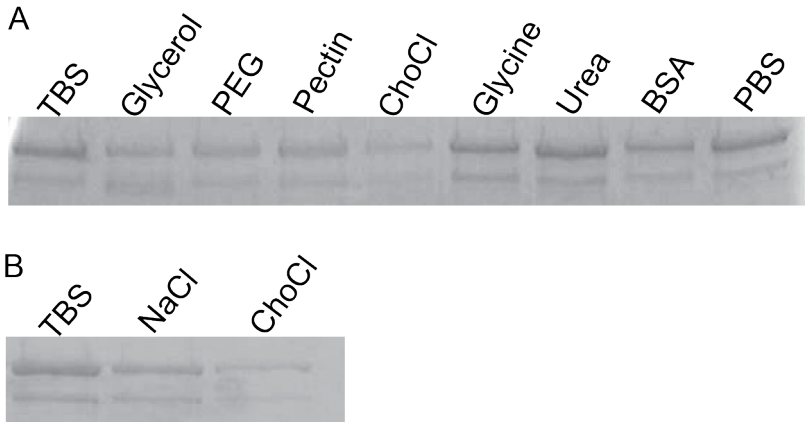

Figure 3.1. Choline chloride stabilizes *Ds*RED-TGD4-His. **(A)** Various reagents were added to 5 µg purified *Ds*RED-TGD4-His in TBS buffer at 4 °C for 2 hours followed by centrifugation at 13,000 ×g for 10 minutes. The protein precipitate was analyzed by SDS-PAGE and stained by Coomassie Brilliant Blue. 20% Glycerol; PEG: 20% polyethylene; 5% Pectin; ChoCl: 1M choline chloride; 1 M Glycine; 1M Urea; 0.5 mg/ml BSA; **(B)** The stabilizing effect of choline chloride upon *Ds*RED-TGD4-His can be attribute to its choline group, not the chloride ion. PBS: phosphate buffered saline; NaCl: sodium chloride.

3.3.4. Protein-lipid overlay assay

The protein-lipid overlay assay was modified from (Awai *et al.*, 2006, Lu and Benning, 2009). PIP2 lipid strips were purchased from Echelon Biosciences (Salt Lake City, UT). The lipids spotted onto membranes were purchased from Avanti Polar Lipids (Alabaster, AL) as well as Larodan Fine Chemicals (Malmö, Sweden). Lipids (10 nmol) were suspended in 20 µl spotting buffer (250 µl chloroform, 500 µl methanol, 200 µl 50 mM HCl, 2 µl 1% (w/v) Ponseau S (Sigma, St. Louis, MO)) and spotted onto Amersham Hybond-C Extra membranes (GE Healthcare, Piscataway, NJ) followed by drying for 1 hour in a fume hood. The lipid membranes were then blocked in 3% (w/v) bovine serum albumin (BSA) in TBST buffer (TBS with 0.25% (v/v) Tween 20) for 2 hours at room temperature. Purified *Ds*RED-TGD4-His fusion proteins were added at 1 µg/ml final concentration and incubated overnight at 4 °C followed by washing 3 times in TBST. Lipid membranes were then incubated with 1:2000 diluted His antibody (Sigma) in blocking buffer for 2 hours at room temperature followed by 2 washes with TBST. The membranes were processed for immunoblotting as described below.

3.3.5. Liposome association assay

The liposome association assay was adapted from (Awai *et al.*, 2006, Lu and Benning, 2009) with minor modifications. Dioleoyl-PtdCho and PtdOH with different acyl chain lengths and desaturation levels or 1-palmitoyl-2-(12-((7-nitro-2-1,3-benzoxadiazol-4-yl)amino)dodecanoyl)-*sn*-glycero-3-phosphate (NBD-PtdOH; Avanti, Alabaster, AL) were mixed at indicated ratios to give a total lipid amount of 250 µg. The lipids were dried under a stream of nitrogen, resuspended in 0.5 ml TBS buffer with 0.2 M choline chloride and hydrated at 37 °C for 1 hour

followed by vigorous vortexing for 2 minutes. The resulting multi-lamellar vesicles were centrifuged at 13,000×g for 10 minutes and then washed once with TBS buffer containing 0.2 M choline chloride. The liposomes were resuspended into 100 µl TBS buffer with 0.2 M choline chloride and incubated with 2 µg purified *Ds*RED-TGD4-His protein and its derivatives. The protein liposome mixture was incubated on ice for 30 minutes followed by centrifugation at 13,000×g for 10 minutes and two washes with 500 µl TBS containing 0.2 M choline chloride. The resulting protein-liposome pellet was resuspended in 20 µl 2×Laemmli buffer (Laemmli, 1970) and processed by SDS-PAGE (Shapiro and Maizel, 1969).

3.3.6. Lipid analysis by two-dimensional TLC and GC

Total lipids were extracted from 300 mg fresh weight seedlings as described (Wang and Benning, 2011) and separated on TLC silica gel plates (EMD Chemicals, Gibbstown, NJ). The first-dimension solvent contained chloroform: methanol: 7 M ammonium hydroxide (65:30:4, v/v/v) and the second-dimension solvent contained chloroform: methanol: acetic acid: water (170:25:25:6, v/v/v/v). Lipids were visualized either by 50% sulfuric acid or by iodine vapor. The iodine-stained lipids were scraped from TLC plates and quantified as previously described (Wang and Benning, 2011).

3.3.7. Production and purification of TGD4-antibodies

For the generation of polyclonal antibodies, 100 µg purified *Ds*RED-ΔTGD4-His was injected three times to immunize rabbits (Cocalico Biologicals, PA). To purify the antibodies from the serum, *Ds*RED-TGD4C-His was conjugated with Affi-Gel 15 (Bio-Rad, Hercules, CA) beads in 0.1 M HEPES, 8 M Urea according to the manufacturer's instruction. Anti-TGD4 crude serum

was incubated with the antigen-coupled beads overnight at 4 °C. After washing seven times with 5 ml phosphate buffered saline each, antibodies were eluted with 0.1 M glycine, pH 2.7 and were immediately neutralized with 1 M Tris-HCl, pH 9.0.

3.3.8. Immunoblot Analysis

Arabidopsis total leaf extracts or isolated chloroplasts were dissolved in 2×Laemmli buffer, and the proteins were separated on SDS-PAGE followed by transfer to the polyvinylidine fluoride (PVDF) membrane (Bio-Rad), which was then blocked with 5% (w/v) non-fat dry milk in TBST buffer at room temperature for 1 hour. Primary antibodies were added to the blocking solution at various dilutions and incubation was continued overnight at 4 °C. The PVDF membrane was then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse (diluted 1:20,000, Bio-Rad) or goat anti-rabbit sera (diluted 1:75,000, Bio-Rad) for 30 minutes at room temperature followed by 6 washes with TBST and detection using a chemiluminescence kit (Sigma). The TGD4 antibodies were diluted 1:500. BIP antibodies (diluted 1:500) were purchased from Santa Cruz Biotechnology; a monoclonal His-tag antibody (diluted 1:2,000) and HA-antibodies (diluted 1:5,000) were purchased from Sigma. SMT1 antibodies (diluted 1:200) were purchased from Agrisera (Vännäs, Sweden). TOC75 (diluted 1:3,000) and TIC110 (diluted 1:3,000) antibodies were kindly provided by Dr. John Froehlich, Michigan State University, while the TOC159 (diluted 1:2000) antibody was kindly provided by Dr. Masato Nakai, Osaka University.

3.3.9. Chloroplast isolation and proteinase digestion

Intact Arabidopsis chloroplasts were purified by discontinuous Percoll (Sigma) gradient (Aronsson and Jarvis, 2002). To perform Thermolysin and Trypsin digestions, 10 µg chlorophyll

equivalent chloroplasts were incubated with 0-4mg/ml Thermolysin (Sigma) or 0-0.8 mg/ml Trypsin (Sigma) in digestion buffer (330 mM sorbitol, 50 mM Hepes-KOH pH 8.0, 5 mM $MgCl_2$) at 100 µl total volume on ice for 30 minutes. 1% (v/v) TritonX-100 was added to the sample containing the lowest amount of either proteinase as the positive control. The digestion was terminated by adding 50 µl 20 mM EDTA or 50 µl 0.2 mg/ml Trypsin inhibitor. After passing through a 40% Percoll cushion and washing with digestion buffer once, proteinase digested intact chloroplasts were dissolved in 10 µl 2×Laemmli buffer and processed for SDS-PAGE and immunoblotting.

To test the interaction strength between TGD4 and the outer envelope, 10 µg chlorophyll equivalent chloroplasts of the wild type were treated with hypotonic buffer (10 mM MOPS-NaOH, 4 mM $MgCl_2$) or reagents as indicated in Figure 5d on ice for 30 minutes followed by centrifugation at 100,000×g for 1 hour. The protein compositions of both the supernatant and the pellet were examined by SDS-PAGE.

3.3.10. Membrane fractionation

Arabidopsis ER enriched microsomes were isolated from 4-week-old seedlings as described (Chen *et al.*, 2002). Briefly, seedlings were homogenized employing a pre-chilled mortar and pestle in grinding buffer containing 50 mM Tris-HCl, pH 8.2, 20% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM EDTA and protease inhibitor cocktail (Roche). The homogenate was then filtered through Miracloth and centrifuged at 12,000×g for 15 minutes. The supernatant was centrifuged again at 100,000×g for 1 hour. The resulting microsomes were resuspended in 0.5 ml buffer containing 10 mM Tris-HCl, pH 7.5, 10% (w/v) sucrose, 5 mM $MgCl_2$, 2 mM EDTA, 1

mM dithiothreitol and protease inhibitor cocktail. The microsome suspension was separated on a 20%-50% (w/v) continuous sucrose gradient at 100,000×g for 16 hours at 4 °C. Fractions of 1 ml were collected and processed for SDS-PAGE and immunoblotting.

3.4. Results

3.4.1. TGD4 binds PtdOH *in vitro.*

If TGD4 is involved in the transfer of lipids from the ER to the plastid as suggested by the mutant phenotype, it might specifically bind membrane lipids. Therefore, the lipid binding properties of TGD4 were investigated by producing TGD4 fused to *Ds*RED. The *Ds*RED protein is a red fluorescent protein of the coral *Discosoma sp.* (Gross *et al.*, 2000) and fusions with TGD2 have been successfully used to produce soluble protein, which was functional in lipid binding assays (Lu and Benning, 2009, Roston *et al.*, 2011). Initially, the *Ds*RED protein was fused to the N-terminus of the full-length TGD4 protein with a C-terminal His-tag (*Ds*RED-TGD4-His) giving rise to a fusion protein that is membrane associated. The *Ds*RED-TGD4-His protein and later its derivatives were solubilized and purified on a nickel-chelate column in the presence of the zwitter-ionic detergent foscholine-12. Removal of detergent from the *Ds*RED-TGD4-His protein preparation resulted in protein precipitation, which was found to be minimized in the presence of choline chloride. Of all compounds tested, choline chloride was found to be a suitable stabilizer (Figure 3.1) and was routinely added to the purified protein.

In protein-lipid overlay assays probing lipids on commercially available membranes (Figure 3.2A), *Ds*RED-TGD4-His was found to specifically bind to PtdOH, but not to any other phospholipids tested. Moreover, when probing different chloroplast lipids manually spotted onto

membranes, *Ds*RED-TGD4-His did not bind to any other lipids but PtdOH (Figure 3.2B). The *Ds*RED-His protein itself did not bind to any of the lipids on both membranes.

To verify PtdOH binding in a different assay and to test whether the protein showed preferences for different molecular species of PtdOH with regard to the acyl composition of the DAG moiety, a liposome binding assay was developed in which binding of the protein to liposomes containing different species of PtdOH could be tested (Figure 3.2C and D). In this assay, liposomes of defined lipid composition were incubated with the *Ds*RED-TGD4-His protein. Following centrifugation, protein bound to the liposomes was detected in the pellet while unbound protein remained in the supernatant. Prerequisite for the liposome binding assay to work was the exclusion of detergent, while at the same time stabilizing the *Ds*RED-TGD4-His fusion protein by adding choline chloride. Thus, choline chloride was a necessary addition to the buffer used for the liposome binding assay. Using this assay, *Ds*RED-TGD4-His was found to bind to dipalmitoyl PtdOH and distearoyl PtdOH although the binding of distearoyl PtdOH appeared to be stronger. For PtdOH species of the same acyl chain length but different desaturation levels, *Ds*RED-TGD4-His showed higher affinity for PtdOH with an increasing number of double bonds. Interestingly, *Ds*RED-TGD4-His appeared to have an even higher affinity to diphytanoyl PtdOH which carries branched acyl chains with four methyl groups. However, *Ds*RED-TGD4-His did not bind PtdOH carrying fluorescently labeled acyl substituents. The secondary band visible for the *D*sRED fusion proteins on the gels (Figure 3.2C to E, and Figure 3.3) was a result of *Ds*RED self-cleavage during denaturation prior to electrophoresis (Gross *et al.*, 2000). Because pH affects protonation of PtdOH and in some instances also PtdOH binding to proteins (Young *et al.*, 2010), the effect of pH was tested.

However, the binding of *Ds*RED-TGD4-His to PtdOH was not affected over a pH range of 6.4-7.8 (Figure 3.2E).

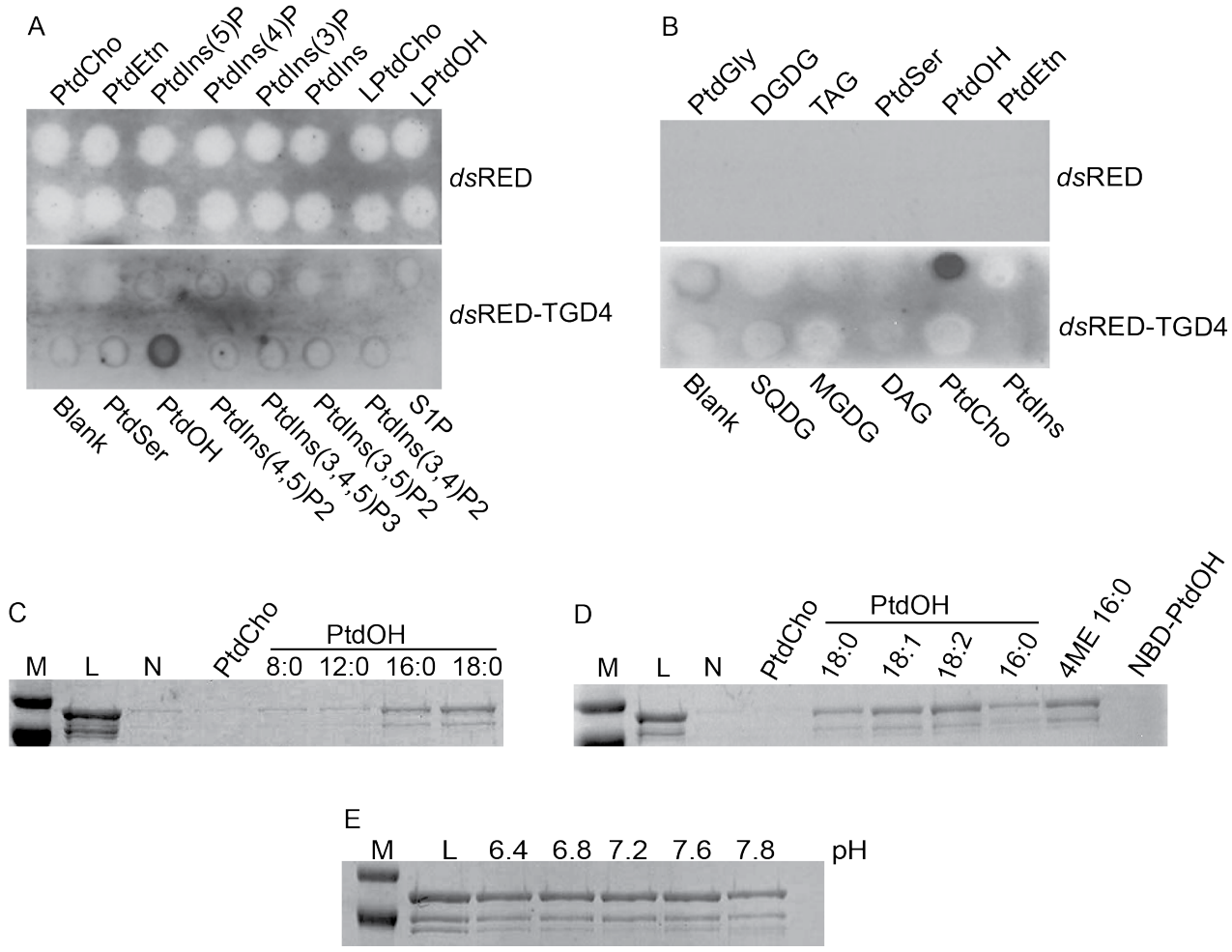

Figure 3.2. The TGD4 protein binds phosphatidic acid *in vitro*. (A) Of all phospholipids tested, the *Ds*RED-TGD4-His protein binds specifically to PtdOH in the lipid overlay assay. An immunoblot is shown with proteins detected using His-tag antibodies. LPtdOH, lysophosphatidic acid; LPtdCho, lysophosphatidylcholine; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-phosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(5)P,

Figure 3.2. (cont'd)

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-triphosphate; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine.

(B) Of all plant lipids tested, *Ds*RED-TGD4-His protein binds to PtdOH in the lipid overlay assay. An immunoblot is shown with proteins detected using His-tag antibodies. DAG, diacylglycerol; TAG, triacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PtdGro, phosphatidylglycerol.

(C) Effect of PtdOH fatty acyl chain length on *Ds*RED-TGD4-His binding affinity in the liposome association assay. Liposomes contained 40 mol% PtdOH and 60 mol% PtdCho. Protein gels stained with CBB (Coomassie Brilliant Blue) are shown. M, protein marker; L, loading control (total protein issued in the assay); N, no liposome control (representing protein precipitation and not lipid binding). Fatty acids are indicated with their number of carbons: number of double bonds.

(D) Effect of PtdOH fatty acyl desaturation level on the *Ds*RED-TGD4-His binding affinity in the liposome association assay. Liposomes contained 40 mol% PtdOH and 60 mol% PtdCho. Protein gels stained with CBB are shown. M, protein marker; L, loading control; N, no liposome control; 4ME 16:0, diphytanoyl phosphatidic acid; NBD-PtdOH, fluorescent NBD group labeled phosphatidic acid.

Figure 3.2. (cont'd)

(E) Effect of pH on PtdOH binding to *Ds*RED-TGD4-His. Liposomes contained 40 mol% PtdOH and 60 mol% PtdCho. Protein gels stained with CBB are shown. M, protein marker; L, loading control.

3.4.2. PtdOH binding is primarily a function of the N-terminal half of TGD4.

To determine the possible location of a PtdOH binding site in TGD4, a series of *Ds*RED-TGD4-His truncation mutants was constructed as shown in Figure 3.3. TGD4 contains a hydrophobic region of 23 amino acids (287D-309F) predicted by Aramemnon (Schwacke *et al.*, 2003). To test whether this region is involved, it was deleted in the *Ds*RED-ΔTGD4-His protein (Figure 3.3A). The N-terminal portion of TGD4 up to the mentioned hydrophobic region was fused to the N-terminus of *Ds*RED giving rise to TGD4N-*Ds*RED-His (Figure 3.3A). The TGD4 part C-terminal of the hydrophobic region was fused to the C-terminus of *Ds*RED giving rise to *Ds*RED-TGD4C-His (Figure 3.3A). Except for *Ds*RED-His alone, all tested recombinant fusion proteins bound to PtdOH-containing liposomes, more so as the fraction of PtdOH in the liposomes increased. Relative to the respective loading control (L) representing the total amount of protein in the assay, the TGD4N-*Ds*RED-His protein showed an affinity to PtdOH liposomes similar to the full-length protein *Ds*RED-TGD4-His (full binding at 40% PtdOH), suggesting that a major PtdOH binding region resides within the N-terminal part of TGD4 (Figure 3.3B). In contrast, the *Ds*RED-TGD4C-His protein had much lower affinity compared to the wild-type protein *Ds*RED-TGD4-His but still bound PtdOH, because only a fraction of the protein in the assay (compared to the loading control L representing total protein) was present in the liposome

pellet at all concentrations tested. Thus PtdOH binding activity does not require the central hydrophobic region of TGD4 and resides primarily, although not exclusively, in the N-terminal portion of TGD4.

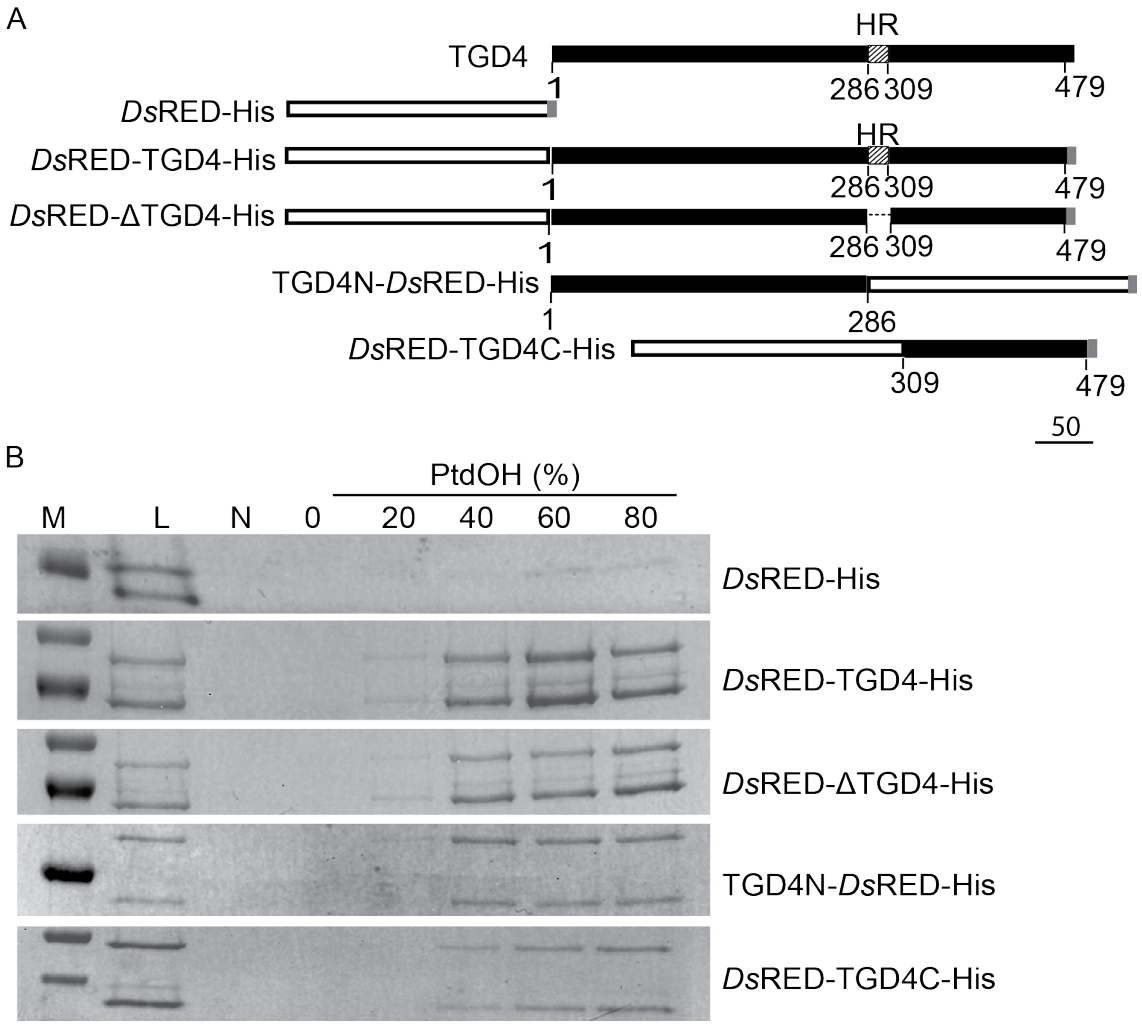

Figure 3.3. PtdOH binds to the N-terminal domain of *Ds*RED-TGD4-His. (A) The primary structure of the TGD4 protein, *Ds*RED-His, *Ds*RED-TGD4-His and truncation mutants. HR,

Figure 3.3. (cont'd)

hydrophobic region (cross-hatched bar); solid bar, TGD4; open bar, *Ds*RED-His; gray bar, His-tag; dashed line, deletion. The numbers refer to amino acids. **(B)** PtdOH binding affinity of *Ds*RED-TGD4-His derivatives in the liposome binding assay. Protein gels stained with CBB are shown. Liposomes were made up of dioleoyl-PtdOH and dioleoyl-PtdCho. The weight percentage of PtdOH in the liposome varied from 0 to 80%. M, protein marker; L, loading control; N, no liposome control.

3.4.3. PtdOH is increased in the *tgd4* mutants.

Previous lipid profiling of the *tgd4* mutant plants did not extend to PtdOH (Xu *et al.*, 2008). If TGD4 is involved in the transfer of PtdOH from the ER to the plastid, one would expect an increase in PtdOH content in the *tgd4* mutant, as was previously observed for *tgd1* (Xu *et al.*, 2005). The *tgd4-1* allele carries a one-amino acid substitution (P20L) while *tgd4-2* and *tgd4-3* are T-DNA knockout lines (Xu *et al.*, 2008). Total lipid extracts from the wild type and the different *tgd4* mutant alleles were separated by two-dimensional thin-layer chromatography (TLC), which allowed clean isolation of PtdOH (Figure 3.4A), and subsequent quantification (Figure 3.4B). All *tgd4* mutant alleles showed increased relative amounts of PtdOH, approximately doubled in the weak *tgd4-1* point mutant allele and tripled in the strong *tgd4-2* allele (Figure 3.4B) compared to wild type. Probing lipids in chloroplasts isolated from the wild type and the weaker *tgd4-1* mutant allele, which was not possible for the stronger T-DNA-alleles due to the limited availability of material, did not reveal PtdOH in mutant chloroplasts (Figure 3.5). Thus it is likely that the additional PtdOH observed in the *tgd4-1* mutant is associated with

extraplastidic membranes. Analyzing the fatty acid composition of PtdOH in the *tgd4-2* mutant revealed an elevated 18:1 and decreased 18:3 acyl group content, similar to observations previously made for the *tgd1* mutant (Xu *et al.*, 2005).

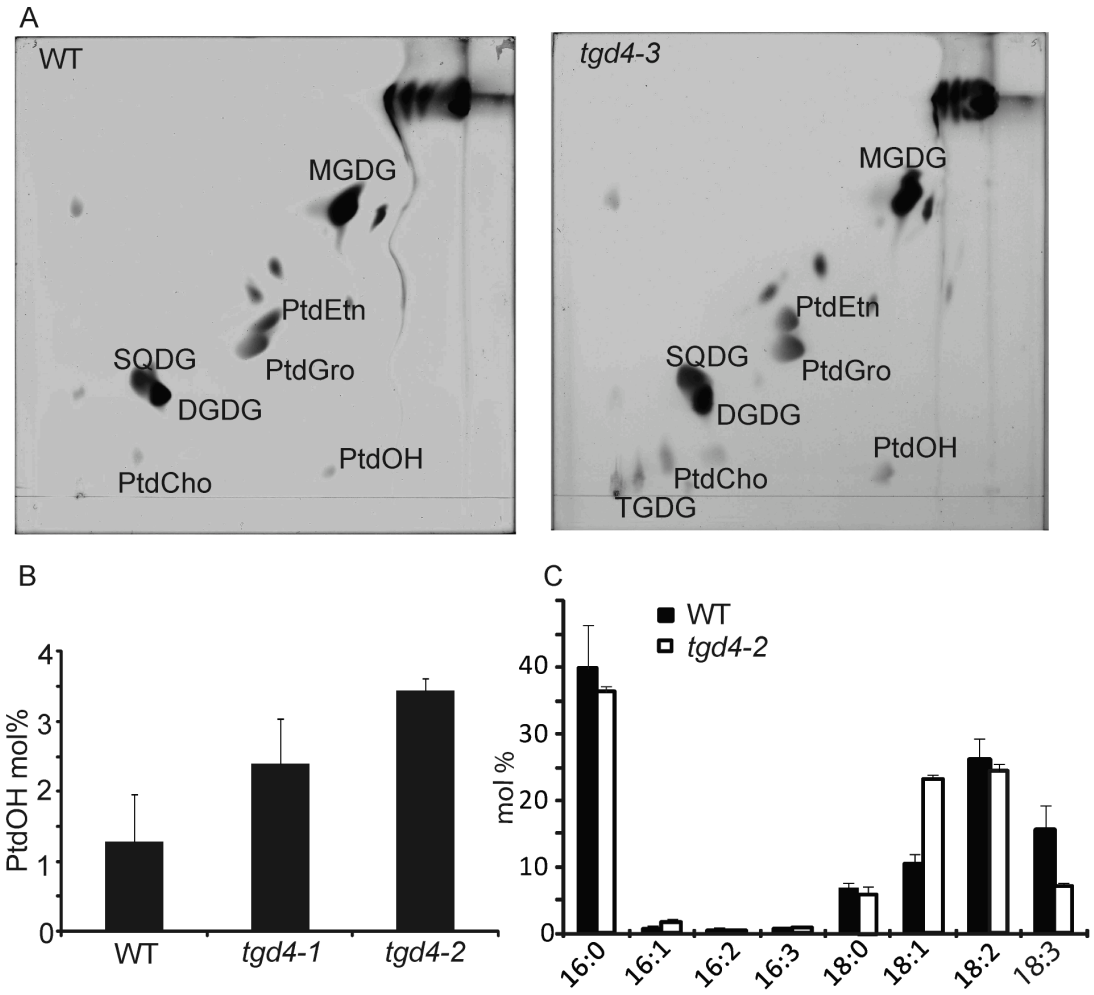

Figure 3.4. The *tgd4* mutants accumulate phosphatidic acid *in vivo*. (A) PtdOH separated by two-dimensional TLC. Wild type (WT) and *tgd4-3* were compared. Lipids were visualized by charring using 50% sulfuric acid. Abbreviations of lipids are as in Figure 3.2. TGDG:

Figure 3.4. (cont'd)

trigalactosyldiacylglycerol. **(B)** Quantification of PtdOH by gas-liquid chromatography. Values represent the molar ratio of PtdOH to total lipids. Error bars indicate the standard deviation of three biological repeats. **(C)** PtdOH fatty acid profile of wild type (WT) and *tgd4-2* mutants. Fatty acid species are designated with numbers of carbon: double bonds. Error bars represent the standard deviation of three biological repeats.

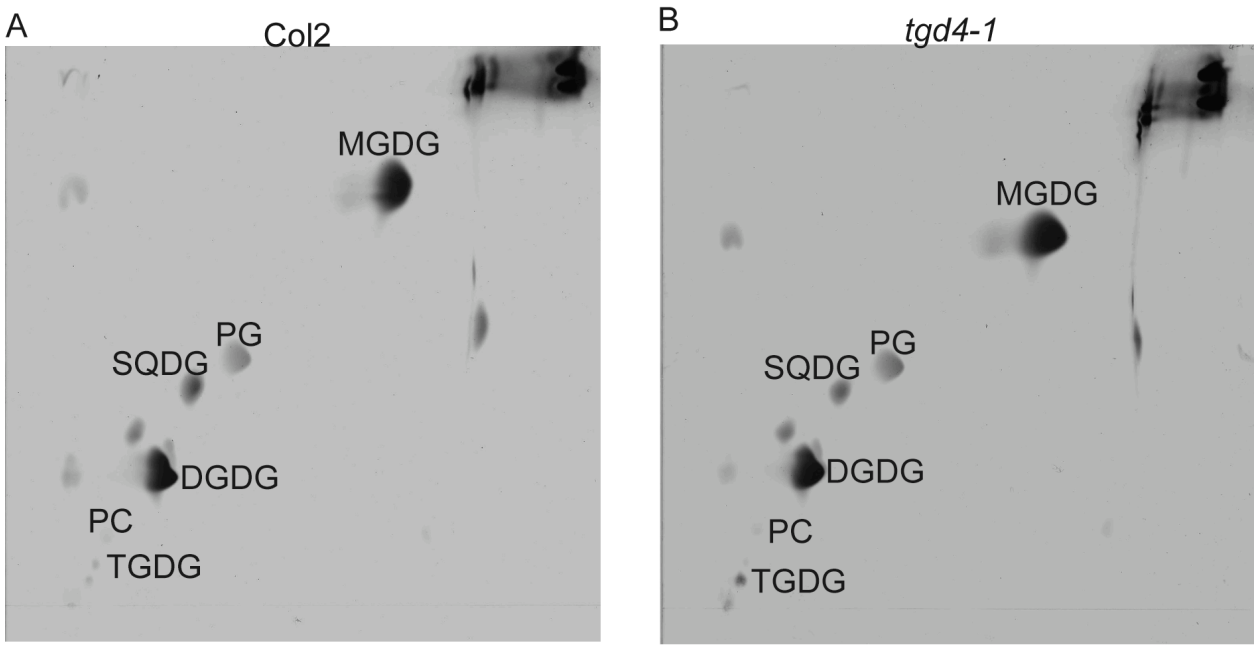

Figure 3.5. PtdOH is not detectable in the chloroplast. Total lipids of isolated chloroplast from **(A)** the wild type and **(B)** *tgd4-1* lipid extracts were separated by two-dimensional TLC. Lipids were visualized by charring using 50% sulfuric acid. Abbreviations of lipids are as in Figure 3.2.

3.4.4. The TGD4 protein is localized in the outer chloroplast envelope membrane

To investigate the location of the native TGD4 protein, a polyclonal TGD4 antiserum was produced in rabbits using purified *Ds*RED-ΔTGD4-His as antigen. From the crude serum, TGD4 antibodies were highly purified. Using immunoblotting, a signal corresponding to the TGD4 protein with a calculated molecular weight of 52.8 kDa, was detected in leaf-extract of the wild type but not of the *tgd4-1* mutants (Figure 3.6A). It is interesting to note that the TGD4 protein was not detectable in this point mutant, suggesting that the respective mutation affects the abundance of TGD4 *in vivo*.

Cell fractionation in combination with protein immunoblotting and detection with the purified TGD4 antibody was employed to localize TGD4. The TGD4 protein was enriched in isolated chloroplasts in wild-type plants (Figure 3.6B) in parallel with the chloroplast outer envelope marker TOC75 (Tranel *et al.*, 1995). However, the ER luminal binding protein marker (BIP) (Oliver *et al.*, 1995), was absent from the isolated chloroplasts. To determine whether TGD4 might also be present in the ER, an Arabidopsis wild-type microsomal preparation was fractionated by a continuous sucrose gradient to separate ER from other membranes (Figure 3.6C). ER microsomes represented by BIP and SMT1, an ER membrane protein (Boutte and Grebe, 2009), were present in the denser fractions, which also contained thylakoid membrane fragments as indicated by the presence of chlorophyll. TOC75 was enriched in the medium dense fractions while TIC110, an inner envelope marker (Inaba *et al.*, 2005), was found in both medium dense and dense fractions. The fractionation profile for TGD4 was most similar to that of TOC75, suggesting that TGD4 is primarily associated with the chloroplast.

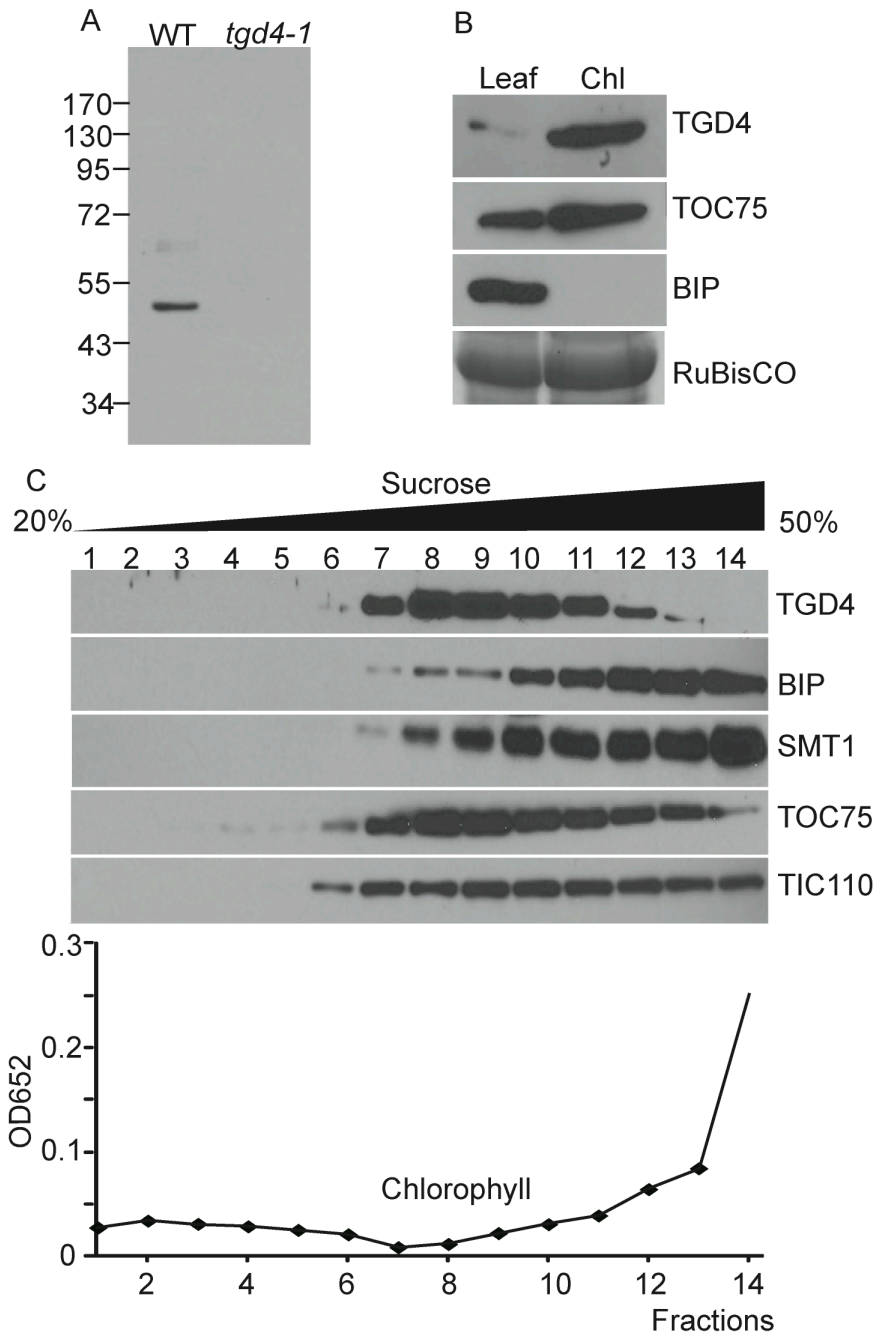

Figure 3.6. TGD4 is localized to the chloroplast. Immunoblots are shown for TGD4, TOC75, and BIP. RuBisCO was visualized by CBB staining. **(A)** Purified polyclonal antibody raised against *Ds*RED-ΔTGD4-His specifically detects TGD4 in wild type (WT) but not in the *tgd4-1*

Figure 3.6. (cont'd)

point mutant line. Numbers on the left indicate the molecular weights of protein markers in kD. **(B)** TGD4 is enriched in chloroplast preparations compared to total leaf extracts. TOC75, chloroplast outer envelope marker; BIP, ER marker; RuBisCO, loading control. **(C)** TGD4 does not co-fractionate with ER markers on a sucrose gradient. TIC110, chloroplast inner envelope marker. Chlorophyll content serves as a thylakoid marker.

To refine the localization of the native TGD4 protein further, chloroplasts isolated from the wild-type leaves were subjected to protease digestion. Thermolysin does not penetrate the chloroplast outer envelope membrane and, therefore, only digests proteins of the outer envelope membrane exposed to the cytosol. On the other hand Trypsin, which is smaller in size, is able to penetrate the outer envelope membrane but not the inner envelope membrane and digests proteins associated with the inner envelope membrane facing the intermembrane space (Joyard *et al.*, 1983). As shown in Figure 3.7A and B, TGD4 protein was susceptible to Thermolysin and Trypsin digestion as was TOC159, an outer envelope membrane protein (Hiltbrunner *et al.*, 2001), while the stroma protein RuBisCO was resistant to both. The addition of TritonX-100 disrupts chloroplast envelopes allowing complete accessibility by both proteases.

To study the topology of TGD4, a transgenic line producing an N-terminally HA-tagged TGD4 protein in the *tgd4-1* mutant background was generated. This construct was able to rescue the lipid phenotype of the *tgd4-1* mutant (Figure 3.8). When chloroplasts isolated from this HA-TGD4-producing line were treated with Thermolysin, the N-terminal HA-tag was susceptible to the protease (Figure 3.7C). Therefore, the N-terminus of TGD4 is presumably facing the cytosol.

Based on these results it is concluded that TGD4 is located in the outer envelope membrane of the chloroplast with its N-terminal portion at least partially exposed to the cytosol.

To determine the strength of the interaction between TGD4 and the outer envelope, isolated wild-type chloroplasts were extracted with sodium chloride, sodium carbonate, or sodium hydroxide (Figure 3.7D). Peripheral or monotopic membrane proteins can be extracted by sodium chloride or sodium carbonate, respectively, while transmembrane proteins are resistant to strongly basic sodium hydroxide (Miege *et al.*, 1999). TGD4, like TOC75, which is a *β*-barrel membrane protein, could not be extracted by any of the three reagents. In contrast, RuBisCO, most of which is peripheral to the thylakoid membrane (Irving and Robinson, 2006), was extracted by all three reagents. Secondary structure prediction of TGD4 by PROF (Rost *et al.*, 2004) suggested that the TGD4 protein most likely forms multiple *β*-sheets especially at the C-terminus corresponding well with regions not accessible to water indicative of a possible *β*-barrel conformation (Figure 3.7E). Taken together, we propose that TGD4 is a transmembrane protein, possibly a *β*-barrel protein, localized in the outer envelope membrane of the chloroplast with its N-terminus partially exposed to the cytosol.

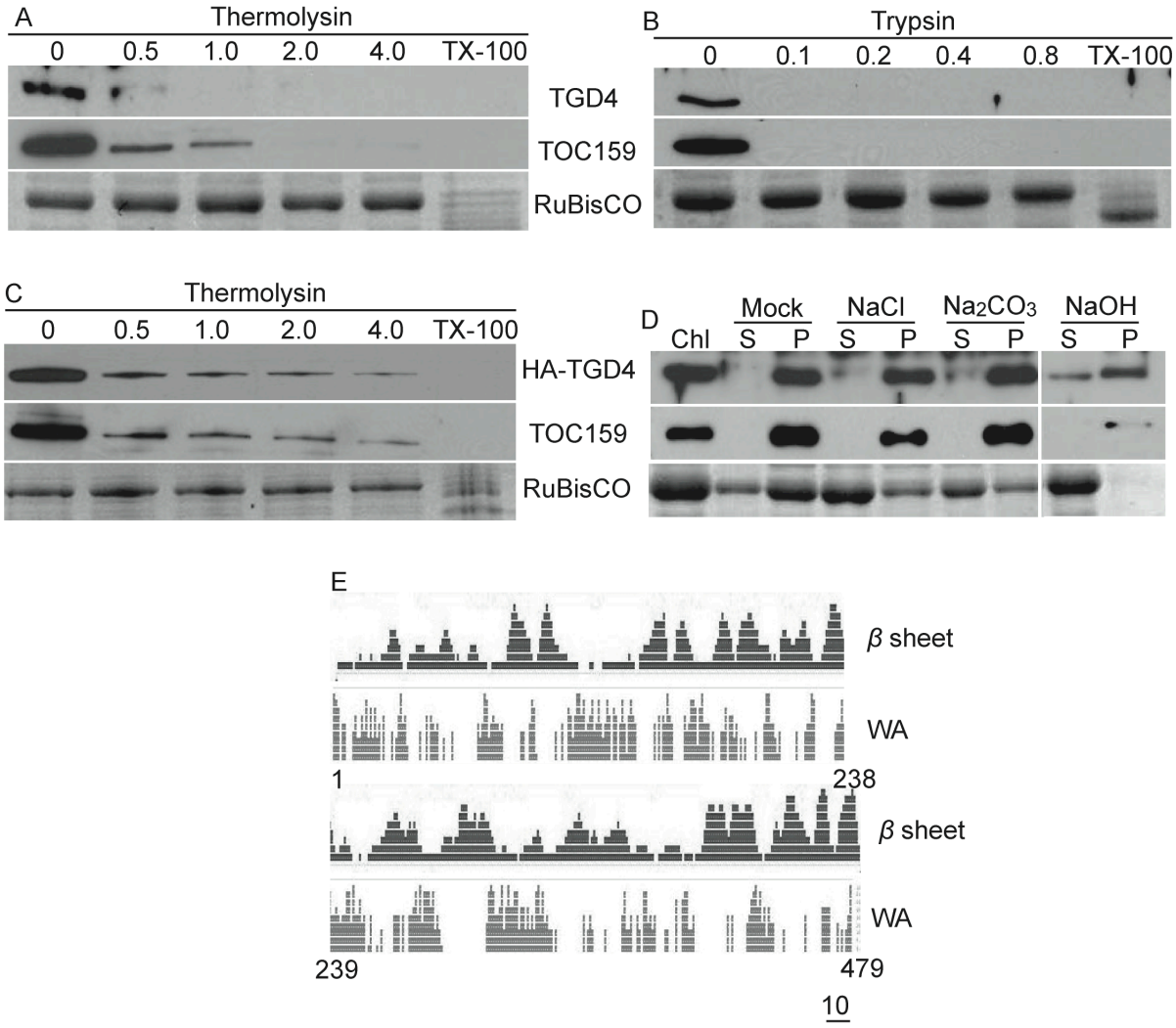

Figure 3.7. TGD4 is a membrane embedded protein of the outer chloroplast envelope. Immunoblots are shown for TGD4 and TOC75. RuBisCO was visualized by CBB staining. **(A)** Wild-type (WT) chloroplasts were treated with 0 to 4 mg/ml Thermolysin. TX-100, tritonX-100; TOC 159, outer envelope marker. RuBisCO, stroma marker. **(B)** Wild-type (WT) chloroplasts were treated with 0 to 0.8 mg/ml Trypsin. **(C)** Chloroplasts isolated from a transgenic line producing an N-terminally HA-tagged TGD4 protein in the *tgd4-1* mutant background were

Figure 3.7. (cont'd)

treated with 0-to-4.0 mg/ml Thermolysin. HA-TGD4 was detected by immunoblotting using a monoclonal HA-antibody. **(D)** Wild-type (WT) chloroplasts were treated with hypotonic buffer only, 2 M NaCl, 0.1 M Na_2CO_3 or 0.1 M NaOH followed by centrifugation. Chl, chloroplast; S, supernatant; P, pellet. **(E)** A histogram of the likelihood of the secondary structure of TGD4 predicted by PROF (PredictProtein). Numbers represent the amino acids. WA, water accessibility.

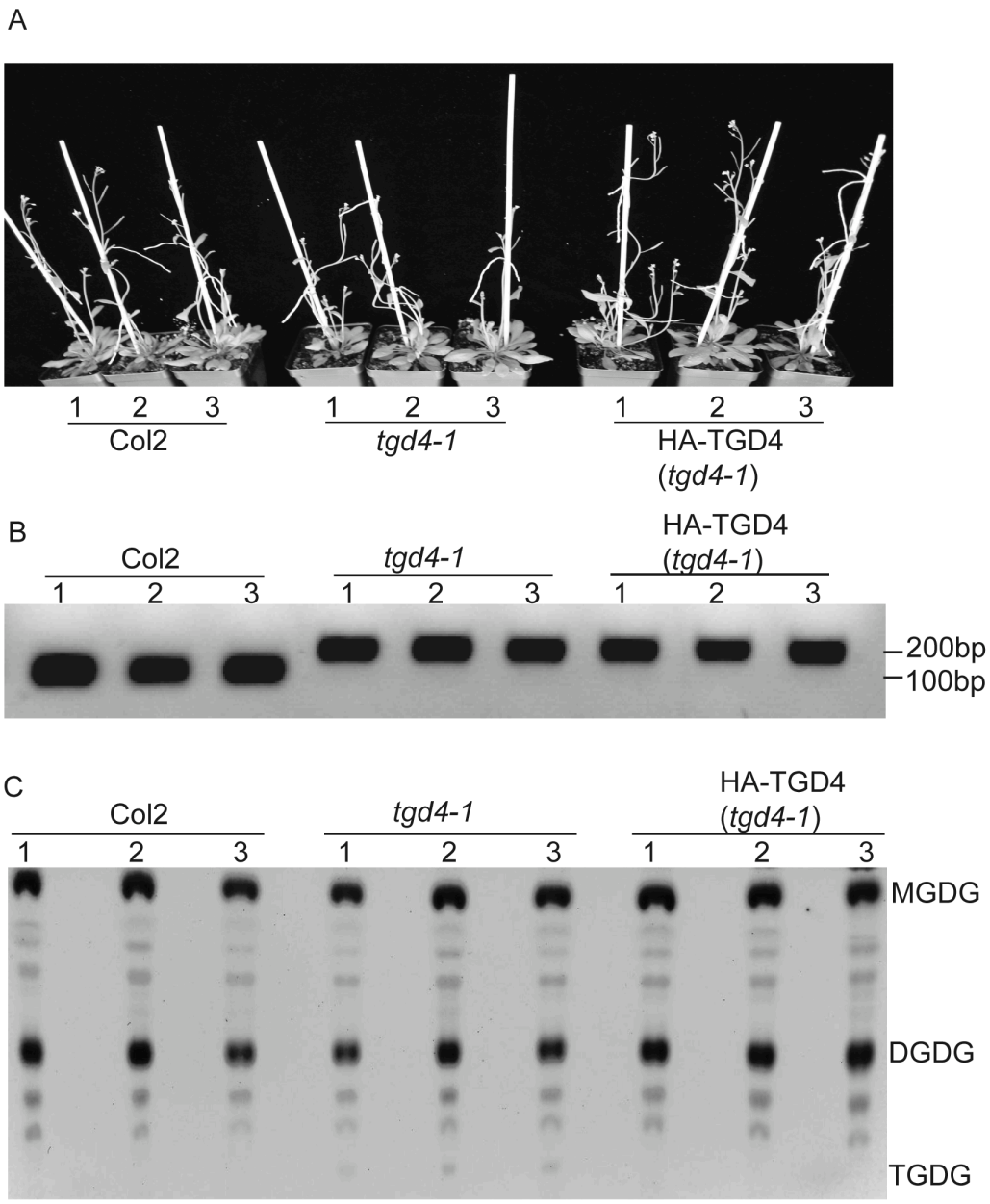

Figure 3.8. HA-TGD4 transgenic line complements the *tgd4-1* phenotype. (A) 6-week-old plants growing on soil. Notice that HA-TGD4 is slightly larger in size compared to *tgd4-1*. **(B)** Genotyping the *TGD4* locus using CAPs marker for the wild type, *tgd4-1* and HA-TGD4 with

Figure 3.8. (cont'd)

three individual lines. **(C)** One dimensional TLC shows that HA-TGD4 does not accumulate TGDG as *tgd4-1*.

3.5. Discussion

In seed plants, the biogenesis of thylakoid lipids requires the import of precursors from the ER. The identity of the transported lipid(s) remains unresolved, but a likely candidate is PtdOH as this lipid specifically binds to TGD2 (Awai *et al.*, 2006, Lu and Benning, 2009), the substrate binding protein associated with the proposed inner envelope TGD1,2,3 complex (Roston *et al.*, 2011). However, while the TGD1,2,3 complex possibly transfers PtdOH from the inter-membrane face of the outer envelope membrane to the stroma face of the inner envelope membrane, the lipid transported from the ER-to-the-outer envelope membrane may be different, e.g. phosphatidylcholine (PtdCho) (Roughan and Slack, 1982). PtdCho could be converted at the outer envelope membrane to PtdOH by the activity of a phospholipase D making PtdOH available for further transfer by the TGD1,2,3 complex (Benning, 2009). Based on results described here, it is suggested that PtdOH might also be the lipid transported to and through the outer envelope from the ER. The finding that TGD4 specifically binds PtdOH *in vitro* is one piece of evidence in favor of the transport of PtdOH all the way from the ER to the stroma face of the inner envelope membrane. The PtdOH binding activity of TGD4 was mostly attributed to its N-terminal fragment (1-286aa), which faces the cytosol and is, therefore, potentially able to contact the ER. The C-terminal fragment was predicted to adopt a secondary structure of

hydrophobic *β*-sheets possibly forming a *β*-barrel (Imai *et al.*, 2011). As the ancestor of chloroplasts is thought to be a Gram-negative bacterium, it is not surprising that outer membrane transporters of chloroplasts are *β*-barrel proteins (Schleiff *et al.*, 2003). In fact, TGD4 is similar to LptD/Imp, an *E.coli β*-barrel outer membrane protein involved in the transfer of Lipid A through the outer bacterial membrane (Sperandeo *et al.*, 2008).

A second piece of evidence in favor of PtdOH transport from the ER to the outer envelope membrane derives from the observation that the different *tgd4* mutant alleles have increased PtdOH content, likely associated with extraplastidic membranes, i.e. the ER. The *tgd1-1* mutant also showed an increase in PtdOH content outside of the chloroplast (Xu *et al.*, 2005). The PtdOH present in *tgd1* and *tgd4* mutants has a very similar fatty acid profile with increased 18:1 and decreased 18:3 fatty acids. The reason for this change in fatty acid profile is not yet understood.

TGD4 lacks a recognizable chloroplast transit peptide and it was tentatively localized to the ER by transiently over-producing a functional GFP-TGD4 fusion protein in tobacco (Xu *et al.*, 2008). Because GFP fused to TGD4 may sequester or expose a signal peptide due to altered folding (Hanson and Kohler, 2001), or because overproduction of the recombinant protein could lead to saturation of the cellular protein-sorting machinery, mistargeting of the majority of the recombinant protein visible by fluorescence microscopy might be possible using this approach. Here we employed specific TGD4 antibodies and determined that the native TGD4 protein is primarily associated with the outer chloroplast envelope membrane. This result was in agreement with previous proteomics studies of chloroplast envelopes (Ferro *et al.*, 2003, Zybailov *et al.*, 2008). However, our new result does not exclude the possibility that a subfraction of TGD4 is also associated with the ER as the microsome preparations inevitably contained microsomes

derived from both the outer envelope membrane and the ER. Moreover, physical membrane contacts between the ER and the chloroplast have been visualized and suggested as the sites of lipid trafficking between the ER and the chloroplast (Andersson *et al.*, 2007). It was previously determined that isolated chloroplasts of the *tgd4-1* mutant do not have a increased number of ER-fragments attached compared to wild-type chloroplasts, suggesting that TGD4 might not be directly involved in the tethering of the two membranes (Xu *et al.*, 2008). However, this result did not exclude the possibility that TGD4 is enriched in ER-outer envelope membrane contact sites. Naturally, localization of proteins in membrane contact sites is bound to be ambiguous. For example, the yeast protein Mmm1, which is an essential component of the tethering complex in ER-mitochondrion contact sites (Kornmann *et al.*, 2009), was first localized to the outer envelope of mitochondria by cellular fractionation (Burgess *et al.*, 1994). However, more recent evidence suggests that without interaction partners, Mmm1 redistributes to the entire ER network (Kornmann *et al.*, 2009).

Based on all data available at this time, we hypothesize that the N-terminal portion of TGD4 extracts PtdOH produced at the ER and transfers it through a β-barrel channel consisting of the C-terminal portion of the protein to the intermembrane face of the outer envelope membrane. Here TGD2, a second PtdOH binding protein involved in the process accepts PtdOH and transfers it to the TGD1/TGD3 ABC transporter complex, which facilitates PtdOH transfer across the inner envelope membrane hydrolyzing ATP. On the stroma face of the inner envelope membrane PtdOH is dephosphorylated to DAG, the ER-derived substrate for thylakoid lipid synthesis by the ER-pathway.

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CHAPTER 4

Structural and functional relationship of TGD4 protein and its complex

4.1. Abstract

Chloroplast membrane lipid synthesis relies on the import of glycerolipids from the ER. The TGD (TriGalactosylDiacylglycerol) proteins are required for this lipid transfer process. The TGD1, 2, and 3 proteins form a putative ABC (ATP-binding cassette) transporter involved in transporting ER-derived lipids through the inner envelope membrane of the chloroplast, while TGD4 binds PtdOH and resides on the outer chloroplast envelope. In this study, we identified two PtdOH binding domains in TGD4, amino acids 110-145 and amino acids 1-80, that are necessary and sufficient for PtdOH binding. Deletion of both domains abolished TGD4 PtdOH binding activity. We also found that TGD4 from 18:3 plants bound specifically to PtdOH and with increased affinity. TGD4 did not interact with other proteins and formed a homodimer complex both *in vitro* and *in vivo*. When reconstituted into liposomes, TGD4 transferred PtdOH from the donor to the acceptor membrane. Our results suggest that TGD4 is an integral dimeric *β*-barrel lipid transfer protein that transfers PtdOH from the ER to the intermembrane space of the chloroplast.

4.2. Introduction

In land plants, glycolipids, such as monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (MGDG), and sulfoquinovosyldiacylglycerol (SQDG), comprise approximately 64% of total leaf membrane lipids (Dörmann and Benning, 2002). Glycolipids are synthesized through two independent pathways: the prokaryotic and the eukaryotic pathways (Benning, 2009). Plastid specific acyltransferases of the prokaryotic pathway are associated with the inner plastid envelope membrane and transfer the acyl groups from acyl-acyl carrier proteins

(acyl-ACPs) *de novo* synthesized in the chloroplast to glycerol 3-phosphate, producing PtdOH. This PtdOH is then dephosphoralated to diacylglycerol (DAG), the direct substrate for glycolipid synthesis. In the eukaryotic pathway, endoplasmic reticulum (ER)-associated acyltransferases use acyl-CoAs derived from plastid-exported acyl groups and glycerol 3-phosphate as substrates, providing PtdOH for the synthesis of extraplastidic phospholipids, such as phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn). A certain portion of DAG moieties return to the chloroplast, where they are incorporated into glycolipids (Wang and Benning, 2012). Glycolipids originating from the prokaryotic pathway have a 16-carbon acyl chain at the *sn*-2 position of the glyceryl backbone, while glycolipids derived from the eukaryotic pathway have an acyl chain of 18 carbons at the same position. This difference is attributable to the different substrate preferences of acyl transferases in the chloroplast and the ER (Frentzen *et al.*, 1983; Heinz and Roughan, 1983). Plants that utilize both pathways, such as *Arabidopsis thaliana*, have 16:3 fatty acids in the *sn-2* position of their thylakoid lipids, and thus are referred to as 16:3 plants. Other plants, such as maize or castor bean, rely exclusively on the eukaryotic pathway and only have mostly 18:3 fatty acids in the *sn-2* position of their thylakoid lipids; these plants are designated 18:3 plants (Mongrand *et al.*, 1998).

Four Arabidopsis proteins, TGD1-4, are currently known to be involved in the ER-to-chloroplast lipid trafficking process (Xu *et al.*, 2003; Awai *et al.*, 2006; Lu *et al.*, 2007; Xu *et al.*, 2008). Plants with mutations in any of the *TGD* genes are impaired in the eukaryotic pathway and accumulate an additional lipid, trigalactosyldiacylglycerol (TGDG). The TGD1, 2, and 3 proteins are proposed to form a bacterial-type ABC transporter complex (Roston *et al.*, 2012). Because TGD2, which is similar to bacterial substrate binding proteins associated with ABC transporters, specifically binds PtdOH (Awai *et al.*, 2006; Lu and Benning, 2009; Roston *et al.*,

2011), it seems possible that this lipid is also transported, even though this has not been directly shown at this time. TGD4 is a predicted *β*-barrel membrane protein (Imai *et al.*, 2011; Haarmann *et al.*, 2010) embedded in the chloroplast outer envelope and binds specifically PtdOH (Wang *et al.*, 2012). It seems likely that the TGD proteins form a lipid transfer conduit to import PtdOH from the ER through the plastid envelopes.

Here we describe the identification of two PtdOH binding domains of TGD4. We also determine the composition of a TGD4 complex and demonstrate that TGD4 can facilitate the transfer of PtdOH between liposomes.

4.3. Experimental procedures

4.3.1. Expression and purification of *Ds*RED-TGD4 proteins

pLW01/*Ds*RED-TGD4 truncation mutants T1-T21 were generated using construct pLW01/*Ds*RED-TGD4 as the template for PCR (Wang *et al.*, 2012). The PCR products were subsequently cloned to the pLW01 vector using SacI and NotI restriction sites. M1-M9 point mutations were constructed by site-directed mutagenesis using pLW01/*Ds*RED-TGD4 T14 as the template and M10-M11 were commercially synthesized (IDT, San Jose, CA). To isolate TGD4 homologs from *Ricinus communis* (*Rc*TGD4) and *Zea Mays* (*Zm*TGD4), cDNAs obtained by reverse transcription from isolated total RNA were used as the template for PCR. *Rc*TGD4 and *Zm*TGD4 were inserted into the BamHI/NotI and EcoRI/NotI restriction sites of vector pLW01, respectively. Sequences of primers used in this study are summarized in Table 4.1. Sequencing was performed by the Research Technology Support Facility (RTSF) at Michigan State University to verify the accuracy of cloned fragments. Recombinant proteins were

produced in *Escherichia coli* strain BL21 (DE3) and purified as previously described (Wang *et al.*, 2012). Briefly, all proteins were produced at 16 °C overnight after adding 100 μM IPTG except T1, T3, T17-T20, M10, M11, which were produced at 28 °C for 3 hours. Cells were lysed by adding 0.2 mg/ml lysozyme (Sigma, St. Louis, Mo) and subsequent sonication in the presence of 1% foscholine-12. Recombinant proteins were purified to homogeneity by Ni-NTA agarose (Qiagen, Valencia, CA) in the presence of 0.1% foscholine-12. The purified proteins were transferred to Tris Buffered Saline (TBS) with 2 M choline chloride following ultrafiltration using Amicon centrifugal filter devices (Millipore, Billerica, MA).

Table 4.1. Primer sequences for cloning. Primer sequences used for all constructs mentioned in this chapter.

Construct	Primer	Sequence
T1	(SacI) TGD4 Fw	CGAGCTCATGAACAGAATGAGATGGTC
T1	(Not1) T1 Rw	ATAGTTTAGCGGCCGCCACAAAAAGTCCAGGCCA
T2	(SacI) T2 Fw	CGAGCTCCTGGGCCAATTCGATGTT
T2	(Not1) T2 Rw	ATAGTTTAGCGGCCGCAATCCCAGATACTGCGAC
T3	(SacI) T3 Fw	CGAGCTCGTG GAT AAA CAT GGT GAA TATT
T3	(NotI) T3 Rw	ATAGTTTAGCGGCCGCAAATGCTTCTAAACTAGGCTG
T4	(SacI) T4 Fw	CGAGCTCATTATCGGCTCTGTGATGAC
T4	(NotI) TGD4 Rw	ATAGTTTAGCGGCCGCTGTCTCAAAGAAACGAAGCTC
T5	(SacI) TGD4 Fw	CGAGCTCATGAACAGAATGAGATGGTC
T5	(NotI) T5 Rw	ATAGTTTAGCGGCCGCCAGAAGAGACACAAGCCA
T6	(SacI) T2 Fw	CGAGCTCCTGGGCCAATTCGATGTT
T6	(NotI) T6 Rw	ATAGTTTAGCGGCCGCACCCCTCCAGAGATCC
T7	(SacI) T2 Fw	CGAGCTCCTGGGCCAATTCGATGTT
T7	(NotI) T7 Rw	ATAGTTTAGCGGCCGCAGGCACTTCCATAGTATCA
T8	(SacI) T2 Fw	CGAGCTCCTGGGCCAATTCGATGTT
T8	(NotI) T8 Rw	ATAGTTTAGCGGCCGCACCAGATTCAGCAGGAAG
T9	(SacI) T2 Fw	CGAGCTCCTGGGCCAATTCGATGTT
T9	(NotI) T9 Rw	ATAGTTTAGCGGCCGCCACAAAAAGTCCAGGCCA
T10	(SacI) T2 Fw	CGAGCTCCTGGGCCAATTCGATGTT

Table 4.1. (cont'd)

T10	(NotI) T10 Rw	ATAGTTTAGCGGCCGCTCTAGGATTCTTATCGAGATC
T11	(SacI) T2 Fw	CGAGCTCCTGGGCCAATTCGATGTT
T11	(NotI) T11 Rw	ATAGTTTAGCGGCCGCCTCAGAACAAAAACCCAATG
T12	(SacI) T2 Fw	CGAGCTCCTGGGCCAATTCGATGTT
T12	(NotI) T12 Rw	ATAGTTTAGCGGCCGCTGTAGACGAAGACCCTC
T13	(SacI) T13 Fw	CGAGCTCACAGTAGCTTCTCGT TTAAAC
T13	(NotI) T11 Rw	ATAGTTTAGCGGCCGCCTCAGAACAAAAACCCAATG
T14	(SacI) T14 Fw	CGAGCTCACGGAGATAGATAAGACTAAAG
T14	(NotI) T14 Rw	ATAGTTTAGCGGCCGC CAATGCGTACAAAGATTTATCC
T15	(EcoRI) T15 Fw	GGAATTCATGAACAGAATGAGATGGGTCG
T15	(SacI) T15 Rw	CGAGCTCTACGAATCTCTGAACATCGAATT
		CGAGCTCCACCACCACCACCACCACGGTTTTTGTTCTGAG
T15	(SacI) T15 Fw	TTTTTGTTATCA
T15	(NotI) TGD4 Rw	ATAGTTTAGCGGCCGCTGTCTCAAAGAAACGAAGCTC
T17	(SacI) TGD4 Fw	CGAGCTCATGAACAGAATGAGATGGTC
T17	(NotI) T17 Rw	CGAGCTCATGAACAGAATGAGATGGTC
T18	(SacI) TGD4 Fw	CGAGCTCATGAACAGAATGAGATGGTC
T18	(NotI) T18 Rw	ATAGTTTAGCGGCCGCTTGCTTAGGGCGAGATAG
T19	(SacI) TGD4 Fw	CGAGCTCATGAACAGAATGAGATGGTC
T19	(NotI) T19 Rw	ATAGTTTAGCGGCCGCTCCACCACCGCCTCC
T20	(SacI) T20 Fw	CGAGCTCCAAGTTGAGTTCTTCCACC
T20	(NotI) T20 Rw	ATAGTTTAGCGGCCGCTCCACCACCGCCTCC
		GGAATTCGGTGGAGCGGGTGGTTTCTCTCTTCAAAGAGT
T21	(EcoRI) T21 Fw	CCTC
T21	(NotI) TGD4 Rw	ATAGTTTAGCGGCCGCTGTCTCAAAGAAACGAAGCTC
*Ds*RED-*Rc*TGD4	(BamH I) *Rc*TGD4 Fw	CGGGATCCATGAAGAAGCTGGGATGGGCA
*Ds*RED-*Rc*TGD4	(NotI) *Rc*TGD4 Rw	ATAGTTTAGCGGCCGCTGTCTCAAAGAATCTTAGTTCTA CCATG
*Ds*RED-*Zm*TGD4	(EcoR I) *Zm*TGD4 Fw	GGAATTCATGTTGCTGCGGCGGATG
*Ds*RED-*Zm*TGD4	(Not I) *Zm*TGD4 Rw	ATAGTTTAGCGGCCGCGGTTTCGTAGAACCGGAGCTC
		GTCTTCGTCTACAGTAGCTGCGCGTTTAAACACAATTG
M1	M1 Fw	GC
		GCCAATTGTGTTTAAACGCGCAGCTACTGTAGACGAAG
M1	M1 Rw	AC
		GTCTT CGTCTACAGT AGCTTCTGCG TTAAACACAA TT
M2	M2 Fw	GGCAAGC
		GCTTGCCAATTGTGTTTAACGCAGAAGCTACTGTAGAC
M2	M2 Rw	GAAGAC
		AGCTTCTCGT TTAAACACAATTGGCGCGCATTTGAAG
M3	M3 Fw	GAT AAATCTTTGT AC

Table 4.1. (cont'd)

M3	M3 Rw	GTACAAAGATTTATCCTTCAAATGCGCGCCAATTGTGT TTAAACGAGAAGCT
M4	M4 Fw	GTTTAAACACAATTGGCAAGCATTTGGCGGATAAATC TTTGTACGCATTGGC
M4	M4 Rw	GCCAATGCGTACAAAGATTTATCCGCCAAATGCTTGCC AATTGTGTTTAAAC
M5	M5 Fw	CAATTGGCAAGCATTTGAAGGCGAAATCTTTGTACGCA TTGGCG
M5	M5 Rw	CGCCAATGCGTACAAAGATTTCGCCTTCAAATGCTTGC CAATTG
M6	M6 Fw	GGCAAGCATTTGAAGGATGCGTCTTTGTACGCATTGGCG
M6	M6 Rw	CGCCAATGCGTACAAAGACGCATCCTTCAAATGCTTGCC
M7	M7 Fw	GAGCTCACGGAGATAGATGCGACTAAAGCTTTTGGTCGA G
M7	M7 Rw	CTCGACCAAAAGCTTTAGTCGCATCTATCTCCGTGAGCTC
M8	M8 Fw	CACGGAGATAGATAAGACTGCGGCTTTTGGTCGAGG GTC
M8	M8 Rw	GACCCTCGACCAAAAGCCGCAGTCTTATCTATCTCCGTG
M9	M9 Fw	GATAAGACTAAAGCTTTTGGTGCGGGGTCTTCGTCTACA GTAGCTT
M9	M9 Rw	AAGCTACTGTAGACGAAGACCCCGCACCAAAAGCTTTA GTCTTATC

4.3.2. Liposome association assay and data quantification

Liposomes composed of dioleoyl-PtdCho and dioleoyl-PtdOH with different w/w ratios as mentioned in figure legends were made as previously described (Wang *et al.*, 2012). Briefly, 250 µg total lipids dried under nitrogen were hydrated by adding 500 µl TBS with 0.2 M choline chloride and incubating at 37 °C for 1 hour. Multi-lamellar liposomes were obtained by vortexing for 2 minutes. Purified proteins (0.25 µM) were incubated with liposomes on ice for 30 minutes. Unbound proteins were separated from liposome-bound proteins by centrifugation at 13,000 × g for 10 minutes at 4 °C followed by two washes with TBS containing 0.2 M choline

chloride. The liposome-protein pellet was examined on a SDS-PAGE gel (16) followed by Coomassie brilliant blue staining. Quantitative analysis was based on densitometry using ImageJ software with three technical repeats. The amount of liposome-bound *Ds*RED-TGD4 was set at 100% and the final data were normalized to *Ds*RED-TGD4 as well as individual loading controls. The results in Figure 6C were fitted to Hill's equation using Origin (OriginLab, Northampton, MA) software:

$n= [L]^n/([L]^n+K_d)$, in which "n" is Hill's number, "L" is ligand concentration and K_d is the dissociation constant.

4.3.3. Protein lipid overlay assay

Lipid strips were made as previously described (Wang *et al.*, 2012). Briefly, 1 nmol lipids in 1 µl buffer (25% v/v chloroform, 50% v/v methanol, 10 µM HCl, 1% Ponseau S) were spotted onto a nitrocellulose membrane (GE Healthcare, Piscataway, NJ) and dried for 1 hour. Finished membranes were blocked in TBST (TBS with 0.1% Tween-20) buffer plus 3% BSA for 2 hours at room temperature. Purified proteins were added to the blocking solution at 1 µg/ml final concentration and incubated with lipid strips at 4 °C overnight followed by washing 3 times and immunoblotting with an anti-His (C-term) antibody (Invitrogen, Grand Island, NY) at a dilution of 1:5000. Secondary mouse anti-HRP antibody (dilution: 1:20,000) was added for 0.5 hours followed by 6 washes. Signal was detected using a chemiluminescence kit from Sigma (St. Louis, Mo).

4.3.4. *In vitro* lipid transport assay

Donor liposomes were made by mixing 150 nmoles of polar lipids (1-palmitoyl-2-linoleoyl-PtdOH; 1-palmitoyl-2-linoleoyl-PtdCho and 1-palmitoyl-2-oleoyl-DAG) with 225 nmoles of 1, 2-phytanoyl-PtdCho and drying the lipid mixture under a stream of nitrogen. After hydration in 100 µl TBS with 0.2 M choline chloride at 37 °C for 1 hour, the lipid was vortexed for 2 minutes. Uni-lamellar liposomes with 100 nm diameter were made by lipid extruder (Avanti Polar Lipids, Alabaster, AL).

To make the acceptor proteoliposome, 250 µg 1,2- dioleoylphosphatidylcholine was dried under nitrogen followed by the addition of 500 µl TBS with 0.2 M choline chloride and 0.25 nmol purified protein (protein to lipid molar ratio: 1 : 1,200). After incubation on ice for 1 hour, the lipid-protein mix was resuspended by vortexing for 2 minutes followed by centrifugation at 13,000 × g for 10 minutes. The resulting multi-lamellar proteoliposome was washed once with 500 µl TBS with 0.2 M choline chloride. Liposome transport assays were conducted by mixing the donor and the acceptor proteoliposome and incubating at room temperature for 1 hour followed by two washes with 500 µl TBS with 0.2 M choline chloride. The multi-lamellar proteoliposome was expected to precipitate, while the uni-lamellar liposome was expected to float after centrifugation at 13,000 × g. Fatty acid methylesters prepared from the acceptor proteoliposome pellet following completion of the reaction were analyzed by gas liquid chromatography using pentadecenoic acid as an internal standard (Wang and Benning, 2011). The palmitic acid initially present only in the donor liposome was quantified.

4.3.5. Plant growth conditions

Arabidopsis thaliana ecotype Col2 transformed with N-terminal HA-tag labeled TGD4 (Wang *et al.*, 2012) were grown on agar-solidified MS medium supplemented with 1% sucrose for 3

weeks as previously described (Xu *et al.*, 2005). Aerial parts were harvested for chloroplast isolation.

4.3.6. Chloroplast preparation

Arabidopsis chloroplasts were prepared as previously described with modifications (Aronsson and Jarvis, 2002). Briefly, 4-week-old seedlings harvested from MS solid medium were ground in grinding buffer (330 mM Sorbitol, 50 mM HEPES-KOH, pH 8.0, 2mM EGTA, 0.1% w/v BSA with 5 mM $MgCl_2$) and separated on a 40% and 80% discontinuous Percoll (Sigma) gradient by centrifugation at 4,000rpm for 10 minutes. The intact chloroplasts were isolated at the interface and were washed once with buffer (330 mM Sorbitol, 50 mM HEPES-KOH, pH 8.0, 5 mM $MgCl_2$).

4.3.7. Blue-native electrophoresis and Immunoblots

Isolated chloroplasts from HA-TGD4 transgenic Arabidopsis or purified *Ds*RED-TGD4 recombinant proteins dissolved in Ni-NTA elution buffer with 0.1% foscholine-12 were suspended in a buffer containing 50 mM Bis-tris/HCl (pH 7.0), 500 mM 6-aminocaproic acid, 10% (v/v) glycerol, 1% (w/v) n-dodecyl-β-D-maltoside (Thermo Fisher, Rockford, IL), and protease inhibitor cocktail (Roche, Indianapolis, IN). The remaining debris was removed by ultracentrifugation at 100,000 × g for 10 minutes. Blue-native electrophoresis was performed as previously described (Schägger and Jagow, 1991). Proteins on the Blue-native gel were denatured in a buffer containing 3.3% (w/v) SDS, 1M Tris-HCl (pH 6.8) and 4% (w/v) β-mercaptoethanol at 85°C for 30 minutes. Denatured proteins were then transferred to a PVDF immuno-blot membrane (Bio-Rad, Hercules, CA) followed by blocking in 5% fat-free milk.

Anti-HA antibody 1: 5,000 dilution (Sigma) was used to identify HA-TGD4 protein. The blot was then incubated with anti-mouse Horse Radish Peroxide (1:20,000 dilution) for 0.5 hours followed by 6 washes. The signal was developed using a chemiluminescence kit (ThermoFisher Scientific, Waltham, MA).

4.3.8. Immuno-pull down assay and mass spectrometry

Isolated chloroplast were incubated with 100 nmol multi-lamellar liposomes [50% (mol%) dioleoyl-PtdOH and 50% (mol%) dioleoyl-PtdCho] or buffer only (330 mM Sorbitol + 50 mM HEPES-KOH, pH 8.0 + 5 mM $MgCl_2$) at room temperature for 30 minutes followed by centrifugation at 3,000 × g for 5 minutes. The chloroplast pellet was solubilized in 1 ml buffer containing 50 mM Bis-tris/HCl (pH 7.0), 500 mM 6-aminocaproic acid, 10% (v/v) glycerol, 1% (w/v) n-dodecyl-β-D-maltoside (Thermo Fisher, Rockford, IL), and protease inhibitor cocktail for 1 hour at 4 °C followed by centrifugation at 100,000 × g at 4 °C for 10 minutes to remove debris. 20 µl anti-HA conjugated agarose (Thermo scientific, Rockford, IL) was added to the solubilized chloroplast and incubated at 4 °C overnight. Washings and eluates were performed according to the manufacturer's instruction with a washing solution that consisted of 50 mM Bis-tris/HCl (pH 7.0), 500 mM 6-aminocaproic acid and 0.1% (w/v) n-dodecyl-β-D-maltoside. The elusions were submitted to the Proteomics Center at Michigan State University for LC/MS/MS analysis.

4.3.9. Accession numbers

Sequence of *Arabidopsis thaliana* TGD4 can be found in The Arabidopsis Information Resource under the name At3g06960. The sequence of *Ricinus communis* and *Zea mays* TGD4 can be found in GeneBank under the accession number: XM_002519286.1 and XM_002519286.1, respectively.

4.4. Results

4.4.1. Amino acids 110-145 are necessary and sufficient for PtdOH binding by TGD4.

Partially overlapping truncation mutants of TGD4 fused with their N-terminus to *Ds*RED to enhance solubility of the protein, T1-T5, were constructed, which cover the length of the protein, to coarse-map the minimal PtdOH binding domain of TGD4 (Figure 4.1.). Recombinant proteins were produced in *E. coli* and purified to homogeneity. The proteins were then subjected to a liposome association assay, wherein proteins capable of binding the PtdOH-containing liposome would co-precipitate after centrifugation. The protein content of the liposome pellet was then examined by SDS-PAGE and quantified by densitometric scanning. As shown in Figure 4.1B and C, the N-terminal 1-300 amino acids accounted for the PtdOH binding activity of TGD4 and the C-terminal portion of the protein binds PtdOH with much decreased affinity.

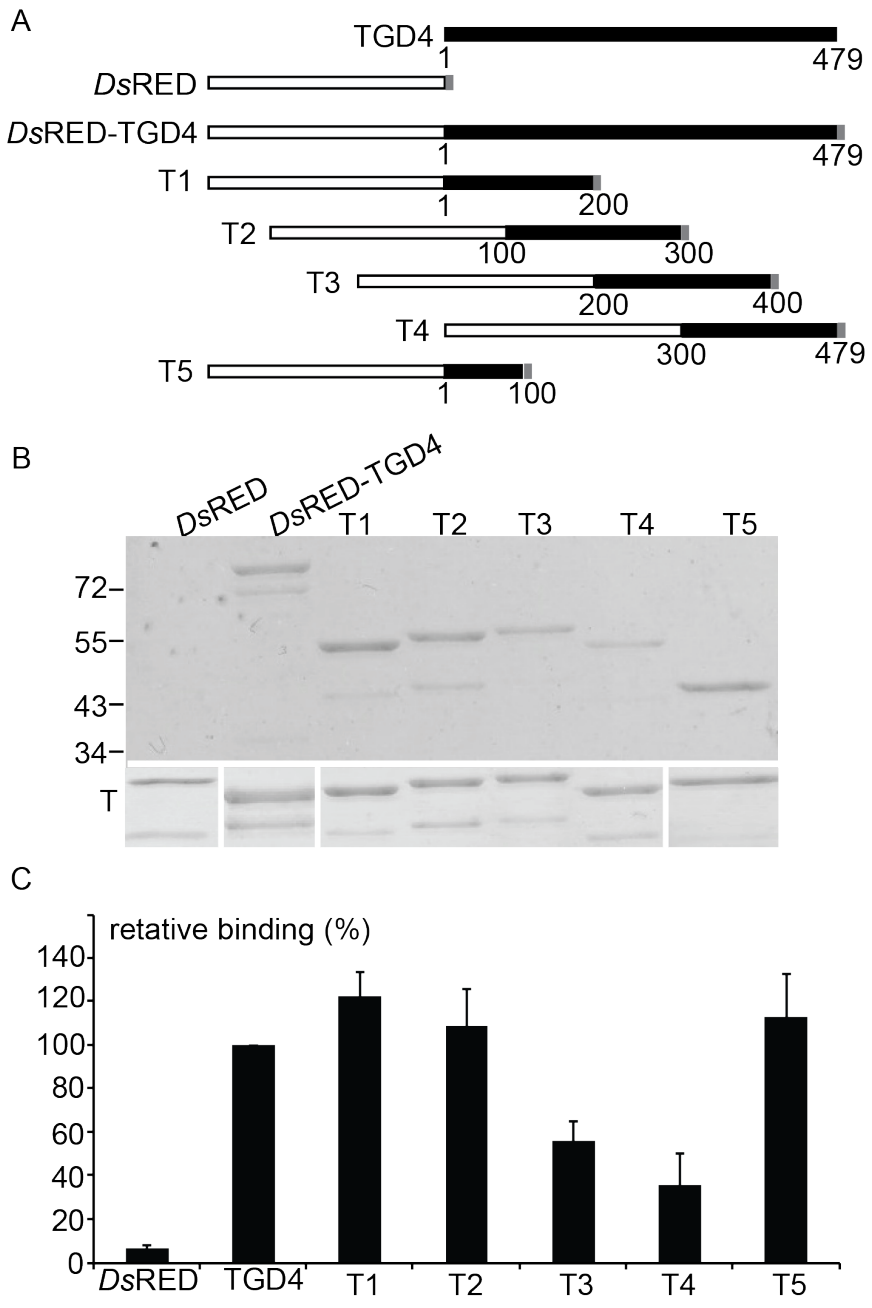


Figure 4.1. Coarse-mapping the TGD4 PtdOH binding region. (A) Schematic representation of TGD4 truncation mutants T1-T5 fused with *Ds*RED on the N-terminus. White box: *Ds*RED; black box: TGD4 fragment; gray box: 6×His tag; numbers below indicate amino acid sequences.

Figure 4.1. (cont'd)

(B) Multi-lameller liposomes consisting of 40% (w/w) PtdOH and 60% (w/w) PtdCho were incubated with purified proteins. Proteins bound to the liposome co-precipitated after centrifugation at 13,000 × g. Protein content of the pellet was analyzed by SDS-PAGE and stained by Coomassie Brilliant Blue R-250. Numbers indicate molecular masses in kD. T: total protein used in this assay. **(C)** Images were quantified by densitometry software. Data were normalized with bound *Ds*RED-TGD4 as 100%. Averages and standard deviations were calculated from 3 technical repeats.

To map the minimum PtdOH binding domain in-depth, additional truncation mutants T7 to T12 spanning amino acids100 to 200 were tested (Figure 4.2A) for their PtdOH binding abilities (Figure 4.2B). Except for T12 (AA 100-125), all truncations up to T11 (AA 100-150) were able to bind PtdOH as strong as T2, indicating that amino acids 100-150 are sufficient and amino acids 125-150 are necessary for PtdOH binding. A computer-generated prediction of the secondary structure suggested that amino acids 100-150 contain an intact soluble loop domain (AA 110-145) (Figure 4.2C). Since many of the identified PtdOH binding domains, such as those in mTOR (mammalian target of rampamycin), Opi1p (overproduction of inositol), and TGD2, adopt a loop conformation (Fang *et al.*, 2001; Loewen *et al.*, 2004; Lu and Benning, 2009), we hypothesized that this soluble loop (AA 110-145) represents the minimum PtdOH binding domain of TGD4.

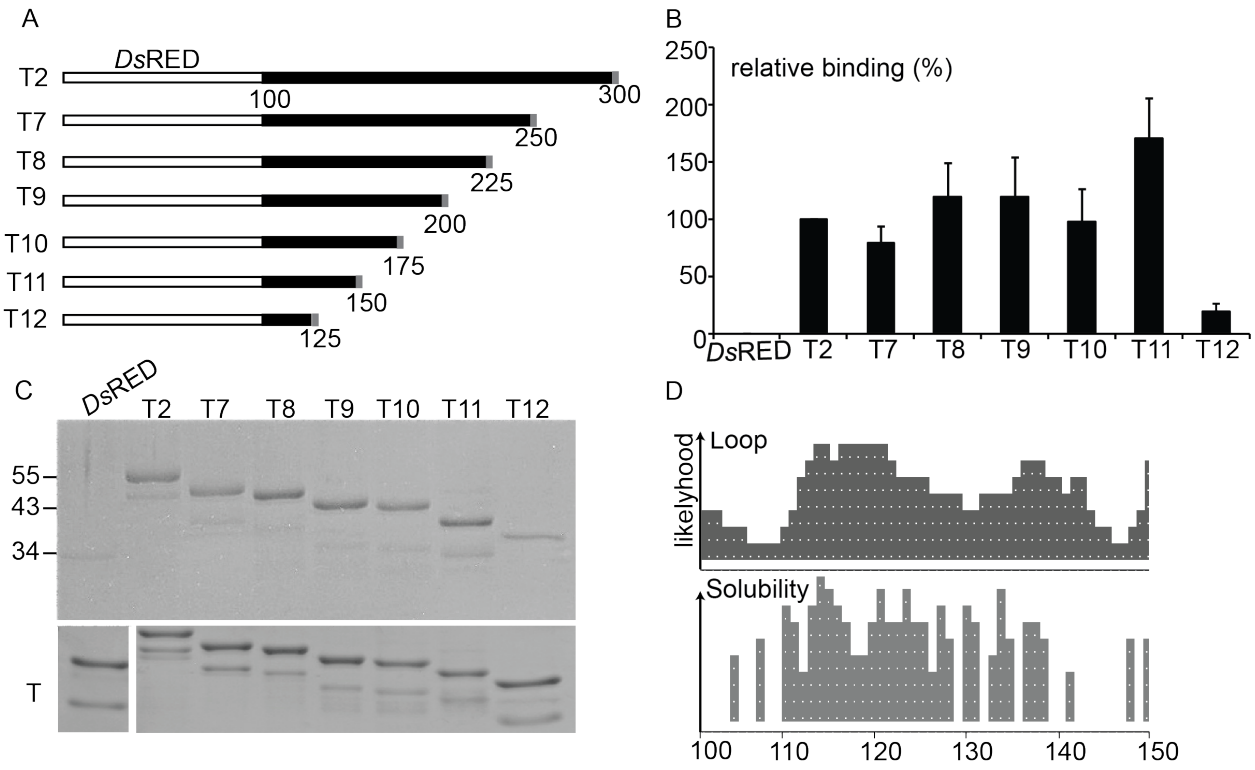


Figure 4.2. Fine-mapping the TGD4 PtdOH binding site. (A) Schematic representation of N-terminal *Ds*RED fused TGD4 truncation mutants T7-T12. White box: *Ds*RED; black box: TGD4 fragment; gray box: 6×His tag; numbers below indicate amino acids. **(B)** Proteins bound to 40% (w/w) PtdOH liposomes were separated by SDS-PAGE and stained by Coomassie Brilliant Blue. Numbers indicate molecular masses in kD. T: total protein used in this assay. **(C)** Images were quantified and data were normalized with liposome-bound T2 protein as 100%. Averages and standard deviations were calculated from 3 technical repeats. **(D)** T14 (100-150) secondary structure predicted by PROF (PredictProtein). The y-axis represents the likelihood of each structural signature. Note that amino acids 110-145 formed a putative loop.

Additional mutants were constructed to test this hypothesis: T13 (AA 125-145), T14 (AA 110-145), and T15, a truncated version of TGD4 that contained the whole protein but substituted the loop with a linker sequence (Figure 4.3A). While T13 only retained partial PtdOH binding activity, T14 still showed full PtdOH binding (Figure 4.3B). To test if these truncations were still specific to PtdOH, a lipid overlay assay was used. The purified proteins were incubated with a hydrophobic membrane onto which different plant lipids were spotted. The proteins were then detected with an anti-His antibody (Figure 4.3C). Both T14 and full-length TGD4 bound exclusively to PtdOH, and with similar strength. T15 bound to PtdOH only as well, but the signal was attenuated suggesting weakened PtdOH binding. In the liposome association assay, T15 lost about 50% of the total PtdOH binding, suggesting the existence of an additional PtdOH binding domain (Figure 4.3D). As positive amino acids are usually essential for PtdOH binding through electrostatic interaction with the negatively charged PtdOH head group, we hypothesized that mutating those positive residues in the minimal PtdOH binding domain of TGD4 would abolish PtdOH binding. Thus, we changed each positively charged amino acids between residues 110 and 145 to alanine (Figure 4.3E) and tested the point mutants' PtdOH binding activity except M6 (AA 110-145, K140A), which we were unable to produce in *E. coli*. We also included a point mutant targeting the highly conserved Serine, M1 (AA 110-145, S128A). No single point mutant abolished PtdOH binding; however, mutants M1-to-M4 retained only about 60% of PtdOH binding activity compared to T14 (AA 110-145) (Figure 4.3F).

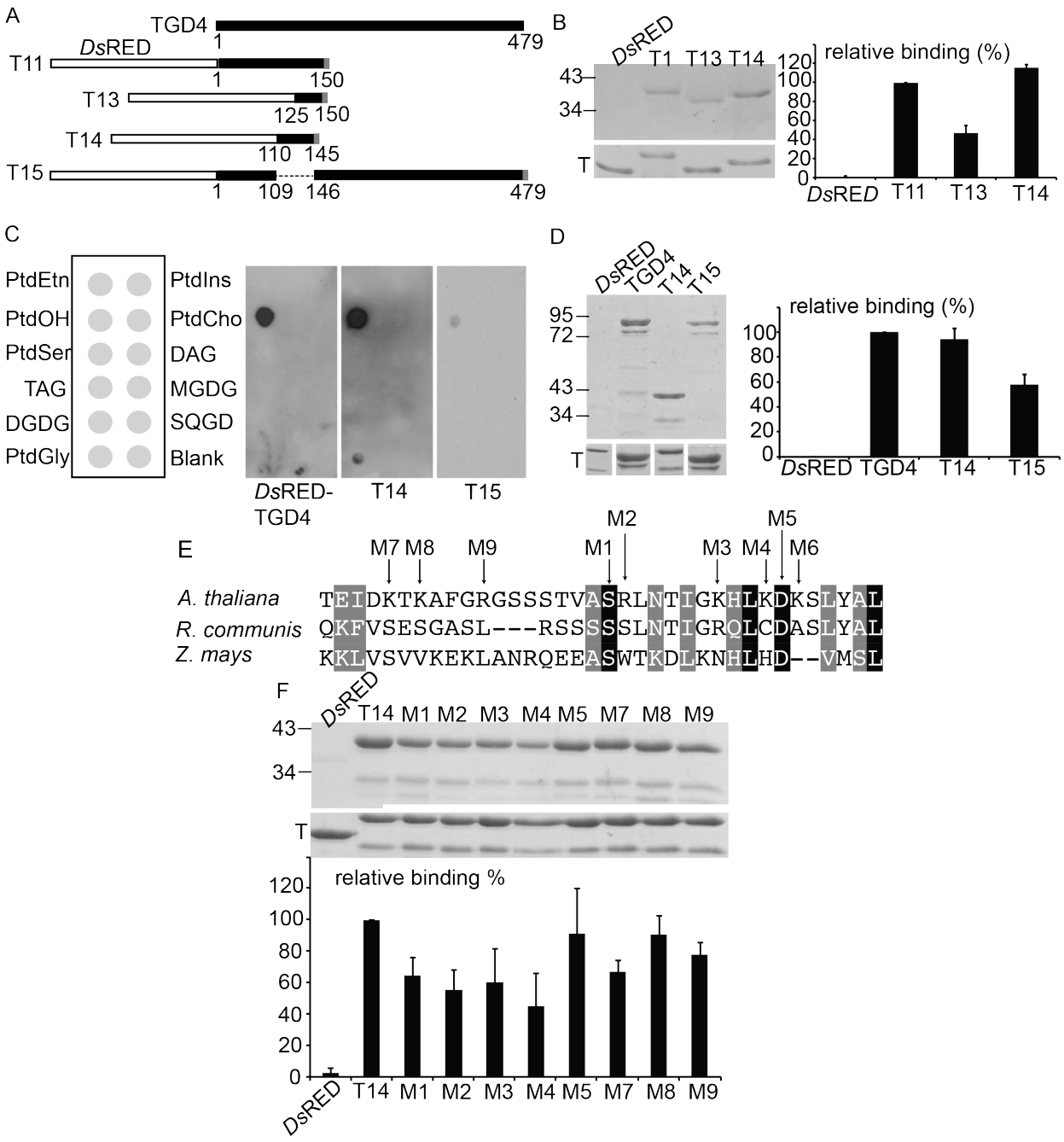


Figure 4.3. TGD4 amino acids 110-145 comprise the minimum PtdOH binding site. (A) Schematic representation of *Ds*RED-TGD4 truncation mutants T13-T15. White box: *Ds*RED; black box: TGD4 fragment; gray box: 6×His tag; numbers below indicate amino acids. **(B)** Proteins bound to 40% (w/w) PtdOH liposomes were separated by SDS-PAGE and stained by

Figure 4.3. (cont'd)

Coomassie Brilliant Blue. Numbers indicate molecular masses in kD. T: total protein. Images were similarly quantified as in Figure 4.2B and normalized with liposome-bound T11 as 100%. **(C)** Membranes spotted with various lipids were incubated with purified proteins. Proteins bound to lipids were detected using an anti-His antibody. PtdEtn: dioleoyl-phosphatidylethanolamine; PtdIns: dioleoyl-phosphatidylinositol; PtdOH: dioleoyl-phosphatidic acid; PtdCho: dioleoyl-phosphatidylcholine; PtdSer: dioleoyl-phosphatidylserine; DAG: dioleoyl-diacylglycerol; TAG: trioleoyl-triacylglycerol; MGDG: monogalactosyldiacylglycerol; DGDG: digalactosyldiacylglycerol; SQDG: sulfoquinovosyldiacylglycerol; PtdGly: phosphatidylglycerol. **(D)** Same liposome binding assay as in **(B)**, except the PtdOH concentration was 30% (w/w). Data was normalized with liposome-bound *Ds*RED-*At*TGD4 as 100%. Numbers indicate molecular masses in kD. T: total protein. **(E)** Alignment of TGD4 minimum PtdOH binding site within three species. Black column: identical amino acids; gray column: similar amino acids; M1-M9: T14 point mutants with indicated amino acid changed to alanine. **(F)** Liposome binding assays with 30% (w/w) PtdOH were conducted with different point mutants. T: total proteins. Numbers indicate molecular masses in kD. Data was quantified by densitometry with 3 technical repeats and normalized with liposome-bound T14 as 100%.

4.4.2. A second PtdOH binding domain is present in the first 80 amino acids of TGD4.

As shown in Figure 4.1C, T5 (AA 1-100) retained wild-type PtdOH binding activity despite lacking the aforementioned, PtdOH-binding domain (AA 110-145). We investigated which residues in this most N-terminal portion of the protein represented by T5 (AA 1-100) were

responsible for PtdOH binding. Additional truncation mutants T17-T19 were constructed (Figure 4.4A) and their PtdOH binding activity was tested by liposome association assay. It was shown that only T19 (AA 1-80) bound to PtdOH while T18 (AA 1-50) and T17 (AA 1-25) did not, indicating that amino acids 50-80 were necessary for PtdOH binding by this fragment (Figure 4.4B). This region was also predicted as a loop structure and was highly conserved among different species (Figure 4.4A and E). Additional mutants were tested, which included a truncation spanning residues 50-80 (T20) and two point mutants in the 50-80 AA region that had an alanine in place of a positively charged arginine (Figure 4.4A). In contrast to T14 (AA 110-145), T20 (AA 50-80) was not sufficient for PtdOH binding (Figure 4.4C). The truncation mutant T21 that lacked both PtdOH binding domains (AA 110-145 and AA 1-80) has minimum PtdOH binding activity, indicating these two regions are necessary in order for TGD4 to bind PtdOH (Figure 4.4C). Like the first PtdOH binding domain (AA 110-145), AA 1-80 also binds only with PtdOH but not any other lipids tested (Figure 4.4D).

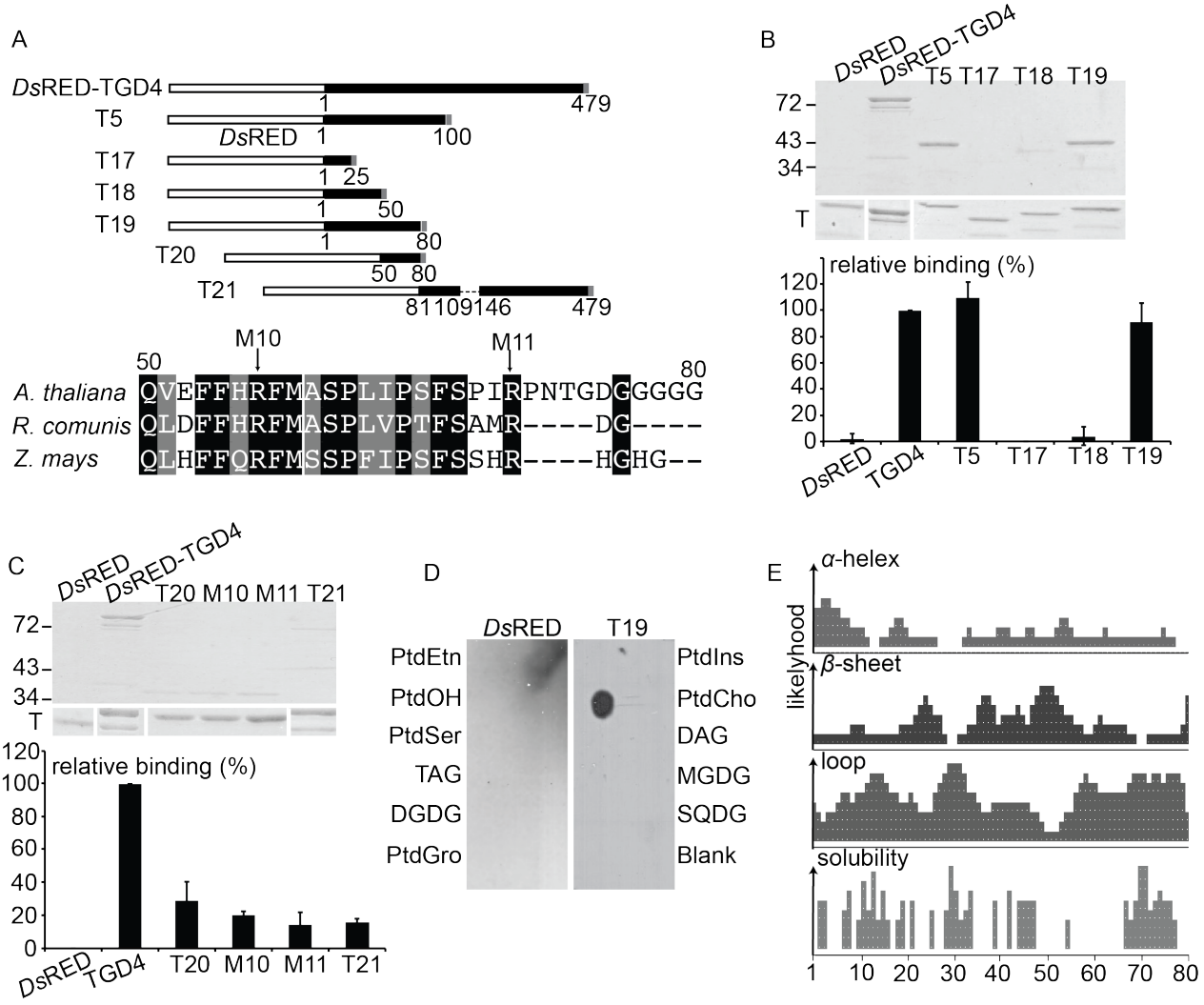


Figure 4.4. Fine-mapping the PtdOH binding region of TGD4 residing in amino acids 1-100. (A) Schematic representation of *Ds*RED-TGD4 truncation mutants T17-T21 and point mutants M10 and M11. White box: *Ds*RED; black box: TGD4 fragment; gray box: 6×His tag; numbers below indicate amino acids. Black column: identical amino acids; gray column: similar amino acids; M1-M9: T20 point mutants with indicated amino acid changed to alanine. **(B)** Liposome binding assay with 40% (w/w) PtdOH was conducted for truncation mutants T17-T19.

Figure 4.4. (cont'd)

T: total proteins; Numbers indicate protein masses in kD. Data was normalized to liposome-bound *Ds*RED-TGD4 as 100% and quantified by densitometry with three technical repeats. **(C)** Liposome binding assay with 30% (w/w) PtdOH was performed for T20, M10, M11 and T21. The results were quantified as above. Note that T20 was not sufficient for PtdOH binding. **(D)** Lipid overlay assay testing the lipid binding specificity of T19 (AA 1-80). Lipid abbreviations are the same as in Figure 4.3C. **(E)** T19 (AA 1-80) secondary structure predicted by PROF (PredictProtein). Y-axis represents the likelihood of each structural signature.

4.4.3. *At*TGD4 homologs from 18:3 plants bind PtdOH with higher affinity.

Unlike 16:3 plants such as *Arabidopsis*, which have two pathways for chloroplast lipid synthesis, 18:3 plants depend solely on the eukaryotic pathway. However, the activity of plastidic PtdOH phosphatase was shown to be very low in the 18:3 plants (Heinz and Roughan, 1983) leading to the suggestion that DAG instead of PtdOH was the lipid transferred from the ER to the chloroplast in 18:3 plants.

To test this hypothesis, cDNAs of *TGD4* orthologs of the 18:3 plants *Ricinus comunis* (*Rc*TGD4) and *Zea mays* (*Zm*TGD4) were isolated and expressed in *E. coli* to produce fusion proteins with *Ds*RED at the TGD4 N-terminus. *At*TGD4 shares about 50% amino acid identity with *Rc*TGD4 and *Zm*TGD4. Lipid overlay assay showed that, similar to *At*TGD4, both *Rc*TGD4 and *Zm*TGD4 bound only to PtdOH and not to DAG (Figure 4.5A). To estimate the relative PtdOH binding affinities, the liposome association assay was employed. Both TGD4 orthologs

from the 18:3 plants, especially *Rc*TGD4, showed much higher PtdOH binding than *At*TGD4 (Figure 4.5B).

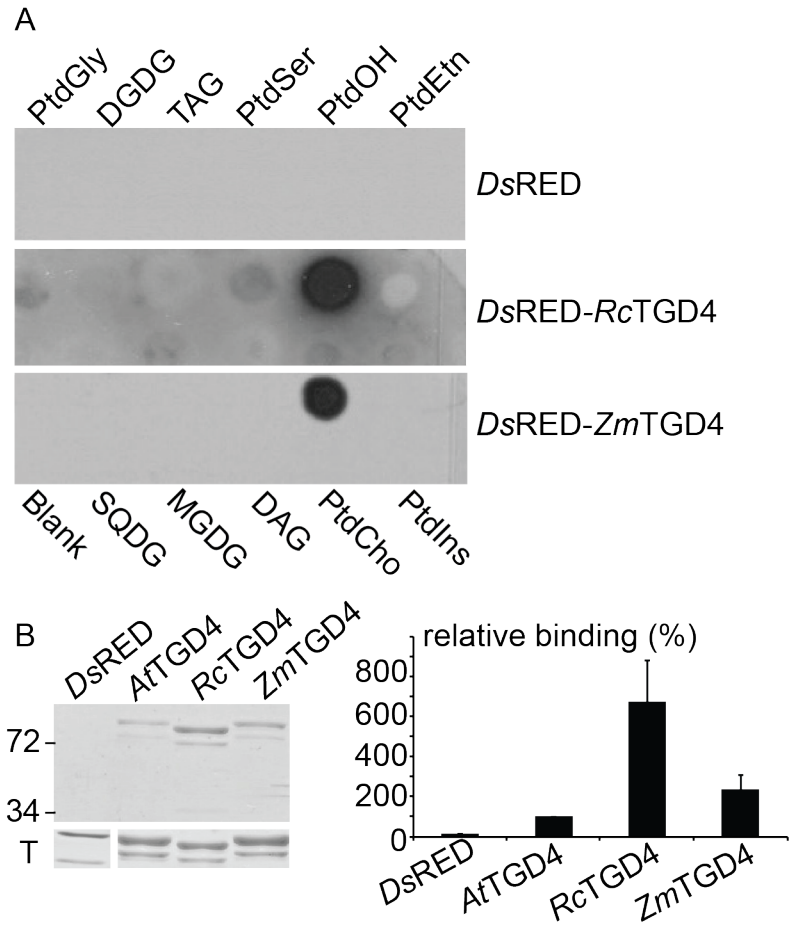


Figure 4.5. TGD4 homologs in 18:3 plants had higher affinity for PtdOH than TGD4 of 16:3 Arabidopsis. (A) The same lipid membrane as in Figure 4.3C was used to study the lipid binding property of *Ds*RED-TGD4 from *R. communis* and *Z. mays*. Proteins were detected by anti-His antibody. **(B)** Liposome binding assay with 20% (w/w) PtdOH was performed. Proteins

Figure 4.5. (cont'd)

bound to liposomes were separated by SDS-PAGE and stained with Coomassie brilliant blue. Images were quantified by densitometry software ImageJ with 3 technical repeats. Data were normalized with liposome-bound *Ds*RED-*At*TGD4 as 100%. Numbers indicate molecular masses in kD. T: total protein.

4.4.4. TGD4 forms a functional homodimer *in vitro* and *in vivo.*

Proteins in a cell often form large complexes with other proteins involved in the same pathway to carry out their biological functions efficiently. These proteins may not be identified through genetic screening due to gene redundancy or mutant lethality. To identify possible additional components involved in lipid transfer from the ER to the plastid as part of the eukaryotic pathway, we studied TGD4 interacting partners *in vivo* using immuno-precipitation followed by mass spectrometry. However, no new proteins were found to interact with TGD4, while TGD4 itself was identified repetitively with high confidence (Table 4.2). The recombinant *Ds*RED-TGD4 protein migrated as oligomers, mainly dimers or pentamers, on a Blue-Native PAGE gel, while *Ds*RED alone behaved as expected for a monomer (Figure 4.6A and B). This oligomerization activity was mainly attributed to the C-terminal portion of TGD4 (*Ds*RED-TGD4C), the region that was not responsible for PtdOH binding (Figure 4.6A and B). Liposome association experiments with increasing amounts of PtdOH showed that TGD4 exhibited positive cooperativity characteristic of allosteric proteins with Hill's number equal to 3 (Figure 4.6C). This behavior is expected for a protein complex that contains more than one substrate-binding

site, which interacts cooperatively upon ligand binding. Thus, we conclude that TGD4 forms homo-oligomers with subunits that are functionally cooperative.

Table 4.2. Representative list of proteins identified by mass spectrometry in HA-TGD4 immuno-precipitation assay. Chloroplasts isolated from either HA-TGD4 (*tgd4-1*) or *tgd4-1* Arabidopsis were solubilized by 1% n-dodecyl-β-D-maltoside. The solubilized total protein was incubated with anti-HA conjugated agarose. The eluates were analyzed by LC-MS/MS. Candidates for TGD4 interacting proteins should only be present in the HA-TGD4 (*tgd4-1*) sample but not the *tgd4-1* sample. Numbers in the *tgd4-1* and HA-TGD4 columns indicate the spectral counts of each protein. This experiment was repeated ten times and a representative data from a single experiment is shown.

Identified Proteins	Accession No.	*tgd4-1*	HA-TGD4
ATPRX Q | Thioredoxin superfamily protein	AT3G26060	7	0
PTAC16 | plastid transcriptionally active 16	AT3G46780	23	16
NPQ4, PSBS | Chlorophyll A-B binding family protein	AT1G44575	16	18
RB, CSP41B, HIP1.3 | chloroplast RNA binding	AT1G09340	22	5
PSBR | photosystem II subunit R	AT1G79040	17	16
CaS | calcium sensing receptor	AT5G23060	20	17
TGD4 | pigment defective 320	AT3G06960	0	29
RCA | rubisco activase	AT2G39730	13	5
ATRAB8D, | RAB GTPase homolog E1B	AT4G20360	21	9
GAPB | glyceraldehyde-3-phosphate dehydrogenase B	AT1G42970	12	3
GAPA, | glyceraldehyde 3-phosphate dehydrogenase A subunit	AT3G26650	14	3
PSBQ, PSBQA, PSBQ-1 | photosystem II subunit QA	AT4G21280	3	1
NAD(P)-binding Rossmann-fold superfamily protein	AT1G24360	3	2
PSAF | photosystem I subunit F	AT1G31330	5	5
RBCL | ribulose-bisphosphate carboxylases	ATCG00490	3	0

To study the TGD4 complex *in vivo*, chloroplasts from HA-TGD4 transgenic plants were isolated and protein content was analyzed by Blue-Native PAGE. The TGD4 complex was specifically detected by immunoblotting using a HA antibody. The size of the complex fell between protein markers 66kDa and 132kDa under both resting and lipid-importing conditions (Figure 4.6D). In the resting condition, isolated chloroplasts were in an iso-osmotic buffer only while in the lipid-importing condition liposomes composed of PtdOH and PtdCho were added to the chloroplast. To better estimate the size of the complex, protein marker sizes were plotted against their migration distances. The approximate size of the complex was estimated to be 135 kD, which is approximately double the size of the TGD4 monomer, 53kD, TGD4 monomer. Since no further interacting partners were identified through mass spectrometry (Table 4.2) and TGD4 did not interact with other known TGD proteins by co-immunoprecipitation, we concluded that TGD4 forms homodimers *in vivo*.

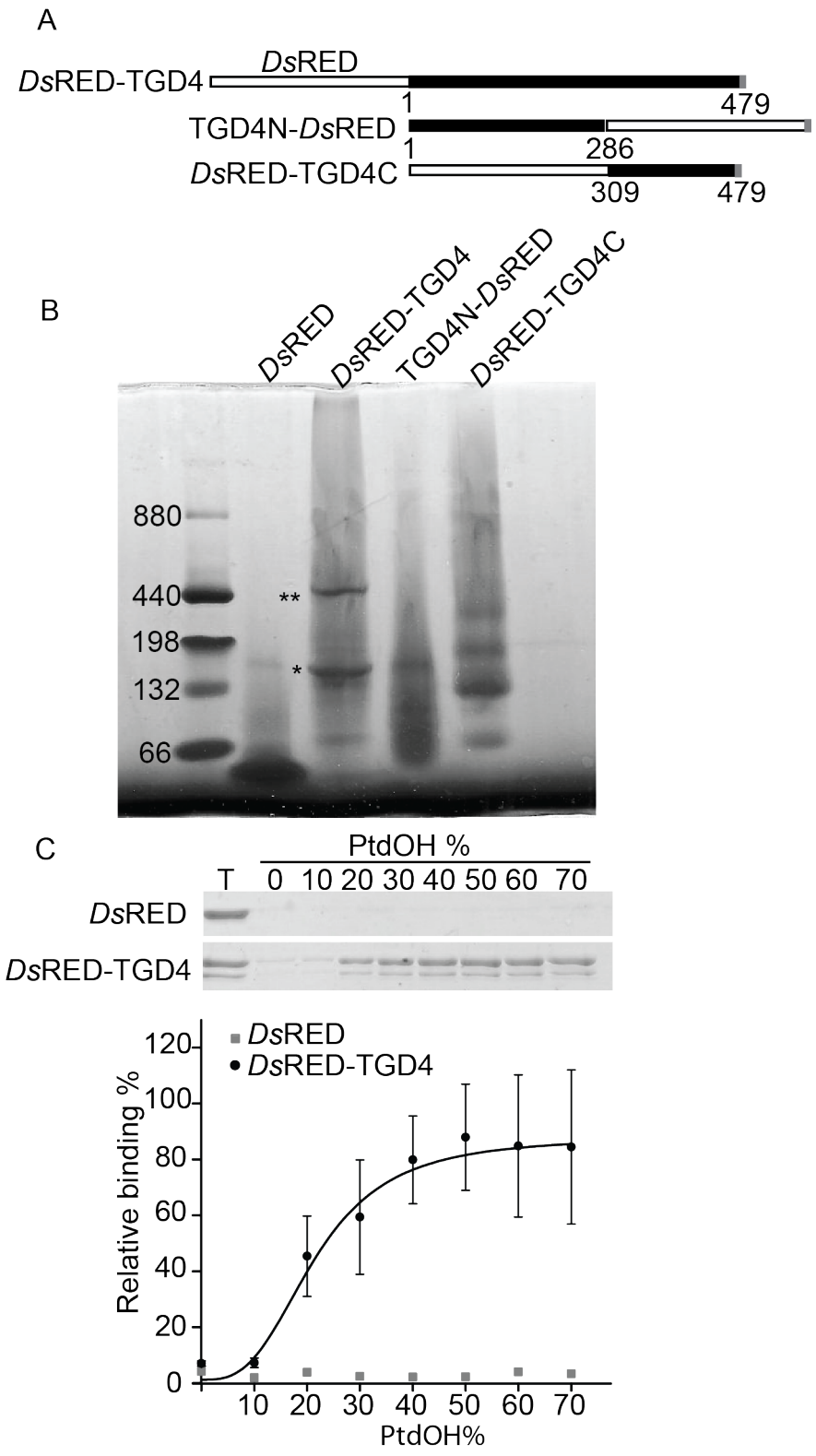
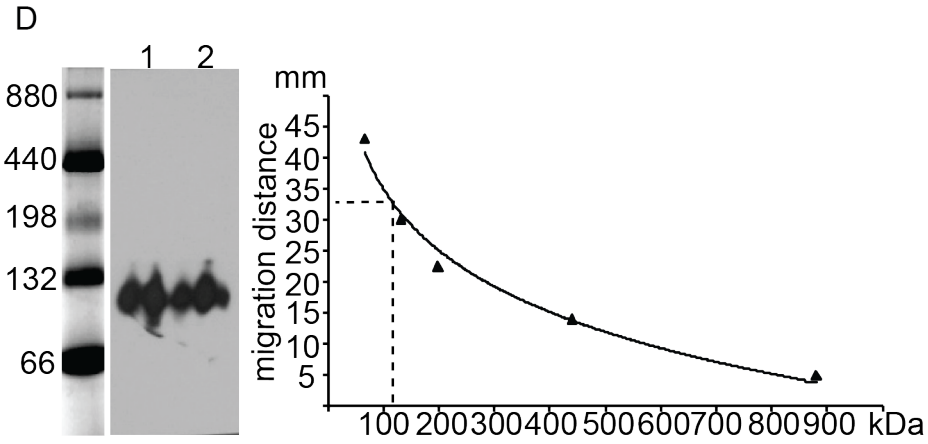


Figure 4.6. TGD4 formed oligomers *in vitro* and dimers *in vivo*. (A) Schematic representation of *Ds*RED-TGD4 N-terminal and C-terminal truncation mutants. White box: *Ds*RED; black box:

Figure 4.6. (cont'd)

TGD4 fragment; gray box: 6×His tag; numbers below indicate amino acids. **(B)** Blue-Native PAGE showed that *Ds*RED-TGD4 proteins oligomerized *in vitro*. Theoretical monomer size of each protein was: *Ds*RED: 26.5 kD; *Ds*RED-TGD4: 80kD; *Ds*RED-TGD4N: 59kD; *Ds*RED-TGD4C: 46kD. Single asterisk: dimer band; double asterisk: pentamer band. **(C)** *Ds*RED-TGD4 bound to PtdOH in a cooperative manner. Liposomes containing different percentage of PtdOH were incubated with purified proteins. Liposome-bound proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Data were normalized to total *Ds*RED-TGD4 as 100% and quantified by densitometry with 3 technical repeats and fitted with Hill's equation with a derived Hill's number equal to 3. Black dots: *Ds*RED-TGD4; gray squares: *Ds*RED. **(D)** TGD4 forms a dimer *in vivo*. Chloroplast proteins from HA-TGD4 transgenic Arabidopsis in *tgd4-1* background were separated on a Blue-Native gel and detected by anti-HA antibody. The monomer size of HA-TGD4: 53kD. 1. HA-TGD4 chloroplast; 2. HA-TGD4 chloroplast incubated with PtdOH/ PtdCho (50/50, w/w) liposomes. Vertical numbers: molecular masses in kD. Migration distance was plotted against molecular mass and fitted with the logarithmic curve. Dashed line showed the position of HA-TGD4 band on the chart.

Figure 4.6. (cont'd)



4.4.5. TGD4 transfers PtdOH from the donor to the acceptor liposome *in vitro.*

Although TGD4 had been shown to bind to PtdOH and is predicted to be a *β*-barrel transporter, whether TGD4 transferred PtdOH was still unclear given the versatile roles of PtdOH as a lipid messenger regulating various enzyme activities. To test if TGD4 transferred PtdOH or any other polar lipids that are popularly speculated to be involved in ER-chloroplast lipid transfer, namely PtdCho and DAG, an *in vitro* lipid transfer assay was developed. Donor liposomes containing the lipid of interest with a specific 16:0 acyl chain were made in the form of small uni-lamellar liposomes. Purified proteins were reconstituted into large multi-lamellar liposomes that were made entirely of dioleoyl-PtdCho. If TGD4 transferred PtdOH, the lipid of interest would be present on the acceptor liposomes after the reaction (Figure 4.7B). Donor and

acceptor liposomes were separated by centrifugation as large multi-lamellar liposomes precipitated while small uni-lamellar liposomes remained in the supernatant. The unique 16:0 acyl chain present only in the lipid of interest was detected and quantified by gas chromatography. Since *Ds*RED was a soluble protein, it did not partition into the liposomes as *Ds*RED-TGD4. Approximately 40% of *Ds*RED-TGD4 was reconstituted on to the acceptor liposomes (Figure 4.7A). Among all three lipids tested, TGD4 transferred PtdOH with the highest efficiency. It also transferred DAG moderately and did not transfer PtdCho at all. The lipid of interest presented in the *Ds*RED sample might have resulted from spontaneous fusion of donor and acceptor liposomes (Figure 4.7C).

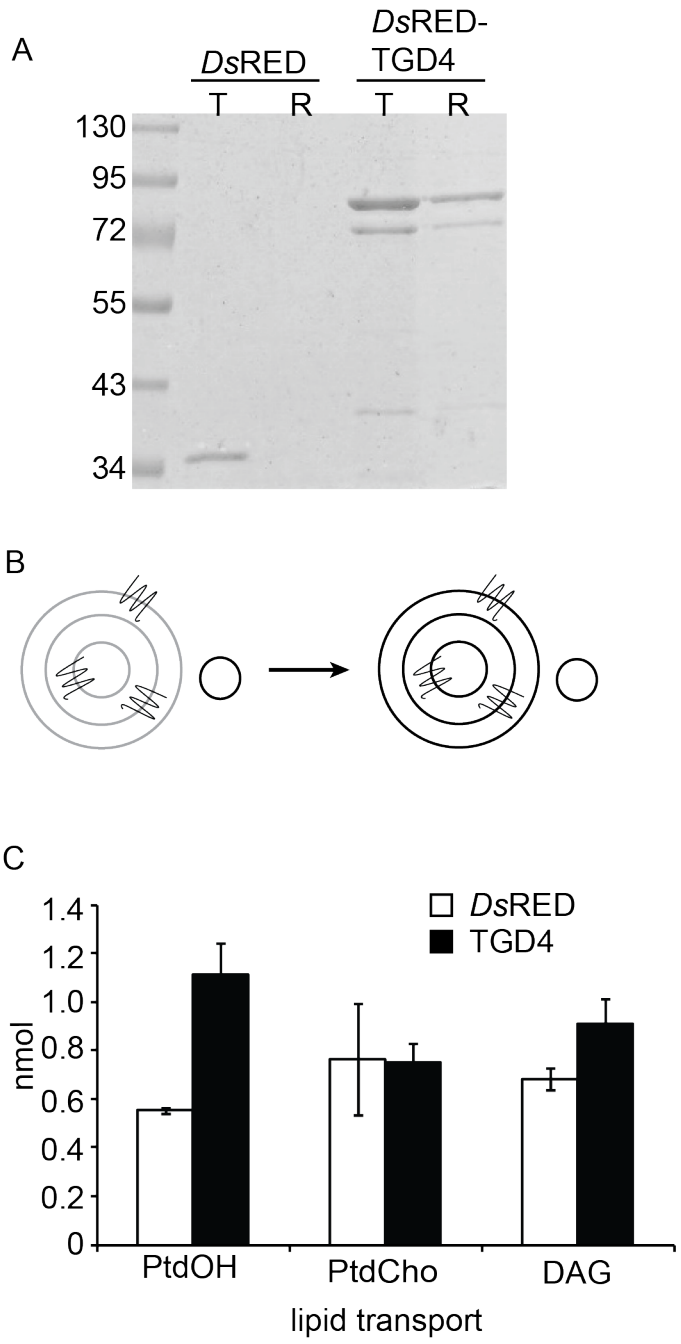


Figure 4.7. TGD4 transported PtdOH from the donor to the acceptor liposome *in vitro*. (A) A Coomassie Brilliant Blue stained SDS-PAGE showing that *Ds*RED-TGD4 could be reconstituted onto the acceptor liposome. T: total protein; R: protein that was reconstituted on to

Figure 4.7. (cont'd)

the liposome. Numbers indicate protein masses in kD. **(B)** A diagram illustrating the *in vitro* lipid transfer assay. Purified *Ds*RED-TGD4 protein was reconstituted onto the multi-lamellar liposome (acceptor, gray circles) made with dioleoyl-PtdCho. The proteoliposome (acceptor) was incubated with small uni-lamellar liposomes (donor, black circles) containing 40% (mol%) polar lipid of choice that would be transferred from the donor to the acceptor liposome in the presence of a transporter. **(C)** The content of acceptor liposome after the reaction was quantified by gas chromatography. The error bars represent the results from three technical repeats.

4.5. Discussion

TGD4 was identified as a protein involved in ER-to-chloroplast lipid trafficking by a genetic screening (Xu *et al.*, 2008). However, its molecular function was unknown until recently. TGD4 was found to be a PtdOH binding protein that formed a *β*-barrel membrane protein embedded in the chloroplast outer envelope (Imai *et al.*, 2011; Wang *et al.*, 2012). In this study, we employed truncation mutagenesis to the *Ds*RED-TGD4 recombinant protein to identify regions that were responsible for its PtdOH binding activity. The *Ds*RED protein enhances the expression and solubility of TGD4. Fusion of GFP, a homolog of *Ds*RED, to the N-terminus of TGD4 rescues the *tgdd4-1* mutants phenotype {Xu:2008kj}. So far, there are only seven PtdOH binding protein described in plants (ABI1, AtPDK1, PEPC, ACBP1, TGD2, TGD4 and MGD1), and four of them have a characterized PtdOH binding region as defined by truncation mutagenesis (Zhang *et al.*, 2004; Anthony *et al.*, 2004; Testerink *et al.*, 2004; Du *et al.*, 2010; Lu and Benning, 2009; Awai *et al.*, 2006; Dubots *et al.*, 2010). Comparing PtdOH binding regions

described for animal and yeast PtdOH binding proteins, some general traits emerged: PtdOH binding regions lack sequence similarity from one to the other, demanding that each be mapped individually; positively charged amino acids were essential for PtdOH binding; and most of the PtdOH binding regions were soluble domains with helix or coiled structures. As a β-barrel membrane protein, TGD4 likely binds PtdOH through its soluble, surface-exposed loops. The β-barrel core might serve as the conduit for PtdOH transfer. Concurrent with these expectations, both 110-145 and 50-80 PtdOH binding loops were predicted to face the cytosol by the software BOCTOPUS, indicating that TGD4 most likely accepts PtdOH outside of the chloroplast (Hayat and Elofsson, 2012).

We also studied the lipid binding properties of TGD4 from 18:3 plants and found that, just like Arabidopsis TGD4, they also bound specifically to PtdOH, suggesting both types of plants import PtdOH from the ER to the chloroplast. PtdOH was considered less likely as the lipid substrate transferred from the ER to the chloroplast compared to DAG and PtdCho due to its extremely low abundance in the chloroplast (Xu *et al.*, 2005). However, the steady-state amount of PtdOH might not accurately reflect its kinetic turnover rate *in vivo*. We were also concerned that 18:3 plants might have different lipid species imported to the chloroplast compared to 16:3 plants, as plastidic PtdOH phosphatase activity appears very low in 18:3 plants. Thus the 18:3 plants are inefficient at converting plastidic PtdOH to DAG, the direct substrate for MGDG synthesis (Heinz and Roughan, 1983). However, the original experiment was done with *de novo* synthesized PtdOH from radiolabeled acetate that only reflected PtdOH with prokaryotic origin. Whether 18:3 plants could utilize PtdOH outside of the chloroplast for MGDG synthesis was unclear at this point. We also found that TGD4 from 18:3 plants had higher PtdOH affinity comparing to TGD4 from 16:3 Arabidopsis. Since 18:3 plants lacked the

prokaryotic pathway for chloroplast lipid synthesis, one could speculate there was increased metabolite flux through the eukaryotic pathway and proteins involved would have higher efficiency in transport or turnover of the eukaryotic metabolites.

By studying the TGD4 protein complex we found that TGD4 forms a homodimer *in vivo*. This finding was unusual as *β*-barrel membrane proteins are mostly observed as monomers (Imai *et al.*, 2011). The homodimer arrangement was advantageous for PtdOH binding as positive cooperation was observed, indicating a dynamic interaction between the two subunits. The C-terminal portion of TGD4 is largely responsible for this dimerization activity, which was separate from the major N-terminal PtdOH binding regions. To gain a comprehensive view of the TGD4 *β*-barrel core, the PtdOH binding loops, and the dimer configuration, a high resolution crystal structure is needed. In addition, we also found that TGD4 did not form a complex with the TGD1, 2, 3 ABC transporter on the inner envelope of the chloroplast.

We developed an *in vitro* lipid transfer assay to test if TGD4 transferred PtdOH in addition to binding, and our results showed that TGD4 was a *bona fide* PtdOH transporter. TGD4 was able to transfer PtdOH from one membrane bilayer to the other, suggesting it could extract PtdOH from the ER and transfer it to the inner leaflet of the chloroplast outer envelope. This result also established that PtdOH likely functions as the lipid substrate transferred from the ER to the chloroplast in both 16:3 and 18:3 plants. TGD4 also transferred DAG but with lower efficiency than PtdOH. It is unclear whether the transferred PtdOH originates from PtdCho hydrolyzed by phospholipase D or from glycerol-3-phosphate that is *de novo* synthesized by acyltransferases. However, the two hypotheses might not be mutually exclusive. Cytosolic phospholipase D had been shown to be involved in the ER-to-chloroplast lipid trafficking, but the identity of this phospholipase D has not yet been revealed due to possible redundancy of this

enzyme, as there are 12 phospholipase D in Arabidopsis genome (Andersson *et al.*, 2004; Eliás *et al.*, 2002). If TGD4 transfers PtdOH to the chloroplast, there must be a PtdOH phosphatase inside the chloroplast to hydrolyze PtdOH for MGDG synthesis. This PtdOH phosphatase might be shared by both prokaryotic and eukaryotic pathways, similar to MGD1 and DGD1, since a major PtdOH phosphatase (*LPPγ*) knockout was lethal in Arabidopsis (Nakamura *et al.*, 2007).

In conclusion, TGD4 homodimer binds to ER/cytosolic PtdOH through its PtdOH binding loops and transfers it across the outer envelope, a process independent of ATP. TGD2 protein of the ABC transporter complex extracts PtdOH from the inner leaflet of the outer envelope and presents it to the TGD1 permease, which transports PtdOH across the inner envelope at the expense of ATP hydrolyzed by TGD3 (Figure 4.8). PtdOH is later converted to DAG, the direct substrate for the bulk synthesis of chloroplast glycolipids.

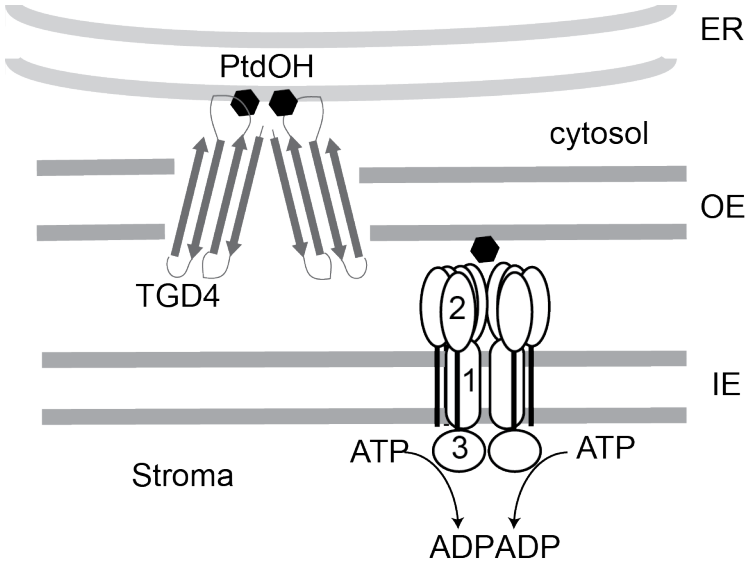


Figure 4.8. A diagram of protein complexes involved in the ER-to-chloroplast lipid trafficking pathway. TGD4 embedded in the outer envelope of the chloroplast (OE) forms a *β*-

Figure 4.8. (cont'd)

barrel dimer complex while the TGD1, 2, 3 complex forms an ABC transporter on the inner envelope of the chloroplast (OE). Together these two complexes import PtdOH from the cytosol to the inside of the chloroplast for the synthesis of dominant plant lipids such as MGDG, DGDG, and SQDG.

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CHAPTER 5

Conclusions and perspectives

5.1. Conclusions and future directions for TGD4 protein study

Before this work, TGD4 was identified during a genetic study, and it was tentatively localized to the ER membrane, but its molecular functions were unknown (Xu *et al.*, 2008). In this thesis work I found that TGD4 binds phosphatidic acid, an important lipid intermediate for chloroplast lipid synthesis. I also found that TGD4 is localized to the outer envelope of the chloroplast, but not to the ER, and is a transmembrane protein with a putative *β*-barrel configuration (Wang *et al.*, 2012). By mapping PtdOH binding domains I found two separate regions responsible for TGD4 PtdOH binding. TGD4 forms a homodimer *in vivo* and it does not have other strongly interacting partners.

In the post-genomics era, one of the major goals in biology is to understand the function of each gene. I characterized the function of the Arabidopsis TGD4 protein that can serve as the prototype for all TGD4 homologs in the plant kingdom. Chloroplast *β*-barrel outer envelope proteins are hard to identify because they lack a common targeting peptide and conserved primary sequence motifs. Nevertheless, researchers predicted 891 putative chloroplast *β*-barrel outer envelope proteins (Schleiff *et al.*, 2003). Thus far however, only five of these have been studied: TOC75, OEP21, OEP24, OEP37, and TGD4 through this work (Waegemann and Soil, 1991; Bölter *et al.*, 1999; Pohlmeyer *et al.*, 1998; Goetze *et al.*, 2006). Among them, only TOC75 and TGD4, which are involved in the import of chloroplast protein and lipids respectively, are essential for the survival of plants.

Future directions for the study of TGD4 include validating its putative function as a *β*-barrel lipid transporter and studying its detailed location with respect to the ER-chloroplast interaction sites.

5.1.1. In-depth study of TGD4 molecular function

To understand the molecular function of TGD4, a crystal structure with high resolution is needed. It can also confirm the PtdOH binding regions identified by truncation mutagenesis and potentially discriminate different hypotheses regarding the role of PtdOH in binding by TGD4. A crystal structure can confirm the predicted β-barrel structure of TGD4 and the dimer composition of the TGD4 complex. The precise dimerization domain can also be identified. Crystallizing membrane proteins is always challenging, but current progress made on TGD4 lays solid ground work to overcome these difficulties. First, β-barrel proteins usually fold correctly in liposomes in the absence of chaperones (Tamm, 2004). Conversely, proteins consisting mostly of α-helixes go through a more complicated two-stage folding process (Popot and Engelman, 2000). In addition, the *Ds*RED-TGD4-His protein has been successfully purified under native conditions, and the zwitterionic surfactant foscholine-12 was shown to be a suitable detergent during purification. Choline chloride serves as a convenient stabilizer of the recombinant TGD4 protein in a buffer without detergent. Constructs pLW01/*At*TGD4-His and pLW01/*Zm*TGD4-His eliminating the *Ds*RED fusion protein have been completed and are available for crystallization purposes. Recombinant *At*TGD4-His and *Zm*TGD4-His proteins have been expressed in *E. coli* strain BL21 (DE3) and partially purified (Figure 5.1.). Dr. R. Michael Garavito's lab at Michigan State University is currently working on optimizing conditions for crystallization. If successful, TGD4 will be the first plant β-barrel membrane protein whose crystal structure has been solved.

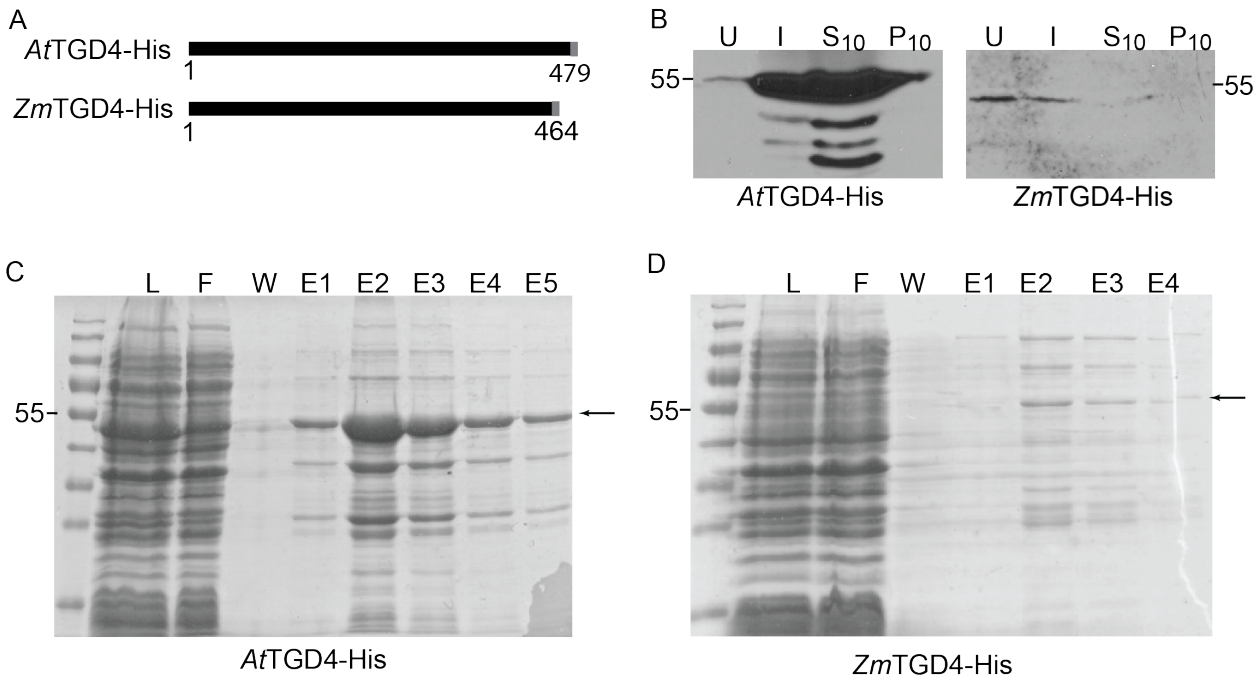

Figure 5.1. Partial purification of *At*TGD4-His and *Zm*TGD4-His for crystallization. (A) Schematic representation of recombinant protein *At*TGD4-His and *Zm*TGD4-His. Black bar: TGD4 protein; Gray bar: 6 × His tag. **(B)** Both proteins are expressed in *E. coli* strain BL21(DE3) and solubilized by the detergent foscholine-12. U: uninduced; I: induced; S_{10}: supernatant after 10,000 × g, 20 minutes centrifugation; P_{10}: pellet after 10,000 × g, 20 minutes centrifugation. Numbers indicate the protein ladder size in kD. **(C)** Ni-NTA purified *At*TGD4-His under the native condition in the presence of foscholine-12. **(D)** Ni-NTA purified *Zm*TGD4-His under the native condition in the presence of foscholine-12. L: loading control; F: flow through; W: wash through; E1-E5: elusion fractions. Arrows indicate the protein of target. Numbers indicate the protein ladder size in kD.

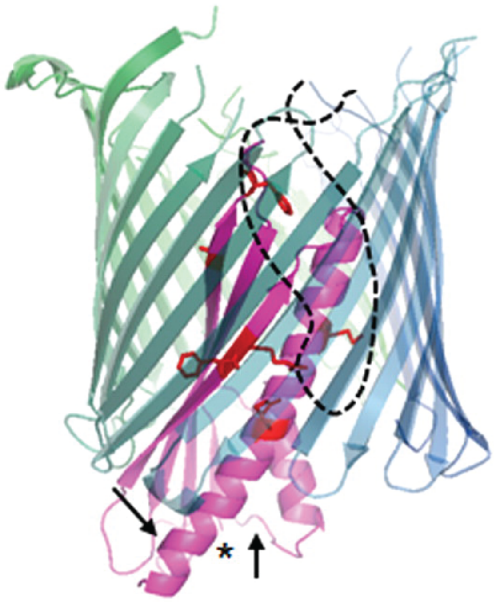

Figure 5.2. Model structure of LptD/LptE complex in *E. coli*. The transmembrane portion of LptD is shown as a hypothetic 22-strand β-barrel in blue-green. The asterisk represents the N-terminus. The lipoprotein LptE is in magenta. Arrows shows two trypsin digestion sites in LptE. Amino aicds in red are residues in LptE that interact with LptD.

5.1.2. Fine localization of the TGD4 protein in plant cell

The investigation of TGD4 localization has been carried out using an N-terminal GFP fusion TGD4 driven by a CaMV35S constitutive promoter. This recombinant protein was transiently produced in tobacco leaves and found to be associated with the ER membrane (Xu *et al.*, 2008). However, chloroplast proteomics data suggested that TGD4 is associated with the chloroplast envelopes (Zybailov *et al.*, 2008; Ferro *et al.*, 2010). My fractionation studies using a

purified antibody specific to TGD4 revealed that it is a membrane protein associated with the outer envelope of the chloroplast, thereby confirming the proteomics data (Wang *et al.*, 2012). GFP fusion may alter the conformation of TGD4 and mask the chloroplast targeting sequences. These findings indicate that using GFP fusion protein may not be a reliable method for precise localization of TGD4.

Plastid associated membranes or PLAMs are contact sites between the ER and the chloroplast and have been suggested to be involved in ER-to-chloroplast lipid transfer (Andersson *et al.*, 2007). If this assumption is true, the TGD4 protein is likely located on these PLAMs. Chloroplasts isolated from transgenic lines producing an ER targeting GFP protein in both the wild-type and the *tgd4-1* background have fluorescent patches attached representing PLAMs. However, *tgd4-1* chloroplast has more PLAMs comparing to the wild type (Xu *et al.*, 2008). This observation indicates that the TGD4 protein is not essential for tethering the two membranes together but may still be enriched at these contact sites. Because of the disruption of ER-chloroplast lipid transfer in the *tgd4-1* mutant, plant cells may increase the number of PLAMs to compensate for the loss of the TGD4 protein.

To test if the TGD4 protein is localized at the PLAMs, immuno-gold labeling can be employed. As GFP fusion affects the localization of TGD4, methods involving large fluorescent fusion proteins are not recommended. Plant leaf tissue from both the wild type and the *tgd4-2* or *tgd4-3* knockout lines can be fixed and embedded. the *tgd4-1* mutant is not suitable as it contains a point mutation and produces protein recognizable by the polyclonal anti-TGD4 antibody. Ultra-thin slices can be incubated with purified TGD4 antibody and later gold-conjugated secondary antibody, following by examination using electron microscopy. ER-chloroplast contact sites have been observed repetitively by electron microscopy (Kaneko and Keegstra,

1996). Thus it seems feasible to precisely localize TGD4 by this method. There is a chance that the TGD4 antibody does not work for immuno-gold labeling. In that case, one can use HA-tagged TGD4 produced in stable transgenic lines and use commercially available anti-HA antibody as this fusion protein complements the mutant phenotype and is correctly localized to the outer envelope of the chloroplast.

5.2. Remaining questions in the study of ER-to-chloroplast lipid trafficking

Although the eukaryotic pathways of chloroplast lipid synthesis have been well characterized, there are still puzzling questions to pursue: Are there additional cytosolic factors involved? What are the proteins that tether ER and chloroplast membranes together? Are there more proteins involved in the ER-to-chloroplast lipid transfer?

5.2.1. Isolation of the cytosolic factor required for ER-to-chloroplast lipid transfer

It has been suggested that cytosolic lipases are involved in ER-to-chloroplast lipid transfer in pea (Andersson *et al.*, 2004). When isolated chloroplasts were incubated with lyso-PtdCho liposomes and ^{14}C labeled acyl-CoA in the presence of UDP-Galactose, labeled MGDG was produced. This *in vitro* transport assay can be stimulated by the addition of cytosol. This activity is retained in the cytosolic fraction containing proteins larger than 100kD and is abolished by phospholipase D (PLD) inhibitor. Experiments conducted using Arabidopsis cytosol and pea chloroplasts showed similar degree of stimulation (Xu *et al.*, 2008). These experiments indicated that a phospholipase, likely PLD or PLC, which uses PtdCho as substrate

is involved in the lipid transfer from the ER to the chloroplast. PLD catalyzes the conversion of PtdCho to PtdOH while PLC hydrolyzes PtdCho to produce DAG. It is unlikely that DAG is the substrate that is transferred through the envelopes of the chloroplast, as DAG import into chloroplasts in the *tgd1-1* mutant is not compromised (Xu *et al.*, 2005). Thus it is likely an unidentified cytosolic PLD, which, is involved in the lipid transfer between ER and the chloroplast.

There are 12 predicted PLDs in the Arabidopsis genome (Eliás *et al.*, 2002). While most of them are yet to be characterized, PLD α1 PLD Z1 and Z2 are involved in stress responses (Zhang *et al.*, 2004; Cruz-Ramírez *et al.*, 2006; Li *et al.*, 2006). I have analyzed the lipid profile of PLD α1 null mutant and the results shows that the ER-to-chloroplast lipid transfer pathway is not disturbed. Since PLDs comprise a large family in Arabidopsis, functional redundancy poses a big challenge for studying their role in lipid transfer from the ER to the chloroplast using genetic tools. A traditional biochemistry method is proposed as follows:

Firstly, perform the radiolabeled PtdCho chloroplast transfer assay in the presence of wild-type cytosol with UDP-galactose and contrast this experiment using radiolabeled PtdOH. If both phospholipids show a comparable degree of conversion to MGDG, it suggests the presence of a cytosolic PLD. For easy access of material, pea chloroplasts can be used instead of Arabidopsis. Cytosol pre-treated with protease such as trypsin should abolish the stimulation effect. If above experiments are successful, one may fractionate cytosolic proteins by HPLC using size-exclusion chromatography. Different fractions will be added with radiolabeled PtdCho and UDP-galactose for the chloroplast lipid import assay. The fraction that retains the activity can be sub-fractionated by HPLC using ion-exchange chromatography. Different sub-fractions will be tested as above. The sub-fraction that contains the active enzyme will be sent for

LC/MS/MS analysis. The resulting protein list may contain the PLD of interest as well as other cytosolic protein factors involving in the ER-to-chloroplast lipid transfer. These candidates can be expressed by *E.coli* and purified to homogeneity. The purified proteins will be added to the PtdCho chloroplast import assay and test its lipid transfer stimulatory effect.

5.2.2. Identify proteins that form ER-chloroplast contact sites

There is clear evidence that proteins are involved in the formation of PLAMs. Isolated chloroplasts have strands of ER attached that cannot be pulled off by optical tweezers (Andersson *et al.*, 2007). However ER strands can be easily separated from chloroplasts pre-treated with protease trypsin indicating protein components are essential for the integrity of PLAMs (Andersson *et al.*, 2007). If the ER-to-chloroplast lipid-transfer process happens on PLAMs, it is reasonable to assume that some of the proteins involved in the pathway, for example TGD4, are located at those PLAMs. Thus it is interesting to investigate the protein composition of PLAMs. Methods for isolating PLAMs have been described (Andersson *et al.*, 2007). However later experiments show that the isolated PLAMs are heavily contaminated with inner and outer envelopes lowering the potential for identifying specific PLAMs proteins through this method.

On the other hand, a genetic screening method has been previously described for the identification of proteins essential for the formation of ER/mitochondria associated membranes (MAMs) in yeast. MAMs contain PtdSer decarboxylase (Psd1) which converts PtdSer to PtdEtn in the mitochondria (Vance, 1990). It is hypothesized that PtdSer traverses from the ER through MAMs to the outer envelope of the mitochondria. Kornmann et. al. reported a mutant screen in

which an artificial tether consisting of an GFP with N-terminal mitochondria targeting signal and C-terminal ER retaining signal was expressed and localized to the MAMs (Kornmann *et al.*, 2009). This transgenic line was subjected to ethylmethasulfonate mutagenesis and mutants that failed to grow in the absence of the plasmid were identified.

A similar strategy can be applied for PLAMs to identify tethering proteins. First, one may construct a GFP with N-terminal chloroplast transit peptide containing a transmembrane helix, for example TGD2 N-terminus, and C-terminal ER signal KDEL (Munro and Pelham, 1987). This fusion protein should be expressed under the regulation of an inducible promoter such as an estrogen inducible promoter. Two popular constructs of choice are: pMDC7 and pER8 for their tight control over downstream gene expression (Zuo *et al.*, 2000; Curtis and Grossniklaus, 2003). Second, the localization of GFP chimera in the transgenic plants should be validated by con-focal microscopy for correct targeting to the PLAMs. Third, an ethylmethanesulfonate mutant pool will be generated using the GFP chimera transgenic line. All of the above experiments should be carried out in the presence of estrogen to allow for tethering by the synthetic tether. Mutants of interest will be screened in the absence of estrogen. If we assume that PLAMs are important for plant growth and development, mutants with impaired PLAMs will be compromised in growth. Once interesting mutants are identified, map-based cloning compbined with genome sequencing will reveal the disrupted gene. Lipid analysis of the mutants will be carried out to test the hypothesis that polar lipids transfer from the ER to the chloroplast proceeds through PLAMs.

An alternative approach to discover proteins on the PLAMs is reverse genetics. Three out of four MAM tethering proteins (Mmm1, Mdm34, Mdm12) have the synaptotagmin-like-mitochondrial-lipid binding protein (SMP) domain (Lee and Hong, 2006). Synaptotagmin is a

membrane-associated protein involved in exocytosis when the synaptic vesicle contacts the plasma membrane (Perin *et al.*, 1991). The SMP domain is widespread in all eukaryotes and enriched in plants (Lee and Hong, 2006). It was found recently that all seven SMP domain-containing proteins in yeast are localized to membrane contact sites (Toulmay and Prinz, 2012). It is intriguing to hypothesize that certain SMP domain-containing proteins are involved in the formation of PLAMs in plants. Arabidopsis has five SMP domain containing proteins. Three of these are localized to the plasma membrane by proteomic studies (At1g05500, At3g61050, At2g20990). At3g18370 is predicted to be a mitochondrial protein. Only At1g50260 is strongly predicted to be on the outer envelope of the chloroplast by ARAMEMNON thus it is the gene of special interest (Schwacke *et al.*, 2003). It also has a paralog At3g19830 that shares 70% identity at the protein level. A T-DNA insertion line of At1g50260 is unavailable indicating mutations in this gene are lethal.

To study the role of this gene in PLAM formation and lipid metabolism, RNAi knockdown construct pGSA1285/*At*SMP1 targeting the coding sequence of both paralogs were constructed and transferred to wild-type Arabidopsis. However, I was unable to identify transgenic lines during the initial screening. Future experiments including retransformation with the pGSA1285/*At*SMP1 construct and/or making new RNAi constructs will be necessary. As ER-chloroplast contact sites can only be studied in plants, Arabidopsis is the best model for this purpose because of the large set of molecular tools.

5.2.3. Identify additional genes by screening for mutants with altered PtdOH level

As revealed by this work and previous reports, PtdOH is a central metabolite for chloroplast lipid synthesis. PtdOH either *de novo* synthesized from glycerol-3-phosphate and acyl-ACP in the prokaryotic pathway or derived from PtdCho in the eukaryotic pathway is dephosphoralated to DAG which serves as the direct substrate for MGDG, DGDG and SQDG synthesis (Benning, 2009). *De novo* synthesized PtdOH from the prokaryotic pathway is activated as CDP-DAG for the synthesis of chloroplast PtdGro (Wang and Benning, 2012). PtdOH also serves as a lipid signal for the regulation of MGD1 enzymatic activity (Dubots *et al.*, 2010). In both *tgd1* and *tgd4* mutants, PtdOH levels are elevated (Xu *et al.*, 2005; Wang and Benning, 2012). Thus, screening for mutants with altered level of PtdOH may reveal unidentified components in the chloroplast synthesis pathway such as PLD and PAP (a PtdOH phosphatase) (Zhang *et al.*, 2004; Nakamura *et al.*, 2007).

PtdOH only accounts for about 1% of total lipids in plant tissue and is not detectable in isolated chloroplasts (Xu *et al.*, 2005). In addition, PtdOH is very hard to separate from other polar lipids using one-dimensional TLC. The minor amount of PtdOH and the difficulties with separation pose challenges for precise quantification of this lipid. The current method is to separate PtdOH by two-dimensional TLC and quantify its fatty acyl methylesters by GLC. However, this method requires large quantity of plant tissue (>300mg or more than six rosette leaves) and is time and labor consuming rendering it impractical for high-throughput screening for PtdOH mutant.

Recently Morita et. al. developed a novel method for PtdOH quantification for animal cell lines that can be readily adapted for plant tissue (Morita *et al.*, 2009). Generally, PtdOH in total lipid extract is completely hydrolyzed by a lipase from *Pseudomonas sp.* to produce glycerol-3-phosphate. The latter is then oxidized by glycerol-3-phosphate oxidase to generate

H_2O_2 that reacts with Amplex Red, a hydrogen peroxide probe, to produce highly fluorescent molecules. The fluorescence can be easily quantified by a spectrofluorometer. This fast and easy assay can be performed in a 96-well plate thus ideal for high-throughput screening. All the reagents are also commercially available by Cayman Chemicals (Cat. 700240). The detection range of this method is 0-125μM, which is suitable for Arabidopsis leaf tissue as 50 mg of leaves (one adult leaf) contain approximately 20 μM PtdOH in 100μL reaction buffer.

An alternative method for PtdOH quantification will be tandem mass spectrometry (MS/MS) as employed by the Kansas Lipidomics Research Center (http://www.k-state.edu/lipid/lipidomics/). Ionized total lipids are injected into the first MS that varies the electrical field systematically to separate total lipid ions which then collide and lose head groups in a collision cell. A second MS later profiles the DAG ion after collision. Like the previous method, this method is also suitable for high-throughput mutant screening and doesn't require pre-separation of PtdOH. The advantage is that not only total PtdOH but also PtdOH with different acyl-chains can be quantified. However, it requires specific expertise to setup the initial system and is potentially more expensive and time-consuming comparing to the previous method.

Once a sensitive and efficient PtdOH quantification method is established, a forward genetic screening can be carried out. For instance, wild-type Arabidopsis can be mutagenized with ethylmethasulfonate and the mutants will be screened for their PtdOH content. Once interesting mutants are identified, map-based cloning will reveal the disrupted gene. This gene and its product will be later characterized by genetic, molecular, cellular and biochemical tools.

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