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Evan LaBrant
University of Nebraska–Lincoln, elabrant2@unl.edu

Allison C. Barnes
University of Nebraska–Lincoln, asiekmanbarnes@gmail.com

Rebecca Roston
University of Nebraska- Lincoln, rroston@unl.edu

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Lipid transport required to make lipids of photosynthetic membranes

Evan LaBrant, Allison C. Barnes, Rebecca L Roston

Department of Biochemistry, University of Nebraska–Lincoln.
1901 Vine St, Lincoln NE 68588

Corresponding author — R. L. Roston, rroston@unl.edu tel 402-472-2936

ORCIDs:

Rebecca Roston	0000-0002-3063-5002
Evan LaBrant	0000-0003-0742-9363
Allison Barnes	0000-0001-9539-3297

Abstract

Photosynthetic membranes provide much of the usable energy for life on earth. To produce photosynthetic membrane lipids, multiple transport steps are required, including fatty acid export from the chloroplast stroma to the endoplasmic reticulum, and lipid transport from the endoplasmic reticulum to the chloroplast envelope membranes. Transport of hydrophobic molecules through aqueous space is energetically unfavorable and must be catalyzed by dedicated enzymes, frequently on specialized membrane structures. Here, we review photosynthetic membrane lipid transport to the chloroplast in the context of photosynthetic membrane lipid synthesis. We independently consider the identity of transported lipids, the proteinaceous transport components, and membrane structures which may allow efficient transport. Recent advances in lipid transport of chloroplasts, bacteria and other systems strongly suggest that lipid transport is achieved by multiple mechanisms which include membrane contact sites with specialized protein machinery. This machinery is likely to include the TGD1, 2, 3 complex with the TGD5 and TGD4/LPTD1 systems, and may also include a number of proteins with domains similar to other membrane contact site lipid-binding proteins. Importantly, the likelihood of membrane contact sites does not preclude lipid transport by other mechanisms including vectorial acylation and vesicle transport. Substantial progress is needed to fully understand all photosynthetic membrane lipid transport processes and how they are integrated.

Keywords: Chloroplast, lipids, lipid transport, photosynthetic lipids, membrane contact sites

Introduction: Chloroplast membranes are sites of specialized membrane lipid synthesis.

Chloroplasts are one type of many forms of plastids, semi-autonomous organelles that likely evolved from an ancestral symbiotic relationship between cyanobacteria and eukaryotes. Modern plastids take multiple forms throughout the plant body, where they are specialized for different functions (Lindquist et al. 2016). All plastids provide critical functions for the plant, including fatty acid, lipid, tetrapyrrole, isoprenoid, and carbohydrate metabolism, signaling and redox processes, among others (Rolland et al. 2012). In addition to these functions, the chloroplast also fixes light energy into carbon skeletons and ultimately supports most life on earth. Perhaps because of its importance, many investigations into lipid synthesis and transport have used photosynthetic tissues and chloroplasts, though the processes described below that involve the chloroplast envelopes are believed to be true in all plastid types.

   The light reactions of photosynthesis occur on a single, continuous thylakoid membrane encapsulated by two envelope membranes, the outer envelope membrane (OEM) and inner envelope membrane (IEM). The lipid composition of these membranes is unique and well documented to be similar to that of modern day cyanobacteria (Wada and Murata 1989), containing abundant sugar-lipids instead of the abundant phospholipids observed in other eukaryotic membranes. The composition of lipid head groups in isolated plastids has been measured from a number of species (Alban et al. 1988; Dorne et al. 1990; Kirchhoff et al. 2002; Yu and Benning 2003). Fig. 1A shows the composition in each of the chloroplast membranes isolated from spinach (Block et al. 1983). The lipids of highest abundance are monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), together comprising greater than 75% of the thylakoid and IEM. They are followed by two anionic lipids, sulfoquinovosyldiacylglycerol (SQDG) and phosphatidylglycerol (PG). Although lower in abundance
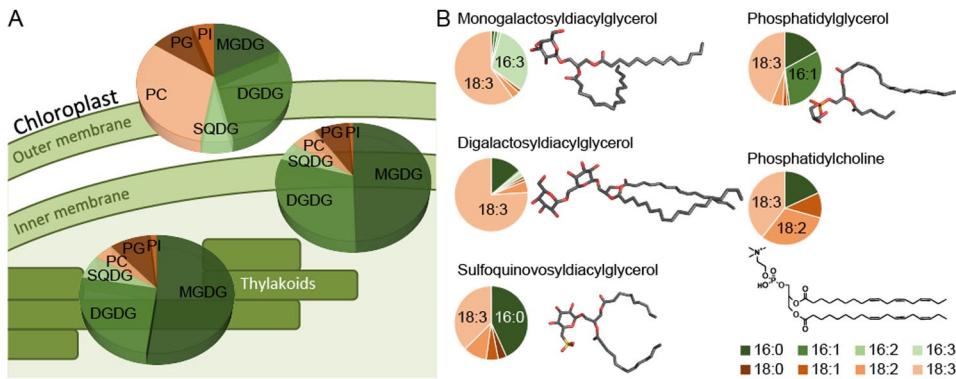
Figure 1. The distribution and composition of chloroplast-specific lipids. A. The composition of lipid headgroups in the chloroplast OEM, IEM and thylakoid membranes from spinach are given (Block et al. 1983). B. An example of the structures of the major lipid classes as crystallized in photosystem II from *Thermoxynechococcus elongatus* (PDB ID: 3KZI), with graphs indicating typical fatty acid abundance from Arabidopsis. Note that PC is not present in photosystems, so a drawn structure is substituted. Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

and each present in varying levels across species, it is clear that anionic lipids are required for photosynthetic capacity (Yu and Benning 2003) and PG specifically seems to be indispensable (Kim et al. 2007; Kobayashi et al. 2015; Tanoue et al. 2014). In addition to its phosphate headgroup, it has a unique trans-fatty acid that may allow it to fill a specific space in photosystems, in which it seems to be overrepresented compared to its overall abundance in thylakoid membranes (Qin et al. 2015; Umena et al. 2011). It is hypothesized that the reduction in phosphate usage inherent in replacing most phospholipids with sugar-lipids is critical for the proliferous photosynthetic membranes (Härtel et al. 2000). Especially since plants are sessile organisms and phosphorous can be limiting in the environment.

MGDG, DGDG, PG and SQDG are made inside the chloroplast from diacylglycerol (DAG) lipid backbones partly or completely transported from the endoplasmic reticulum (ER). The origin of the lipid backbones can be distinguished by the fatty acid length at the *sn-2* position on the glycerol. 18 carbon fatty acids are esterified primarily in the ER, while 16 carbons in the chloroplast (Frentzen 1986; Roughan and Slack 1982). Plants with an appreciable amount of chloroplast-derived or "prokaryotic" lipids are frequently referred to as 16:3 plants,

while plants with less than 2% of 16:3 fatty acids in the total lipids are referred to as 18:3 plants, in a reference to the highly desaturated fatty acids appearing on MGDG (Heinz and Roughan 1983; Mongrand et al. 1998). Even in 16:3 plants, more than 50% of chloroplast lipids are derived from the ER, an example of the fatty acid distribution among chloroplast lipids in a 16:3 plant is given in Fig. 2. Even in 16:3 plants, the ER-lipid derived pathway appears to be more important as genetic manipulation resulting in almost complete loss of 16:3 fatty acids has little effect on the plant phenotype (Kunst et al. 1988). Thus, lipid transport from the ER to the chloroplast is a requirement of photosynthetic lipid synthesis in all known plants. Here, we review lipid transport to the chloroplast, from the perspective of photosynthetic lipid synthesis. Because many investigations have focused on these topics from the perspective of sugar lipid synthesis, we do the same, discussing other lipids as appropriate. Readers looking for Arabidopsis gene identifiers of most discussed proteins are referred to the Arabidopsis Acyl-lipid metabolism database (http://aralip.plantbiology. msu.edu) and associated review (Li-Beisson et al. 2013).

Synthesis of fatty acids in the chloroplast stroma. Production of chloroplast lipids begins with fatty acid synthesis in the chloroplast stroma. Fatty acids are synthesized on a prokaryotic-type multi-subunit fatty acid synthase, in repeated cycles of condensation, dehydration and reduction on an acyl-carrier protein. Ultimately, some acyl-carrier protein-bound 16:0 is released, while most is extended in a final round to produce 18:0 which is desaturated to 18:1 by stearoyl-ACP desaturase. Fatty acids are released from the acyl-carrier protein as CoA esters by acyl-ACP thioesterases (FATA, B). The order of fatty acid abundance from production alone is 18:1, 16:0, with trace levels of 18:0 (Browse et al. 1986). Recent reviews of fatty acid production highlight the metabolism (Li-Beisson et al. 2013), the regulation (Marchive et al. 2014) and the protein chemistry involved (Troncoso-Ponce et al. 2016).

Beginning chloroplast sugar lipid synthesis. Inside the chloroplast, a glycerol-phosphate acyltransferase (GPAT) esterifies a fatty acid onto the *sn-1* position of glycerol-3-phosphate with preference for 18:1 acyl-ACP (Frentzen et al. 1983). Then a lyso-phosphatidic acid acyltransferase (LPAAT) esterifies a second fatty acid onto the glycerol backbone at the *sn-2* position. Chloroplast LPAAT strongly prefers to use 16:0-ACP
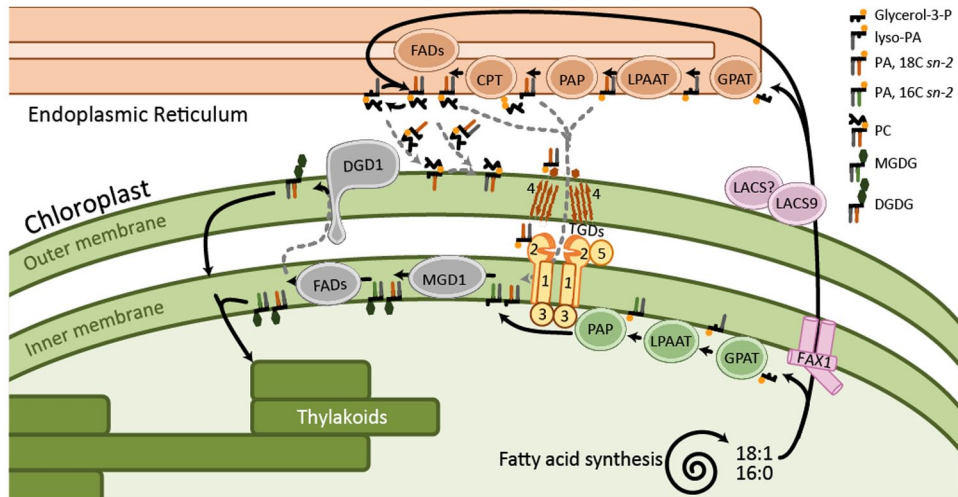
Figure 2. Transport of lipids to and within the chloroplast envelope membranes.
Fatty acid synthesis occurs inside the chloroplast, where acyl-ACPs can be directly integrated into lipids. These lipids include a 16 carbon acyl chain at the *sn-2* position, depicted in green. Alternatively, free fatty acids can be exported using the FAX1/LACS mechanisms to reach the cytoplasm as acyl-CoAs where they are incorporated into ER lipids through acyl-editing or the Kennedy pathway. Both pathways prefer to add an 18 carbon acyl chain at *sn-1*, depicted in orange. Potential transport mechanisms to and within the chloroplast envelope membranes are depicted as dashed grey lines, because many portions of them remain hypothetical. Specifically, lipids transport through the TGD complexes may include PC, PA, or DAG; PC and lyso-PC may have alternate transport mechanisms to the chloroplast outer envelope membrane with unknown protein components. The TGD complexes each bind PA specifically, denoted by a PA near appearing near them. MGDG is synthesized from both chloroplast-derived and ER-derived lipids, after which it can be desaturated by chloroplast-specific FADs. The N-terminus of DGD1 likely recruits MGDG from the inner membrane to the outer membrane though it is possible other proteinaceous components are needed. Abbreviations: FADs, fatty acid desaturases; DGD1, digalactosyldiacylglycerol synthase 1; MGD1, monogalactosyldiacylglycerol synthase 1; CPT, diacylglycerol-choline phosphotransferase; PAP, phosphatidic acid phosphatase; LPAAT, lyso-phosphatidic acid acyltransferase, GPAT, glycerol-phosphate acyltransferase; TGD, trigalactosyldiacylglycerol; LACS, long-chain acyl-CoA sythetases; FAX1, Fatty acid export 1; PA, phosphatidic acid; PC, phosphatidylcholine; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol.

as its substrate, and incorporates greater than 95% 16:0 when given a choice between 16:0- and 18:1-ACP (Frentzen et al. 1983). The resulting phosphatidic acid (PA) is dephosphorylated by a phosphatidic acid phosphatase (PAP) to generate DAG (Joyard and Douce 1977; Nakamura et al. 2007). Plastidic PAP is associated with the inner envelope of the chloroplast, known to have an alkaline pH optimum and

to be inhibited by Mg^{2+}, the opposite of ER localized PAPs which generally are independent of or require Mg^{2+} (Joyard and Douce 1979; Malherbe et al. 1992). At this time, the plastidic PAP has not been purified or used in recombinant studies to allow further enzyme characterization and conformation of its sub-organellar location. After its creation, DAG can be immediately incorporated into MGDG or SQDG with the characteristic 16 carbon fatty acid at the *sn-2* position. Collectively, this process is known as "anterograde" or "prokaryotic" lipid synthesis because it remains entirely within the plastid, and a visual summary can be seen in Fig. 2. MGDG is synthesized at the chloroplast inner envelope membrane (Miege et al. 1999) from activated galactose in the form of UDP-gal and a DAG from the chloroplast inner envelope (Coves et al. 1986; Marechal et al. 1994; Marechal et al. 1995). SQDG is synthesized by SQD2 from UDP-sulfoquinovosyl and DAG, also at the chloroplast inner envelope membrane (Seifert and Heinz 1992; Yu et al. 2002).

Export of fatty acids from the chloroplast. The majority of fatty acids released from the fatty acid synthase are exported to the ER, where they are also incorporated into lipids. The mechanism by which fatty acids are exported is not completely clear, though recent progress has been made and was recently excellently reviewed (Li-Beisson et al. 2017; Li et al. 2016). Briefly, a family of seven transporters broadly similar to yeast fatty acid transporters has been identified, four of which have chloroplast-targeting sequences. Fatty acid export 1, or FAX1, has been confirmed to transport fatty acids when expressed in yeast, and its lack increases the number of 16 carbon fatty acids incorporated into MGDG (Li et al. 2015). FAX1 is located in the chloroplast inner envelope where it is likely only one part of the machinery to export fatty acids. Other portions must exist, because plants lacking FAX1 continue to transport some fatty acids and are much healthier than plants lacking all ER-derived lipids (Xu et al. 2003). Paralogs of FAX1 in the chloroplast are potential sources of additional fatty export. Fatty acids must also be transported across the chloroplast OEM, and to date the most likely mechanism is vectorial acylation by acyl-CoA synthetases (Koo et al. 2004). Vectorial acylation is a well-established mechanism for fatty acid movement in yeast (Black and DiRusso 2007; Zou et al. 2003), where free fatty acids are transported across a membrane by a fatty acid exporter, Fat1p, and then retained on the

far side of the membrane by combining them with coenzyme A, thus preventing their return. If this mechanism occurs in plants, the identity of the long-chain acyl-CoA sythetases (LACS) remains unclear, and it is likely that multiple LACS are involved. Although the major LACS associated with the outside of the chloroplast envelope membrane was identified as LACS9, Arabidopsis plants lacking LACS9 are indistinguishable from wildtype in both visible and lipid phenotypes. Further, approximately 10% of the original acyl-CoA synthetase activity remains associated with the chloroplast (Schnurr et al. 2002), and other members of the LACS family have overlapping functions (Jessen et al. 2015; Zhao et al. 2010). A visual summary is provided in Fig. 2.

Lipid synthesis in the ER. Once fatty acids have been exported to the ER, there are two pathways for incorporating them into lipids. The first is known as the "Kennedy" pathway, and is broadly similar to fatty acid incorporation in the chloroplast (Kennedy 1961). Fatty acids in the form of acyl-CoAs are esterified onto a glycerol-3-phosphate by a GPAT, then an LPAAT with a strong preference for 18:1 acyl-CoA as its substrate esterifies a fatty acid into the *sn-2* position (Frentzen 1990; Frentzen et al. 1983). The resulting PA is dephosphorylated by a PAP to generate DAG, which is then incorporated into various lipids, including phosphatidylcholine (PC). PC is made by action of Diacylglycerol-Choline phosphotransferase (DAG-CPT) which uses DAG and CDP-choline as substrates (Goode and Dewey 1999). The second pathway is known as "acyl-editing" (Bates et al. 2007). In this pathway, fatty acids are added directly to lyso-PC to regenerate PC, which is cycled back into lyso-PC. The removal of fatty acids to make lyso-PC is again specific for the *sn-2* position. Both the Kennedy pathway and acyl-editing are depicted in Fig. 2, though in most tissues and for most fatty acid types, acyl editing is the predominant pathway for incorporation of new fatty acyl-CoAs. Fatty acid desaturases (FADs) in the ER prefer to use PC as a substrate, FAD2 desaturates 18:1 to 18:2 (Miquel and Browse 1992), and FAD3 desaturates 18:2 to 18:3 (Browse et al. 1993). Through acyl editing and head group conversion desaturated fatty acids can be distributed throughout all lipids, including chloroplast lipids when chloroplast desaturases are absent (Miquel and Browse 1992), another piece of evidence for lipid transport to the chloroplast.

Lipid transport to the chloroplast

Lipid backbones must be transported to the chloroplast to generate the most common sugar lipid structures with 18 carbon acyl groups on the *sn-2* position of glycerol. This process is commonly referred to as "retrograde" or "eukaryotic" plastid lipid synthesis. For incorporation into plastid lipids, lipid backbones must be present at the IEM as a DAG for use by MGD1 and SQD2. Between the ER and the chloroplast IEM are the cytoplasm, the OEM and the intermembrane space of the chloroplast envelope. There are multiple hypotheses that describe the mechanisms by which lipid transport occurs through these spaces and which lipid species are involved at each step. The following sections describe the current state of knowledge for the identity of the transported lipid, proteins known to assist in the transport, and finally the possibilities for membrane structures that would best support transport of hydrophobic molecules through aqueous spaces.

Identity of the transported lipid. To date, four lipids are candidates to shuttle lipid backbones with an 18 carbon in the *sn-2* position from the ER to the chloroplast OEM: lyso-PC, PC, PA, and DAG. Lyso-PC and PC are hypothesized to only make the transition from the ER to the chloroplast, as they are abundant in the chloroplast OEM outer leaflet (see Fig. 1A), but are in very low abundance or absent from the IEM (Dorne et al. 1985; Dorne et al. 1990). They are likely converted to PA or DAG to finish transport to the IEM. PA and DAG may be transported for any fraction of the total transport from ER to OEM to IEM; they are present in all of the membranes at low amounts (Alban et al. 1988; Muthan et al. 2013). At the IEM, PA too must be converted into DAG for incorporation into sugar lipids. Currently, no evidence suggests that only a single lipid is transported, so all the above lipids may be important. Similarly, lipids may also be transported and present in the chloroplast without contributing to bulk sugar-lipid synthesis.

Evidence for transport of PC. *De novo* lipid synthesis in the chloroplast does not include a protein capable of adding CDP-choline to DAG to make PC (Joyard et al. 2010), yet PC exists in the chloroplast OEM (Fig. 1A, Bessoule et al. 1995; Dorne et al. 1985). The likely origin of PC is where it is synthesized in the ER, and therefore PC must be transported from the ER to the chloroplast. Some time ago, it was shown that PC can be a precursor to chloroplast lipid synthesis.

Isolated chloroplasts incubated with labeled PC liposomes were shown to take up PC. Subbsequent incubation of the chloroplasts with phospholipase C generated DAG from PC, which allowed synthesis of labeled MGDG (Oursel et al. 1987). Further work confirmed that ER membranes can donate labeled phospholipids to isolated chloroplasts in a time and temperature dependent fashion without any cytosolic factors (Andersson et al. 2004; Xu et al. 2008; Yin et al. 2015). Additionally, protease digestion to remove chloroplast OEM proteins impaired PC uptake, though proteins responsible have yet to be identified (Yin et al. 2015). It is also possible that soluble lipid transfer proteins are involved, as soluble proteins were previously observed to increase transfer of PC to the chloroplast (Miquel et al. 1988). Another consideration is the concentration of PC available for transfer. Recently, an ER flippase named ALA10 was shown to effect fatty acid composition of chloroplast-specific lipids (Botella et al. 2016). ALA10 is likely to interact with PC, and the authors speculate that it may increase the concentration of PC on the outer leaflet of the ER, or at specific sites of contact between the ER and plastids to enhance lipid transfer (Botella et al. 2017).

Evidence for transport of lyso-PC. An alternative to transport of the fully acylated PC molecule is transport of lyso-PC which fractionates more readily into soluble spaces and can be re-esterified to PC at the chloroplast surface. The enzymes involved, phospholipase A to make lyso-PC, and LPCAT to convert lyso-PC to PC, have been shown to be present at ER-chloroplast membrane contact sites (MCSs) and the chloroplast envelope, respectively (Bessoule et al. 1995; Tan et al. 2011). A clever set of experiments was designed to determine the transported lipid by simultaneously measuring precursor PC and lyso-PC labels in the ER and in then subsequent incorporation into chloroplast lipids (Mongrand et al. 2000). They showed that transfer by PC alone cannot explain the concentration of label at the *sn-1* position in galactolipids, and concluded that lyso-PC is more likely to be transported. However, they considered only lyso-PC and PC, and assumed that all transferred PC would be a precursor for galactolipids. Also, the study assumed that bulk PC would be transported. However we now know that ALA10 might reasonably be expected to generate a specific pool of PC for lipid transport. A similar study that was expanded to include analysis of PA and DAG might be more conclusive, though it would still be difficult to measure specific lipid pools.

Evidence for transport of PA. PA is a central intermediate in lipid synthesis that is easily convertible to and from PC and DAG. It is also a non-bilayer forming lipid and would therefore require less energy to be removed from bilayer membranes (Testerink and Munnik 2011), making it an attractive transport candidate. Biochemical evidence for PA transport is limited, however when DAG kinase was introduced to force synthesis of PA in various chloroplast envelope membranes, PA did not accumulate. Instead, it was converted into other lipid species, some of which required transport to other membranes to access converting enzymes (Muthan et al. 2013). This data strongly implies that PA *can* be transported. PA was suggested to be a transported lipid because the TGD complexes, the only confirmed lipid importing systems in the chloroplast, were shown to bind to PA (Lu and Benning 2009; Wang et al. 2012). However, transport of PA by the TGD complexes has not been definitively shown and binding PA may be more important for locally destabilizing the membrane bilayer structure to reduce the energy barrier to removal of another lipid.

Evidence for transport of DAG. DAG is an obvious candidate for lipid transport because DAG itself is a substrate for MGDG synthesis. Further, DAG is non-bilayer forming (Jouhet 2013) and can flip leaflets of a bilayer membrane at biologically relevant rates (Hamilton et al. 1991). Thus, DAG has a low energy barrier to both extraction from a membrane and crossing a leaflet, reducing its need for enzymatic assistance during transport. A recent modeling study predicted that DAG is more likely to be the lipid precursor than PA, although the two have a highly connected relationship (Marechal and Bastien 2014). Because DAG inhibits plastidic PAP and PA stimulates MGD1 activity (Dubots et al. 2010), if both DAG and PA are transported as precursors, two regulatory loops would exist controlling incorporation into sugar lipids (Marechal and Bastien 2014). Physical evidence for DAG transport exists from the OEM to the IEM, from the same studies referenced above that supported PC as a precursor to MGDG. Treatment of isolated chloroplasts with a phospholipase generates DAG from PC at the OEM. The DAG backbone was then incorporated into MGDG, presumably at the IEM (Andersson et al. 2004; Oursel et al. 1987). However, these studies did not exclude the possibility that MGDG synthases on the OEM were responsible for producing the MGDG seen. MGDG synthesis can occur at the OEM during times of stress (Benning and Ohta 2005), and may have been activated when DAG was ectopically produced.

Protein machinery involved in lipid transport from the ER to the chloroplast. The first proteins identified as chloroplast lipid transporters were identified by a forward genetic screen, and were named *tgd* for their constant production of the unusual trigalactolipids (Awai et al. 2006; Xu et al. 2008; Xu et al. 2003), normally seen only during stress conditions (Barnes et al. 2016; Moellering et al. 2010). Identified as point mutants, severe reduction of *TGD1* was lethal (Xu et al. 2005). All *tgd* mutants had reduced levels of 18 carbon fatty acids in the galactolipids, particularly at the *sn-2* position. They were also paler than wildtype, had compromised photosynthetic capacity, reduced thylakoid membranes, compromised growth and embryogenesis. Investigations into photosynthetic defects suggested that *tgd1* and *tgd2* mutants had unaffected carbon assimilation, electron transfer, and photosystem quantum efficiency, while the total proton motive force was reduced, likely because of increased proton conductance (Li et al. 2012). Most significantly, the *tgd* mutants showed defects in lipid transport: when fed radiolabeled acetate, *tgd1* mutants failed to efficiently convert labeled PC into MGDG at rates similar to wildtype (Xu et al. 2003). Similarly, chloroplasts isolated from *tgd1* or *tgd4* mutants were less efficient at converting exogenously supplied PA into galactolipids (Wang et al. 2012; Xu et al. 2005). Since their initial genetic isolation, additional TGD proteins have been described and all have been further biochemically characterized until they now are a potentially complete set of lipid transporters in the chloroplast OEM and IEM, spanning the intermembrane space. Recent evidence even suggests that the TGD system likely works similarly in *Chlamydomonas reinhardtii* algal cells that do not have the same 16 carbon / 18 carbon preferences differentiated between organelles (Warakanont et al. 2015).

At the chloroplast OEM, TGD4 is a dimeric β-barrel Lipid A transporter (LptD)-family protein that also binds PA (Wang et al. 2013; Wang et al. 2012; Xu et al. 2008). A second LptD-family homolog appears to be common among land plants, and the properties of its Arabidopsis homolog, atLPTD1, were recently described (Hsueh et al. 2017). Its presence may explain the previous observation that *tgd4* loss-of-function mutant phenotypes are less severe than those of the *tgd1* mutant (Xu et al. 2008). RNAi reduction of atLPTD1 caused similar phenotypic changes to *tgd4*, including an increase in trigalactolipids, and an altered the ratio of 16 to 18 carbon fatty acids in chloroplast lipids. Phenotypic results of reduced atLPTD1 levels were most

obvious during phosphate deprivation, when atLPTD1 normally seems to increase in abundance, prompting the authors to suggest that TGD4 and atLPTD1 may have functionally diverged (Hsueh et al. 2017). The mechanism by which either TGD4 or atLPTD1 transports lipids has yet to be shown. The homologous bacterial LptD is part of a seven-membered transport system that has a continuous hydrophobic groove from the bacterial inner membrane, across the periplasmic space to directly contact the outer envelope membrane through LptD and LptE (Okuda et al. 2012). LptD itself forms a β-barrel inside of which LptE forms a "plug", and simulations suggest that lipids are transferred into the barrel, passing through two strands to enter the membrane laterally (Dong et al. 2014). A somewhat similar mechanism could be inferred for the TGD4/atLPTD1 system, potentially passing a lipid from the outer leaflet of the OEM directly to the TGD1, 2, 3 transporter of the inner envelope. Arguments against this hypothesis include the apparent lack of an LptE homolog and that the TGD1, 2, 3 and TGD4 complexes do not appear to directly interact under normal isolation conditions (Roston et al. 2012; Wang et al. 2013).

At the chloroplast IEM, TGD1, 2 and 3 form a prokaryotic-like multi-subunit ATP-binding cassette (ABC) transporter complex (Roston et al. 2012; Roston et al. 2014). TGD1 contains a multi-membrane spanning permease domain (Xu et al. 2003). TGD3 contains the ABC transporter ATPase domain, and is peripherally associated with the inner envelope membrane from the stromal side (Lu et al. 2007). TGD2 contains an atypical substrate-binding domain (Awai et al. 2006) that binds specifically to PA (Lu and Benning 2009). Unlike typical bacterial substrate binding domains, TGD2 is anchored in the membrane with a single transmembrane domain and is capable of disrupting membranes (Roston et al. 2011). Most ABC transporters have two permease domains, two ATPase domains, and a single substrate binding protein (Roston et al. 2014). The TGD1, 2, 3 transporter has 8 – 12 TGD2 substrate-binding domain proteins for every two TGD1 and TGD3 proteins (Roston et al. 2012). Based on this finding, the homologous bacterial MlaDEF transporter was recently shown to also have six copies of MlaD, the substrate binding domain, per two each of MlaE and F. Notably MlaD is also has a single membrane spanning domain (Thong et al. 2016). In the bacterial system MlaD also modulates the activity of the entire complex by affecting ATP hydrolysis. The similarity in the unusual oligomerization of the substrate binding domain protein indicates that this may be a requirement for lipid transport.

The rationale for this requirement is less clear. One hypothesis is that because TGD2 oligomerizes while binding PA, it effectively concentrates a non-bilayer forming lipid, allowing easier disruption of the nearby membrane to promote lipid removal for transport (Roston et al. 2012). Another hypothesis is that oligomerization of the substrate binding domain allows increased affinity for substrate over a soluble transporter, thus promoting passing of the substrate to the substrate binding domain (Thong et al. 2016). Other remaining questions about the TGD1, 2, 3 complex include the identity of the lipid transported (see above section) and the type of transport that occurs. Although it is likely that the TGD1, 2, 3 complex is directly involved in transporting an ER-derived lipid precursor of MGDG, it may accept it from a soluble lipid transfer protein, or directly from the OEM, and transfer it to the outer leaflet of the IMS. It may flip a lipid from the outer leaflet of the IEM to the inner leaflet. Finally, it may do both simultaneously, resulting in lipid transfer from the OEM to the inner leaflet of the IEM. To that end, it has been proposed that the TGD2 proteins form an extended, closed channel projecting from the surface of the IEM towards the OEM, through which lipids may travel (Ekiert et al. 2017). Experiments testing these questions likely require *in vitro* reconstitution of the entire complex, which has yet to be accomplished.

A potential bridge between the chloroplast OEM and IEM has recently been described. It was named TGD5 because its loss of function mutant *tgd5* is phenotypically similar to the other *tgd* mutants in every measurable way, including production of trigalactolipid, reduced levels of 18 carbon fatty acids in the galactolipids, paleness, compromised growth and embryogenesis (Fan et al. 2015). When transiently overexpressed in tobacco, TGD5 could interact both with the TGD1, 2, 3 complex and with TGD4, enticingly suggesting that it may bridge the gap between the complexes (Fan et al. 2015; Li et al. 2016). One currently conflicting piece of evidence is that TGD5 is protected from thermolysin and trypsin digestion, and therefore is either intrinsically protease resistant, in a protease-resistant complex, or protected by the chloroplast IEM. TGD2 is somewhat protease resistant (Awai et al. 2006), so it is tempting to propose that TGD5 may be tightly associated with the TGD1, 2, 3 complex, however was not copurified with the complex (Roston et al. 2012). The nature of TGD5s role in lipid transport is an exciting new development since it does not have direct analogs in bacterial lipid transport systems and may represent a novel lipid transport mechanism.

Physical structures that support lipid transfer. There is an energetic barrier to moving a hydrophobic lipid across an aqueous space. Membrane contact sites, vesicular transport and membrane hemifusion are three mechanisms that have been suggested to reduce that barrier for lipid transport to the chloroplast envelopes.

Membrane Contact Sites

In multiple systems, it has been shown that two membranes in close proximity have more efficient trafficking of large or insoluble metabolites such as calcium or lipids (Block and Jouhet 2015; Manjarrés et al. 2011; Szabadkai et al. 2006), channeling of metabolites or peptides (Brdiczka et al. 2006; Pain et al. 1988; Reichert and Neupert 2002; Schnell and Blobel 1993; Schumann et al. 2007), and trans-activity of lipid modifying enzymes (Stefan et al. 2011; Tavassoli et al. 2013; Tong et al. 2013). A substantial body of work has been devoted to studying the role of membrane contact sites (MCS) and their components in mammals and yeast, while MCS research in plants is more nascent. Three key determinants of true MCSs have been used to distinguish them from simple juxtaposition: 1) membranes from two compartments are separated by less than 30 nanometers, 2) the membranes do not undergo complete fusion to unite luminal compartments, and 3) specialized proteins are located or concentrated at the site (Prinz 2014), for recent reviews of membrane contact sites in other systems, please see (Bayer et al. 2017; Pérez-Sancho et al. 2016; Quon and Beh 2015) In mature plant cells close association of organelles is expected as the vacuole occupies 80-90% of mesophyll cell volume (Wink 1993; Zhang et al. 2014). Thus, organellar apposition does not imply the formation of a functional contact site and verification of a MCS must rely on biochemical evidence of points 2 and 3. To date, proteins involved in MCS formation and biochemical cross-talk between the chloroplast and other organelles have not been identified, with the exception of one protein necessary for chloroplast:peroxisome interaction (Schumann et al. 2007), however other data implies that these proteins exist.

Evidence for Contacts between Chloroplast Envelopes and the Endoplasmic Reticulum Reports of the close relationship between the chloroplast and ER membranes have been presented in numerous transmission electron microscopy (TEM) studies of plant cell ultrastructure (Cran and Dyer 1973; Crotty and Ledbetter 1973; Renaudin and Capdepon 1977). Measurements of membrane connective forces has also suggested the formation of ER:chloroplast contact sites (Andersson et al. 2007). Isolated chloroplasts were shown to retain vesicles of intact ER, which remained attached to the chloroplast envelope even when a >400 pN stretching force was applied. Trypsin treatment reduced the force required to separate the ER and chloroplast to 130 pN, indicating the likely involvement of tethering proteins. Finally, when plastid associated ER membranes were isolated, they had a unique lipid composition from the remainder of the ER or chloroplast OEM (Andersson et al. 2007). Thus, it seems likely that an ER:chloroplast contact site exists that satisfies all of the criteria of a true MCS.

More indication of interconnection between the ER and chloroplasts has been provided through experiments describing stromules, a stroma-filled tubule projected from the chloroplast body (Delfosse et al. 2016). While abundant stromules have been associated with non-photosynthetic tissues (Köhler and Hanson 2000; Kwok and Hanson 2004) accruing experimental data show that chloroplast protrusions are present in all cell types and under a range of conditions (Natesan et al. 2005). Several colocalization studies have indicated significant interactions between the chloroplast body, stromules, and ER tubules (Schattat et al. 2011a; Schattat et al. 2011b). The authors showed that ER and stromule branching were highly correlated, and that extension and retraction of stromules coincide with changes in cortical ER structure. This may merely suggest that stromules and ER tubules stream along the same cytoskeletal lattice, but confocal data shows tight spatial association reminiscent of MCSs. Using 3-dimensional rendering of confocal images, they also modeled "ER baskets" enclosing the chloroplast body. Interestingly, the formation of stromules and stroma-filled "beaks" can be observed when plants experience stresses pertinent to lipid homeostasis such as high temperature (Buchner et al. 2007), oxidative stress (Brunkard et al. 2015), osmotic stress (Gray et al. 2012), and phosphate limitation (Vismans et al. 2016). Further

evidence is needed to confirm the formation of contact sites where the ER and stromules associate, and a better understanding of their relationship may help provide insight into ER:chloroplast lipid trafficking.

Contact Sites Between Chloroplasts and Mitochondria A host of TEM images define the close association of chloroplasts and mitochondria in mesophyll cells, including (Holzinger et al. 2008; Jouhet et al. 2004; Vigani et al. 2015), and their proximity may play an important role in lipid homeostasis. Under phosphate-limiting conditions, cells often substitute DGDG for phospholipids in extra-plastidic membranes (reviewed in: Dormann and Benning 2002; Nakamura 2013). This allows for mobilization of phosphate from phospholipids which act as a phosphate reservoir (Cruz-Ramirez et al. 2006; Tjellström et al. 2008), and mutants in phospholipid recycling genes show abnormal phenotypes on low-phosphate growth media (Angkawijaya and Nakamura 2017; Gaude et al. 2008; Hsueh et al. 2017).

Mobilization of the phospholipid phosphate pool requires transport of DGDG to extra-plastidic membranes, including mitochondrial membranes which show an increase in DGDG and decrease in PE/PC content (Jouhet et al. 2004). There are two potential pathways: from chloroplast to ER to mitochondria, or directly from chloroplast to mitochondria. The presence of DGDG in the ER and plasma membrane during phosphate limitation (Andersson et al. 2005; Andersson et al. 2003) suggests that DGDG is distributed through vesicles and ER:PM/ER:mitochondrial MCS, which may support transfer through the ER. Two studies have provided strong evidence that DGDG can also be directly transferred to mitochondria at chloroplast:mitochondria MCS (Jouhet et al. 2004; Michaud et al. 2016). In their 2004 paper, Jouhet et al. showed that DGDG present in mitochondrial isolations was not due to chloroplast lipid contamination. Further, they documented a statistically significant increase in the association of mitochondria and chloroplasts during phosphate limitation using TEM. Finally, they showed that pure chloroplast envelope fractions could transfer DGDG to mitochondria *in vitro*, indicating that endomembrane trafficking is not necessary for phospholipid substitution in mitochondria. Michaud et al. have proposed that a component of the mitochondrial protein import machinery is a mediator of DGDG trafficking to the mitochondria (Michaud et al. 2016). They show that loss of AtMic60, an inner membrane protein involved in crista junction formation (Hessenberger et al. 2017;

Sastri et al. 2017), induces a significant decrease in mitochondrial glycolipids. Further, AtMIC60 is associated with a megacomplex that is enriched with DGDG during phosphate limitation, and DGDG association with the complex is dependent on presence of AtMIC60. Despite this, mutation of AtMIC60 did not lead to complete loss of DGDG in mitochondrial membranes, suggesting another mechanism of transfer also exists. Overall, there may be two mechanisms for lipid transfer from chloroplasts to mitochondria.

Chloroplast OEM:IEM Contact Sites As with other membrane appositions, close contact between OEM and IEM was reported in early TEM data which show the two membranes are usually within 30 nm of each other (Pain et al. 1988; Schnell and Blobel 1993; Schnell et al. 1990). That the membranes define separate compartments has been shown in a number of ways. It was established early on through use of radiolabeled metabolites and water that the inner envelope was a permeability barrier that the outer membrane was not (Heldt and Sauer 1971). Each membrane also has distinct lipid compositions, implying that the membranes are not fused (Block et al. 1983). The last pieces of evidence for determining a real contact site are specialized proteins, and there are multiple reports of protein complexes that span the OEM and IEM. The protein translocons of the OEM and IEM have been shown to be a part of a protein megacomplex spanning both membranes (Kikuchi et al. 2009), and recent evidence shows the complexes are stable structures (Chen and Li 2017). Thus, the chloroplast OEM:IEM meets the standards of a true MCS.

Do chloroplast OEM:IEM contact sites transport lipids? It seems likely. Recent developments in lipid transport suggest that TGD5 may mediate contact between the TGD1,2,3 transporter at the chloroplast IEM and TGD4 of the chloroplast OEM (Fan et al. 2015). The TGD components have multiple lines of evidence supporting their involvement in lipid transport, as detailed above. In addition, the N-terminus of chloroplast OEM protein DGD1 has been shown to be required for recruiting MGDG substrate from the chloroplast IEM to produce DGDG (Kelly et al. 2016), additional details below. Interestingly, no data currently suggest that either the TGD system or DGD1 are associated with the protein translocons known to bridge the OEM:IEM gap. Instead, native gels of the TGD systems support that they are in their own complexes with no stable bridges to larger complexes (Roston et al. 2012; Wang et al. 2013). The lipid transfer components may occur

at additional contact sites independent of the protein translocon, or they may group without direct protein-protein interaction at regions of membrane apposition.

Potential Components Involved in Chloroplast MCS Formation Because the chloroplast OEM contains lipid amounts comparable to the plasma membrane that can interact with MCS components of the ER like phosphoinositides, phospholipids, and sterols (see above sections, and Daum and Vance 1997; Rolland et al. 2009; Schwertner and Biale 1973), it is possible that ER-resident proteins could form MCS at the OEM with no additional protein components (for a recent review of predicted ER MCS proteins in plants, see Pérez-Sancho et al. 2016). However, most MCS identified in mammals and yeast have components on both membranes involved in a contact site (Eisenberg-Bord et al. 2016; Phillips and Voeltz 2016). Given the relative paucity of information available regarding plant MCS proteins, we generated a group of potential Arabidopsis MCS proteins that may reside in the chloroplast and bind to, or transport lipids. Using a non-redundant list of confirmed MCS proteins and functional lipid binding domains from other systems extracted from the following reviews (Pérez-Sancho et al. 2016; Prinz 2014; Quon and Beh 2015), a bidirectional approach was taken to minimize false negatives. First, reciprocal BLAST was used to identify homologous Arabidopsis proteins. Second, lipid binding/transport domain-containing proteins were identified using the InterPro database (Finn et al. 2017). All identified Arabidopsis sequences were analyzed for presence in chloroplast-specific proteomics studies or presence of chloroplast transit peptides using the Plant Proteome Database (Sun et al. 2009) and TargetP/ChloroP (Emanuelsson et al. 2007). Finally, expression of candidate genes was visualized using ePlant (Waese et al. 2017) to confirm their expression in photosynthetic tissues. Resulting Arabidopsis gene identifiers are given in Table 1. It is important to note that this list is far from complete. Chloroplast MCS proteins may have evolved independently from other MCS proteins in the cell, and thus share no identifiable sequence similarity. This is supported by the fact that mitochondrial MCS proteins have a diverse evolutionary history, with core components being lost and novel components evolving independently while maintaining proper mitochondrial function and morphology (Flinner et al. 2013; Huynen et al. 2016; Kozjak-Pavlovic 2017; Michaud et al. 2016). Finally, given that suborganellar prediction is not currently possible, the location of

Table 1. Putative Chloroplast-Targeted MCS Components

Lipid Interaction Domain	AGI	Name (TAIR)	Predicted Function
SMP/C2 (SMP:IPR031468) (C2:IPR035892)	AT1g50260 AT3G19830	NTMC2 DOMAIN TYPE 5.1 NTMC2 DOMAIN TYPE 5.2	Phospholipid binding[a]
OSBP (IPR000648)	AT5G02100 AT3G09300 AT5G59420	ORP3A ORP3B ORP3C	Oxysterol binding and/or transfer[b]
PH (IPR001849)	AT4G11790 AT4G23895	PH domain superfamily protein PH domain-containing protein	Phosphatidylinositol binding[c]
OSBP/PH	AT2G31020 AT1G13170 AT1G77730	ORP1A ORP1D PH domain superfamily protein	
CaLB (C2)	AT1G48590 AT5G55530 AT5G11100	C2-DOMAIN ABA-RELATED 5 CaLB domain family protein SYNAPTOTAGMIN 4	Calcium-dependent binding of phospholipids and phosphoinositides[d]

[a]Ref: (AhYoung et al. 2015; Schauder et al. 2014)
[b]Ref: (Im et al. 2005; Saravanan et al. 2009)
[c]Ref: (Shaw 1996; Stefan et al. 2011; Watt et al. 2002)
[d]Ref: (Davletov and Sudhof 1993; Pérez-Sancho et al. 2015)

the above proteins and their potential contact sites remains to be determined. However, the simple fact that so many proteins can be predicted to be involved in lipid binding in the chloroplast implies that documented MCS components involving lipids are likely far from complete, and more study is needed.

Thus far, studies of chloroplast MCSs have been limited by the techniques used. A particular disadvantage of TEM is that samples are fixed and represent a single time point for a specimen, preventing observation of cellular dynamics in real time. In contrast, traditional confocal microscopy can visualize living cells in real-time, but lacks the resolution needed to view nanoscale membrane structures (Pawley 1991). Newer super-resolution microscopy techniques have been employed to image membrane interactions in other systems (Hua et al. 2017; Jans et al. 2013; Sastri et al. 2017), and similar studies could refine contact site models in plants.

Vesicle Transfer

A classic method of moving lipids generated in the ER to other cellular membranes is through the secretory pathway. Evidence for secretory

vesicles fusing with the chloroplast envelopes comes from the transport of fully glycoslyated proteins to the chloroplast stroma (Kitajima et al. 2009; Nanjo et al. 2006; Villarejo et al. 2005). A subset of these proteins require glycosylation for function, further evidence of their transport through the secretory pathway (Buren et al. 2011; Kaneko et al. 2016). Although it seems likely that the proteins are transported through vesicles, the mechanism by which vesicles fuse with the chloroplast is unknown (Baslam et al. 2016). Therefore, it is unclear if lipids may also be transported through this mechanism.

Membrane Hemifusion

To this point we have focused on transport mechanisms proposed to explain molecules which are transported to the chloroplast under normal conditions. Recently, the general accessibility of the chloroplast to hydrophobic molecules in the ER was tested using an approach termed "transorganellar complementation" (Mehrshahi et al. 2013). In this approach, multiple enzymes of the tocopherol synthetic pathway or an enzyme of the carotenoid synthetic pathway were independently relocated from the chloroplast to the ER using Arabidopsis lines lacking expression of native genes and expressing cDNAs fused with ER targeting information. Resultant plants produced significant levels of the tocopherols and carotenoids, implying that these compounds were efficiently shuttled between the two organelles. This was a surprising result because neither carotenoids nor tocopherols need to be exchanged between the ER and chloroplast under normal circumstances, and transport was bi-directional. The authors suggested that should MCSs between the ER and chloroplast go through a stable hemifusion intermediate, enzymes from either organelle would be able to act on hydrophobic molecules on either side. The suggestion is attractive from the standpoint of transport, because many diverse molecules could be freely transported at sites of hemifusion. It is somewhat less attractive from the perspective that very few stable membrane hemifusion events are known to exist in nature, and none of the machinery promoting these has been well-characterized. Lacking known hemifusion stabilizing proteins, the hemifusion hypothesis is difficult to test at this time.

Finishing sugar lipid synthesis in the chloroplast. Much of the MGDG produced at the chloroplast IEM from the prokaryotic or eukaryotic pathways must be desaturated prior to use in the thylakoid membranes. FAD5, 6, 7 and 8 desaturate MGDG 16:0 or 18:1 to 16:3 or 18:3 (Falcone et al. 1994; Heilmann et al. 2004; Iba et al. 1993; McConn et al. 1994). Interestingly, when ER desaturases FAD2 or FAD3 are absent, chloroplast FADs appear to also desaturate ER lipids, presumably through lipid backbones which are exported from the chloroplast under normal conditions (Browse et al. 1993; Miquel and Browse 1992). Like the transorganellar complementation results, this surprising result implies that bulk lipids are easily transported between the chloroplast and ER.

After desaturation, MGDG can be converted into DGDG. The majority of the DGDG under normal conditions is produced by DGD1 (Dörmann et al. 1999; Kelly et al. 2003). DGD1 is anchored in the chloroplast OEM facing the cytoplasm (Froehlich et al. 2001). During phosphate stress DGDG can also be produced by DGD2, also in the chloroplast OEM (Kelly and Dörmann 2002). Finally, during severe abiotic stress including wounding and freezing in Arabidopsis, DGDG can be made by a progressive galactosyltransferase known as SFR2 or GGGT (Moellering et al. 2010). The commonality in all of these mechanisms is that their lipid precursor is made in the IEM while they reside in the OEM. Thus, lipid transport is again required. A possible exception is the case of DGD2, because during phosphate stress MGDG can also be made at the OEM by MGD2 and MGD3 (Benning and Ohta 2005). Exploiting this difference, a recent study investigated the function of an N-terminal extension present in DGD1 and its homologs but missing from DGD2. They showed that the N-terminus of DGD1 is required for efficient MGDG transport to the chloroplast OEM (Kelly et al. 2016). Like TGD2, it can bind to PA and distort membranes, implying that it too may directly extract a lipid from or deliver it to a membrane. Still unknown is whether the N-terminus of DGD1 operates on its own or if it works with a protein or proteins of the IEM. These final steps of lipid synthesis and the transport events that make them possible are summarized in Fig. 2.

Concluding Remarks

Membrane hemifusion was raised as a possibility because of how many precursors we know are transported between the ER and plastid, and how little we know about the enzymes and membrane structures providing transport routes. To better understand the generation of the photosynthetic membranes, more research is needed to understand how the gregarious chloroplast shares hydrophobic molecules efficiently with the rest of the cell.

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