CHLOROPLAST LIPID IMPORT AND METABOLISM, AND THEIR EFFECT ON CHLOROPLAST STABILITY

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ABSTRACT

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Much is already known about plant lipid metabolism and the basic enzymes required for lipid assembly. However, lipid exchange between subcellular compartments remains a challenging area, which this thesis helps to address. Currently, it is known that the chloroplast synthesizes fatty acids that are subsequently used by lipid biosynthetic pathways in both the chloroplast and the endoplasmic reticulum. Fatty acid export to the endoplasmic reticulum (ER) and lipid transport from the ER to the chloroplast are both known to occur, but the participating membrane proteins and transporters have proven to be difficult to work with due to their hydrophobic nature. Many *in vivo*, genetic, and *in vitro* techniques have been employed to carry out these studies, but the field has reached a point at which new creative techniques need to be developed in order to answer the remaining questions regarding the nature of transported lipid substrates and the mechanisms of intermembrane lipid transfer. In this thesis, both *in vivo* and *in vitro* methods were developed in order to study lipid transport, acyl editing, and other aspects of lipid metabolism for which our knowledge is incomplete. An *in vivo* method using a "tag and track" approach introducing an ER-located fatty acid desaturase into Arabidopsis, which is distinct from any of the native fatty acid desaturases, led to a subtle modification of the acyl group composition of phosphatidylcholine. This "tag and track" approach proved useful in studying lipid trafficking with high sensitivity in different lipid mutant backgrounds affected in lipid trafficking and assembly. It clearly demonstrated the import of lipid precursors from the ER for phosphatidylglycerol biosynthesis and extensive acyl editing of chloroplast lipids as described in Chapter 2.

In vitro methods to study lipid exchange between different membranes using isolated chloroplasts and envelope membranes from different lipid trafficking mutants with either radio- or fluorescently labeled lipids as described in Chapter 3, yielded promising results, further suggesting candidates for lipid molecular species that might be transported from the ER to the chloroplast. However, there were many caveats with the interpretations of the results using these methods that will require further consideration. One example is the differing stability of isolated chloroplasts from different lipid mutants which affected the interpretation of the results. On the other hand, the discovery of differences in the stability of isolated chloroplasts for different lipid mutants, as described in Chapter 4, provides a new avenue to study the specific physiological effects of altered chloroplast lipid composition.

As a whole, the work presented here not only yielded new information about plant lipid metabolism, but it also provides a new tool set for further studies of lipid trafficking and lipid metabolism in general.

ACKNOWLEDGMENTS

I came to Michigan State University in 2011 to pursue my PhD in Biochemistry and Molecular Biology because my undergraduate advisor, Clint Chapple, had told me MSU is the best place to study plant biochemistry with the leading scientists in the field and a huge number of resources. He had told me who some of the best professors to rotate with would be, and among those people was Christoph Benning.

The first time I met Christoph at a conference as an undergraduate, I found him very serious and intimidating. I thought there was no way I would fit into his lab, but I went ahead and did my first rotation with Christoph taking on a challenging rotation project studying interorganellar lipid transport using radiolabeled lipids. This was my first experience with radioactivity, and the experiments were lengthy, delicate, and frustrating. Christoph trusted in my abilities allowing me to work on this project as a beginning student, and for that, I am thankful. I moved onto my next rotation thinking the lab environment and project in Christoph's lab were going to be too intense for my permanent lab as I was worn out from my first rotation, but for some reason, I just could not get that pesky plant lipid transport project out of my head. I was hooked on plant lipid metabolism, and I missed being a part of the Benning lab and all of the hard-working people that provided extra support and advice. I joined Christoph's lab not only because of the project, but also because his mentorship style allows for independence and fosters an environment where everyone works as equals providing different perspectives to each person's project. Christoph deserves a special thanks for accepting me into his lab and supporting me through all of the ups and downs of my project.

I would also like to thank my committee members for providing extra support and guidance. John Ohlrogge has been a huge help with all of my radiolabeling assays and with interpreting my data. As a plant lipid expert, he has been a resource of knowledge that is not easily pulled up in a literature search, and my depth of knowledge has grown more thanks to

him. I also appreciate that he agreed to be on my committee despite being so close to retirement when I started graduate school. Tom Sharkey has provided me with suggestions and resources for experiments, and he even taught me how to use an oxygen electrode for measurement of oxygen evolution from chloroplasts which he had built himself. Gregg Howe has challenged me to think from a different perspective with his comments and questions, and I also had a great rotation experience in his lab. Finally, I appreciate that Shelagh Fergason-Miller stepped in to fill a vacancy on my committee so late in my career as a graduate student.

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KEY TO ABBREVIATIONS

PA	Phosphatidic Acid
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PI	Phosphatidylinositol
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
MGDG	Monogalactosyldiacylglycerol
DGDG	Digalactosyldiacylglycerol
TGDG	Trigalactosyldiacylglycerol
SQDG	Sulfoquinovosyldiacylglycerol
GLA	γ-Linolenic Acid
SDA	Stearidonic Acid

CHAPTER 1

Introduction: Lipid Trafficking in Plant Cells - a Literature Review[1]

[1]This review was published: Hurlock AK, Roston RL, Wang K, & Benning C (2014) Lipid trafficking in plant cells. *Traffic* 15(9):915-932. The sections which were relevant to this review were decided by me with guidance from Christoph Benning and Rebecca Roston. Rebecca Roston contributed the sections "Inner Envelope to Thylakoid Transport" and "Transport to the Vacuole." Kun Wang contributed the sections "Chloroplast Export" and "Lipid Trafficking Related to Lipid Droplets." I contributed the rest of the sections and all of the figures as well as performed extensive editing of the other sections. I also added some extra information into the review since publication in order to cover advances in knowledge after publication.

Abstract

Plant cells contain unique organelles such as chloroplasts with an extensive photosynthetic membrane. In addition, specialized epidermal cells produce an extracellular cuticle composed primarily of lipids, and storage cells accumulate large amounts of storage lipids. As lipid assembly is associated only with discrete membranes or organelles, there is a need for extensive lipid trafficking within plant cells, more so in specialized cells and sometimes also in response to changing environmental conditions such as phosphate deprivation. Due to the complexity of plant lipid metabolism and the inherent recalcitrance of membrane lipid transporters to molecular characterization, the mechanisms of lipid transport within plant cells are far from fully understood. Recently, several new proteins have been implicated in different aspects of plant lipid trafficking. While these proteins provide only first insights into limited aspects of lipid transport phenomena in plant cells, they represent exciting opportunities for further studies.

Introduction

Plant cells have many membranes that are generally comparable to those in animal cells including the plasma, mitochondrial, nuclear, and peroxisomal membranes. In addition, plant cells contain unique membrane-bound compartments such as the chloroplast, vacuole, and symbiosome (1) and have other unique cellular structures composed of lipids, e.g. the cuticle of epidermal cells. As the key photosynthetic organelle in plants, one focus of plant lipid research has been on the chloroplast. It is surrounded by the outer and the inner envelope membranes and encompasses one of the most extensive membrane systems found in nature, the photosynthetic membrane organized into thylakoids. This membrane is unique in its lipid composition with the two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), being predominant (2, 3). Unlike animal cells which synthesize fatty acids in the cytoplasm and assemble glycerolipids primarily in the endoplasmic

reticulum (ER), golgi apparatus and mitochondria (4), plant cells synthesize fatty acids in the stroma of the chloroplast and assemble glycerolipids mainly by two pathways, the prokaryotic/plastid pathway in the chloroplast envelope membranes and the eukaryotic pathway predominantly in the ER (5). It should be noted that while lipid metabolism occurs in plastids other than chloroplasts, for example chromoplasts in fruits and flowers or leucoplasts in storage tissues, most research on lipid trafficking has focused on chloroplasts.

The origin of specific glycerolipids from either the ER or the chloroplast pathways in plants can be determined due to the specificity of the respective acyltransferases (6, 7). The ER localized acyltransferase preferentially transfers 18 carbon fatty acids to the *sn*-2 (carbon 2) position of the glycerol backbone while the chloroplast localized acyltransferase preferentially transfers 16 carbon fatty acids to the *sn*-2 position of the glycerol backbone (6, 7). Because of this difference in enzyme specificity, it has been well established that plants use both chloroplast and ER derived lipids as precursors in the assembly of chloroplast-specific galactolipids (7-9). Different plants utilize each pathway for the synthesis of chloroplast lipids to different extents (10); Arabidopsis uses each pathway nearly equally while many other plants, e.g. grasses, nearly exclusively use the ER pathway (8, 9). Based on lipid acyl composition alone, the unicellular green alga, Chlamydomonas, has been reported to use only the plastid pathway for the synthesis of chloroplast lipids (11); however, the specificity of the different acyltransferases involved has not yet been determined and may not be the same as for seed plants.

Certainly in all seed plants, the presence of ER derived chloroplast lipids requires the transport of ER lipid precursors to the chloroplast. Lipids, being hydrophobic molecules, must be trafficked from their place of synthesis to their destination whether that is another membrane or the extracellular space in the case of the cuticle. Because this is a review focused on plant cells, lipid trafficking between organelles largely similar to those found in animal cells will not be covered. This includes trafficking to the plasma membrane, mitochondria, and peroxisome,

which are covered in recent reviews on these topics (12-15). There are several possible modes of lipid movement within cells which will be mentioned throughout such as vesicular movement, transport by soluble proteins, flipping across bilayer membranes, and movement at membrane contact sites. Covering the basic mechanistic principles of different modes of lipid movement goes beyond this review, and the reader is referred for further details to the following publications (14, 16-19).

Chloroplast Export

Fatty acids (FAs) synthesized in chloroplasts need to be exported to the ER for the biosynthesis of phospholipids and the major storage lipid, triacylglycerol (TAG), and to provide lipid precursors for export to the extracellular cuticle. FA synthase is a multi-enzyme complex that catalyzes a series of condensation and elongation steps to produce C16 and C18 acyl chains attached to an acyl carrier protein (ACP) (5). A soluble Δ9-desaturase converts 18:0-ACP (carbons: double bonds) to 18:1-ACP (5). The 16:0, 18:0, and 18:1 acyl-ACPs are hydrolyzed by two thioesterases designated FATA and FATB, and it is generally assumed that the free FAs have to be activated to acyl-CoA at some step during export to the ER (5, 20-23). This reaction is thought to be catalyzed by long-chain acyl-CoA synthases (LACSs) (24, 25). The Arabidopsis genome contains nine *LACS* genes, and only LACS9 is known to be localized in the chloroplast envelope (26, 27). Labeling experiments using ^{14}C-18:1 or ^{14}C-16:0 with chloroplasts isolated from the *lacs9* null mutant showed a reduction in measurable LACS activity suggesting LACS9 contributes much of this activity in chloroplasts (26). However, the mutant plant was indistinguishable from wild type in growth and morphology as well as lipid and FA composition, which suggests that functionally redundant orthologs of LACS9 exist (26). It is also possible that the conversion of FA to acyl-CoA is not a rate-limiting step in FA export. LACS8 shows high sequence similarity with LACS9, but it is associated with the ER (28). No changes in seed FA content or growth phenotype were detected in the double knock-out of *lacs8lacs9*, but lipids in

other tissues have not been analyzed (28). Like LACS8, LACS1 is also ER localized, but it is primarily involved in providing FAs for cuticle lipid synthesis (28, 29). However, while none of the single mutants have seed FA phenotypes, both the double *lacs1lacs9* and triple *lacs1lacs8lacs9* knock-out mutants have similarly decreased seed FAs suggesting LACS1 and LACS9 may have overlapping functions (28). These LACS proteins warrant further study in non-seed tissues, although it seems that one or more chloroplast-associated LACS involved in FA export remain to be identified.

Other than the LACS proteins, another protein has been implicated in FA export from chloroplasts, Fatty Acid Export 1 (FAX1) (30). FAX1 is an inner envelope protein, and mutants have decreased ER derived lipid species and increased plastid derived lipids (30). This mutant phenotype combined with the fact that FAX1 can complement fatty acid transport in yeast mutants makes FAX1 the protein with the most concrete evidence for being directly involved in FA export from chloroplasts (30); however, due to its inner envelope location, it is likely other proteins are still involved in chloroplast FA export.

ATP-binding cassette (ABC) transporter proteins can transfer lipophilic compounds through membranes, and it is possible that this class of transporters is involved in acyl export from the chloroplast or in the import of acyl derivatives into the ER (31). For example when *AtABCA9*, an ER-localized ABC transporter in Arabidopsis, was knocked-out (32), the seed TAG amount was decreased while overexpression of *AtABCA9* increased the TAG amount (32). Labeling experiments with ^{14}C-acetate, ^{14}C-18:1, or ^{14}C-18:1-CoA suggested that AtABCA9 plays a role in supplying free FAs or acyl-CoA substrates for TAG biosynthesis in the ER (32). Although the specific substrate for AtABCA9 is unknown, analysis of the mutant indirectly suggested that acyl trafficking to the ER might be limiting TAG biosynthesis in this mutant (32). Another ABC transporter, AtABCD2/AtPMP1, which is plastid localized, was hypothesized to be involved in chloroplast FA export based on its similarity to AtABCD1, which is a peroxisomal localized ABC transporter that is required for FA import into peroxisomes (31, 33, 34). However,

the function of AtABCD2 has not been experimentally verified, and the transport of hormone precursors, carotenoids, or tocopherols by this protein have also been proposed (31, 33). During a global phenotyping screen to determine functions of chloroplast proteins, no strong phenotypes were detected in two characterized T-DNA insertion lines fully disrupted in AtABCD2 (35) making it unlikely that this protein is responsible for major fluxes of acyl groups from the plastid to the ER.

Acyl-CoA-binding proteins (ACBPs) have also been suggested to play a role in FA export from plastids (36, 37). Studies of yeast and mammalian ACBPs provide evidence for ACBPs acting as acyl-CoA transporters (38, 39). Many of the ACBPs in plants have been implicated in stress response, but others such as BnACBP, AtACBP4, and AtACBP5 may be involved in transfer of acyl-CoAs from the chloroplast to the ER (37, 40, 41). An ACBP from *Brassica napus*, BnACBP, enhances acyl exchange between phosphatidylcholine (PC) and the acyl-CoA pool, particularly with 18:1-CoA (41). Two of the 6 Arabidopsis ACBPs, ACBP4 and ACBP5, are localized to the cytosol and are of particular interest as they have high binding affinity for 18:1 acyl-CoA *in vitro*, can also bind PC, and have increased expression in response to light (36, 40, 42, 43). Analyses of T-DNA lines of ACBP5 have yet to be published, but lipid analysis of the T-DNA *acbp4* mutant showed reduced levels of membrane lipids, including MGDG, DGDG, PC, phosphatidylethanolamine (PE), and phosphatidylinositol (PI) in leaves (42). The decreased ER phospholipid levels and perhaps the galactolipids derived from ER precursors suggest a possible role of ACBP4 in the transport of acyl-CoA from plastids to the ER, but further FA analysis of the MGDG and DGDG would need to be done in this mutant to support this hypothesis (36, 42, 44). Also, there is some evidence supporting the role of ACBP4 in stress response instead as it has been shown to co-express under stress conditions, co-localize with, and bind to AtEBP, an ethylene-responsive element binding protein (45).

Direct membrane contact sites between the ER and the chloroplast outer envelope could provide possible conduits for lipid transfer (46). Rapid labeling studies using ^{14}C-acetate or ^{14}C-

glycerol indicated that FAs *de novo* synthesized in the chloroplast are first incorporated into PC, which is present in the ER as well as in the outer envelope membrane of chloroplasts (47), before entering the cytosolic acyl–CoA pool. Lyso-PC acyltransferase activity likely involved in this acyl exchange process is associated with isolated chloroplasts (48, 49). Thus, PC might serve as the first intermediate for FAs exported from the chloroplast, and extensive acyl-exchange through chloroplast envelope PC into cytosolic acyl-CoAs might provide the acyl groups needed for the assembly of phospholipids at the ER (Figure 1.1). Phosphatidylglycerol (PG) may also be a source of acyl groups for synthesis of other lipids. A chloroplast specific lipase, Plastid Lipase 1 (PLIP1), has been shown to hydrolyze acyl groups from PG, which are then shuttled through PC before being used for seed oil biosynthesis (50). While this process is

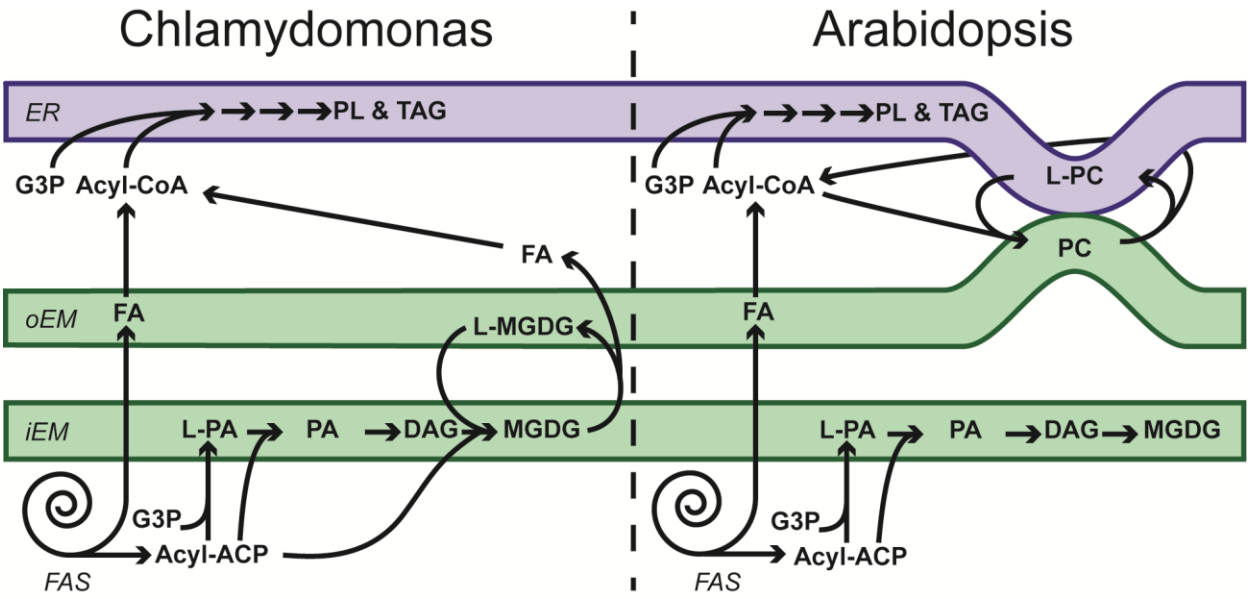

Figure 1.1 Generation of the Acyl-CoA Pool for ER Lipid Synthesis in *Chlamydomonas reinhardtii* and *Arabidopsis thaliana*. Hypotheses for how FAs enter the acyl-CoA pool are presented. A long standing hypothesis has been that FAs are released from acyl-ACP and converted to acyl-CoAs at the oEM as shown for both organisms. In Arabidopsis, a high rate of acyl exchange between PC and the acyl-CoA pool has been observed. As PC is also located in the oEM as well as in the ER, it is likely acyl-exchange may occur at both the ER and the oEM. In Chlamydomonas, which lacks PC, there is evidence of newly synthesized FAs being incorporated into MGDG first, and then hydrolyzed from MGDG by the enzyme PGD1 and used for TAG synthesis in the ER. The models presented are simplified and may not be exclusionary with regard to plants, as a possible role of MGDG in acyl exchange and FA export still needs to be investigated. It should be noted that TAG biosynthesis can also occur at the chloroplast envelope in both organisms but is not shown here. ER, endoplasmic reticulum; oEM, outer envelope membrane; iEM, inner envelope membrane; FAS, fatty acid synthase; FA, fatty acid; G3P, glycerol 3-phosphate; acyl-CoA, acyl-coenzyme A; PL, phospholipid; TAG, triacylglycerol; L-PA, lyso-phosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; L-MGDG, lyso-monogalactosyldiacylglycerol; MGDG, monogalactosyldiacylglycerol; L-PC, lyso-phosphatidylcholine; PC, phosphatidylcholine.

most active in the seeds, it does not mean a similar process could not be occurring in leaf tissue by way of one of the many other uncharacterized lipases.

A similar mechanism of using a membrane lipid intermediate for FA export from the chloroplast to the ER has been proposed for Chlamydomonas which lacks PC (51) (Figure 1.1). The MGDG lipase, Plastid Galactolipid Degradation 1 (PGD1), is involved in the production of TAG from *de novo* synthesized FAs exported from the chloroplast when N is limited (51). PGD1 specifically releases the newly incorporated FA from the *sn*-2 position of the glycerol backbone of MGDG before it gets further desaturated, thereby making it available for TAG biosynthesis either at the ER or the outer chloroplast envelopes (51). A lyso-MGDG acyltransferase has not yet been identified, but together with PGD1, it could provide a metabolic cycle shuttling *de novo* synthesized FAs from the inside of the plastid to the outer leaflet of the outer chloroplast envelope. PGD1 is thought to have access to its substrate at the outer envelope (Figure 1.2) as it does not have a predicted plastid transit peptide, but its exact location has not yet been verified. This hypothesis implies that acyl groups attached to lipids - in this case MGDG - are passed between and through the envelope membranes. In thylakoid lipid biosynthesis, it is also necessary to transfer galactolipids from their site of synthesis on the intermembrane face of the inner and the outer membrane face of the outer envelope back to the thylakoids inside plastids. Whether this process is related to FA export and how galactolipids move between the plastid membranes remains to be defined.

In plants, lipid metabolism and trafficking can be greatly altered under nutrient stress. For example, under normal growth conditions, DGDG is exclusively localized in chloroplasts (52). However, when the availability of phosphate is limited, DGDG biosynthesis is elevated partially due to the transcriptional induction of MGDG synthases, MGD2 and 3, and DGDG synthase, DGD2 (53, 54). To maintain the proper function of membranes, DGDG is transported from the chloroplast envelopes to the extraplastidic membranes where DGDG replaces phospholipids (52, 55-57). While the transporters which are responsible for DGDG export from

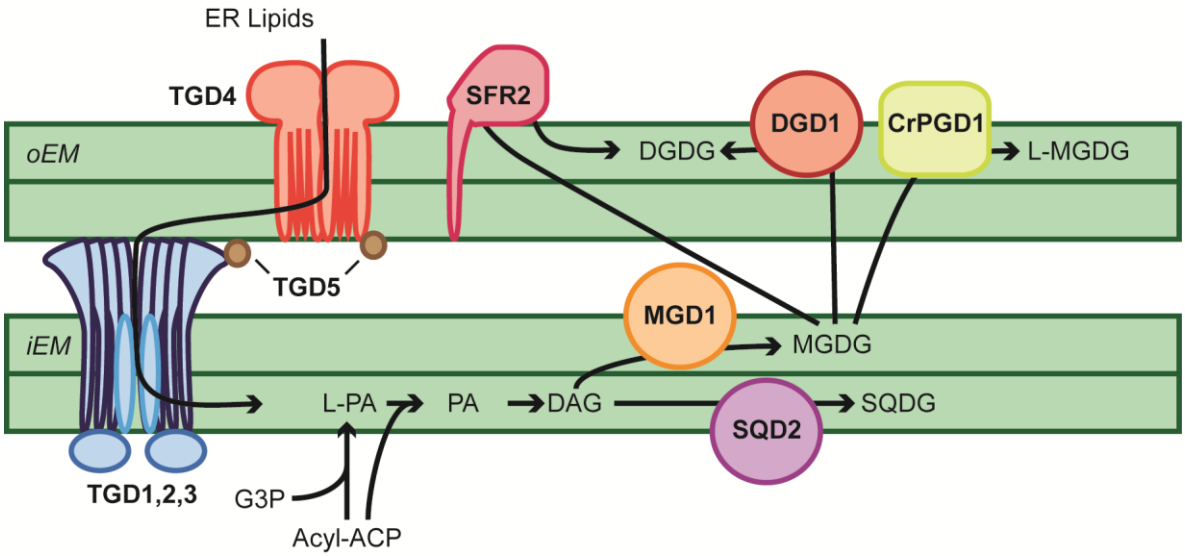

Figure 1.2 Subcellular Locations of Lipid Synthesis and Transport Proteins for the Major Chloroplast Lipids. The subcellular locations of the chloroplast envelope associated enzymes for final assembly of the major chloroplast lipids are depicted along with known lipid transport proteins. Presumed movement of lipid precursors for the synthesis of major thylakoid lipids between and through the envelope membranes is indicated as necessitated by the location of the respective enzymes. All proteins represented have been studied in Arabidopsis except for PGD1 which was discovered in Chlamydomonas but has orthologs in Arabidopsis. ER, endoplasmic reticulum; oEM, outer envelope membrane; iEM, inner envelope membrane; G3P, glycerol 3-phosphate; ACP, acyl carrier protein; L-PA, lyso-phosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; L-MGDG, lyso-monogalactosyldiacylglycerol; MGDG, monogalactosyl-diacylglycerol; DGDG, digalactosyldiacylglycerol

the plastid have not yet been reported, several putative lipid binding or transport proteins with unknown function are predicted to be present in Arabidopsis chloroplasts (33). Interestingly, when the bacterial lipid, glucosylgalactosyldiacylglycerol, was introduced into Arabidopsis DGDG-deficient mutants, *dgd1* and *dgd1dgd2*, the accumulation of this lipid in extraplastidic membranes was observed, which indicated the transport mechanism does not discriminate the glucose and galactose in the lipid head group *in vivo* (58).

Characterization of the Arabidopsis *fad3* mutant, provides another example for the possible export of desaturated lipids from chloroplast (59). The *fad3* mutant is deficient in the ER 18:2 desaturase activity, but in ^{14}C-acetate labeling experiments, the *fad3* mutant showed delayed and reduced accumulation of 18:3 in PC and PE, suggesting that plastid desaturated 18:3 FAs can find their way into ER lipids (59). However, in this study the possibility that another 18:2 desaturase exists in the ER or that the *fad3* point mutant allele is leaky could not be ruled out. FAD7 and FAD8 are the two chloroplast localized isoforms of *fad3* (60, 61). When

comparing the lipid phenotype of two triple mutants *fad3-2 fad7-2 fad8* and *fad3-2 fad7-1 fad8*, the latter of which carries a leaky *fad7* allele, higher 18:3 amounts in PC and PE were found in the *fad7* leaky triple mutant (62). This indicates that ER phospholipids containing18:3 acyl chains are dependent on the precursors from the chloroplast, supporting the hypothesis that 18:3 FAs can transfer from the chloroplast to the ER. However, the mechanism of this export and the lipid molecules transported remain to be determined.

ER to Chloroplast Envelope and Inter-Envelope Transport

The chloroplast has the ability to synthesize the major lipid components of its membranes, the galactolipids, MGDG and DGDG, and the two anionic lipids, sulfoquinovosyldiacylglycerol (SQDG) and PG (5, 53, 63). Positional analysis of acyl chains of the major plastid lipids (see Introduction of this chapter) suggests that in all plants a fraction of these lipids is derived from ER-synthesized precursors, although this proportion varies between different plant species (9). In addition, PC, which the chloroplast cannot assemble, is a significant component of the chloroplast outer envelope membrane (64, 65). Thus, the presence of ER-derived thylakoid lipids and PC in the chloroplast requires the transport of ER lipids to this organelle. Because of the localization of the enzymes required for the synthesis of the major chloroplast lipids in the two envelope membranes, both the ER-derived and chloroplast-derived lipids used for the synthesis of the major chloroplast lipids must be transported between the envelopes (Figure 1.2). ER-derived lipids must first be transported to the outer envelope and then to the outer leaflet of the inner envelope where the predominant MGDG synthase, MGD1, is localized or to the inner leaflet of the inner envelope where the SQDG synthase, SQD2, is localized (66-68). Lipids originating in the chloroplast are first synthesized by the transfer of acyl-chains to glycerol-3-phosphate to make phosphatidic acid (PA) at the inner leaflet of the inner envelope where PA can then be further metabolized to PG or SQDG (5). Like the ER lipids, the chloroplast derived lipid precursors must be transported to the outer leaflet of the inner envelope

for MGDG synthesis, and then MGDG made from both ER and plastid precursors must be transported to the outer leaflet of the outer envelope where the DGDG synthase, DGD1, is localized (5, 54, 69). It should also be noted that MGD2 and 3 are not considered here because, as mentioned previously, they are induced under nutrient deprivation (63). The exact mechanisms of transport of lipid precursors and lipids from the ER to the chloroplast and between envelopes are still not fully understood, but recent progress has led to a refined hypothesis.

A first step towards gaining a comprehensive understanding of ER to chloroplast lipid transport has been the discovery and characterization of the TriGalactosylDiacylglycerol (TGD) proteins of Arabidopsis. The TGDs were identified in a mutant screen for suppressors of *dgd1*, a mutant deficient in DGDG synthesis, and the *tgd* mutants were named for their production of oligogalactolipids such as trigalactosyldiacylglycerol (70). The *tgd* mutants present a range of other phenotypes including an increase in the 16-to-18 carbon ratio in total FA composition consistent with a decrease in ER-derived membrane lipids (70-73). Further analysis of chloroplast-specific lipids showed that the total fatty acid change in the 16-to-18 carbon ratio is due to decreases in galactolipids derived from ER precursors (70-75). Other *tgd* mutant phenotypes include reductions in growth, fertility, germination, and formation of photosynthetic membranes, as well as lethality of null-alleles of specific *TGD* genes (70-75). Taken together, these phenotypes implicate the TGDs in ER to chloroplast transport and demonstrate the importance of ER-derived chloroplast lipids to the formation of photosynthetic membranes and the overall health and viability of plants.

Five TGDs have been identified and extensively studied. TGD1, TGD2, and TGD3 form an ABC transporter complex in the inner envelope membrane, while TGD4 forms a homodimer in the outer envelope membrane, and TGD5 is a small protein which can associate with the other TGD proteins (76-80). TGD1 is predicted to be the permease of the ABC transporter and is present in 2 copies per complex (70, 78). TGD3 is a confirmed ATPase and is also present in

2 copies per complex (72, 78). TGD2 is the substrate binding domain of the complex and is known to bind PA (73, 78, 79, 81). TGD2 makes the TGD1,2,3 complex unique among ABC transporters as it is present in 8-12 copies per complex, while most substrate binding domains of bacterial ABC transporters are present in 1-2 copies if present at all (31, 78). TGD4 is a predicted β-barrel protein which, like TGD2, binds PA (76, 77). TGD5 was shown to interact with the other TGD proteins through co-immunoprecipitation when heterologously expressed in tobacco, but it is still unclear how TGD5 mechanistically works with the other TGD proteins (80). TGD4 and the TGD1,2,3 complex do not form any stable interactions (78), so if TGD5 helps to connect TGD4 and TGD1,2,3, it may be a transient interaction.

Although two TGD proteins bind PA, there is no direct evidence demonstrating PA as the transported lipid species of either the TGD4 or the TGD1,2,3 complexes. PA seems to be a reasonable substrate for transport as it can support multiple membrane conformations and is a non-bilayer forming lipid, so it may require less energy to be removed from membranes (82). Several other candidates for the transported lipid species have been suggested including diacylglycerol (DAG), PC, and lyso-PC (3). DAG is considered because, like PA, it is a non-bilayer forming lipid, but it also has the unique ability to flip between leaflets of a bilayer at biologically relevant rates (83). PC and lyso-PC have both been proposed due to the inability of chloroplasts to produce PC, but this hypothesis would only be relevant for TGD4 as PC is only present in the outer envelope membrane (3, 64, 65).

Despite extensive forward genetic screening approaches that mostly yielded a sizable number of additional alleles of the TGD proteins, the TGD proteins are the only proteins thus far directly implicated in ER to chloroplast lipid transport (79). It is hypothesized that they work by forming a lipid conduit through the outer and inner envelope membranes facilitating transport (84). TGD2 has the ability to disrupt membranes *in vitro* which provides the basis for the hypothesis that the TGD1,2,3 complex works by removing lipids from the outer envelope membrane and inserting them into the inner envelope membrane, although there is no direct

evidence that the TGD1,2,3 complex contacts the outer envelope membrane *in vivo* (79). While TGD4 is associated with the outer envelope membrane, a portion cofractionates with ER markers and hence TGD4 may contact the ER membrane (76). The possibility that the TGD complexes act as flippases moving lipids across leaflets within the same bilayer must also be considered. Although DAG can flip between bilayers at a reasonable rate, flippases may be required to facilitate this process to keep up with flux demands. In fact, DAG pools at different chloroplast membrane leaflets can be distinguished *in vivo* (66) suggesting that DAG does not rapidly equilibrate between the different leaflets.

Related hypotheses discussed in the field for both ER to chloroplast envelope and inter-envelope transport involve PLastid Associated Membranes (PLAMs) and hemifusion. PLAMs involve stable membrane contact between the ER and the chloroplast and have been observed by microscopy and optical manipulation (85-89). While information on PLAMs is still scarce, they are likely similar to Mitochondria Associated Membranes (MAMs), which have been extensively studied and reviewed (90, 91). MAMs are known to be involved in lipid transport between the ER and mitochondria (92, 93), so it is hypothesized that PLAMs may be involved in lipid transport between the ER and chloroplasts. Transorganellar complementation studies suggest there can be considerable transport of diverse hydrophobic compounds, e.g. tocopherol precursors, between the ER and chloroplast envelopes (94). To accommodate the perceived lack of specific transporters for the number of molecules that are likely exchanged between the chloroplast and the ER, it has been suggested that intermembrane hemifusions of the outer leaflets of the ER and outer envelope membranes and of the inner leaflet of the outer envelope with the outer leaflet of the inner envelope membrane provide a mechanism for lipohilic compound exchange between membranes without transporters (94, 95). However, hemifusion of membranes has yet to be directly observed in plant cells, although hemifused membranes have been shown to form *in vivo* during specific cellular events in other organisms (i.e. vesicle fusion, egg fertilization) (96-98). A confirmation of the hemifusion hypothesis will require

identifying proteins that one might predict are involved in initiating and stabilizing membrane hemifusions and in preserving the unique protein and lipid compositions of the membranes involved in hemifusion. A protein complex which tethers ER to mitochondria has been identified in yeast (99), and similar complexes must be involved if contact sites between the ER and the plastid facilitate the exchange of membrane lipids and other membrane soluble small molecules.

Inner Envelope to Thylakoid Transport

As proplastids from meristematic cells differentiate into chloroplasts, thylakoid membranes proliferate greatly. When mature, thylakoids are the major membrane system of the leaf cell and form the boundary of a continuous aqueous compartment called the thylakoid lumen. The lipids required to make the thylakoids are themselves made in the chloroplast envelopes (see above, 84). Accordingly, the thylakoids are assumed to derive directly from the chloroplast inner envelope membrane, an idea which is further supported by the similarity of their respective lipid compositions (100).

Three non-exclusive hypotheses for the mechanism of thylakoid expansion and maintenance have been proposed. The first is that thylakoid lamellae derive from an invagination of the inner envelope membrane which expands until it fuses with other such invaginations and is then separated from the inner envelope. Observations of young chloroplasts by transmission electron microscopy clearly show long inner envelope invaginations protruding into the stroma (101). This hypothesis is favored for early development of thylakoids but has not been observed in mature chloroplasts and may be unlikely as a mechanism for mature thylakoid maintenance because ATP generation from photosynthesis requires a stable, low pH in the lumen. The second hypothesis states that a vesicle transport system exists between the inner envelope and thylakoid membranes. Vesicle transport is supported by the observation of stromal vesicles in multiple plant species under cool conditions which slow vesicle fusion (102), by assay (103-105), and by recent molecular biology studies

described in detail below. Finally, thylakoid membranes may be maintained by non-vesicular lipid transport. MGDG was observed to have high rates of transport compared to other thylakoid lipids, in spite of the fact that the steady-state composition of both the inner envelope and thylakoid membranes is similar (106). This may indicate that it is moved to the thylakoids preferentially through a separate soluble lipid transport system.

To date, few gene products have been described as direct effectors of thylakoid biogenesis. For the most part, these have been identified because the respective mutants lack normal thylakoid membranes, though confirmation that their direct effect is on the membrane itself has been tenuous. Examples include THylakoid Formation 1 (THF1), which was later found to have a direct effect on photosystem II (107), and Plastid Fusion and/or Translocation Factor (PFTF), which was shown to be a FtsH protease (108). It seems likely in these cases that the proteins in question are not also directly involved in thylakoid formation. Candidates more likely to have direct roles have also been identified. First, a protein named Vesicle-Inducing Protein in Plastids 1 (VIPP1) was shown to be required to form cold-induced vesicles at the inner envelope membrane (109) and was localized to both the inner envelope and thylakoid membranes (110). Direct evidence that VIPP1 induces vesicles is still lacking, (reviewed in 111), and there is conflicting evidence from its homolog in *Synechocystis* which suggests that aberrant thylakoid structure in knockouts may be due to a direct effect on photosynthetic ability (112). Likewise, Snow-White Leaf1 (SWL1) has been described as involved in thylakoid biogenesis in rice (113). It was shown to be important for development of chloroplasts from proplastids, but not for amyloplasts or etioplasts, indicating a specific role in thylakoid biogenesis; however, its direct function has not yet been shown. Alternative approaches to forward genetic screens include bioinformatics, which have identified multiple chloroplast proteins similar to the proteins required for COPII vesicle trafficking (114, 115). Each of these studies has fallen short of identifying a chloroplast homolog of every protein required for cytosolic COPII trafficking, though the chloroplast vesicle system may well be a simpler one.

There has been some confirmation of the COPII hypothesis using reverse genetic tools; these studies showed that cpSAR1A is in the chloroplast and essential for viability (116) and identified cpRABA5e as a chloroplast located Rab GTPase which may be involved in trafficking of stromal vesicles (117). Both studies located the respective proteins as distributed between the stroma and thylakoid membranes and suggest they are found on stromal vesicles. At this point, suggesting the mechanism of vesicle transport would be premature, though it is worth pointing out that no proteins have been suggested for the alternative thylakoid biogenesis hypotheses. Thus, the vesicle hypothesis is the most substantiated, though all require further verification.

Once lipids have arrived at the thylakoid membrane, they are maintained asymmetrically between the inner and outer leaflets. It has been shown both through multiple methods and in several species that MGDG and PG are more populous in the stromal leaflet, while DGDG and SQDG are more prominent in the lumenal leaflet of the membrane (118, 119). The asymmetry is present at the earliest testable stage of thylakoid development (119), raising the hypothesis that asymmetry precedes thylakoid integration of lipids and thus may be a result of passive maintenance. However, rates of MGDG and DGDG incorporation into thylakoid membranes were observed to differ considerably from their steady-state levels (106), discrediting the passive maintenance hypothesis. Currently, the mechanism by which lipid asymmetry is maintained, like the mechanisms by which thylakoid membranes are built, remains at the hypothesis stage.

Transport to the Vacuole

In plants, vacuoles have developed some functions dissimilar from vacuoles of most motile species. In seeds, a specific type of vacuole stores protein for the embryo, while in most other tissues, a “lytic” vacuole is the largest cellular structure. Lytic vacuole functions include maintaining turgor pressure, providing pH homeostasis, detoxifying the cytosol, degrading waste, sequestering ions and participating in multiple signaling pathways (120). The unique

membrane composition of the vacuole (121) is known to affect its function. In plants capable of withstanding high salt stress, vacuolar membrane fatty acids and overall protein levels change to make a less permeable membrane (122). Similarly, during phosphate stress, the vacuolar membrane is extensively remodeled, partially through the action of a specific phospholipase D (PLDζ2) (123). Finally, the lipid composition of the vacuolar membrane is likely to directly affect its morphology. It was shown that disruption or overexpression of genes encoding enzymes that modify the number of phosphates on PI affect both vacuolar shape and vesicle traffic destined for the vacuole (124).

Lipid transport to support lytic vacuolar membrane composition is likely to occur by vesicle transport through the endomembrane system (120). Vacuole membrane lipids are synthesized in the ER, though the diversity of their vacuole delivery methods has only recently been appreciated. Vacuole development occurs during cell differentiation, when a tubular “provacuolar” network found in undifferentiated meristematic cells fuses and expands to form the large central vacuole (125). Recent developments show that vacuole biogenesis begins in a specific area of the ER (125), shedding doubt on an earlier hypothesis that provacuole membranes originate from fusions of vesicles from the trans-Golgi network (126). Similarly for mature vacuoles, we now know that transport of sterols and some proteins occurs independently of a functioning Golgi (125, 127-129), though the Golgi network and multivesicular body are essential for delivery of others (reviewed in 130). Maintenance of the vacuole membrane has not been well studied. It was shown to have only slight membrane asymmetry in sycamore (131), which could have originated from asymmetry inherent in its progenitor membranes. Vesicular transport to the vacuole has been studied primarily as a means to identify targeting methods of proteins, thus whether additional mechanisms of lipid transport may occur is unknown.

Lipid Trafficking Related to Lipid Droplets

Lipid droplets (LDs) are dynamic organelles containing lipid storage compounds, typically TAGs. LDs are surrounded by a phospholipid monolayer into which diverse proteins are inserted. Although LDs were initially regarded as inert storage depots, they have drawn extensive attention since the discovery that disturbances in LD metabolism are at the basis of many human diseases and that LD formation holds potential for biofuel production in plants. LDs are observed in nearly all cell types in plants, and they have been shown to play important roles in stress responses, pathogen resistance, and hormone metabolism, as well as in anther development (132). Here the focus will be on lipid metabolism/trafficking during LD biogenesis and other trafficking roles in which LDs may be involved. LD turnover and FA transport to the peroxisome will not be covered as it is discussed in the peroxisomal review mentioned previously (13).

Several models have been proposed for LD biogenesis in eukaryotic cells (133). In plant seeds, the sequestration of TAG during biogenesis of LDs is believed to occur at specialized domains of the ER similar to proposals for animal LD biogenesis (133-135). Evidence derives from ultrastructural studies showing an intimate association of the ER and LDs (136, 137). In addition, the biosynthesis of TAGs in the LD core and of the major LD membrane lipids, PC and PE, is accomplished by ER associated enzymes (138). Accordingly, it is proposed that during seed LD biogenesis, specific LD membrane proteins such as DAG AcylTransferase (DGAT) and LD associated proteins, generally called perilipins, i.e. oleosins in plants, first aggregate in ER specific domains (132, 135). DGAT is one of the enzymes responsible for the final step of TAG biosynthesis (138), and oleosin is the most abundant membrane protein found in plant seed LDs (132). In plants, TAG biosynthesis can occur by the conventional Kennedy Pathway or by acyl-CoA-independent mechanisms involving Phospholipid : DAG AcylTransferase (PDAT) as reviewed in (138). Oleosin stabilizes the LDs and also controls the size of the LD by preventing fusion between LDs (139). Therefore, after oleosins are recruited to the ER domains, they

stabilize the ER domains and support the budding process (132, 140). Localized DAG formation is particularly important to LD biogenesis as shown in yeast and mammalian cells. Yeast mutants impaired in punctate DAG synthesis, but with no changes in total DAG amounts, showed a decrease in LD number, while mammalian cells with ER enriched in DAG showed increased recruitment of perilipins (134, 141, 142). Despite what is known in other eukaryotes, the mechanism of LD biogenesis in plant non-seed tissues is poorly understood. Non-seed tissues are known to accumulate LDs in response to certain stresses such as freezing (132, 143). One protein known to provide DAG for LD biogenesis under freezing conditions is Sensitive to Freezing 2 (SFR2) which removes MGDG from the chloroplast outer envelope by forming oligogalactolipids and DAG (143). Since oleosins are absent, those ER domains that are specific for TAG sequestration during LD biogenesis in seeds may not exist. Therefore, an oleosin-independent model has been proposed (132). In this model, LD biogenesis is thought to be solely dependent on the coalescence of small LDs forming, e.g., in the chloroplast envelopes (132), but how these small LDs precisely fuse with other LDs instead of other organelles and how LD size is controlled remain elusive.

Another class of perilipins are lipid-droplet associated proteins (LDAPs) identifed in the mesocarp of avocado (*Persea americana*) (144). The protein level of LDAPs correlates well with the fatty acid level of the TAG core, indicating this LDAP protein may be directly involved in the accumulation of TAG for LD biogenesis or the regulation of TAG metabolism (144). Similarly, a protein called major lipid droplet protein (MLDP) may have perilipin function in Chlamydomonas cells that accumulate TAG following nutrient deprivation (145). When *MLDP* was knocked down by RNA interference, increased LD size was observed (145). This supports the hypothesis that perilipins may have roles in the control of LD size and likely in LD movement and fusion with other LDs. Recruitment of enzymes for TAG synthesis or degradation to the LD may be another function of plant lipid droplet specific proteins. For example, introducing a lipid droplet specific protein from the unicellular algae Nannochloropsis into an Arabidopsis oleosin mutant reverted

LD size to be normal but did not rescue biochemical phenotypes (146), perhaps because the algal LD protein lacked the specificity to recruit Arabidopsis proteins to the LD.

Recent studies have revealed that LDs may be more complex organelles than originally thought (147, 148). One of the proposed functions for LDs is to transport neutral lipids and phospholipids between different organelles (148). Proteomic and genomic studies of mammalian and yeast LDs show that the LD membranes are rich in vesicular traffic related proteins, such as the Rabs, SNAREs and Arf (147, 149). In plants, COP (coatomer protein) vesicular traffic proteins have also been found in LD membranes, and it has been suggested that these proteins may be involved in LD biogenesis (150). In *Drosophila* and mammalian cells, cytoskeleton-dependent LD trafficking has been found, and the fusion between LDs is highly related to microtubule activities (148). Moreover, intimate contacts between LDs and other organelles have been observed with different microscopy techniques in various cell types, and the biological relevance of these contacts has been discussed as reviewed in (148). Even though the mechanism and regulation of this general transport remains unknown, this evidence suggests the size and location of LDs is able to be controlled and likely to be well regulated. In addition, it has been shown that LDs can deliver a group of 1,3-β-glucanases to the plasmodesma channel connecting two plant cells, and it is proposed that this LD-dependent transport mechanism could be an alternative pathway to transport cargoes to adjacent cells and even cell-cell signal transduction (151, 152).

Transport to the Cuticle

Plant epidermal cells have a high demand for lipid transport as they synthesize the outer cuticle at the surface of the plant which serves as a protective layer against the environment. The cuticle is known to play an important role in resistance to pathogens, and thus, plants impaired in cuticle formation have been identified not only by visually reduced waxes but also by increased susceptibility to certain pathogens (153, 154). In epidermal cells, FA export from the

chloroplast to the ER is extensive to support the synthesis of waxes (usually very long chain FA derivatives) and cutin precursors (e.g. hydroxy fatty acids and dicarboxylic acids) at the ER. These cuticle components need to be transported from the ER to the plasma membrane and then across the plasma membrane through the cell wall in order to form the cuticle where the waxes crystallize and the cutin monomers polymerize to cutin (155). While monoacylglycerol cutin precursors may be sufficiently soluble so that they may not require the use of a transporter, waxes and other precursors likely require a transport system (155). Thus far, there is little to no biochemical or molecular evidence for a mechanism of cuticular lipid precursor trafficking from the ER to the cuticle, but several plasma membrane localized ABC transporters have been genetically characterized and implicated in cutin precursor and wax transport across the plasma membrane. Plants with mutated forms of ABCG12/WBC12/CER5 have a cuticle with reduced wax components, in particular C29 alkanes (154, 156). ABCG12 is hypothesized to function as a wax transporter because wax lacking in the cuticle accumulates within the cell (154, 156). When ABCG11/WBC11 is mutated or knocked-out, plants have reduced levels of both cuticular waxes and cutin (156, 157). Like with *abcg12* mutants, waxes were found to accumulate in the cells of *abcg11* mutants (156, 157). The double mutants of ABCG11 and ABCG12 do not completely deplete the cuticular wax load, and the two mutations do not have additive effects (157). This suggests the involvement of other transporters as well as a possible interaction between ABCG11 and ABCG12, which are both half transporters with similar mutant phenotypes (156). ABCG12 has been confirmed to require the formation of a heterodimer with ABCG11 in order to be trafficked to the plasma membrane while ABCG11 can form a homodimer and does not require ABCG12 for proper localization to the plasma membrane (158). This dimerization pattern and the mutant phenotypes suggest the ABCG11/ABCG12 heterodimer is responsible for transport of cuticular waxes while the ABCG11 homodimer is responsible for transport of cutin precursors, but this has yet to be confirmed at the biochemical level. Other ABC transporters with genetic evidence of involvement in cuticular lipid transport

include ABCG13 and ABCG32 (159, 160). Mutants carrying *abcg13* alleles have reduced levels of cutin monomers, but this phenotype is restricted to flower petals (159). The knockout of ABCG32 (*permeable cuticle1, pec1*) has reduced levels of cutin monomers, but the cuticle is only structurally impaired in the layer closest to the cell wall suggesting ABCG32 is only involved in transport to this layer (160). Homologs of ABCG32 have been identified in both rice and barley with mutants of each showing similar phenotypes as that of Arabidopsis suggesting a conserved function across plant species (161). There are several other ABC transporters encoded by genes with high expression in the epidermal cells that may be involved in cuticular lipid transport, but they have yet to be investigated (156).

Another group of proteins implicated in cuticular lipid transport include the Lipid Transfer Proteins (LTPs). By definition, LTPs are small (~9 kDa) proteins which have the ability to bind and transfer lipids *in vitro* (162). Historically, LTPs were proposed to be involved in intracellular lipid transfer, but more recently LTPs are thought to more likely mediate cuticular lipid transport as a large number of LTPs have been localized to the plasma membrane, cell wall, and surface wax (163-167). Mutants carrying mutations in two GPI-anchored LTPs, LTPG1 and LTPG2, have been shown to have reduced stem cuticular wax with further wax reduction in the double mutant over either single mutant (168-170). Both LTPs were chosen for study based on the high expression of the respective genes in epidermal tissue (169-171). LTPG1 has been confirmed to have the ability to bind lipids, and it has been localized to the extracellular space of epidermal cells (169, 171). While this evidence supports a role for LTPG1 and LTPG2 in cuticular lipid transport, direct biochemical evidence is still lacking.

Transport to the Symbiosome

One membrane unique to a subset of plants is the symbiosome formed around symbiotic bacteria in root nodules of legumes. The symbiosome is derived from the plasma membrane of the plant, but its lipid composition is modified by incorporation of considerable amounts of

DGDG which is produced in the chloroplast (172-174). As bacteria replicate, more symbiosome membrane must be formed which requires the transport of plant lipids (172, 173). Lipid transport to the symbiosome may occur by both vesicular and non-vesicular mechanisms. It is not known how vesicles are specifically targeted to the symbiosome rather than the plasma membrane or vacuoles, which both share similar membrane properties to the symbiosome (172, 173). SNAREs are proteins well known for their involvement in directing vesicles to target membranes (175).There is some evidence that symbiosome specific SNARE proteins, such as MtSYP132, may be involved in vesicular trafficking to the symbiosome, but this hypothesis is based only on bioinformatics and immunolocalization to the symbiosome (176).

For non-vesicular lipid transport to the symbiosome, LTPs are thought to be major contributors. One LTP implicated in lipid transport to the symbiosome, AsE246, was discovered in Chinese milk vetch (177). AsE246 gene expression is specific to root nodules, the protein has been localized to the symbiosome membrane and binds several membrane lipids *in vitro* and DGDG *in vivo* (177). Both overexpression and knockdown lines for AsE246 were analyzed. The overexpression lines showed increased nodule formation while the knockdown lines had impaired symbiosome development with lower lipid levels in the nodules (177). Another LTP specifically expressed in nodules of Medicago, MtN5, was identified as increasing nodulation and decreasing nodulation in overexpression and knockdown lines, respectively, and it was also shown to bind lyso-lipids *in vitro* (178). These findings suggest that AsE246 and MtN5 are involved in lipid transport to the symbiosome.

Sphingolipid Trafficking

Most of this review has focused on the trafficking of glycerolipids, but as sphingolipids have many important roles in plant cells (e.g. protein trafficking, pathogen defense, signaling, structure, etc.), current knowledge about their trafficking is relevant to plant cell biology. Sphingolipid synthesis takes place in both the ER and the golgi with ceramide and

glucosylceramide (GlcCer) synthesis in the ER and glycosyl inositolphosphoceramide (GIPC) synthesis in the golgi (179). Because of this compartmentalization of synthesis, ceramide must be transported from the ER to the golgi. This transport may occur by vesicular or non-vesicular means. In mammals, this ER to golgi ceramide transport occurs non-vesicularly by way of ceramide transfer protein (CERT), however, no CERT-like proteins have been identified in plants (179-181). Plant sphingolipids are most abundant in the plasma and vacuolar membranes, and this means sphingolipids must be transported from both the ER and golgi in order to accumulate in these other membranes (179, 182). This transport could also occur by vesicular or non-vesicular means. A major hypothesis in the field is that much of the transport occurs through the vesicular pathway. The vesicular pathway has been well-studied in yeast and mammals, but much information is lacking in plants. The reader is referred to the following papers for information on vesicular trafficking in plants and how it may differ from that in yeast and mammals (183, 184). There is evidence sphingolipids are involved in targeting vesicular trafficking by forming distinct microdomains, but the exact mechanisms of microdomain sorting and specific membrane and protein targeting remain elusive (185-190). Thus far, only two proteins have been implicated in sphingolipid transport in plants. The first protein, Accelerated Cell Death 11 (ACD11), is known to cause programmed cell death when absent and was originally characterized as having sphingosine transfer activity *in vitro*, but the rates were low and sphingosine can move through an aqueous phase on its own (191). ACD11 is unable to bind either ceramide or glycosphingolipids (191). ACD11 is localized to the cytosol, but it is able to interact with membrane associated proteins BPA1, PRA7, and PRA8 *in vitro* and BPA1 *in vivo* (192). BPA1 is of unknown function, but PRA7 and PRA8 may have roles in ER to golgi trafficking based on their relation to mammalian proteins localized to the golgi (192). Recently ACD11 was shown to be a ceramide-1-phosphate (C1P) transfer protein by *in vitro* binding assays, crystallization, and accumulation of C1P in the *acd11* mutant (193). As C1P is usually kept at low levels under normal conditions and is involved in the regulation of programmed cell

death, it is unlikely ACD11 has a role in normal trafficking of sphingolipids to destination membranes (193). The second protein implicated in sphingolipid transfer in plants is Glycolipid Transfer Protein 1 (GLTP1). GLTP1 has the ability to transfer glycosphingolipids *in vitro*, but there is no genetic or *in vivo* data available (194). Another GLTP, *At1g21360*, is predicted to have possible sphingolipid transfer abilities, but no work has been published on this protein (194). It is possible these GLTPs could have roles in the trafficking of sphingolipids in plants.

Concluding Remarks

Plant cells have many unique requirements for lipid trafficking, and the underlying mechanisms are just beginning to be uncovered. Few transporters have been confirmed, and non-vesicular trafficking may account for much of the transport. Some of the major questions remaining include the following: What is the mechanism for transporting FAs to the ER? How are lipids exported from the chloroplast to other organelles? Which lipid(s) is/are transported from the ER to the chloroplast for galactolipid synthesis? How are thylakoid membranes assembled from envelope derived lipids and maintained? How are lipids trafficked specifically to other structures such as the vacuole, cuticle, and symbiosome? What role do lipid droplets play in lipid trafficking? How are the bulk of sphingolipids transported to their destination membranes? In pursuing these questions, lipid transfer phenomena in plants cells provide rich opportunities for discovery of novel mechanisms and proteins catalyzing lipid transport.

REFERENCES

REFERENCES

1. Oldroyd GE, Murray JD, Poole PS, & Downie JA (2011) The rules of engagement in the legume-rhizobial symbiosis. *Annu Rev Genet* 45:119-144.

2. Dormann P & Benning C (2002) Galactolipids rule in seed plants. *Trends Plant Sci* 7(3):112-118.

3. Andersson MX & Dörmann P (2009) Chloroplast membrane lipid biosynthesis and transport. *The Chloroplast,* Plant Cell Monographs, eds Sandelius A & Aronsson H (Springer Berlin Heidelberg), Vol 13, pp 125-158.

4. Fagone P & Jackowski S (2009) Membrane phospholipid synthesis and endoplasmic reticulum function. *J Lipid Res* 50 Suppl:S311-316.

5. Li-Beisson Y*, et al.* (2013) Acyl-lipid metabolism. in *The Arabidopsis book / American Society of Plant Biologists*, p e0161.

6. Frentzen M, Heinz E, McKeon TA, & Stumpf PK (1983) Specificities and selectivities of glycerol-3-phosphate acyltransferase and monoacylglycerol-3-phosphate acyltransferase from pea and spinach chloroplasts. *Eur J Biochem* 129(3):629-636.

7. Frentzen M (1986) Biosynthesis and desaturation of the different diacylglycerol moieties in higher plants. *J Plant Physiol* 124(3-4):193-209.

8. Browse J, Warwick N, Somerville CR, & Slack CR (1986) Fluxes through the prokaryotic and eukaryotic pathways of lipid synthesis in the '16:3' plant Arabidopsis thaliana. *Biochem J* 235(1):25-31.

9. Mongrand S, Bessoule JJ, Cabantous F, & Cassagne C (1998) The C-16 : 3/C-18 : 3 fatty acid balance in photosynthetic tissues from 468 plant species. *Phytochemistry* 49(4):1049-1064.

10. Heinz E & Roughan PG (1983) Similarities and differences in lipid metabolism of chloroplasts isolated from 18:3 and 16:3 plants. *Plant Physiol* 72(2):273-279.

11. Giroud C, Gerber A, & Eichenberger W (1988) Lipids of *Chlamydomonas reinhardtii* - analysis of molecular species and intracellular site(s) of biosynthesis. *Plant Cell Physiol* 29(4):587-595.

12. Tatsuta T, Scharwey M, & Langer T (2014) Mitochondrial lipid trafficking. *Trends Cell Biol* 24(1):44-52.

13. Hu J*, et al.* (2012) Plant peroxisomes: biogenesis and function. *Plant Cell* 24(6):2279-2303.

14. van Meer G, Voelker DR, & Feigenson GW (2008) Membrane lipids: where they are and how they behave. *Nat Rev Mol Cell Biol* 9(2):112-124.

15. Zachowski A (2012) New insights in membrane lipid asymmetry in animal and plant cells *Transmembrane Dynamics of Lipids*, ed Devaux PF, Herrmann, A. (John Wiley & Sons, Inc., Hoboken, New Jersey), pp 47-64.

16. Raggers RJ, Pomorski T, Holthuis JC, Kalin N, & van Meer G (2000) Lipid traffic: the ABC of transbilayer movement. *Traffic* 1(3):226-234.

17. Baldridge RD & Graham TR (2013) Two-gate mechanism for phospholipid selection and transport by type IV P-type ATPases. *Proc Natl Acad Sci USA* 110(5):E358-367.

18. Sprong H, van der Sluijs P, & van Meer G (2001) How proteins move lipids and lipids move proteins. *Nat Rev Mol Cell Biol* 2(7):504-513.

19. Holthuis JC & Levine TP (2005) Lipid traffic: floppy drives and a superhighway. *Nat Rev Mol Cell Biol* 6(3):209-220.

20. Dormann P, Voelker TA, & Ohlrogge JB (1995) Cloning and expression in *Escherichia coli* of a novel thioesterase from *Arabidopsis thaliana* specific for long-chain acyl-acyl carrier proteins. *Arch Biochem Biophys* 316(1):612-618.

21. Salas JJ & Ohlrogge JB (2002) Characterization of substrate specificity of plant FatA and FatB acyl-ACP thioesterases. *Arch Biochem Biophys* 403(1):25-34.

22. Bonaventure G, Salas JJ, Pollard MR, & Ohlrogge JB (2003) Disruption of the FATB gene in Arabidopsis demonstrates an essential role of saturated fatty acids in plant growth. *Plant Cell* 15(4):1020-1033.

23. Pollard M & Ohlrogge J (1999) Testing models of fatty acid transfer and lipid synthesis in spinach leaf using in vivo oxygen-18 labeling. *Plant Physiol* 121(4):1217-1226.

24. Roughan PG & Slack CR (1977) Long-chain acyl-coenzyme A synthetase activity of spinach chloroplasts is concentrated in the envelope. *Biochem J* 162(2):457-459.

25. Joyard J & Stumpf PK (1981) Synthesis of long-chain acyl-CoA in chloroplast envelope membranes. *Plant Physiol* 67(2):250-256.

26. Schnurr JA, Shockey JM, de Boer GJ, & Browse JA (2002) Fatty acid export from the chloroplast. Molecular characterization of a major plastidial acyl-coenzyme A synthetase from Arabidopsis. *Plant Physiol* 129(4):1700-1709.

27. Shockey JM, Fulda MS, & Browse JA (2002) Arabidopsis contains nine long-chain acyl-coenzyme a synthetase genes that participate in fatty acid and glycerolipid metabolism. *Plant Physiol* 129(4):1710-1722.

28. Zhao LF, Katavic V, Li FL, Haughn GW, & Kunst L (2010) Insertional mutant analysis reveals that long-chain acyl-CoA synthetase 1 (LACS1), but not LACS8, functionally overlaps with LACS9 in Arabidopsis seed oil biosynthesis. *Plant J* 64(6):1048-1058.

29. Lu S*, et al.* (2009) Arabidopsis CER8 encodes LONG-CHAIN ACYL-COA SYNTHETASE 1 (LACS1) that has overlapping functions with LACS2 in plant wax and cutin synthesis. *Plant J* 59(4):553-564.

30. Li N*, et al.* (2015) FAX1, a novel membrane protein mediating plastid fatty acid export. *PLoS Biology* 13(2):e1002053.

31. Roston RL, Hurlock AK, & Benning C (2014) Plastidic ABC Proteins. *Plant ABC Transporters,* Signaling and Communication in Plants, ed Geisler M (Springer, Cham), Vol 22, pp 103-136.

32. Kim S*, et al.* (2013) AtABCA9 transporter supplies fatty acids for lipid synthesis to the endoplasmic reticulum. *Proc Natl Acad Sci USA* 110(2):773-778.

33. Jouhet J, Marechal E, & Block MA (2007) Glycerolipid transfer for the building of membranes in plant cells. *Prog Lipid Res* 46(1):37-55.

34. Baker A, Graham IA, Holdsworth M, Smith SM, & Theodoulou FL (2006) Chewing the fat: beta-oxidation in signalling and development. *Trends Plant Sci* 11(3):124-132.

35. Ajjawi I, Lu Y, Savage LJ, Bell SM, & Last RL (2010) Large-scale reverse genetics in Arabidopsis: case studies from the Chloroplast 2010 Project. *Plant Physiol* 152(2):529-540.

36. Xiao S & Chye ML (2009) An Arabidopsis family of six acyl-CoA-binding proteins has three cytosolic members. *Plant Physiol Biochem* 47(6):479-484.

37. Xiao S & Chye ML (2011) New roles for acyl-CoA-binding proteins (ACBPs) in plant development, stress responses and lipid metabolism. *Prog Lipid Res* 50(2):141-151.

38. Schjerling CK*, et al.* (1996) Disruption of the gene encoding the acyl-CoA-binding protein (ACB1) perturbs acyl-CoA metabolism in *Saccharomyces cerevisiae*. *J Biol Chem* 271(37):22514-22521.

39. Faergeman NJ & Knudsen J (1997) Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. *Biochem J* 323:1-12.

40. Xiao S, Chen QF, & Chye ML (2009) Light-regulated Arabidopsis ACBP4 and ACBP5 encode cytosolic acyl-CoA-binding proteins that bind phosphatidylcholine and oleoyl-CoA ester. *Plant Physiol Biochem* 47(10):926-933.

41. Yurchenko OP*, et al.* (2009) A 10-kDa acyl-CoA-binding protein (ACBP) from *Brassica napus* enhances acyl exchange between acyl-CoA and phosphatidylcholine. *Plant Biotech J* 7(7):602-610.

42. Xiao S, Li HY, Zhang JP, Chan SW, & Chye ML (2008) Arabidopsis acyl-CoA-binding proteins ACBP4 and ACBP5 are subcellularly localized to the cytosol and ACBP4 depletion affects membrane lipid composition. *Plant Mol Biol* 68(6):571-583.

43. Leung KC, Li HY, Mishra G, & Chye ML (2004) ACBP4 and ACBP5, novel Arabidopsis acyl-CoA-binding proteins with kelch motifs that bind oleoyl-CoA. *Plant Mol Biol* 55(2):297-309.

44. Yurchenko OP & Weselake RJ (2011) Involvement of low molecular mass soluble acyl-CoA-binding protein in seed oil biosynthesis. *N Biotechnol* 28(2):97-109.

45. Li HY, Xiao S, & Chye ML (2008) Ethylene- and pathogen-inducible Arabidopsis acyl-CoA-binding protein 4 interacts with an ethylene-responsive element binding protein. *J Exp Bot* 59(14):3997-4006.

46. Wang Z & Benning C (2012) Chloroplast lipid synthesis and lipid trafficking through ER-plastid membrane contact sites. *Biochem Sos Trans* 40(2):457-463.

47. Bates PD, Ohlrogge JB, & Pollard M (2007) Incorporation of newly synthesized fatty acids into cytosolic glycerolipids in pea leaves occurs via acyl editing. *J Biol Chem* 282(43):31206-31216.

48. Mongrand S, Cassagne C, & Bessoule JJ (2000) Import of lyso-phosphatidylcholine into chloroplasts likely at the origin of eukaryotic plastidial lipids. *Plant Physiol* 122(3):845-852.

49. Andersson MX, Kjellberg JM, & Sandelius AS (2004) The involvement of cytosolic lipases in converting phosphatidyl choline to substrate for galactolipid synthesis in the chloroplast envelope. *Biochim Biophys Acta* 1684:46-53.

50. Wang K, Froehlich JE, Zienkiewicz A, Hersh HL, & Benning C (2017) A Plastid Phosphatidylglycerol Lipase Contributes to the Export of Acyl Groups from Plastids for Seed Oil Biosynthesis. *Plant Cell* 29(7):1678-1696.

51. Li XB*, et al.* (2012) A galactoglycerolipid lipase is required for triacylglycerol accumulation and survival following nitrogen deprivation in *Chlamydomonas reinhardtii*. *Plant Cell* 24(11):4670-4686.

52. Hartel H, Dormann P, & Benning C (2000) DGD1-independent biosynthesis of extraplastidic galactolipids after phosphate deprivation in Arabidopsis. *Proc Natl Acad Sci USA* 97(19):10649-10654.

53. Awai K*, et al.* (2001) Two types of MGDG synthase genes, found widely in both 16:3 and 18:3 plants, differentially mediate galactolipid syntheses in photosynthetic and nonphotosynthetic tissues in *Arabidopsis thaliana*. *Proc Natl Acad Sci USA* 98(19):10960-10965.

54. Kelly AA, Froehlich JE, & Dormann P (2003) Disruption of the two digalactosyldiacylglycerol synthase genes DGD1 and DGD2 in Arabidopsis reveals the existence of an additional enzyme of galactolipid synthesis. *Plant Cell* 15(11):2694-2706.

55. Andersson MX, Stridh MH, Larsson KE, Liljenberg C, & Sandelius AS (2003) Phosphate-deficient oat replaces a major portion of the plasma membrane phospholipids with the galactolipid digalactosyldiacylglycerol. *FEBS Lett* 537(1-3):128-132.

56. Jouhet J*, et al.* (2004) Phosphate deprivation induces transfer of DGDG galactolipid from chloroplast to mitochondria. *J Cell Biol* 167(5):863-874.

57. Andersson MX, Larsson KE, Tjellstrom H, Liljenberg C, & Sandelius AS (2005) The plasma membrane and the tonoplast as major targets for phospholipid- to-glycolipid replacement and stimulation of phospholipases in the plasma membrane. *J Biol Chem* 280(30):27578-27586.

58. Holzl G*, et al.* (2009) The role of diglycosyl lipids in photosynthesis and membrane lipid homeostasis in Arabidopsis. *Plant Physiol* 150(3):1147-1159.

59. Browse J, McConn M, James D, Jr., & Miquel M (1993) Mutants of Arabidopsis deficient in the synthesis of alpha-linolenate: biochemical and genetic characterization of the endoplasmic reticulum linoleoyl desaturase. *J Biol Chem* 268(22):16345-16351.

60. Browse J, McCourt P, & Somerville C (1986) A mutant of Arabidopsis deficient in c(18:3) and c(16:3) leaf lipids. *Plant Physiol* 81(3):859-864.

61. McConn M, Hugly S, Browse J, & Somerville C (1994) A mutation at the Fad8 locus of Arabidopsis identifies a 2nd chloroplast omega-3 desaturase. *Plant Physiol* 106(4):1609-1614.

62. McConn M & Browse J (1996) The critical requirement for linolenic acid is pollen development, not photosynthesis, in an Arabidopsis mutant. *Plant Cell* 8(3):403-416.

63. Benning C & Ohta H (2005) Three enzyme systems for galactoglycerolipid biosynthesis are coordinately regulated in plants. *J Biol Chem* 280(4):2397-2400.

64. Dorne AJ, Joyard J, Block MA, & Douce R (1985) Localization of phosphatidylcholine in outer envelope membrane of spinach chloroplasts. *J Cell Biol* 100(5):1690-1697.

65. Bessoule JJ, Testet E, & Cassagne C (1995) Synthesis of phosphatidylcholine in the chloroplast envelope after import of lysophosphatidylcholine from endoplasmic reticulum membranes. *Eur J Biochem* 228(2):490-497.

66. Muthan B, Roston RL, Froehlich JE, & Benning C (2013) Probing Arabidopsis chloroplast diacylglycerol pools by selectively targeting bacterial diacylglycerol kinase to suborganellar membranes. *Plant Physiol* 163(1):61-74.

67. Miege C*, et al.* (1999) Biochemical and topological properties of type A MGDG synthase, a spinach chloroplast envelope enzyme catalyzing the synthesis of both prokaryotic and eukaryotic MGDG. *Eur J Biochem* 265(3):990-1001.

68. Tietje C & Heinz E (1998) Uridine-diphospho-sulfoquinovose: diacylglycerol sulfoquinovosyltransferase activity is concentrated in the inner membrane of chloroplast envelopes. *Planta* 206(1):72-78.

69. Froehlich JE, Benning C, & Dormann P (2001) The digalactosyldiacylglycerol (DGDG) synthase DGD1 is inserted into the outer envelope membrane of chloroplasts in a manner independent of the general import pathway and does not depend on direct interaction with monogalactosyldiacylglycerol synthase for DGDG biosynthesis. *J Biol Chem* 276(34):31806-31812.

70. Xu C, Fan J, Riekhof W, Froehlich JE, & Benning C (2003) A permease-like protein involved in ER to thylakoid lipid transfer in Arabidopsis. *EMBO J* 22(10):2370-2379.

71. Xu C, Fan J, Cornish AJ, & Benning C (2008) Lipid trafficking between the endoplasmic reticulum and the plastid in Arabidopsis requires the extraplastidic TGD4 protein. *Plant Cell* 20(8):2190-2204.

72. Lu B, Xu C, Awai K, Jones AD, & Benning C (2007) A small ATPase protein of Arabidopsis, TGD3, involved in chloroplast lipid import. *J Biol Chem* 282(49):35945-35953.

73. Awai K, Xu C, Tamot B, & Benning C (2006) A phosphatidic acid-binding protein of the chloroplast inner envelope membrane involved in lipid trafficking. *Proc Natl Acad Sci USA* 103(28):10817-10822.

74. Xu CC, Moellering ER, Muthan B, Fan JL, & Benning C (2010) Lipid transport mediated by Arabidopsis TGD proteins is unidirectional from the endoplasmic reticulum to the plastid. *Plant Cell Physiol* 51(6):1019-1028.

75. Xu C, Fan J, Froehlich JE, Awai K, & Benning C (2005) Mutation of the TGD1 chloroplast envelope protein affects phosphatidate metabolism in Arabidopsis. *Plant Cell* 17(11):3094-3110.

76. 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(4):614-623.

77. Wang Z, Anderson NS, & Benning C (2013) The phosphatidic acid binding site of the Arabidopsis Trigalactosyldiacylglycerol 4 (TGD4) protein required for lipid import into chloroplasts. *J Biol Chem* 288(7):4763-4771.

78. Roston RL, Gao J, Murcha MW, Whelan J, & Benning C (2012) TGD1, -2, and -3 proteins involved in lipid trafficking form ATP-binding cassette (ABC) transporter with multiple substrate-binding proteins. *J Biol Chem* 287(25):21406-21415.

79. Roston R, Gao J, Xu C, & Benning C (2011) Arabidopsis chloroplast lipid transport protein TGD2 disrupts membranes and is part of a large complex. *Plant J* 66(5):759-769.

80. Fan J, Zhai Z, Yan C, & Xu C (2015) Arabidopsis TRIGALACTOSYLDIACYLGLYCEROL5 Interacts with TGD1, TGD2, and TGD4 to Facilitate Lipid Transfer from the Endoplasmic Reticulum to Plastids. *Plant Cell* 27(10):2941-2955.

81. Lu BB & Benning C (2009) A 25-Amino Acid Sequence of the Arabidopsis TGD2 Protein Is Sufficient for Specific Binding of Phosphatidic Acid. *J Biol Chem* 284(26):17420-17427.

82. Testerink C & Munnik T (2011) Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. *J Exp Bot* 62(7):2349-2361.

83. Hamilton JA, Bhamidipati SP, Kodali DR, & Small DM (1991) The interfacial conformation and transbilayer movement of diacylglycerols in phospholipid bilayers. *J Biol Chem* 266(2):1177-1186.

84. Benning C (2009) Mechanisms of lipid transport involved in organelle biogenesis in plant cells. *Annu Rev Cell Del Biol* 25:71-91.

85. Whatley JM, Mclean B, & Juniper BE (1991) Continuity of chloroplast and endoplasmic reticulum membranes in *Phaseolus vulgaris*. *New Phytol* 117(2):209-217.

86. Mclean B, Whatley JM, & Juniper BE (1988) Continuity of chloroplast and endoplasmic reticulum membranes in *Chara* and *Equisetum*. *New Phytol* 109(1):59-65.

87. Wooding FBP & Northcot.Dh (1965) Association of endoplasmic reticulum and plastids in Acer and Pinus. *Am J Bot* 52(5):526-531.

88. Tan XL*, et al.* (2011) A Brassica napus lipase locates at the membrane contact sites involved in chloroplast development. *PLoS One* 6(10):e26831.

89. Andersson MX, Goksor M, & Sandelius AS (2007) Optical manipulation reveals strong attracting forces at membrane contact sites between endoplasmic reticulum and chloroplasts. *J Biol Chem* 282(2):1170-1174.

90. Fujimoto M & Hayashi T (2011) New insights into the role of mitochondria-associated endoplasmic reticulum membrane. *Int Rev Cell Mol Biol* 292:73-117.

91. Raturi A & Simmen T (2013) Where the endoplasmic reticulum and the mitochondrion tie the knot: the mitochondria-associated membrane (MAM). *BBA Mol Cell Res* 1833(1):213-224.

92. Vance JE & Shiao YJ (1996) Intracellular trafficking of phospholipids: import of phosphatidylserine into mitochondria. *Anticancer Res* 16(3B):1333-1339.

93. Shiao YJ, Lupo G, & Vance JE (1995) Evidence that phosphatidylserine is imported into mitochondria via a mitochondria-associated membrane and that the majority of mitochondrial phosphatidylethanolamine is derived from decarboxylation of phosphatidylserine. *J Biol Chem* 270(19):11190-11198.

94. Mehrshahi P*, et al.* (2013) Transorganellar complementation redefines the biochemical continuity of endoplasmic reticulum and chloroplasts. *Proc Natl Acad Sci USA* 110(29):12126-12131.

95. Mehrshahi P, Johnny C, & Dellapenna D (2014) Redefining the metabolic continuity of chloroplasts and ER. *Trends Plant Sci* Advance Online Publication:10.1016/j.tplants.2014.1002.1013.

96. Chernomordik LV & Kozlov MM (2005) Membrane hemifusion: crossing a chasm in two leaps. *Cell* 123(3):375-382.

97. Chernomordik LV & Kozlov MM (2008) Mechanics of membrane fusion. *Nat Struct Mol Biol* 15(7):675-683.

98. Hernandez JM*, et al.* (2012) Membrane fusion intermediates via directional and full assembly of the SNARE complex. *Science* 336(6088):1581-1584.

99. Kornmann B*, et al.* (2009) An ER-mitochondria tethering complex revealed by a synthetic biology screen. *Science* 325(5939):477-481.

100. Marechal E, Block MA, Dorne AJ, Douce R, & Joyard J (1997) Lipid synthesis and metabolism in the plastid envelope. *Physiol Plant* 100(1):65-77.

101. Muhlethaler K & Freywyssling A (1959) Entwicklung und struktur der proplastiden. *J Biophys Biochem Cytol* 6(3):507-512.

102. Morre DJ, Sellden G, Sundqvist C, & Sandelius AS (1991) Stromal low temperature compartment derived from the inner membrane of the chloroplast envelope. *Plant Physiol* 97(4):1558-1564.

103. Andersson MX, Kjellberg JM, & Sandelius AS (2001) Chloroplast biogenesis: regulation of lipid transport to the thylakoid in chloroplasts isolated from expanding and fully expanded leaves of pea. *Plant Physiol* 127(1):184-193.

104. Rantfors M, Evertsson I, Kjellberg JM, & Sandelius AS (2000) Intraplastidial lipid trafficking: regulation of galactolipid release from isolated chloroplast envelope. *Physiol Plant* 110(2):262-270.

105. Westphal S, Soll J, & Vothknecht UC (2001) A vesicle transport system inside chloroplasts. *FEBS Lett* 506(3):257-261.

106. Rawyler A, Meylan M, & Siegenthaler PA (1992) Galactolipid export from envelope to thylakoid membranes in intact chloroplasts: I. characterization and involvement in thylakoid lipid asymmetry. *Biochim Biophys Acta* 1104(2):331-341.

107. Keren N, Ohkawa H, Welsh EA, Liberton M, & Pakrasi HB (2005) Psb29, a conserved 22-kD protein, functions in the biogenesis of photosystem II complexes in *Synechocystis* and *Arabidopsis*. *Plant Cell* 17(10):2768-2781.

108. Hugueney P*, et al.* (1995) Identification of a plastid protein involved in vesicle fusion and/or membrane protein translocation. *Proc Natl Acad Sci USA* 92(12):5630-5634.

109. Kroll D*, et al.* (2001) VIPP1, a nuclear gene of *Arabidopsis thaliana* essential for thylakoid membrane formation. *Proc Natl Acad Sci USA* 98(7):4238-4242.

110. Li HM, Kaneko Y, & Keegstra K (1994) Molecular cloning of a chloroplastic protein associated with both the envelope and thylakoid membranes. *Plant Mol Biol* 25(4):619-632.

111. Vothknecht UC, Otters S, Hennig R, & Schneider D (2012) Vipp1: a very important protein in plastids?! *J Exp Bot* 63(4):1699-1712.

112. Gao H & Xu X (2009) Depletion of Vipp1 in *Synechocystis sp.* PCC 6803 affects photosynthetic activity before the loss of thylakoid membranes. *FEMS Microbiol Lett* 292(1):63-70.

113. Hayashi-Tsugane M*, et al.* (2014) A mutable albino allele in rice reveals that formation of thylakoid membranes requires the SNOW-WHITE LEAF1 gene. *Plant Cell Physiol* 55(1):3-15.

114. Andersson MX & Sandelius AS (2004) A chloroplast-localized vesicular transport system: a bio-informatics approach. *BMC Genomics* 5(1):40.

115. Khan NZ, Lindquist E, & Aronsson H (2013) New putative chloroplast vesicle transport components and cargo proteins revealed using a bioinformatics approach: an arabidopsis model. *PLoS One* 8(4):e59898.

116. Brandizzi F (2011) Is there a COPII-mediated membrane traffic in chloroplasts? *Traffic* 12(1):9-11.

117. Karim S*, et al.* (2014) A novel chloroplast localized Rab GTPase protein CPRabA5e is involved in stress, development, thylakoid biogenesis and vesicle transport in Arabidopsis. *Plant Mol Biol* 84(6):675-692.

118. Gounaris K, Barber J, & Harwood JL (1986) The thylakoid membranes of higher plant chloroplasts. *Biochem J* 237(2):313-326.

119. Rawyler A*, et al.* (1987) The transmembrane distribution of galactolipids in chloroplast thylakoids is universal in a wide variety of temperate climate plants. *Photosynth Res* 11(1):3-13.

120. Viotti C (2014) ER and vacuoles: never been closer. *Front Plant Sci* 5(20):1-7.

121. Yoshida S & Uemura M (1986) Lipid composition of plasma membranes and tonoplasts isolated from etiolated seedlings of mung bean (*Vigna radiata L.*). *Plant Physiol* 82(3):807-812.

122. Leach RP, Wheeler KP, Flowers TJ, & Yeo AR (1990) Molecular markers for ion compartmentation in cells of higher plants 2: lipid composition of the tonoplast of the halophyte *Suaeda maritima (L) Dum*. *J Exp Bot* 41(230):1089-1094.

123. Yamaryo Y, Dubots E, Albrieux C, Baldan B, & Block MA (2008) Phosphate availability affects the tonoplast localization of PLDzeta2, an *Arabidopsis thaliana* phospholipase D. *FEBS Lett* 582(5):685-690.

124. Nováková P*, et al.* (2014) SAC phosphoinositide phosphatases at the tonoplast mediate vacuolar function in Arabidopsis. *Proc Natl Acad Sci USA* 111(7):2818-2823.

125. Viotti C*, et al.* (2013) The endoplasmic reticulum is the main membrane source for biogenesis of the lytic vacuole in Arabidopsis. *Plant Cell* 25(9):3434-3449.

126. Taiz L (1992) The plant vacuole. *J Exp Biol* 172(Pt 1):113-122.

127. Feraru E*, et al.* (2010) The AP-3 beta adaptin mediates the biogenesis and function of lytic vacuoles in Arabidopsis. *Plant Cell* 22(8):2812-2824.

128. Batistic O, Waadt R, Steinhorst L, Held K, & Kudla J (2010) CBL-mediated targeting of CIPKs facilitates the decoding of calcium signals emanating from distinct cellular stores. *Plant J* 61(2):211-222.

129. De Marchis F, Bellucci M, & Pompa A (2013) Traffic of human alpha-mannosidase in plant cells suggests the presence of a new endoplasmic reticulum-to-vacuole pathway without involving the golgi complex. *Plant Physiol* 161(4):1769-1782.

130. Pedrazzini E, Komarova NY, Rentsch D, & Vitale A (2013) Traffic routes and signals for the tonoplast. *Traffic* 14(6):622-628.

131. Tavernier E & Pugin A (1995) Transbilayer distribution of phosphatidylcholine and phosphatidylethanolamine in the vacuolar membrane of *Acer pseudoplatanus* cells. *Biochimie* 77(3):174-181.

132. Chapman KD, Dyer JM, & Mullen RT (2012) Biogenesis and functions of lipid droplets in plants: thematic review series: lipid droplet synthesis and metabolism: from yeast to man. *J Lipid Res* 53(2):215-226.

133. Walther TC & Farese RV, Jr. (2012) Lipid droplets and cellular lipid metabolism. *Annu Rev Biochem* 81:687-714.

134. Brasaemle DL & Wolins NE (2012) Packaging of fat: an evolving model of lipid droplet assembly and expansion. *J Biol Chem* 287(4):2273-2279.

135. Murphy DJ (2012) The dynamic roles of intracellular lipid droplets: from archaea to mammals. *Protoplasma* 249(3):541-585.

136. Frey-Wyssling A, Grieshaber E, & Mühlethaler K (1963) Origin of spherosomes in plant cells. *J Ultrastruct Res* 8(5–6):506-516.

137. Wanner G & Theimer RR (1978) Membranous appendices of spherosomes (oleosomes): possible role in fat utilization in germinating oil seeds. *Planta* 140(2):163-169.

138. Chapman KD & Ohlrogge JB (2012) Compartmentation of triacylglycerol accumulation in plants. *J Biol Chem* 287(4):2288-2294.

139. Siloto RM*, et al.* (2006) The accumulation of oleosins determines the size of seed oilbodies in Arabidopsis. *Plant Cell* 18(8):1961-1974.

140. Murphy DJ & Vance J (1999) Mechanisms of lipid-body formation. *Trends Biochem Sci* 24(3):109-115.

141. Adeyo O*, et al.* (2011) The yeast lipin orthologue Pah1p is important for biogenesis of lipid droplets. *J Cell Biol* 192(6):1043-1055.

142. Skinner JR*, et al.* (2009) Diacylglycerol enrichment of endoplasmic reticulum or lipid droplets recruits Perilipin 3/TIP47 during lipid storage and mobilization. *J Biol Chem* 284(45):30941-30948.

143. Moellering ER, Muthan B, & Benning C (2010) Freezing tolerance in plants requires lipid remodeling at the outer chloroplast membrane. *Science* 330(6001):226-228.

144. Gidda SK*, et al.* (2013) Lipid droplet-associated proteins (LDAPs) are involved in the compartmentalization of lipophilic compounds in plant cells. *Plant Signal Behav* 8(11).

145. Moellering ER & Benning C (2010) RNA interference silencing of a major lipid droplet protein affects lipid droplet size in *Chlamydomonas reinhardtii*. *Eukaryot Cell* 9(1):97-106.

146. Vieler A, Brubaker SB, Vick B, & Benning C (2012) A lipid droplet protein of Nannochloropsis with functions partially analogous to plant oleosins. *Plant Physiol* 158(4):1562-1569.

147. Yang L*, et al.* (2012) The proteomics of lipid droplets: structure, dynamics, and functions of the organelle conserved from bacteria to humans. *J Lipid Res* 53(7):1245-1253.

148. Zehmer JK*, et al.* (2009) A role for lipid droplets in inter-membrane lipid traffic. *Proteomics* 9(4):914-921.

149. Murphy DJ*, et al.* (1991) A class of amphipathic proteins associated with lipid storage bodies in plants: possible similarities with animal serum apolipoproteins. *Biochim Biophys Acta* 1088(1):86-94.

150. Kalantari F, Bergeron JJM, & Nilsson T (2010) Biogenesis of lipid droplets - how cells get fatter. *Mol Membr Biol* 27(8):462-468.

151. Paul LK, Rinne PLH, & Van Der Schoot C (2014) Refurbishing the plasmodesmal chamber: a role for lipid bodies? *Front Plant Sci* 5(40):1-10.

152. van der Schoot C, Paul LK, Paul SB, & Rinne PL (2011) Plant lipid bodies and cell-cell signaling: a new role for an old organelle? *Plant Signal Behav* 6(11):1732-1738.

153. Sohal AK*, et al.* (1999) Cauliflower Mosaic Virus infection stimulates lipid transfer protein gene expression in Arabidopsis. *J Exp Bot* 50(341):1727-1733.

154. Pighin JA*, et al.* (2004) Plant cuticular lipid export requires an ABC transporter. *Science* 306(5696):702-704.

155. Yeats TH & Rose JK (2013) The formation and function of plant cuticles. *Plant Physiol* 163(1):5-20.

156. Bird DA (2008) The role of ABC transporters in cuticular lipid secretion. *Plant Sci* 174(6):563-569.

157. Bird D*, et al.* (2007) Characterization of Arabidopsis ABCG11/WBC11, an ATP binding cassette (ABC) transporter that is required for cuticular lipid secretion. *Plant J* 52(3):485-498.

158. McFarlane HE, Shin JJ, Bird DA, & Samuels AL (2010) Arabidopsis ABCG transporters, which are required for export of diverse cuticular lipids, dimerize in different combinations. *Plant Cell* 22(9):3066-3075.

159. Panikashvili D, Shi JX, Schreiber L, & Aharoni A (2011) The Arabidopsis ABCG13 transporter is required for flower cuticle secretion and patterning of the petal epidermis. *New Phytol* 190(1):113-124.

160. Bessire M*, et al.* (2011) A member of the PLEIOTROPIC DRUG RESISTANCE family of ATP Binding Cassette transporters is required for the formation of a functional cuticle in Arabidopsis. *Plant Cell* 23(5):1958-1970.

161. Chen GX*, et al.* (2011) An ATP-binding cassette subfamily G full transporter is essential for the retention of leaf water in both wild barley and rice. *Proc Natl Acad Sci USA* 108(30):12354-12359.

162. Yeats TH & Rose JK (2008) The biochemistry and biology of extracellular plant lipid-transfer proteins (LTPs). *Protein Sci* 17(2):191-198.

163. Pyee J, Yu H, & Kolattukudy PE (1994) Identification of a lipid transfer protein as the major protein in the surface wax of broccoli (*Brassica oleracea*) leaves. *Arch Biochem Biophys* 311(2):460-468.

164. Pyee J & Kolattukudy PE (1995) The gene for the major cuticular wax-associated protein and three homologous genes from broccoli (*Brassica oleracea*) and their expression patterns. *Plant J* 7(1):49-59.

165. Thoma S*, et al.* (1994) Tissue-specific expression of a gene encoding a cell wall-localized lipid transfer protein from Arabidopsis. *Plant Physiol* 105(1):35-45.

166. Sterk P, Booij H, Schellekens GA, Vankammen A, & Devries SC (1991) Cell-specific expression of the carrot EP2 lipid transfer protein gene. *Plant Cell* 3(9):907-921.

167. Suh MC*, et al.* (2005) Cuticular lipid composition, surface structure, and gene expression in Arabidopsis stem epidermis. *Plant Physiol* 139(4):1649-1665.

168. Lee SB*, et al.* (2009) Disruption of glycosylphosphatidylinositol-anchored lipid transfer protein gene altered cuticular lipid composition, increased plastoglobules, and enhanced susceptibility to infection by the fungal pathogen *Alternaria brassicicola*. *Plant Physiol* 150(1):42-54.

169. Debono A*, et al.* (2009) Arabidopsis LTPG is a glycosylphosphatidylinositol-anchored lipid transfer protein required for export of lipids to the plant surface. *Plant Cell* 21(4):1230-1238.

170. Kim H*, et al.* (2012) Characterization of glycosylphosphatidylinositol-anchored lipid transfer protein 2 (LTPG2) and overlapping function between LTPG/LTPG1 and LTPG2 in cuticular wax export or accumulation in *Arabidopsis thaliana*. *Plant Cell Physiol* 53(8):1391-1403.

171. Ambrose C, DeBono A, & Wasteneys G (2013) Cell geometry guides the dynamic targeting of apoplastic GPI-linked lipid transfer protein to cell wall elements and cell borders in *Arabidopsis thaliana*. *PLoS One* 8(11):e81215.

172. Verma DP & Hong Z (1996) Biogenesis of the peribacteroid membrane in root nodules. *Trends Microbiol* 4(9):364-368.

173. Jones KM, Kobayashi H, Davies BW, Taga ME, & Walker GC (2007) How rhizobial symbionts invade plants: the Sinorhizobium-Medicago model. *Nat Rev Microbiol* 5(8):619-633.

174. Gaude N*, et al.* (2004) The galactolipid digalactosyldiacylglycerol accumulates in the peribacteroid membrane of nitrogen-fixing nodules of soybean and lotus. *J Biol Chem* 279(33):34624-34630.

175. Sanderfoot AA & Raikhel NV (1999) The specificity of vesicle trafficking: coat proteins and SNAREs. *Plant Cell* 11(4):629-642.

176. Catalano CM, Czymmek KJ, Gann JG, & Sherrier DJ (2007) *Medicago truncatula* syntaxin SYP132 defines the symbiosome membrane and infection droplet membrane in root nodules. *Planta* 225(3):541-550.

177. Lei L*, et al.* (2014) A nodule-specific lipid transfer protein AsE246 participates in transport of plant-synthesized lipids to symbiosome membrane and is essential for nodule organogenesis in Chinese Milk Vetch. *Plant Physiol* 164(2):1045-1058.

178. Pii Y*, et al.* (2009) The *Medicago truncatula* N5 gene encoding a root-specific lipid transfer protein is required for the symbiotic interaction with *Sinorhizobium meliloti*. *Mol Plant Microbe In* 22(12):1577-1587.

179. Chen M, Cahoon EB, Saucedo-Garcia M, Plasencia J, & Gavilanes-Ruiz M (2009) Plant sphingolipids: structure, synthesis and function. *Lipids in Photosynthesis: Essential and Regulatory Functions*, eds Wada H & Murata N (Springer Netherlands), Vol 30, pp 77-115.

180. Hanada K, Kumagai K, Tomishige N, & Kawano M (2007) CERT and intracellular trafficking of ceramide. *Biochim Biophys Acta* 1771(6):644-653.

181. Kudo N*, et al.* (2008) Structural basis for specific lipid recognition by CERT responsible for nonvesicular trafficking of ceramide. *Proc Natl Acad Sci USA* 105(2):488-493.

182. Markham JE, Li J, Cahoon EB, & Jaworski JG (2006) Separation and identification of major plant sphingolipid classes from leaves. *J Biol Chem* 281(32):22684-22694.

183. Ueda T & Nakano A (2002) Vesicular traffic: an integral part of plant life. *Curr Opin Plant Biol* 5(6):513-517.

184. Nebenfuhr A (2002) Vesicle traffic in the endomembrane system: a tale of COPs, Rabs and SNAREs. *Curr Opin Plant Biol* 5(6):507-512.

185. Yang H*, et al.* (2013) Sterols and sphingolipids differentially function in trafficking of the Arabidopsis ABCB19 auxin transporter. *Plant J* 74(1):37-47.

186. Markham JE*, et al.* (2011) Sphingolipids containing very-long-chain fatty acids define a secretory pathway for specific polar plasma membrane protein targeting in Arabidopsis. *Plant Cell* 23(6):2362-2378.

187. Klemm RW*, et al.* (2009) Segregation of sphingolipids and sterols during formation of secretory vesicles at the trans-golgi network. *J Cell Biol* 185(4):601-612.

188. Laloi M*, et al.* (2007) Insights into the role of specific lipids in the formation and delivery of lipid microdomains to the plasma membrane of plant cells. *Plant Physiol* 143(1):461-472.

189. Breslow DK & Weissman JS (2010) Membranes in balance: mechanisms of sphingolipid homeostasis. *Mol Cell* 40(2):267-279.

190. Lingwood D & Simons K (2010) Lipid rafts as a membrane-organizing principle. *Science* 327(5961):46-50.

191. Brodersen P*, et al.* (2002) Knockout of Arabidopsis ACCELERATED-CELL-DEATH11 encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Gene Dev* 16(4):490-502.

192. Petersen NHT*, et al.* (2009) Identification of proteins interacting with Arabidopsis ACD11. *J Plant Physiol* 166(6):661-666.

193. Simanshu Dhirendra K*, et al.* (2014) Arabidopsis Accelerated Cell Death 11, ACD11, Is a ceramide-1-phosphate transfer protein and intermediary regulator of phytoceramide levels. *Cell Rep* 6(2):388-399.

194. West G*, et al.* (2008) Identification of a glycosphingolipid transfer protein GLTP1 in *Arabidopsis thaliana*. *FEBS J* 275(13):3421-3437.

CHAPTER 2

In Vivo Lipid "Tag and Track" Approach Shows Acyl Editing of Plastid Lipids and Chloroplast Import of Phosphatidylglycerol Precursors in *Arabidopsis thaliana*[1]

[1]This work has been reviewed and is under revision for publication in The Plant Journal with coauthors Kun Wang, Patrick Horn, and Christoph Benning. Kun Wang took the confocal picutures and performed the mass spectrometry after I prepared the samples. Patrick Horn contributed to the interpretation of results and writing strategy.

Abstract

In plant lipid metabolism, the synthesis of many intermediates or end products often appears overdetermined with multiple synthesis pathways acting in parallel. Lipid metabolism is also dynamic with interorganelle transport, turnover, and remodeling of lipids. To explore this complexity *in vivo*, we developed an *in vivo* lipid “tag and track” method. Essentially, we probed lipid metabolism in *Arabidopsis thaliana* by heterologously expressing a coding sequence for a fatty acid desaturase from *Physcomittrella patens* (Δ6D). Δ6D places a double bond after the 6th carbon from the carboxyl end of an acyl group attached to phosphatidylcholine at its *sn*-2 glyceryl position providing a subtle, but easily trackable modification of the glycerolipid. Phosphatidylcholine is a central intermediate in plant lipid metabolism as it is modified and converted to precursors for other lipids throughout the plant cell. Taking advantage of the exclusive location of Δ6D in the endoplasmic reticulum (ER) and its known substrate specificity for one of the two acyl groups on phosphatidylcholine, we were able to “tag and track” the distribution of lipids within multiple compartments and their remodeling in transgenic lines of different genetic backgrounds. Key findings were the presence of ER-derived precursors in plastid phosphatidylglycerol and prevalent acyl editing of thylakoid lipids derived from multiple pathways. We expect that this "tag and track" method will serve as a tool to address several unresolved aspects of plant lipid metabolism, such as the nature and interaction of different subcellular glycerolipid pools during plant development or in response to adverse conditions.

Introduction

Plant membrane lipids not only provide the structural basis for cell boundaries and sub-cellular compartmentalization, but they are also involved in signaling and hormone production (1-5), are integral to the photosynthetic machinery (6-11), and provide energy storage (12). Plant cells have a unique lipid composition coinciding with specialized organelles, chloroplasts, to carry out photosynthesis. While most cellular membranes are composed primarily of phospholipids and

sphingolipids, chloroplast membranes are lacking sphingolipids and many classes of phospholipids, and they are primarily composed of glycoglycerolipids, with monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) being the most abundant followed by the sulfolipid, sulfoquinovosyldiacylglycerol (SQDG). The outer envelope membrane of the chloroplast does contain phosphatidylcholine (PC) (13), but the only phospholipid abundantly found in the photosynthetic membranes, the thylakoids, is phosphatidylglycerol (PG).

Complementing the unique chloroplast lipid composition, plants stand out amongst eukaryotes in their synthesis of chloroplast lipids through the cooperation of two assembly pathways. Fatty acid synthesis takes place in the stroma of chloroplasts, but chloroplast glycerolipid biosynthesis occurs both in chloroplasts (plastid pathway) and in the endoplasmic reticulum (ER pathway). Products from each of these pathways have historically been distinguished due to the specificity of the respective acyltransferases catalyzing the second step of glycerolipid assembly, the transfer of an acyl group to *sn*-1-acylglyceryl *sn*-3-phosphate (lyso-phosphatidic acid) (14, 15). The plastid acyltransferase prefers to add 16 carbon (16C) acyl groups to the *sn*-2 position of the glyceryl backbone, while the ER acyltransferase prefers to add 18 carbon (18C) acyl groups to the same position (14, 15). Based on this distinction, it has been posited that nearly 50% of chloroplast lipids of *Arabidopsis thaliana* are derived from ER precursors with 18C fatty acids in the *sn*-2 position (16). This fraction varies among species with plants, such as Arabidopsis, using both chloroplast and ER pathways, therefore, being designated as 16:3 plants (17, 18). In contrast, plants designated as 18:3 plants almost entirely use the ER pathway. However, plastid PG has almost exclusively a 16C fatty acid in the *sn*-2 position and is, therefore, presumed to be made nearly exclusively by the plastid pathway in both 16:3 and 18:3 plants (16-18). The use of ER precursors to produce chloroplast lipids also implies the presence of ER-to-chloroplast lipid transport. While key proteins involved in this

transport have been identified, TriGalactosylDiacylglycerol (TGD) 1-5, the transport mechanism and the transported lipid species remain to be determined (19-26).

Because membrane lipids are important to so many different cellular processes, extensive efforts have been made to gain a comprehensive understanding of plant lipid metabolism. However, lipid metabolism has proven to be complex with not only multiple pathways and transport between organelles but also with acyl editing by way of transacylases, lipases, and acyltransferases. Acyl editing, in which de novo synthesized fatty acids are transferred from the acyl-CoA pool to PC, has been characterized by *in vivo* kinetic radiolabeling for PC of pea leaves (27) and may occur at the ER or plastid envelope membrane (28). The acyl groups undergo desaturation on PC, and the now polyunsaturated fatty acids are returned to the acyl-CoA pool in leaves (27). This acyl editing process actually receives the majority of the flux from the *de novo* synthesized acyl-CoA pool derived from chloroplast fatty acid export (27). Because there are many predicted plastid lipases of unknown function that could be involved in acyl editing, it is reasonable to consider acyl editing of plastid lipids as part of the plastid pathway, as has already been hinted at by others (27-29). However, the existence of extensive acyl editing within the chloroplast could affect the currently accepted quantitative indicator for identifying lipid precursor origin, the 16C versus 18C occupation in the plastid lipid *sn*-2 position. It also raises new questions regarding the origin of plastid PG, which based on the above-mentioned criterion, appears to be derived from the plastid pathway to a larger extent than any other lipid.

Thus far, methods involving radiolabeling and positional analysis of lipids have been the most informative to determine flux through the different pathways and the transport between organelles, but conclusions based on these data rely on the *sn*-2 position 16C versus 18C standard for distinguishing pathway origin. Here, *in vivo* lipid tagging was employed using heterologous production of an ER-located Δ6 desaturase from *Physcomitrella patens* (Δ6D) in Arabidopsis. Arabidopsis lacks a Δ6 desaturase, and this approach led to the formation of

uniquely traceable Δ6 fatty acyl groups as they appear in different lipids. This Δ6D is well characterized with a substrate preference for the *sn*-2 position of PC (30, 31). Expression of the *Δ6D*-encoding cDNA in Arabidopsis resulted in lipids with γ-linolenic acid (GLA, $18{:}3^{\Delta6,9,12}$; carbon number: double bond number, Δ double bond position from the carboxyl terminus) and stearidonic acid (SDA, $18{:}4^{\Delta6,9,12,15}$). Tracking of GLA and SDA in the wild type (WT) and different, well-characterized lipid mutant backgrounds validated this "tag and track" approach as an *in vivo* method for detailed study of plant lipid metabolism. Furthermore, it enabled us to directly test the current standard of using the *sn*-2 position occupation by 16C versus 18C in lipid molecular species for the extrapolation of the origin of chloroplast lipids.

Results

Δ6D Is External to the Chloroplast when Heterologously Produced.

In *Physcomitrella patens*, the Δ6 desaturase is predicted to be located in the ER based on the presence of a cytochrome B5 domain which is considered indicative of ER fatty acid desaturases (30). For this reason, the Δ6D was also predicted to be in the ER when heterologously produced. For the "tag and track" approach to give conclusive results, it was critical to demonstrate that Δ6D is not targeted to the chloroplast. Therefore, the subcellular location of Δ6D was determined by cellular fractionation and subsequent immunoblot analysis of Arabidopsis plants expressing a Δ6D-encoding sequence in the *pEarleyGate203* vector producing the recombinant enzyme with an N-terminal Myc tag (Myc-Δ6D) (32). Protein extract from *Saccharomyces cerevisiae* containing Myc-Δ6D was used as a control, and when mixed with ER protein from non-transgenic plants, the signal for the yeast Myc-Δ6D faded substantially but remained detectable (Figure 2.1A). The Myc-Δ6D full-length protein and a presumed degradation product were similarly detectable in two independent ER extracts from transgenic plants, but both were absent from the transgenic chloroplast sample and the non-transgenic ER sample (Figure 2.1A). Markers for the chloroplast fraction (oxygen evolving complex 33, OE33)

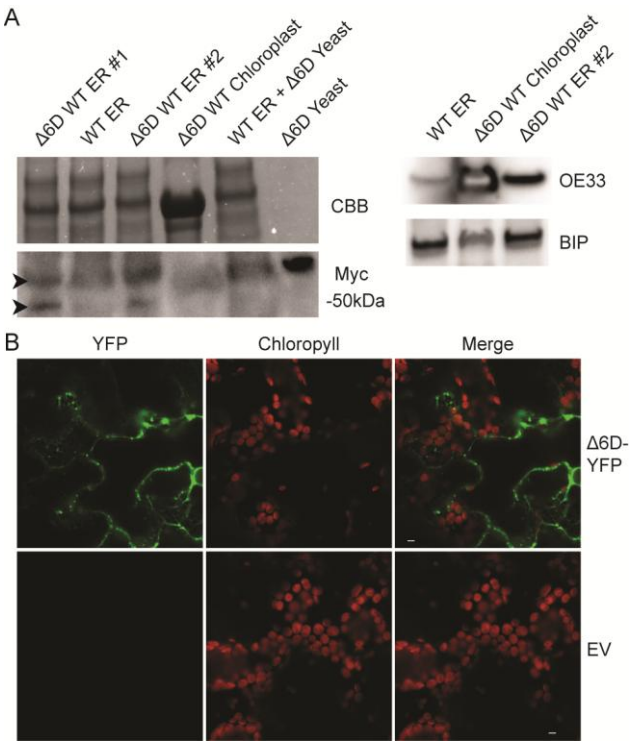

Figure 2.1 Determination of Δ6D Subcellular Location. (A) The portion of Coomassie Brilliant Blue (CBB) stained gel where Myc-Δ6D runs is shown. The immunoblots for OE33 (33kDa) and BIP (74kDa) show the extent of cross-contamination in each organelle preparation. The immunoblot for Myc-Δ6D (62 kDa) shows presence of the recombinant protein in the ER but not the chloroplast. The arrows point to the Myc-Δ6D and a presumed degradation product, which is only present in the transgenic ER samples. (B) Confocal images of Δ6D-YFP (top) and empty vector (EV, bottom) are shown with YFP detection (green), chlorophyll detection (red), and merged images (from left to right). Scale bars = 5μm. WT, wild-type.

and the ER (binding immunoglobulin protein, BIP) were included to indicate purity of the fractions. Taken together, these results were consistent with an exclusive extra-plastidic location of the recombinant protein.

For independent corroboration of subcellular location, the *Δ6D* cDNA was inserted into the *pEarleyGate101* vector (32) leading to a C-terminal YFP fusion protein (Δ6D-YFP). This vector as well as the *pEarlyGate101* empty vector (EV) were transiently expressed in tobacco (*Nicotiana benthamiana*), and the tissue was examined by confocal laser microscopy. The YFP signal in the tissue producing Δ6D-YFP showed a webbing-like pattern (similar to the pattern usually associated with the ER) with some solid structures resembling ER cisternae (Figure 2.1B). In no instance did the YFP signal overlap with the chlorophyll auto-fluorescence of chloroplasts, and the tissue with *pEarleyGate101* EV showed no YFP signal (Figure 2.1B).

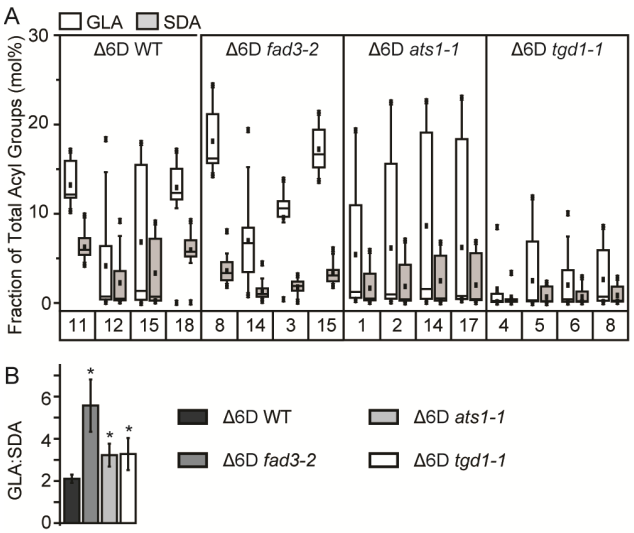

Figure 2.2 GLA and SDA Content of Each Independent Line. (A) The spread of GLA and SDA levels as a mole percentage of total acyl groups between individual plants of each line in each genetic background is shown. 12 ≤ n ≤58. The Δ6D wild-type (WT) and Δ6D *fad3-2* plants are of the T2 generation, and the Δ6D *ats1* and Δ6D *tgd1-1* plants are of the F3 generation. (B) The ratio of GLA to SDA is shown for each background using only data from individuals producing > 0.5 mol% SDA. Student's t-test was performed for each background against the WT, n > 33, * p-value < 0.005. GLA, γ-linolenic acid; SDA, stearidonic acid. Error bars denote SD.

Based on these two lines of independent evidence, we concluded that the Δ6D-YFP was exclusively located outside the chloroplast, primarily associated with the reticular structure of the ER as previously predicted (30).

Plants Producing Δ6D Accumulate GLA and SDA.

Potential Arabidopsis Myc-Δ6D-producing T1 plants were screened for Basta resistance, and survivors were subsequently rescreened by PCR. Positive transformants were then subjected to total fatty acid methylester analysis. Four transgenic lines were isolated that produced high levels of GLA and SDA in the WT background (Figure 2.2A). The best line in the WT background produced 10-18 mol% GLA of total acyl groups and 5-10 mol% SDA (Figure 2.2A). Progeny of the plants with the highest levels of GLA and SDA were investigated in further detail.

Myc-Δ6D was also produced in three different, well-characterized lipid metabolism mutants, *fad3-2*, *ats1-1*, and *tgd1-1*, to explore the potential of and to provide proof-of-concept for the lipid “tag and track" approach based on the Δ6 acyl group redistribution from PC into different cellular lipids. The *fad3-2* mutant is lacking the ER Δ15 desaturase (33) leaving only

the plastid Δ15 desaturases. Therefore, any SDA detected in *fad3-2* must have received the Δ6 double bond in the ER and the Δ15 double bond in the chloroplast. The dual location of Δ15 desaturases accounts for SDA biosynthesis in the ER and the plastid in WT. However, with the exclusive origin of GLA in the ER of transgenic lines, we hypothesized that the SDA distribution between ER and chloroplast lipids as well as the GLA-to-SDA ratio are diagnostic of changes in the relative contribution of the ER and plastid pathways in the different mutants. Accordingly, eliminating the ER Δ15 desaturase in the *fad3-2* mutant allowed us to verify this hypothesis.

The *ats1-1* mutant is deficient in the plastid pathway for lipid assembly as it is strongly reduced or even lacking the enzyme activity which transfers the first acyl group from an acyl carrier protein to glycerol-3-phosphate (34, 35). We expected that when Myc-Δ6D is present in this mutant background, GLA and SDA content in chloroplast lipids should be enhanced due to the increased ER contribution to plastid lipid assembly compared to the WT background. On the contrary, the *tgd1-1* mutant is impaired in ER-to-chloroplast lipid transport (26), which decreases the ER contribution to chloroplast lipid assembly and, therefore, should decrease GLA and SDA content in chloroplast lipids in the presence of Myc-Δ6D.

To develop these lines, the *Myc-Δ6D* construct was introduced directly into both the WT and *fad3-2*, and good producers in the WT background were crossed with the other two mutants, *ats1-1* and *tgd1-1*. Four independent lines, which were each capable of segregating plants with high levels of GLA and SDA, were chosen for each genetic background and subjected to detailed lipid analysis (Figure 2.2A). The levels of GLA and SDA produced were somewhat variable between individuals within each line, and while only data for the T2 and F3 generations are shown (Figure 2.2A), this trend carried into future generations. Because of this variability, total GLA and SDA levels were routinely determined for each plant before carrying out analyses that were more detailed.

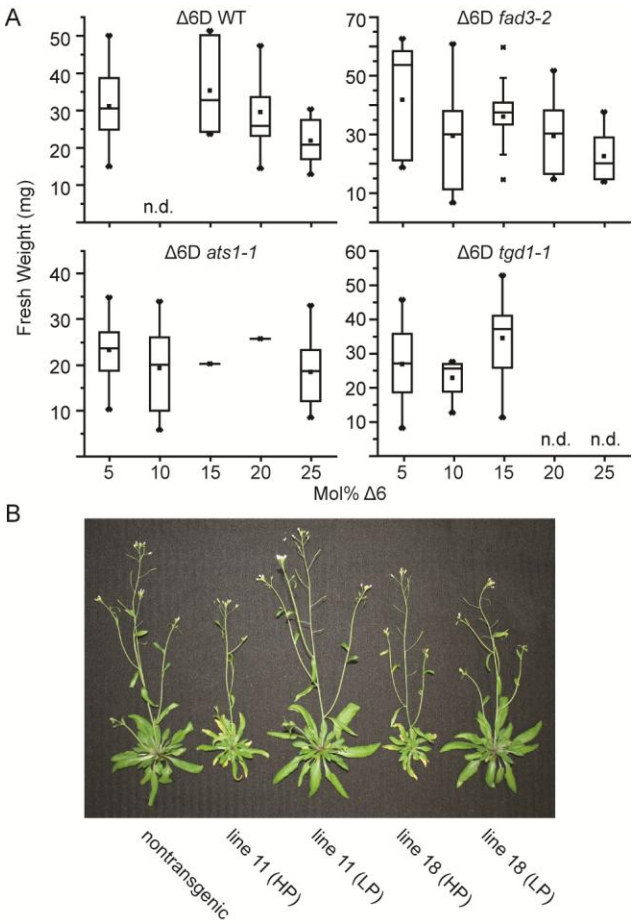

Figure 2.3 Phenotypes of Transgenic Plants. (A) The fresh weight of whole rosettes (20-day-old plants) is plotted against the mole percentage of Δ6 acyl groups in total lipid is shown. Samples are binned with the upper limit of each category shown on the x-axis, n.d. = no data (B) Morphology of non-transgenic plants and 2 lines of transgenic plants. For each line of transgenic plants, a high producer (HP) of Δ6 acyl groups (>15 mol%) and a low producer (LP) of Δ6 acyl groups (<1 mol%) are shown.

Total lipid analysis in each of the different mutant backgrounds revealed different relative levels of GLA and SDA. The *Myc-Δ6D fad3-2* plants accumulated less SDA than the *Myc-Δ6D* WT plants resulting in a 2 to 3 fold higher GLA-to-SDA ratio (Figure 2.2B) as would be expected for a mutant without a functioning ER Δ15 desaturase, the same location as for the Δ6 desaturase. The GLA-to-SDA ratio was also slightly increased in the *Myc-Δ6D ats1-1* and *Myc-Δ6D tgd1-1* lines (Figure 2.2B). While these mutants introduce a disruption in lipid fluxes between the ER and the plastid in different ways, it seems likely that the reduced availability of GLA substrate to the Δ15 desaturases in either the ER or the plastid is reducing overall GLA conversion to SDA in both mutants leading to the observed increase in GLA-to-SDA ratios.

Plants Producing Myc-Δ6D Have a Subtle Secondary Phenotype.

The fresh weight and combined relative fraction (mol%) of Δ6-containing acyl groups, GLA and SDA, were measured in 3-week-old plants for four independent Myc-Δ6D-producing lines in each background, WT, *fad3-2*, *ats1-1*, and *tgd1-1*. The plants were binned based on the mol% fraction of Δ6 acyl groups (Figure 2.3A). The fresh weight of the plants remained within the same range for the <5 mol% category plants as the Δ6 acyl group mol% fraction increased, but Myc-Δ6D-producing plants in the WT and *fad3-2* backgrounds reached a weight threshold when Δ6 acyl levels reached close to 25 mol%. Therefore, relatively high levels of GLA and SDA and/or the corresponding reduction in other acyl groups had a small but discernible effect on plant growth.

Some sets of plants showed a more perceptible growth phenotype after reaching the reproductive stage with less or sometimes delayed growth as well as early yellowing of leaves (Figure 2.3B). This phenotype was not always seen and may have been related to the respective plants' hypersensitivity to minor environmental fluctuations experienced in the growth chambers. In the following, all lipid analyses were conducted on young plants that were indistinguishable in their growth and appearance from WT.

The presence of GLA and SDA acyl groups was also accompanied by a subtle secondary lipid phenotype. Myc-Δ6D-producing plants in WT, *fad3-2*, and *ats1-1* backgrounds had an increased 16C to 18C ratio in some polar lipids (Figure 2.4). This observation could indicate that the TGD1 complex discriminates slightly against the import of Δ6 acyl group containing molecular species leading to the observed increase in16C/18C ratio. While this increase was statistically significant, it was too small to affect any conclusions drawn from GLA and SDA distribution results discussed below.

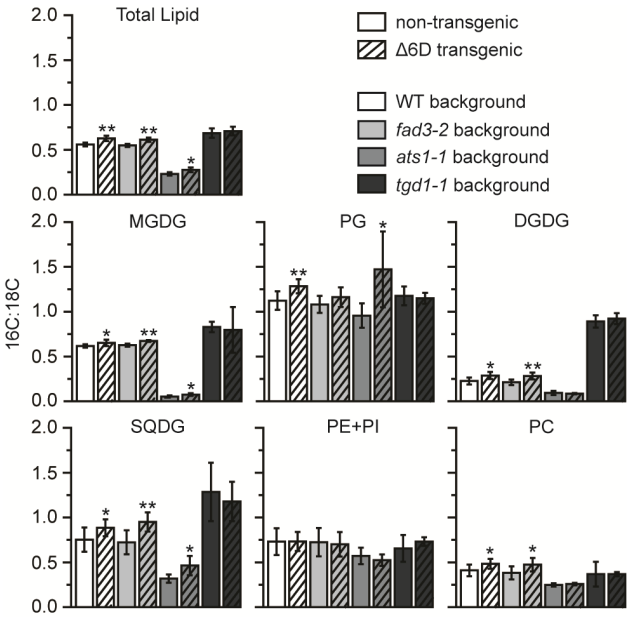

Figure 2.4 16C to 18C Ratio. The ratio of 16C to 18C acyl groups is represented for total lipid and each polar lipid for each genetic background, both transgenic and non-transgenic. The key is included in the figure. Student's t-test was performed comparing Δ6D transgenic to nontransgenic, n = 9-10, * p-value < 0.05, ** p-value < 0.005. Error bars denote SD. MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PE+PI, phosphatidylethanolamine+ phosphatidylinositol; PC, phosphatidylcholine; WT, wild-type.

Δ6 Acyl Groups Are Present in Plastid PG.

The distribution of Δ6 acyl groups among polar lipid classes was determined in leaf tissues from transgenic plants of each genetic background (Figure 2.5A). In general, Myc-Δ6D-producing plants in each of the tested genetic backgrounds showed the predicted distribution patterns of Δ6 acyl groups providing proof-of-concept for the described lipid “tag and track” method. The pattern in *Myc-Δ6D fad3-2* was very similar to that of *Myc-Δ6D* in WT (Figure 2.5A), with the primary predicted phenotype being the increased GLA to SDA ratio as discussed above (Figure 2.2B). *Myc-Δ6D tgd1-1* plants had lower levels of Δ6 acyl groups in the major chloroplast lipids, MGDG and DGDG, and elevated Δ6 acyl group levels in ER-associated lipids, PC and phosphatidylethanolamine (PE) and phosphatidylinositol (PI), due to decreased ER-to-chloroplast lipid transport (Figure 2.5A). This result also indicates that the TGD complex transports Δ6 acyl group-containing lipid species from the ER to the inside of the chloroplast even though at perhaps reduced efficiency as already mentioned above. The *Myc-Δ6D ats1-1*

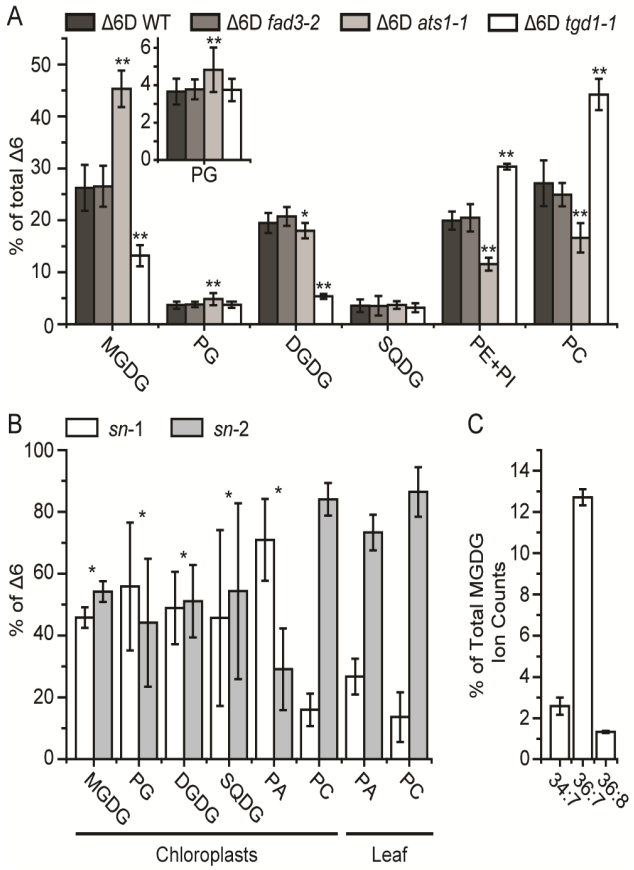

Figure 2.5 Distribution of Δ6 Acyl Groups Among Polar Glycerolipids. (A) The distribution of Δ6 acyl groups among the different polar glycerolipids is shown as a % of the total Δ6 acyl groups in all of the polar lipids. (B) The % of Δ6 acyl groups in each position of the glycerol backbone is shown for each lipid in the Δ6D transgenic wild-type (WT) background. Student's t-test was performed comparing each lipid to whole leaf PC, n = 3-7 for isolated chloroplast samples, and n = 3-4 for whole leaf samples, * p-value < 0.05. (C) The 18:4 containing MGDG species are shown as relative % of total MGDG ion counts. 34:7 (18:4/16:3), 36:7 (18:4/18:3), 36:8 (18:4/18:4), n = 8. Abbreviations as in the Figure 3 legend. All error bars denote SD.

plants had elevated levels of Δ6 acyl groups in MGDG compared to the *Myc-Δ6D* WT plants and decreased levels in PC and PI+PE because the ER pathway was compensating for the loss of the plastid pathway in these plants. The Δ6 acyl group level of DGDG was not affected in the *Myc-Δ6D ats1-1* plants (Figure 2.5A) consistent with the previous observation that DGDG is almost exclusively produced from ER precursors (16).

Each of the four genetic backgrounds tested showed the presence of Δ6 acyl groups in PG, and the mol% fraction of the Δ6 acyl groups in PG increased in the *Myc-Δ6D ats1-1* background, which is deficient in the plastid pathway (Figure 2.5A inset) and in which the import of ER-derived lipid species is increased. Lipids were also analyzed from isolated chloroplasts in

order to observe the presence of Δ6 acyl groups specifically in plastid PG, and Δ6 acyl group presence was corroborated (Figure 2.5B).

Δ6 Acyl Groups in Thylakoid Lipids Are Evenly Distributed Between the Glyceryl *sn*-1 and *sn*-2 Positions.

Based on the substrate preference of the Δ6D for the acyl group at the *sn*-2 position of PC (30, 31), Δ6 acyl groups were expected to be predominantly present in the *sn*-2 position of chloroplast lipids, assuming that the chloroplast-imported lipid species containing Δ6 acyl groups is derived without remodeling from PC assembled at the ER. Positional analysis of PC from both whole leaf tissue and isolated chloroplasts showed a positional distribution of Δ6 acyl groups with approximately 90% being in the *sn*-2 position (Figure 2.5B). However, the four thylakoid lipids differed from PC with Δ6 acyl groups being nearly equally abundant in the *sn*-1 and *sn*-2 positions (Figure 2.5B).

Phosphatidic acid (PA) is a crucial intermediate of lipid assembly and a proposed lipid species imported into plastids (36). Therefore, PA was isolated from whole leaf tissues and isolated chloroplasts and subjected to positional analysis. PA from whole leaf tissues showed a similar profile to PC with the majority of Δ6 acyl groups in the *sn*-2 position, while PA from isolated chloroplasts had an altered distribution of Δ6 acyl groups in the *sn*-1 and *sn*-2 positions more similar to that of the chloroplast lipids (Figure 2.5B). This result suggests that the observed plastid PA is not directly or exclusively derived from extraplastidic PC, and that at least a fraction of PA either imported or assembled in the plastid is likely remodeled by acyl editing.

SDA and 16:3 Are Found in the Same MGDG Molecule.

The acyl group compositional analysis of bulk lipids shown above does not provide information on the presence of different acyl groups within a single molecule. We hypothesized that direct evidence for acyl editing will be immediately apparent if exclusively ER- and plastid-derived acyl

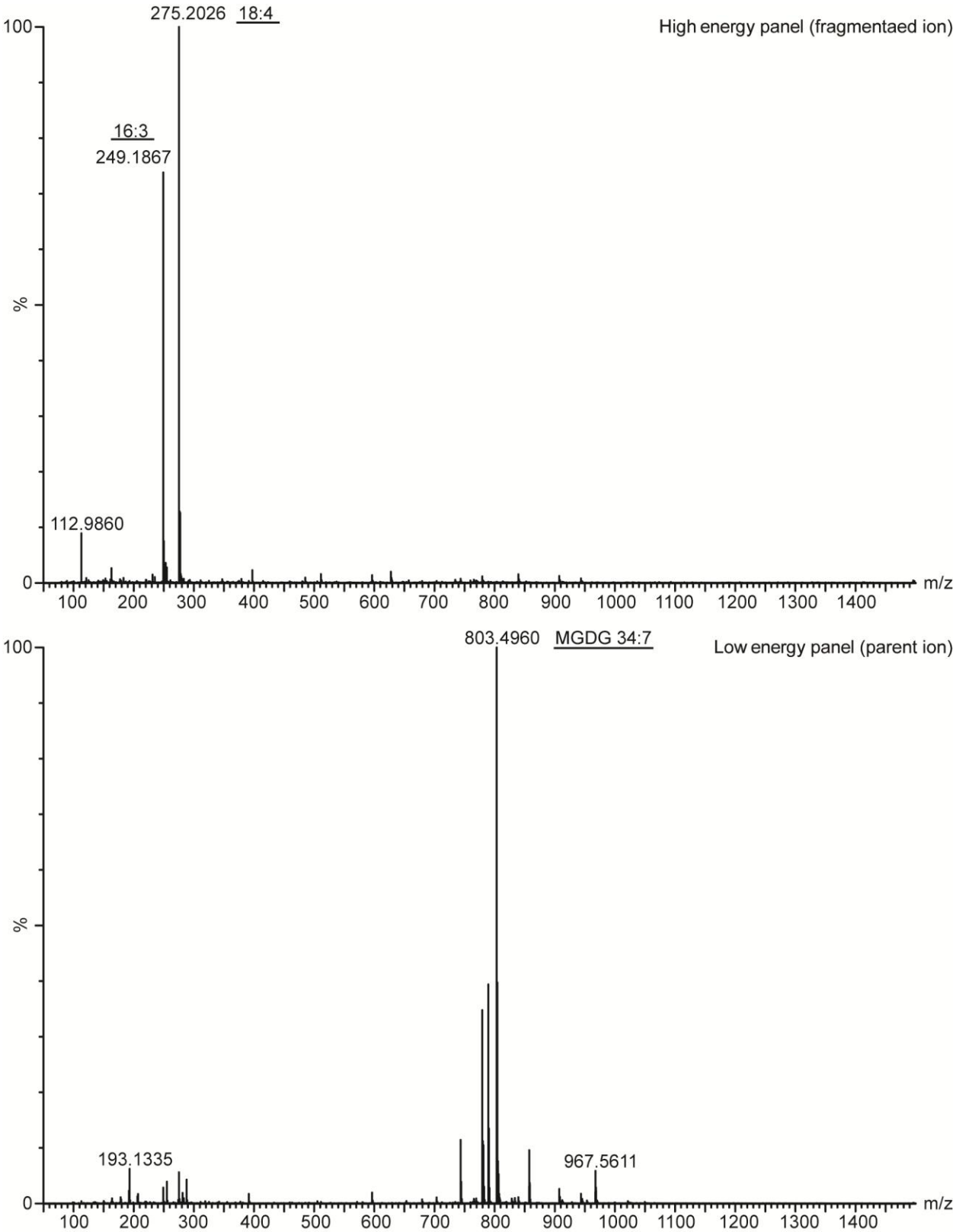

Figure 2.6 Ion Chromatograms for MGDG. The fragmented and the parent ion chromatograms used to identify 34:7 MGDG are shown.

groups are found in the same chloroplast lipid molecule. Therefore, liquid chromatography mass spectrometry (LC/MS) performed on lipids extracted from transgenic plants with high levels of Δ6 acyl groups was employed to determine if 18:4 (SDA), derived from the ER, can be found in the same MGDG molecule as 16:3, an acyl group made exclusively in the chloroplast by the action of FAD5 (37). Focusing on the most abundant chloroplast lipid, 2% of MGDG was determined to be a 34:7 molecular species containing one 16:3 and one 18:4 acyl group each (Figure 2.5C & 2.6). As argued above, identification of this molecular species of MGDG provides strong supporting evidence for acyl editing of an exclusive chloroplast lipid with ER-derived acyl groups.

The Effect of *Myc-Δ6D* on the Metabolism of Specific Lipids.

A pulse-chase labeling experiment using whole leaves and ^{14}C-acetate was conducted in order to determine if the rates of synthesis and turnover of specific polar lipids are affected by the presence of 15-18 mol% Δ6 acyl groups in Δ6D transgenic plants. Both the non-transgenic and transgenic plants showed similar patterns of ^{14}C accumulation in each of the lipids during the pulse and the chase phases of the experiment (Figure 2.7A). However, the transgenic plants did incorporate approximately two-fold higher total levels of ^{14}C-acetate per FW in polar lipids than non-transgenic plants (Figure 2.7B) suggesting an increase in the rate of fatty acid biosynthesis in the transgenic plants affecting all lipids. The amount of lipid per FW only slightly increased in transgenic plants (Figure 2.7C) suggesting lipid turnover was likely increased in transgenic plants to account for the two-fold higher radiolabeling.

Discussion

Differentiation of the two synthesis pathways for chloroplast lipids based on the specificities of the respective acyltransferases resulting in the glyceryl *sn*-2 position 16C/18C diagnostic rule has long been the accepted paradigm. For example, this distinction between lipid species

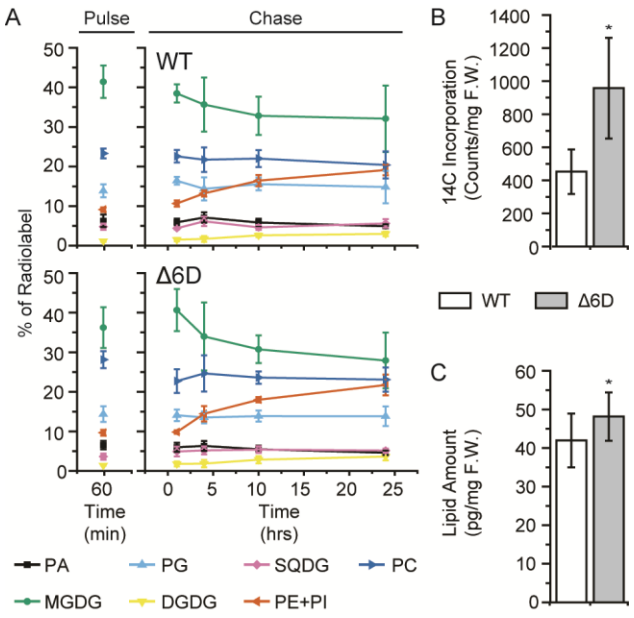

Figure 2.7 ^{14}C-Acetate Labeling. (A) The results of a pulse-chase experiment with ^{14}C-acetate are shown for polar lipids in both transgenic and non-transgenic plants in the WT background. (B) Levels of radiolabel incorporated per mg of tissue fresh weight (F.W.) after a 40 min pulse are shown for transgenic and non-transgenic plants in the WT background. (C) Acyl amount (pg) per mg of tissue F.W. is shown for the same samples used in (B). For (B) and (C), Student's t-test was performed between Δ6D transgenic and non-transgenic, n = 14, * $p < 0.05$. All error bars denote SD. PA, phosphatidic acid; other lipid abbreviations are as in the Figure 3 legend.

derived from the two pathways led to the conclusion that the majority of plastid PG is of plastid origin (16), despite the apparent contradiction that the *ats1-1* mutant disrupted in the plastid pathway retains 75% of WT plastid PG levels (34, 35). The question was whether the *ats1-1* mutant had residual ATS1 acyl transferase activity in the plastid. Based on glycerol labeling of isolated *ats1-1* chloroplasts, Xu and coworkers concluded that PA and PG are still being synthesized in chloroplasts of the *ats1-1* mutant, even though the recombinant mutant protein had very little residual activity (35). Furthermore, when labeled palmitate was fed to leaves, its incorporation was similar in the *ats1-1* mutant and the WT leading to the conclusion that the ER pathway is likely not responsible for maintaining the observed high plastid PG levels in the *ats1-1* mutant (35). However, the 16C acyl group palmitate may not be the right fatty acid substrate for detecting lipid precursor assembly at the ER and subsequent lipid import into plastids, because most chloroplast-imported lipid species contain only 18C acyl groups. Our current observation of an increase in Δ6 acyl groups in PG in the *ats1-1* mutant background points

towards the incorporation of a fraction of acyl groups from the ER pathway into chloroplast PG (Figure 2.5A inset). Based on comparison of the ratio of Δ6 acyl groups-to-total acyl groups between MGDG and PG, we estimate this fraction for PG to be approximately 18% in the WT, although we cannot rule out an additional ATS1-independent assembly pathway for PA and PG as suggested by Xu and coworkers (35). Thus based on our analysis, which is independent of the 16C/18C rule, a substantial fraction of PG is derived from the ER. It should be noted that the majority of plastid PG contains an unusual C16 fatty acid at its *sn*-2, $16{:}1^{\Delta 3t}$, making it the target of a specific lipase, PLIP1 (29). The partial ER-origin of plastid PG in Arabidopsis is likely obscured using traditional approaches by specific acyl group remodeling of chloroplast PG but is clearly apparent in the *Myc-Δ6D* transgenic lines using the "tag and track" approach.

The lipid "tag and track" approach also provided strong evidence for acyl editing of thylakoid lipids. The observed distribution of Δ6 acyl groups in PG, DGDG, MGDG, and SQDG with nearly equal distribution of Δ6 acyl groups between the *sn*-1 and *sn*-2 positions, while 90% of the Δ6 acyl groups in PC occupy the *sn*-2 position (Figure 2.5B), provided one piece of evidence. A second piece of evidence is the observation of SDA and 16:3 acyl groups in the same MGDG molecule as the first acyl group originates in the ER and the second in the plastid (Figure 2.5C). Acyl editing has been described for PC at the ER (27) or the plastid envelope (28), for plastid MGDG in Chlamydomonas (38), and recently for plastid PG during seed development (29). Each of these reactions are proposed to contribute to the export of acyl groups from the plastid. It should be noted that acyl editing within the chloroplast might be increased in Δ6D-producing plants triggered by the presence of unusual fatty acids in the thylakoid lipids. In fact, the increased incorporation of labeled acetate in transgenic plants (Figure 2.7B) in parallel with only slight increases in total acyl content (Figure 2.7C) may be an indication of higher lipid turnover in the transgenic lines compared to WT. Nevertheless, the lipid "tag and track" approach described here provides strong evidence for extensive acyl editing of

thylakoid lipids in the transgenic lines, which likely also occurs in WT leaves, but perhaps to a lower extent.

The observed change in acyl group composition between PC in the ER or the outer plastid envelope and the thylakoid lipids might suggest that PC is not the source of the ER substrate for plastid lipid synthesis. PA has been proposed as the lipid species imported from the ER based on its specific binding to proteins of the TGD complex involved in chloroplast lipid import (39, 40). However, the positional analysis of PA was not conclusive because of its differing positional profiles between whole leaf PA and PA of isolated chloroplasts (Figure 2.5B). In both cases, we have to assume that the imported lipid species is modified by acyl editing, and this could be true for PC or PA derived from PC at the outer envelope leaving the question of the nature of the imported lipid species still unanswered.

Based on the evidence provided by the lipid “tag and track” approach shown here, we propose a mechanism that includes a combination of direct backbone import and acyl editing within the chloroplast. Assuming that this mechanism based on Δ6D transgenic plants also applies to WT plants, one might call into question whether the glyceryl *sn*-2 position 16C/18C diagnostic rule for identifying relative fluxes through the ER and plastid pathways still holds true in view of the observed extensive chloroplast acyl editing in the transgenic lines. Evidence as outlined above suggests that plastid PG does not entirely follow this rule. However, the ”tag and track” approach shown here does not allow us to assess fully the extent of chloroplast acyl editing in WT plants, which could be much lower than in the transgenic plants that have lipids with unusual fatty acids that could trigger an acyl editing or repair response.

Materials and Methods

Plant Growth and Selection.

Arabidopsis (*Arabidopsis thaliana*) were grown under a 16h light/8h dark cycle at 22°C/20°C (light/dark) with a light intensity of 100-120 μmol $m^{-2}s^{-1}$. The T1 generation of Δ6D plants was

screened by spraying 2-3 week-old soil grown plants with 0.01% Basta solution (BioWorld 1% stock, Cat#30632006). Subsequent screening was done by PCR with internal primers for Δ6D: forward - GAA GTT GTC GGG TAT GTG, reverse - CAT CCC ATT GTG GCT AAG. T2, T3, F3, and F4 populations were screened for stable transgenic lines by selection on MS medium (41) + 1% sucrose containing 7mg/L glufosinate ammonium (Sigma Cat# 45520) and subsequent PCR screening with the aforementioned primers. *Arabidopsis* for organelle isolation were grown to 3-4 weeks old on 150x15mm petri plates containing MS medium + 1% sucrose with approximately 30 mg of seed per plate.

Nicotiniana benthamiana were grown under a 16h light/8h dark cycle at a constant 25°C with a light intensity of 100-120 μmol $m^{-2}s^{-1}$.

_Δ6D_ Plasmid Construction.

Δ6D coding sequence (30) was amplified from the p35-d6Physco plasmid provided by Dr. Simone Zäuner using primers compatible for use in the *pENTR D-TOPO* (Thermo Fisher) entry vector and subsequent cloning into both *pEarleyGate101* and *pEarleyGate203* (32) destination vectors. The same forward primer was used for amplification intended for both *pEarleyGate* destination vectors: CAC CAT GGT ATT CGC GGG CGG T. A reverse primer containing a stop codon was used for *pEarleyGate203*: TTA ACT GGT GGT AGC ATG CTG CT, and a reverse primer with no stop codon was used for *pEarleyGate101*: ACT GGT GGT AGC ATG CTG CTC T. For yeast expression, a N-terminal Myc tag and a BamHI site were added by PCR using the forward primer: GAC GGA TCC AAA ATG GAA CAG AAG TTG ATT TCC GAA GAA GAC CTC ATG GTA TTC GCG GGC, and an ApaI site was added with the reverse primer: TTA GGG CCC TTA ACT GGT GGT AGC ATG. BamHI and ApaI restriction enzymes were used to clone the PCR product into the yeast expression vector, pESC-His and create *Δ6D pESC-His*.

Arabidopsis Transgenic Lines.

Arabidopsis Columbia-2 (Col-2) and *fad3-2* plants were transformed with *Δ6D pEarleyGate203* using the floral dip method as described in (42). Four lines from each background were chosen for further analysis based on total GLA and SDA content. The four lines in the WT Col-2 background were also crossed with the *ats1-1* and *tgd1-1* mutants. Four lines from independent crosses in each of these mutant backgrounds were also chosen for further analysis based on total GLA and SDA content.

***Nicotiniana benthamiana* Transient Expression.**

Transient expression of *Δ6D pEarleyGate101* was performed according to (43).

Recombinant Protein Production in *Saccharomyces cerevisiae.*

Δ6D pESC-His and *pESC-His* empty vector were introduced into *S. cerevisiae* (InvSc1) using the Frozen EZ Yeast Transformation II kit from Zymo. Yeast cultures were grown in SD-His selection medium to OD_{600} = 0.4-0.6, and the cells were then pelleted and resuspended in the same volume of YPG induction medium. Cells were harvested at 0, 4, 8 and 24 h after induction and used for protein extraction as described in (44).

Confocal Microscopy.

Confocal imaging was performed on 4-6 week-old tobacco leaves 36 hours post infiltration of agrobacterium culture using a Nikon A1Rsi confocal laser scanning microscope. YFP fusions were excited at 514 nm, and the emissions were detected with a 530-600 nm band pass filter. Chlorophylls were excited at 647 nm, and the emissions were detected with a 660 nm long pass filter. Images were merged and pseudocolored using Nikon NIS Elements AR software (version 4.30.01).

Organelle Isolation and Protein Preparation.

3-5 plates of 3-4 week old seedlings (details above) were used for each chloroplast and ER isolation. Chloroplast isolation was carried out as described in (22), while ER and protein extraction were carried out as described here. Protein was extracted from the isolated chloroplasts by adding 200 µL of extraction buffer (0.1 M Tris-HCl pH6.8, 1% SDS, 15% glycerol, 5% 2-mercaptoethanol) to approximately 250 µg chlorophyll equivalents of pelleted chloroplasts. The chloroplast suspension was heated to 95°C for 10 min and re-pelleted in a microfuge at 14000 x g for 5 min. The supernatant was transferred to a new tube and mixed 1:1 with 2X SDS-PAGE sample buffer (0.1 M Tris-HCl pH6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.05% bromophenol blue) before loading on a protein gel. ER isolation was done by grinding seedlings and 20 mL of ER grinding buffer (10 mM 2-mercaptoethanol, 5 mM EDTA, 10mM Tris-HCl pH7.5) plus 2 cOmplete, mini, EDTA-free protease inhibitor tablets (Roche) into a paste with a mortar and pestle on ice. The whole procedure was carried out on ice or at 4°C. Solid tissue was filtered out through a double layer of Miracloth, and the liquid was centrifuged at 3000 x g for 5 min. The supernatant was centrifuged again at 12,000 x g for 10 min and then again at 100,000 x g for 10 min. The pellet was resuspended in 300 µL of ER grinding buffer and loaded onto a step gradient of 5 mL 25% sucrose in ER grinding buffer and 5 mL 18% sucrose in ER grinding buffer and then centrifuged at 100,000 x g for 1 h. The interface was collected into a new tube, diluted with 10 mL of ER grinding buffer, and centrifuged at 100,000 x g for 10 min. The final pellet was resuspended in 100 µL of import buffer (330 mM sorbitol, 50 mM HEPES pH 8.0-KOH) and mixed 1:1 with 2X SDS-PAGE sample buffer before loading on a protein gel.

SDS-PAGE and Immuno Blotting.

Protein samples (15 µg plant protein extracts or 20 µL of 32-fold diluted yeast protein extract) were run on 10% Mini-Protean TGX 10 well, 50µL gels (BioRad cat# 4561034). Gels were either

subjected to Coomassie Brilliant Blue stain or used for transfer to polyvinylidene fluoride membrane (90 V for 50 min at 4°C). Blocking of membranes was done in 5% milk in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% TWEEN 20) while all antibody incubations were done in 5% BSA in TBST (filter sterilized). The primary antibody used for a chloroplast marker was rabbit αOE33 (Agrisera Cat#AS14 2824), 1:5000. The primary antibody for an ER marker was rabbit αBIP (Santa Cruz Biotech Cat#sc33757, lot#H1806), 1:200. The primary antibody for detection of Myc-Δ6D was rabbit αMyc (Cell Signaling Cat#2278, Lot#5), 1:1000. The same secondary antibody was used in all cases, goat-anti-rabbit HRP, 1:10000. Visualization substrate used for αOE33 and αBIP membranes was Clarity Western ECL substrate (BioRad Cat# 1705060). A more sensitive substrate was needed for detection of Myc-Δ6D, so SuperSignal West Dura substrate (Thermo Fisher Cat#34075) was used.

Lipid Analysis.

Whole tissue lipid analysis was done by subjecting leaf tissue to fatty acid methyl ester (FAME) preparation as described in (45). Lipid extraction, TLC separation, FAME preparation for scrape silica, and GC analysis were also carried out as described in (45). Modifications to the referenced protocol are noted as follows. The FAME preparation for whole tissue lipid analysis was started with whole leaf tissue rather than scraped silica. The 80°C incubation step for whole leaf FAME preparation was extended to 45 min. For TLC separation of individual lipids, Sil G-25 20x20 TLC plates (Macherey Nagal Cat#809013) were treated with 0.15M ammonium sulfate solution at least 2 days prior to use and baked at 250°C for 2.5 h directly before use.

Positional Analysis.

Positional analysis was carried out by first extracting lipids from both isolated chloroplasts and whole leaf tissue and running them on a TLC as described above for all lipids except PA. The TLC solvent system of chloroform:methanol:concentrated ammonium hydroxide (65:25:5 v/v/v)

was used for PA separation. Silica from areas with the desired lipids was scraped into tubes and re-extracted with 2 mL of methanol:chloroform (2:1 v/v) and then 1 mL of 0.2 M H_3PO_4, 1 M KCl. The organic layer was filtered through glass wool and dried under a nitrogen gas stream. Lipids were resuspended in 300 µL PBS (phosphate-buffered saline), 1% Triton X-100 and sonicated 3 x 10 s at power level 0.5. 10 µL of 4 mg/mL Rhizopus lipase stock solution in PBS was added to the lipid suspension and incubated at room temperature for 1 h. Lipids were extracted with 1.1 mL methanol:chloroform (2:1 v/v) and then 400 µL 0.2 M H_3PO_4, 1 M KCl. The organic layer was concentrated under a nitrogen gas stream and run on an ammonium sulfate treated TLC. MGDG, DGDG, PG, SQDG, and PA were run with acetone:toluene:water (91:30:7 v/v/v) solvent, and PC was run with chloroform:methanol:acetic acid:water (60:30:8:5 v/v/v/v).

Lipid preparation and LC/MS measurement.

Total lipids were extracted from 100 mg (fresh weight) leaf tissue high Δ6 acyl producing plants with 1 mL of an extraction buffer composed of methyl-tertiary-butyl-ether:methanol (3:1 v/v). Samples were shaken vigorously for 5 min, and 500 µL of water:methanol (3:1 v/v) was added and well mixed. Phases were completely separated by 5 min centrifugation at 15,000xg. The upper phase of the separation was isolated and dried by nitrogen gas. The concentrated lipids were finally resuspended in 100 µl acetonitrile:isopropanol (7:3 v/v) and diluted 30-fold for LC/MS measurement.

The LC/MS measurement method was modified from (46). Lipid samples were analyzed using a Waters Xevo G2-XS Q-TOF mass spectrometer interfaced with a Waters Acquity binary solvent manager and Waters 2777c autosampler. 10 µl samples were injected onto an Acquity UPLC CSH C18 column (100 x 2.1 741 mm, Waters Corp) held at 55˚C. The mobile phases consist of 0.1% acetic acid and 10 mM NH_4OAc in water (Solvent A) and methanol/isopropanol (7:3, v/v containing 0.1% acetic acid and 10 mM NH_4OAc) (Solvent B). A gradient of mobile phase was applied in a 20-min program with a flow rate of 0.4 ml / min. The gradient profile was

performed as follows: hold for one min after injection at 45% A / 55% B, followed by a one-minute linear ramp to 25% A / 75% B and another two-minute linear ramp to 11% A / 89% B. Then, a 13-minute linear gradient was applied to further decrease to 1% A/ 99% B and equilibrate for one minute, followed by a two-minute isocratic period at 45% A / 55% B. LC separated analytes were ionized by negative ion mode electrospray ionization and mass spectra were acquired using an MSe method in continuum mode over m/z 50 to 1500 to provide data under non-fragmenting and fragmenting conditions (collision energy ramp from 20-80 V).

Processing and alignment of chromatograms, peak identification and integration were performed using Progenesis QI (version 2.2). MGDG peaks were filtered out from all peaks based on their m/z and their expected retention time. The acyl composition of the MGDG peaks was determined by identification of the fragmented fatty acid ions (the m/z value of SDA is 275.21). The ion intensities of SDA containing MGDG species were normalized to the ion intensity of all identified MGDG species.

Radiolabeling.

The leaf pulse-chase was performed as described in (29) with adjustments noted as follows. MS salts and vitamins were added to the incubation medium, and the medium was filter-sterilized before use or addition of ^{14}C-acetate. Incorporation levels per mg of tissue were determined in a separate experiment following the same general protocol as for the pulse-chase, but the only time point taken was the 40 min pulse time point. 10 µL of the lipid extract was also directly used for scintillation counting instead of running on TLC for individual lipid separation.

REFERENCES

REFERENCES

1. Kooijman EE & Burger KN (2009) Biophysics and function of phosphatidic acid: a molecular perspective. *Biochim Biophys Acta* 1791(9):881-888.

2. Zhang W*, et al.* (2003) The oleate-stimulated phospholipase D, PLDdelta, and phosphatidic acid decrease H2O2-induced cell death in Arabidopsis. *Plant Cell* 15(10):2285-2295.

3. van der Schoot C, Paul LK, Paul SB, & Rinne PL (2011) Plant lipid bodies and cell-cell signaling: a new role for an old organelle? *Plant Signal Behav* 6(11):1732-1738.

4. Feussner I & Wasternack C (2002) The lipoxygenase pathway. *Annu Rev Plant Biol* 53:275-297.

5. Janda M*, et al.* (2013) Phosphoglycerolipids are master players in plant hormone signal transduction. *Plant Cell Reports* 32(6):839-851.

6. Stroebel D, Choquet Y, Popot JL, & Picot D (2003) An atypical haem in the cytochrome b(6)f complex. *Nature* 426(6965):413-418.

7. Jordan P*, et al.* (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 A resolution. *Nature* 411(6840):909-917.

8. Liu Z*, et al.* (2004) Crystal structure of spinach major light-harvesting complex at 2.72 A resolution. *Nature* 428(6980):287-292.

9. Loll B, Kern J, Saenger W, Zouni A, & Biesiadka J (2005) Towards complete cofactor arrangement in the 3.0 A resolution structure of photosystem II. *Nature* 438(7070):1040-1044.

10. Jones MR (2007) Lipids in photosynthetic reaction centres: structural roles and functional holes. *Prog Lipid Res* 46(1):56-87.

11. Holzl G*, et al.* (2009) The role of diglycosyl lipids in photosynthesis and membrane lipid homeostasis in Arabidopsis. *Plant Physiol* 150(3):1147-1159.

12. Chapman KD, Dyer JM, & Mullen RT (2012) Biogenesis and functions of lipid droplets in plants: thematic review series: lipid droplet synthesis and metabolism: from yeast to man. *J Lipid Res* 53(2):215-226.

13. Dorne AJ, Joyard J, Block MA, & Douce R (1985) Localization of phosphatidylcholine in outer envelope membrane of spinach chloroplasts. *J Cell Biol* 100(5):1690-1697.

14. Frentzen M (1986) Biosynthesis and Desaturation of the Different Diacylglycerol Moieties in Higher-Plants. *J Plant Physiol* 124(3-4):193-209.

15. Frentzen M, Heinz E, McKeon TA, & Stumpf PK (1983) Specificities and selectivities of glycerol-3-phosphate acyltransferase and monoacylglycerol-3-phosphate acyltransferase from pea and spinach chloroplasts. *Eur J Biochem* 129(3):629-636.

16. Browse J, Warwick N, Somerville CR, & Slack CR (1986) Fluxes through the prokaryotic and eukaryotic pathways of lipid synthesis in the '16:3' plant Arabidopsis thaliana. *Biochem J* 235(1):25-31.

17. Mongrand S, Bessoule JJ, Cabantous F, & Cassagne C (1998) The C-16 : 3/C-18 : 3 fatty acid balance in photosynthetic tissues from 468 plant species. *Phytochemistry* 49(4):1049-1064.

18. Heinz E & Roughan PG (1983) Similarities and differences in lipid metabolism of chloroplasts isolated from 18:3 and 16:3 plants. *Plant Physiol* 72(2):273-279.

19. Awai K, Xu C, Tamot B, & Benning C (2006) A phosphatidic acid-binding protein of the chloroplast inner envelope membrane involved in lipid trafficking. *Proc Natl Acad Sci U S A* 103(28):10817-10822.

20. Fan J, Zhai Z, Yan C, & Xu C (2015) Arabidopsis TRIGALACTOSYLDIACYLGLYCEROL5 Interacts with TGD1, TGD2, and TGD4 to Facilitate Lipid Transfer from the Endoplasmic Reticulum to Plastids. *Plant Cell* 27(10):2941-2955.

21. Lu B, Xu C, Awai K, Jones AD, & Benning C (2007) A small ATPase protein of Arabidopsis, TGD3, involved in chloroplast lipid import. *J Biol Chem* 282(49):35945-35953.

22. Roston R, Gao J, Xu C, & Benning C (2011) Arabidopsis chloroplast lipid transport protein TGD2 disrupts membranes and is part of a large complex. *Plant J* 66(5):759-769.

23. Roston RL, Gao J, Murcha MW, Whelan J, & Benning C (2012) TGD1, -2, and -3 proteins involved in lipid trafficking form ATP-binding cassette (ABC) transporter with multiple substrate-binding proteins. *J Biol Chem* 287(25):21406-21415.

24. Xu C, Fan J, Cornish AJ, & Benning C (2008) Lipid trafficking between the endoplasmic reticulum and the plastid in Arabidopsis requires the extraplastidic TGD4 protein. *Plant Cell* 20(8):2190-2204.

25. Xu CC, Moellering ER, Muthan B, Fan JL, & Benning C (2010) Lipid transport mediated by Arabidopsis TGD proteins is unidirectional from the endoplasmic reticulum to the plastid. *Plant Cell Physiol* 51(6):1019-1028.

26. Xu C, Fan J, Riekhof W, Froehlich JE, & Benning C (2003) A permease-like protein involved in ER to thylakoid lipid transfer in Arabidopsis. *EMBO J* 22(10):2370-2379.

27. Bates PD, Ohlrogge JB, & Pollard M (2007) Incorporation of newly synthesized fatty acids into cytosolic glycerolipids in pea leaves occurs via acyl editing. *J Biol Chem* 282(43):31206-31216.

28. Tjellstrom H, Yang Z, Allen DK, & Ohlrogge JB (2012) Rapid kinetic labeling of Arabidopsis cell suspension cultures: implications for models of lipid export from plastids. *Plant Physiol* 158(2):601-611.

29. Wang K, Froehlich JE, Zienkiewicz A, Hersh HL, & Benning C (2017) A Plastid Phosphatidylglycerol Lipase Contributes to the Export of Acyl Groups from Plastids for Seed Oil Biosynthesis. *Plant Cell*:doi: 10.1105/tpc.1117.00397.

30. Girke T, Schmidt H, Zahringer U, Reski R, & Heinz E (1998) Identification of a novel delta 6-acyl-group desaturase by targeted gene disruption in Physcomitrella patens. *Plant J* 15(1):39-48.

31. Domergue F*, et al.* (2003) Acyl carriers used as substrates by the desaturases and elongases involved in very long-chain polyunsaturated fatty acids biosynthesis reconstituted in yeast. *J Biol Chem* 278(37):35115-35126.

32. Earley KW*, et al.* (2006) Gateway-compatible vectors for plant functional genomics and proteomics. *Plant J* 45(4):616-629.

33. Shah S, Xin Z, & Browse J (1997) Overexpression of the FAD3 desaturase gene in a mutant of Arabidopsis. *Plant Physiol* 114(4):1533-1539.

34. Kunst L, Browse J, & Somerville C (1988) Altered regulation of lipid biosynthesis in a mutant of Arabidopsis deficient in chloroplast glycerol-3-phosphate acyltransferase activity. *Proc Natl Acad Sci USA* 85(12):4143-4147.

35. Xu C, Yu B, Cornish AJ, Froehlich JE, & Benning C (2006) Phosphatidylglycerol biosynthesis in chloroplasts of Arabidopsis mutants deficient in acyl-ACP glycerol-3-phosphate acyltransferase. *Plant J* 47(2):296-309.

36. Hurlock AK, Roston RL, Wang K, & Benning C (2014) Lipid trafficking in plant cells. *Traffic* 15(9):915-932.

37. Kunst L, Browse J, & Somerville C (1989) A mutant of Arabidopsis deficient in desaturation of palmitic Acid in leaf lipids. *Plant Physiol* 90(3):943-947.

38. Li X*, et al.* (2012) A Galactoglycerolipid Lipase Is Required for Triacylglycerol Accumulation and Survival Following Nitrogen Deprivation in Chlamydomonas reinhardtii. *Plant Cell* 24(11):4670-4686.

39. Lu BB & Benning C (2009) A 25-Amino Acid Sequence of the Arabidopsis TGD2 Protein Is Sufficient for Specific Binding of Phosphatidic Acid. *J Biol Chem* 284(26):17420-17427.

40. 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(4):614-623.

41. Murashige T & Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiol Plantarum* 15(3):473-497.

42. Clough SJ & Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J* 16(6):735-743.

43. Ma W*, et al.* (2015) Deletion of a C-terminal intrinsically disordered region of WRINKLED1 affects its stability and enhances oil accumulation in Arabidopsis. *Plant J* 83(5):864-874.

44. Kushnirov VV (2000) Rapid and reliable protein extraction from yeast. *Yeast* 16(9):857-860.

45. 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.

46. Burgos A*, et al.* (2011) Analysis of short-term changes in the Arabidopsis thaliana glycerolipidome in response to temperature and light. *Plant J* 66(4):656-668.

CHAPTER 3

Lipid Composition Affects the Stability of Isolated Chloroplasts

Abstract

Chloroplasts are often isolated to allow for studies which are free of the influence of all other components. Here I investigate the effect of lipid composition on the stability of isolated chloroplasts during extended experiments by comparing chloroplasts of wild-type Arabidopsis to those of three lipid metabolism mutants, *sfr2-3*, *tgd1-1*, and *sfr2-3 tgd1-1*. The results suggest both the presence of oligogalactolipids and the double bond index of the acyl groups have an effect on isolated chloroplast stability, but the double bond index seems to have a stronger and dominant effect.

Introduction

Chloroplasts are the complex organelles which house the machinery required to carry out photosynthesis. They have a unique double membrane as an envelope, the outer and the inner envelope membrane, enclosing the extensive thylakoid membranes, which contain the photosynthetic protein complexes embedded in lipids. In addition to photosynthesis, chloroplasts also carry out much of the lipid metabolism within the plant cell (1). Fatty acids are produced in the chloroplast and are either used within the organelle for glycerolipid biosynthesis or are exported for glycerolipid biosynthesis in the endoplasmic reticulum (ER) (1, 2). The glycerolipid precursors produced in both the chloroplast and the ER serve in the assembly of glycerolipids with galactose sugar head groups which are exclusive to the chloroplast under normal growth conditions (3).

The combination of the presence of specialized chloroplast lipids and the unique membrane arrangement allow each subchloroplast membrane to have a different lipid composition. The outer envelope, which is the most permeable membrane (4), is the only chloroplast membrane which contains phosphatidylcholine (PC) above levels of contamination from ER membranes during isolation (5, 6). PC is actually located specifically in the outer leaflet of the outer envelope membrane but makes up as much as 40% of the total lipid composition of

the outer envelope membrane (5, 7). Both envelopes as well as the thylakoid membranes are composed of substantial quantities of the galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), with all three membranes containing close to 30% DGDG and levels of MGDG increasing from close to 10% in the outer envelope to up to 50% in the inner envelope or 65% in the thylakoid membranes (7). Because chloroplasts are largely lacking in phospholipids abundantly found in extraplastidic membranes, phosphatidylinositol (PI), phosphatidylethanolamine (PE), and phosphatidylserine (PS), the only other major lipids found in the chloroplast are the sulfolipid, sulfoquinovosyldiacylglycerol (SQDG), and phosphatidylglycerol (PG) (7). SQDG and PG occur in the range of 5-15% in each chloroplast membrane, and are slightly more abundant in the thylakoid membranes (7).

Because of the unique biochemistry which takes place within the chloroplasts, these organelles are often isolated for further study of everything from photosynthesis and protein import to lipid metabolism. During my studies done with isolated chloroplasts from Arabidopsis mutants disrupted in lipid metabolism, *tgd1-1* and *sfr2-3*, I observed that chloroplasts with different lipid composition were more or less stable over time when compared to those isolated from wild-type (WT) plants. Aside from practical experimental implications, the further study of this phenomenon may provide novel insights into the physiological function of specific chloroplast lipids. The *tgd1-1* mutant caries a leaky allele and is impaired in ER to chloroplast lipid transport which is known to affect the ratio of 16C to 18C fatty acids. It was named for its constitutive production of oligogalactolipids, specifically trigalactosyldiacylglycerol (8, 9). The production of oligogalactolipids is due to the activation of SFR2 (Sensitive to Freezing 2), which removes galactose head groups from MGDG and repeatedly adds them to other MGDG moieties to make oligogalactolipids of which hexagalactosyldiacylglycerol is currently known as the oligogalactolipid with the most galactosyl residues in its head group (10, 11). While the *tgd1-1* mutant has a constitutively activated SFR2 for not yet known reasons (8, 9), chloroplasts isolated from WT plants also have SFR2 activated (12). The *sfr2-3* mutant lacks functional

SFR2 and, therefore, cannot produce oligogalactolipids even after chloroplast isolation. Thus studying these mutants, I hoped to address the question of whether oligogalactolipids stabilize chloroplasts.

Once isolated, chloroplasts remain intact for a limited amount of time before breaking. For this reason, experiments requiring isolated chloroplasts to maintain the integrity of their membranes for the duration of the experiment are limited to short time spans of usually less than 1-2 hrs. Depending on the type of experiment being conducted, the chloroplasts of different mutants may need to be compared to those of WT. In the case of lipid metabolism mutants, there is a question of whether the isolated chloroplasts of plants with altered lipid composition remain intact for the same amount of time as those of wild type. Different levels of chloroplast breakage could potentially alter the conditions of experiments by leaking stromal contents into the buffer system as well as giving proteins access to compounds in membranes with which they do not normally come in contact. Overall, the aim of this study was to identify the differences in chloroplast intactness over time between isolated chloroplasts of Arabidopsis with different lipid compositions using specific mutants and to correlate these differences with the lipid phenotype through detailed lipid analysis of isolated chloroplasts. I expected to gain novel insights into the function of specific lipids and to develop a novel system to study the physiological function of chloroplast membrane lipids.

Results

Isolated Chloroplasts With Altered Lipid Composition and Metabolism Differ in Stability.

In order to determine if the experimental conditions for isolated chloroplasts with altered lipid composition are similar to those of wild type, the percentage of chloroplasts which remain intact after a 2 h room temperature incubation was determined for WT, *tgd1-1*, *sfr2-3*, and *sfr2-3 tgd1-1* chloroplasts (Figure 3.1A). As noted above, *tgd1-1* plants are defective in ER to chloroplast lipid transport causing an increase in 16C to 18C ratio as well as production of TGDG and other

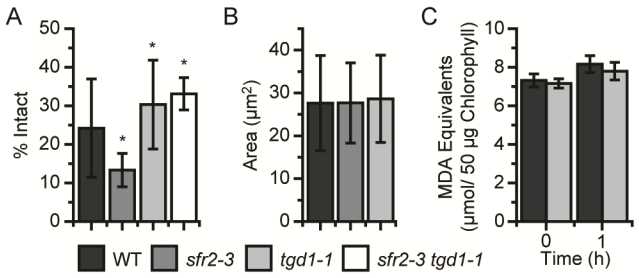

Figure 3.1 Measurement of Properties of Isolated Chloroplasts. (A) The percent of chloroplasts which remained intact after a 2 h incubation period is shown for chloroplasts from WT and each mutant. WT, n = 41; *sfr2-3*, n = 22; *tgd1-1*, n = 44; *sfr2-3 tgd1-1*, n = 4. (B) The average area of the isolated chloroplasts is shown. 185 ≤ n ≤ 222. (C) The MDA equivalents measured for WT and *tgd1-1* are shown for 0 and 1 hour after initial isolation. MDA, malondialdehyde. n = 3. All error bars denote SD. Student's t-test was performed comparing mutant to wild-type (WT) results, * $p < 0.05$.

oligogalactolipids (8, 9). The *sfr2-3* mutant is known to be more similar to WT in its lipid composition other than the inability to produce oligogalactolipids under inducing conditions (11, 13), and the *sfr2-3 tgd1-1* mutant was included to distinguish between chloroplast stability differences caused by oligogalactolipid production or the lipid compositional changes specific to the *tgd1-1* mutant. Chloroplasts were aliquoted into equal chlorophyll equivalents for the experiment. After 2 h at room temperature in the light, chloroplasts remaining intact were re-isolated on Percoll cushions, and their chlorophyll was extracted and measured. The chlorophyll content of re-isolated chloroplasts was compared to the chlorophyll content of aliquots of the same type, which had not been re-isolated after the 2 h incubation in order to determine the percentage of chloroplasts which remained intact. Approximately 25% of the WT chloroplasts were intact after 2 h compared to less than 15% of *sfr2-3* chloroplasts (Figure 3.1A). In contrast, approximately 30% of the *tgd1-1* and *sfr2-3 tgd1-1* mutant chloroplasts could be re-isolated after the incubation period (Figure 3.1A).

WT and *tgd1-1* Mutant Chloroplasts Show No Differences in Size or Production of Lipid Oxidation Products.

Other than lipid composition, presumably differences in size of the chloroplasts or amounts of reactive oxygen species (ROS) produced could affect the stability of chloroplasts in solution. In order to check the size of the isolated chloroplasts, they were extracted from WT, *tgd1-1*, and

sfr2-3 plants and examined under a light microscope. A series of pictures were captured for chloroplasts of each type, and ImageJ was used to determine the apparent area projections of individual chloroplasts. Based on this measure, no apparent size differences were seen between the WT and mutant chloroplasts (Figure 3.1B). For the ROS measurement, a TBARS (thiobarbituric acid reactive substances) assay was used, which measures the malondialdehyde (MDA) content (14). MDA is formed by some lipid peroxidation products, so this method is a general estimate of lipid oxidation levels (14, 15). Only WT and *tgd1-1* chloroplasts were used for this assay as representative samples. Results showed no differences in MDA levels between WT and mutant chloroplasts (Figure 3.1C).

Polar Lipid Amounts Change in the Chloroplasts from Different Mutant Backgrounds

While analysis of the polar lipids in whole leaves for the WT, *sfr2-3*, *tgd1-1*, and *sfr2-3 tgd1-1* plants has been published (8, 9, 11, 13, 16), these analyses needed to be repeated on isolated chloroplasts in order to determine how chloroplast specific differences in lipid composition might affect chloroplast intactness. Chloroplast amounts used for lipid measurement were aliquoted based on chlorophyll equivalents, and the lipids were extracted and used for polar lipid analysis. When the amounts of polar lipid species were analyzed as a percent of the total polar lipid amount, not many statistical differences were seen between WT and the three mutants, but the *tgd1-1* and *sfr2-3 tgd1-1* mutants did show small increases in the percentage of PC or oligogalactolipids (Figure 3.2A). It should be noted that the solvent, which was used for polar lipid separation, could not separate the oligogalactolipids of higher order than TGDG from PC (Figure 3.2D). Therefore, the apparent increase in PC could also indicate an increase in oligogalactolipids, although, this would not be expected to be the case in the *sfr2-3 tgd1-1* chloroplasts as they lack functional SFR2 protein. The solvent system also could not always resolve TGDG from PE and PI (Figure 3.2D). Therefore, all three lipids were consistently isolated together. The amount of lipid detected for the PE+PI+TGDG spot appeared to be

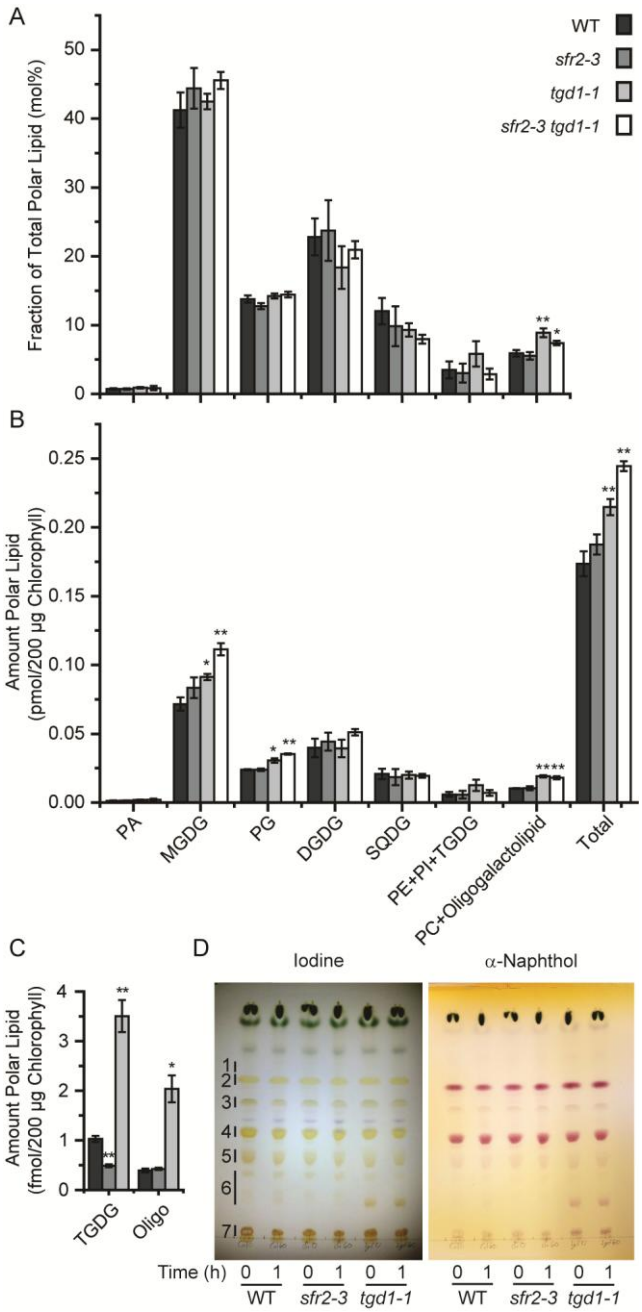

Figure 3.2 Polar Lipid Analysis. The individual polar lipid amounts are represented as a percent of total polar lipid. (A) and as pmol per 200 µg chlorophyll (B) n = 3. (C) TGDG and other oligogalactolipid amounts are show as fmol per 200 µg chlorophyll, n = 3. (D) Polar lipid separation on TLC for chloroplasts 0 and 1 hr after isolation is shown with both iodine (all lipid) and α-naphthol (glyclolipid) stains. 1, PA, phosphatidic acid; 2, MGDG, monogalactosyldiacylglycerol; 3, PG, phosphatidylglycerol; 4, DGDG, diglactosyldiacylglycerol; 5, SQDG, sulfoquinovosyldiacylglycerol; 6, PE+PI+TGDG, phosphatidylethanolamine+phosphatidylinsitol+trigalactosyl-diacylglycerol; 7, PC+Oligogalactolipid, phosphatidylcholine; Oligo, oligogalactolipid. Error bars denote SD. Student's t-test was performed comparing mutant to wild-type (WT) results, * $p < 0.05$, ** $p < 0.005$.

increased in the *tgd1-1* mutant based on visual examination of the TLC plates (Figure 3.2D) as would be expected with constitutive production of TGDG, but with n = 3, the apparent difference was not statistically significant (Figure 3.2A).

The polar lipid amounts were also represented as absolute amounts normalized to the chlorophyll equivalents used for analysis (Figure 3.2B). While no differences in polar lipid amounts were detected between WT and *sfr2-3* chloroplasts, the *tgd1-1* and *sfr2-3 tgd1-1* chloroplasts showed increased total polar lipids (relative to chlorophyll) due to increases in MGDG, PG, and PC+oligogalactolipid (Figure 3.2B). As for the representation as relative mol% fractions, the PE+PI+TGDG visually seemed to be increased in the *tgd1-1* mutant background (Figure 3.2D), but again, this difference was not statistically significant (Figure 3.2B). This increase in absolute amounts of polar lipids for *tgd1-1* and *sfr2-3 tgd1-1* chloroplasts is opposite of the results obtained previously for leaf tissues based on dry weight (16). The *tgd1-1* mutant plants are known to be slightly paler than WT plants (8, 9), so this increase is probably an indication of decreased pigment in the chloroplasts rather than increased lipid since these results are normalized to chlorophyll equivalents. It should be noted that while the *tgd1-1* plants appear pale, previously performed ultrastructural studies showed no differences in the appearance or abundance of the thylakoid membranes (8).

In order to get a better measure of TGDG and the other oligogalactolipids, a different solvent system was used to resolve and analyze them separately. The *sfr2-3* chloroplasts showed a small base level of TGDG and higher order oligogalactolipids, which was probably due to slight overlap with PA on the TLC plate (Figure 3.2C). The WT chloroplasts had detectable amounts above the *sfr2-3* background levels of TGDG but not higher order oligogalactolipids (Figure 3.2C), which was expected based on the known activation of SFR2 upon isolation of chloroplasts (12). The *tgd1-1* chloroplasts had significantly increased levels of both TGDG and higher order oligogalactolipids compared to WT levels as expected with constitutively active SFR2 (Figure 3.2C).

The appearance of lyso-lipids was also examined on the polar lipid TLC plates. WT, *sfr2-3*, and *tgd1-1* chloroplast lipids were extracted from chloroplast aliquots immediately after isolation as well as after a 1 hr room temperature incubation. It was expected that chloroplasts which retained lower levels of intactness over time, like those of the *sfr2-3* mutant, may show signs of lipid degradation, but no lyso-lipids were detectable for any of the types of chloroplasts before or after the 1 h incubation (Figure 3.2D).

The Fatty Acid Ratios in Polar Lipids Change as Expected for the Different Mutant Backgrounds.

Detailed analysis of the mole percentages of different fatty acids in each polar lipid was determined for WT, *sfr2-3*, *tgd1-1*, and *sfr2-3 tgd1-1* isolated chloroplasts (Figure 3.3). No differences were detected in the fatty acid composition of any lipids between the WT and *sf2-3* chloroplasts (Figure 3.3). This result for isolated chloroplasts is consistent with the published results analyzing lipids of whole leaves under normal growth conditions (11, 13). The *tgd1-1* and *sfr2-3 tgd1-1* mutant chloroplasts showed decreases in 18:3 (carbon #: double bond #) and increases in 18:1Δ9 (Δ# = double bond position from the carboxyl end) in all lipids except PE+PI+TGDG in the *tgd1-1* background did not show the decrease in 18:3 (Figure 3.3). PG, DGDG, and PC+oligogalactolipids also had increased 16:3 levels in the *tgd1-1* and *sfr2-3 tgd1-1* chloroplasts, while 16:3 was decreased in MGDG (Figure 3.3). Also in these two types of chloroplasts, both MGDG and DGDG showed increased 16:0, 16:1, and 16:2 levels, while PC+oligogalactolipids had increased 16:1 and 16:2 but decreased 16:0 levels (Figure 3.3). All of these fatty acid changes were consistent with what is known about the *tgd1-1* mutant and indicated that the lipid phenotype of *sfr2-3 tgd1-1* replicates that of *tgd1-1* in all aspects but oligogalactolipid production (8, 9).

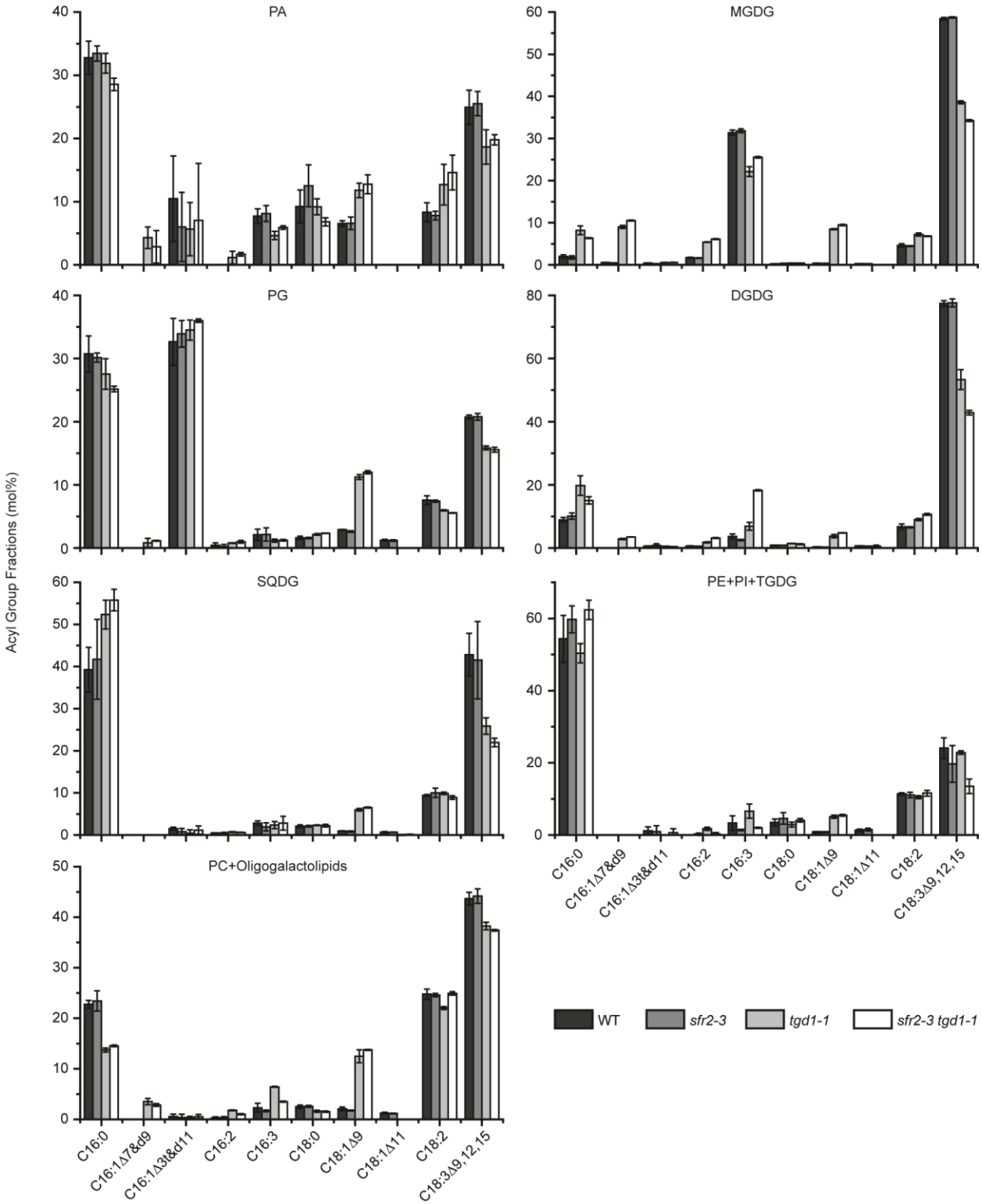

Figure 3.3 Acyl Group Profiles for Polar Lipids. The fractions of different acyl groups are shown as mole fractions for each polar lipid. PA, phosphatidic acid; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; DGDG, diglactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PE+PI+TGDG, phosphatidylethanolamine+phosphatidylinsitol+trigalactosyldiacylglycerol; PC+Oligogalactolipid, phosphatidyl-choline. Error bars denote SD and n = 3. Student's t-test was performed comparing mutant to wild-type (WT) results, * $p < 0.05$, ** $p < 0.005$.

Chloroplasts from plants with the *tgd1-1* Mutation Have Changes in Double Bond Index as well as 16C to 18C and MGDG to DGDG Ratios

In order to get a more complete view of the chloroplast lipid composition differences between WT, *sfr2-3*, *tgd1-1*, and *sfr2-3 tgd1-1* chloroplasts, the double bond index (DBI) and both 16C to 18C and MGDG to DGDG ratios were calculated. The DBI represents the level of unsaturation of the acyl groups with a lower number meaning fewer double bonds and, therefore, less unsaturation. The DBI of all polar lipids was the same between WT and *sfr2-3*, but *tgd1-1* and *sfr2-3 tgd1-1* both had similar decreases in the DBI of MGDG, PG, DGDG, and SQDG (Figure 3.4A). No changes were seen in either 16C to 18C or MGDG to DGDG ratio between WT and *sfr2-3* chloroplasts (Figure 3.4B&C). However, both the *tgd1-1* and *sfr2-3 tgd1-1* chloroplasts had increased 16C to 18C ratios for MGDG, DGDG, and SQDG (Figure 3.4B), which is consistent with published data for *tgd1-1* (17). The MGDG to DGDG ratio was significantly increased in the *tgd1-1* chloroplasts, but the *sfr2-3 tgd1-1* chloroplasts did not show a statistically significant increase in this ratio with only n = 3 (Figure 3.4C). It should be noted that the MGDG to DGDG ratio was neither statistically significant from WT nor *tgd1-1*.

Discussion

Assays done with isolated chloroplasts require much care to assure comparable conditions between samples as well as consideration as to how the conditions may change throughout the duration of the experiment. Here, isolated chloroplasts with different lipid compositions were shown to have different levels of stability over time (Figure 3.1A). Because size of the chloroplasts (Figure 3.1B) and levels of the lipid oxidation product, malondialdehyde (Figure 3.1C), were unchanged between the different types of chloroplasts and no lipid degradation products were detected (Figure 3.2D), it is likely that the differences in lipid composition are the direct cause of the stability differences. However, because the method of re-isolation on a Percoll cushion cannot distinguish between chloroplasts truly remaining intact and chloroplasts

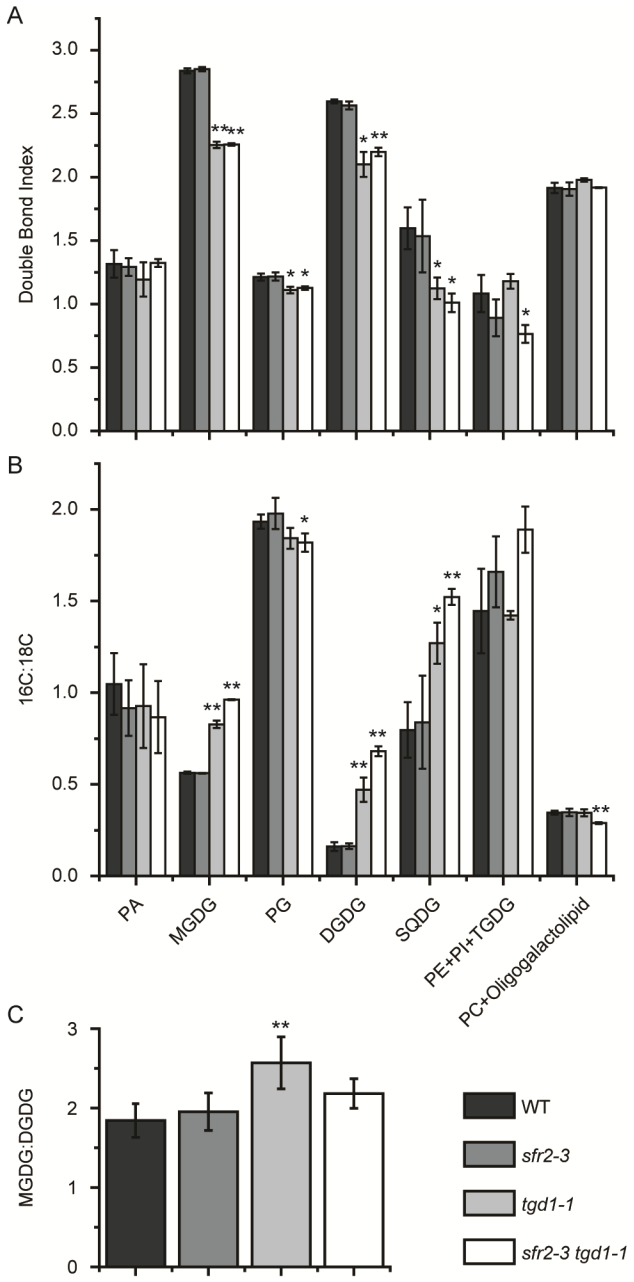

Figure 3.4 Lipid Related Ratios and Double Bond Index. (A) The 16C to 18C acyl composition of each polar lipid is shown for each type of chloroplast, n = 3. (B) The double bond index is shown for each polar lipid of each type of chloroplast, n = 3. (C) The MGDG to DGDG ratio is shown for each type of chloroplast, 3 ≤ n ≤ 6. PA, phosphatidic acid; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; DGDG, diglactosyl-diacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PE+PI+TGDG, phosphatidylethanolamine+phosphatidyl-insitol+trigalactosyldiacylglycerol; PC+Oligogalactolipid, phosphatidylcholine. Error bars denote SD. Student's t-test was performed comparing mutant to wild-type (WT) results, * $p < 0.05$, ** $p < 0.005$.

breaking to release stromal contents but then resealing, the stability differences measured here could also be differences in the ability of the membranes to reseal following damage. In order to distinguish between these two possibilities a different test for chloroplast intactness can be

used. For example, a test which measures for enzyme activity which is normally associated with the stroma of chloroplasts, such as 6-phosphogluconate dehydrogenase, in the buffer (18), might provide information about stroma leakage as a consequence of chloroplast rupture followed by resealing.

Even without further study using a different intactness test, the current results indicate that the differences in lipid composition of the chloroplasts are causing the chloroplasts to act differently once isolated. The only lipid change between WT and *sfr2-3* chloroplasts is the reduced amount of TGDG (Figure 3.2C). Since the *sfr2-3* chloroplasts are more prone to breakage than those of WT (Figure 3.1A), TGDG and higher order oligogalactolipids must be able to confer stability to the membrane whether that be prevention of breakage or the ability to reseal. SFR2 is activated under freezing conditions, which can disrupt cellular membranes (11), so it is understandable that oligogalactolipids would serve to protect the intactness of the membrane.

In contrast to the *sfr2-3* chloroplasts, chloroplasts from *tgd1-1* and *sfr2-3 tgd1-1* show increased stability once isolated when compared to WT chloroplasts (Figure 3.1A). Because the *sfr2-3 tgd1-1* chloroplasts resemble the phenotype of *tgd1-1* rather than that of *sfr2-3*, something other than the presence of oligogalactolipids must affect the stability of isolated chloroplasts, and it must be able to completely compensate for lack of oligogalactolipids. Both *tgd1-1* and *sfr2-3 tgd1-1* chloroplasts have decreased DBI in certain lipids (Figure 3.4A), an increased 16C to 18C ratio (Figure 3.4B), and increased ratios of lipid to chlorophyll (Figure 3.2B). The *tgd1-1* chloroplasts also show an increased MGDG:DGDG ratio, while the results for *sfr2-3 tgd1-1* were inconclusive (Figure 3.4B). Since there is no indication of decreased amounts of membrane damaging lipid oxidation in *tgd1-1* (Figure 3.1C), it is unlikely the stability is due to less chlorophyll and photosynthetic activity. Also, MGDG is a non-bilayer forming lipid, so an increased MGDG to DGDG ratio would more likely destabilize a membrane than stabilize it. This leaves the decreased DBI and increased 16C to 18C ratio as the most likely reasons for

increased chloroplast stability following isolation. Study of the effect of unsaturation levels on the propensity for MGDG to form the hexagonal-II phase rather than a bilayer has shown that decreased levels of unsaturation result in reduced formation of the hexagonal-II phase (19). The degree of unsaturation has this effect on MGDG because fewer double bonds allow for the acyl chains to take up less space and help to balance the smaller head group to form more of a cylindrical than a cone shape. The levels of unsaturation are not known to have an effect on bilayer formation for PG, DGDG, or SQDG (19). The decreased DBI in MGDG for *tgd1-1* and *sfr2-3 tgd1-1* chloroplasts indicates a lower degree of unsaturation (Figure 3.4A). Therefore, MGDG would presumably be less likely to form a hexagonal-II phase thereby decreasing the probability of chloroplast disruption during phase transition of the membrane. It is also possible that an increase in shorter acyl chains, like 16C, may have the same effect on MGDG because this may also decrease the amount of space occupied by the acyl chains. In order to further investigate how DBI and 16C to 18C ratio affect chloroplast membrane stability, the next step would be to run tests on chloroplasts isolated from *ats1-1* mutants which have a decreased 16C to 18C ratio but a similar DBI of MGDG compared to WT (20, 21). Chloroplasts of the *ats1-1* mutant would be expected to have reduced stability compared to those of WT if the 16C to 18C ratio is at least partially responsible for the increased stability of *tgd1-1* and *sfr2-3 tgd1-1* isolated chloroplasts or no change in stability, if DBI is the only factor responsible.

This work on isolated chloroplasts speaks to how important lipid composition can be to the physical characteristics of membranes, but additional work needs to be done in order to substantiate the conclusions. Moving forward, data should be collected for chloroplasts from *ats1-1* plants for all of the experiments already presented here. It is also important to perform a different type of intactness test, such as measurement for 6-phosphogluconic acid dehydrogenase activity release into buffer, in order to distinguish between actual resistance to breakage and the ability to reseal a broken membrane. Having these data in hand, it should be possible to determine the relative importance of the different factors discussed above for the

integrity of isolated chloroplasts and extrapolate these results towards the behavior of chloroplasts in intact plants exposed to different abiotic stresses that could affect membrane integrity.

Materials and Methods

Plant Growth and Genotyping

Arabidopsis (*Arabidopsis thaliana*) were grown on petri plates containing MS medium as described for organelle isolation in Chapter 2. The genotypes of all of the mutant plants used for seed stock were also confirmed with established methods for both *sfr2-3* and *tgd1-1* mutations (9, 11). This confirmation of genotype was needed because plants with the *tgd1-1* mutation in particular are prone to contamination due to poor pollen quality, so each plant from which seeds are collected and used must be checked to ensure homozygosity.

Chloroplast Isolation

Chloroplast isolation was carried out as described in Chapter 2.

Chloroplast Intactness

Isolated chloroplasts were aliquoted into 50 µg chlorophyll equivalents and pelleted by centrifugation at 700 x g for 5 min at 4°C. The pellets were resuspended in 50 µL of import buffer (330 mM sorbitol, 50 mM HEPES, pH 8.0 KOH) for 1 µg chlorophyll equivalents/µL import buffer. The chloroplast aliquots were incubated at room temperature in the light for 2 h. After incubation, half of the aliquots were centrifuged at 5000 rpm in a microfuge for 5 min at room temperature, and the pellet was extracted with 800 µL of methanol:chloroform (2:1 v/v). These aliquots were used to determine the absorbance at 652 nm, a measurement for chlorophyll, for the total amount of chloroplasts in each aliquot. The other half of the aliquots were loaded onto 3 mL 40% Percoll cushions in import buffer and centrifuged at 1500 x g for 5 min at 4°C in order

to pellet only the chloroplasts which remained intact for the duration of the incubation. Each pellet was resuspended in 1 mL of import buffer, centrifuged at 5000 rpm in a microfuge for 5 min at room temperature, and extracted with 800 µL of methanol:chloroform (2:1 v/v). The absorbance at 652 nm was measured for the re-isolated chloroplast extracts and taken as a percent of the total absorbance for the chloroplasts which were not re-isolated. This percent was considered the percent of chloroplasts which remained intact.

Measurement of Chloroplast Area

Isolated chloroplasts were centrifuged at 700 x g for 5 min at 4°C, and the pellets were resuspended in import buffer (same as above) to a concentration of 1 mg chlorophyll equivalents per mL import buffer. Images were taken using a Leica DMi8 inverted microscope and HCX PL S-APO 63x/1.30 oil objective. The area of individual chloroplasts was then determined from the images using ImageJ software for analysis.

Measurement of Reactive Oxygen Species by TBARS

Isolated chloroplasts were aliquoted into 50 µg chlorophyll equivalents and pelleted by centrifugation at 700 x g for 5 min at 4°C. Half of the chloroplast aliquots were resuspended in 50 µL import buffer and incubated for 1 h at room temperature in the light. The other aliquots were immediately resuspended in 1 mL of either TCA solution (4.06% trichloroacetic acid in water) as the negative control or TBA solution (0.31% thiobarbituric acid in TCA solution) for measurement of thiobarbituric acid reactive substances (TBARS). The chloroplasts subjected to 1 h incubation were re-pelleted and resuspended in either TCA or TBA solution after the incubation step. The suspensions were heated at 95°C for 15 min along with 2 tubes of TBA solution to use as blanks for the spectrophotometric measurements. The samples were allowed to cool completely and then centrifuged at maxium speed in a microfuge for 3 min at room

temperature. The absorbances for each sample were taken at 440 nm, 532 nm, and 600 nm. The MDA equivalents were calculated as in (15).

Lipid Analysis

Isolated chloroplasts were aliquoted into 200 µg chlorophyll equivalents (or 100 µg chlorophyll equivalents for thin layer chromatography (TLC) separation only meant for staining and visualization) and pelleted by centrifugation at 2000 x g in a microfuge for 3 min at room temperature. Each pellet was then extracted with 800 µL methanol:chloroform:formic acid (2:1:0.1 v/v/v) followed by 400 µL of 0.2 M H_3PO_4, 1 M KCl. The organic layer was transferred to a new tube and dried under a N_2 gas stream. The TLC plates used were the same as described in Chapter 2. Samples were first resuspended in 25 µL chloroform and loaded onto TLC followed by a second and third resuspensions and loading of 15 µL and 10 µL, respectively, in order to ensure loading of the entire sample. Two different solvent systems were used for lipid separation. The solvent used for general polar lipid separation was acetone:toluene:water (91:30:7 v/v/v), and the solvent used for better separation of the oligogalactolipids was chloroform:methanol:concentrated ammonium hydroxide (50:25:6 v/v/v). Fatty acid methyl ester (FAME) production and GC analysis as well as iodine and α-naphthol staining were carried as described in (22).

REFERENCES

REFERENCES

1. Ohlrogge J & Browse J (1995) Lipid biosynthesis. *Plant Cell* 7(7):957-970.

2. Li-Beisson Y*, et al.* (2013) Acyl-lipid metabolism. *Arabidopsis Book* 11:e0161.

3. Dormann P & Benning C (2002) Galactolipids rule in seed plants. *Trends Plant Sci* 7(3):112-118.

4. Heldt HW & Sauer F (1971) The inner membrane of the chloroplast envelope as the site of specific metabolite transport. *Biochim Biophys Acta* 234(1):83-91.

5. Dorne AJ, Joyard J, Block MA, & Douce R (1985) Localization of phosphatidylcholine in outer envelope membrane of spinach chloroplasts. *J Cell Biol* 100(5):1690-1697.

6. Dorne AJ, Joyard J, & Douce R (1990) Do thylakoids really contain phosphatidylcholine? *Proc Natl Acad Sci U S A* 87(1):71-74.

7. Andersson MX & Dörmann P (2009) Chloroplast Membrane Lipid Biosynthesis and Transport. *The Chloroplast,* Plant Cell Monographs, eds Sandelius A & Aronsson H (Springer Berlin Heidelberg), Vol 13, pp 125-158.

8. Xu C, Fan J, Froehlich JE, Awai K, & Benning C (2005) Mutation of the TGD1 chloroplast envelope protein affects phosphatidate metabolism in Arabidopsis. *Plant Cell* 17(11):3094-3110.

9. Xu C, Fan J, Riekhof W, Froehlich JE, & Benning C (2003) A permease-like protein involved in ER to thylakoid lipid transfer in Arabidopsis. *EMBO J* 22(10):2370-2379.

10. Roston RL, Wang K, Kuhn LA, & Benning C (2014) Structural determinants allowing transferase activity in SENSITIVE TO FREEZING 2, classified as a family I glycosyl hydrolase. *J Biol Chem* 289(38):26089-26106.

11. Moellering ER, Muthan B, & Benning C (2010) Freezing tolerance in plants requires lipid remodeling at the outer chloroplast membrane. *Science* 330(6001):226-228.

12. Wintermans JFGM, van Besouw A, & Bögemann G (1981) Galactolipid formation in chloroplast envelopes II. isolation-induced changes in galactolipid composition of envelopes. *Biochim Biophys Acta* 663(1):99-107.

13. Barnes AC, Benning C, & Roston RL (2016) Chloroplast membrane remodeling during freezing stress is accompanied by cytoplasmic acidification activating SENSITIVE TO FREEZING2. *Plant Physiol* 171(3):2140-2149.

14. Janero DR (1990) Malondialdehyde and thiobarbituric acid-reactivity as diagnostic indices of lipid peroxidation and peroxidative tissue injury. *Free Radical Biol. Med.* 9(6):515-540.

15. Hodges DM, DeLong JM, Forney CF, & Prange RK (1999) Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta* 207(4):604-611.

16. Fan J, Yan C, Roston R, Shanklin J, & Xu C (2014) Arabidopsis lipins, PDAT1 acyltransferase, and SDP1 triacylglycerol lipase synergistically direct fatty acids toward beta-oxidation, thereby maintaining membrane lipid homeostasis. *Plant Cell* 26(10):4119-4134.

17. Yang Y, Zienkiewicz A, Lavell A, & Benning C (2017) Co-evolution of Domain Interactions in the Chloroplast TGD1, 2, 3 Lipid Transfer Complex Specific to Brassicaceae and Poaceae Plants. *Plant Cell*.

18. Journet EP & Douce R (1985) Enzymic capacities of purified cauliflower bud plastids for lipid synthesis and carbohydrate metabolism. *Plant Physiol* 79(2):458-467.

19. Webb MS & Green BR (1991) Biochemical and biophysical properties of thylakoid acyl lipids. *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1060(2):133-158.

20. Kunst L, Browse J, & Somerville C (1988) Altered regulation of lipid biosynthesis in a mutant of Arabidopsis deficient in chloroplast glycerol-3-phosphate acyltransferase activity. *Proc Natl Acad Sci U S A* 85(12):4143-4147.

21. Xu C, Yu B, Cornish AJ, Froehlich JE, & Benning C (2006) Phosphatidylglycerol biosynthesis in chloroplasts of Arabidopsis mutants deficient in acyl-ACP glycerol-3-phosphate acyltransferase. *Plant J* 47(2):296-309.

22. 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. 10.3791/2518.

CHAPTER 4

Development of *In Vitro* Lipid Transfer Assays for Determining the Lipid Molecular Species Transferred from the Endoplasmic Reticulum to the Chloroplast

Abstract

Because plants use both ER and chloroplast based lipid synthesis pathways for the production of chloroplast lipids, a lipid transport mechanism is needed between the two organelles. The TriGalactosylDiacylglycerol (TGD) proteins have been identified to fulfill this need, but the identity of the transported lipid species remains elusive. Here, *in vitro* transport assays are developed in an effort to determine the identity of this transported lipid species. Many variations of radiolabeled donor liposome representing the ER membrane to acceptor membranes represented by isolated chloroplasts were tested. Labeled phosphatidic acid, phosphatidylcholine, and phosphatidylethanolamine + phosphatidylinositol were all transferred at a decreased rate to *tgd* mutant chloroplasts compared to those of wild type. Assays measuring the transport of lipids tagged with a fluorophore to isolated chloroplast envelopes were also explored. Fluorescent studies showed the TGD1,2,3 complex had a slight preference for phosphatidic acid, while the TGD4 dimer had a slight preference for phosphatidylcholine. Both radiolabel and fluorescent assays yielded promising results, but unexpected control results in many cases questioned whether the *in vitro* data observed truly reflect the situation *in vivo.*

Introduction

Chloroplasts are most well-known for housing the photosynthetic machinery which can utilize solar energy for carbon fixation. These organelles use an extensive membrane system, the thylakoids, providing a scaffold for the photosynthetic proteins and the thylakoid lipids that are also found as integral components of the protein complexes (1-6). Plants use two biosynthetic pathways in order to produce all of the membrane lipids needed for the chloroplast. One pathway, the plastid pathway, is located in the chloroplast, which is also the site of fatty acid synthesis for plant cells. The other pathway, the endoplasmic reticulum (ER) pathway, uses fatty acids exported from the chloroplast to then make membrane lipids, which are exported from the ER to other organelles including the chloroplast. Membrane lipids require transport mechanisms

to transverse the aqueous space between organelles or membrane contact sites between different organelles which generally still have up to 30 nm of aqueous space in between (7). The only identified transport mechanism to move lipids from the ER to the chloroplast is facilitated by TriGalactosylDiacylglycerol (TGD) proteins (8-14). Five TGD proteins have been identified in genetic screens (8-15). The *tgd* mutants all show impaired ER to chloroplast lipid transfer, which can be deduced by an increased 16 carbon to 18 carbon fatty acid ratio (16C:18C) in these mutants (14). This fatty acid ratio is diagnostic for the relative contribution of the two pathways because the different substrate specificities of the plastid and ER based acyl transferases, result in the presence of 16C fatty acids in the *sn*-2 glyceryl position of plastid derived lipids whereas 18C fatty acids reside in the *sn*-2 position of ER derived lipids (16, 17).

The TGD proteins have been well characterized in regards to mutant phenotypes and complex formation. Plants with defective or missing TGD proteins show a distinctive increase in the 16C:18C ratio as well as growth and reproductive phenotypes, in addition to the accumulation of oligogalactolipids (which gave rise to their name) (8-11, 14, 18). TGD4 is a β-barrel protein, which forms a homodimer in the outer envelope membrane of the chloroplast and has the ability to bind phosphatidic acid (PA) (11, 19, 20). TGD1, 2, and 3 form an ABC-transporter in the inner envelope membrane of the chloroplast with TGD1 and 3 being present in 2 copies each and TGD2 in 8-12 copies (13). TGD1 is the predicted permease while TGD3 has been confirmed as the ATPase (10, 14). TGD2 is the substrate-binding component which has been shown to both disrupt membranes and bind PA, and it likely spans the intermembrane space forming a lipid conduit (8, 15, 18, 21). TGD5 is a small (9.2 kDa) protein which has been shown to associate with both TGD4 and TGD2 proteins despite the lack of any detectable association between the actual TGD4 and TGD1,2,3 complexes (9). While these studies provide detailed characterization of the five TGD proteins, it is still unclear what lipid or lipids the TGD complexes transport. TGD2 and TGD4 both have the ability to bind PA, but it has not been shown that either complex can actually transport PA. It is possible that the binding of PA is just

a mechanism for membrane contact or disruption to facilitate the transfer of specific lipid species.

Hypotheses for the transported lipid species between the ER and chloroplast include not only PA but also phosphatidylcholine (PC), lyso-PC (L-PC), and diacylglycerol (DAG) (22). It is also possible that more than one lipid species is transferred from the ER to the chloroplast. PC has long been considered because it is present in the outer leaflet of the outer envelope of the chloroplast, but the chloroplast lacks the required machinery to produce PC independently (22, 23). L-PC is a candidate for the same reason as PC, but its biophysical properties may allow it to move more readily between membranes (24-26). DAG is a good candidate because not only is it the precursor for galactolipid synthesis, but it also has the ability to flip between leaflets of a membrane at biologically relevant rates (27, 28). This study focused on developing methods to determine the transported lipid species from the ER to the chloroplast by way of the TGD proteins.

Results

Isolated Chloroplasts from *tgd1-1* Mutants Receive Less Radiolabeled ER Lipids than Wild-type Chloroplasts.

In order to closely mimic the natural environment for ER to chloroplast lipid transport *in vitro,* radiolabeled ER microsomes were incubated with nonlabeled isolated chloroplasts from both wild type (WT) and the most severe, but still viable *tgd* mutant carrying the *tgd1-1* allele. To obtain labeled ER, *Pisum sativum* (pea) plants were incubated with ^{14}C-acetate. ^{14}C-acetate is a lipid precursor that is incorporated almost exclusively into fatty acyl chains of all glycerolipid classes. Pea plants were used because they could readily incorporate the label into lipids, and ER isolation from pea had a higher yield than that from Arabidopsis. The labeled ER microsomes were incubated with isolated chloroplasts from WT and *tgd1-1* plants for 30 min. Chloroplasts which remained intact throughout the duration of the incubation period were re-

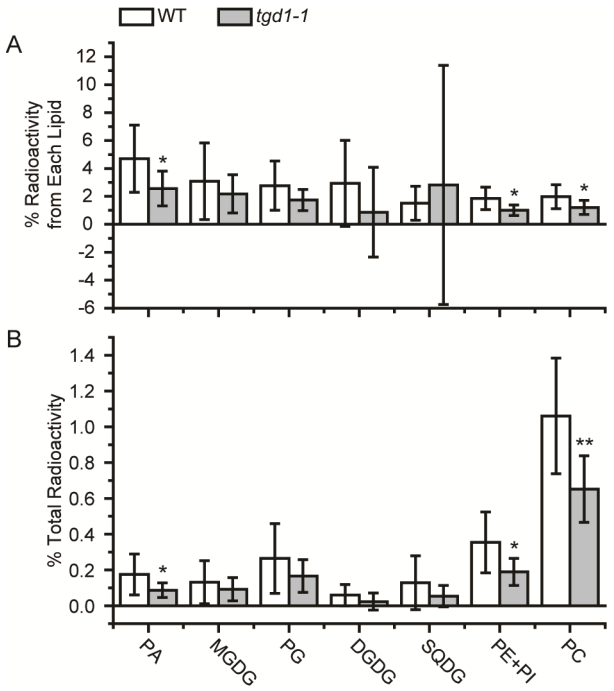

Figure 4.1 ^{14}C Labeled ER Microsome to Intact Chloroplast Lipid Transfer. The % of radiolabel transferred from ER microsomes extracted from ^{14}C-acetate labeled pea plants to isolated Arabidopsis chloroplasts from both wild-type (WT) and lipid transport mutant, *tgd1-1*. (A) The percentage is represented as the percent of radioactivity from each individual labeled lipid in the ER microsomes. (B) The percentage is represented as the percent of the total amount of radioactivity in the ER microsomes regardless of lipid class. Student's t-test was performed comparing WT to *tgd1-1* for each lipid, n = 10, * p-value < 0.05, ** p-value < 0.005, error bars denote SD. PA, phosphatidic acid; MGDG, monogalactosyldiacylglycerol; PG, phosphatidylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PE+PI, phosphatidylethanolamine+phosphatidylinositol; PC, phosphatidylcholine.

isolated for lipid analysis, and results were normalized based on chlorophyll equivalents. The amount of ^{14}C label measured in each polar lipid from the chloroplasts was compared to the amount of the ^{14}C label in each corresponding lipid from the ER microsomes (Figure 4.1A) as well as the total ^{14}C label in the ER microsomes (Figure 4.1B). The *tgd1-1* chloroplasts received significantly less label in PA and PC than the wild-type chloroplasts, but they also had less label in phosphatidylethanolamine (PE) and phosphatidylinositol (PI). The PE+PI fraction was considered a control as chloroplasts lack these phospholipids, and therefore, they should not be transported at all. Because labeled PE+PI represented the second highest fraction of the total radioactivity in the chloroplasts (as it is in the ER microsomes alone) and was lower in the *tgd1-1* mutant chloroplasts, the radiolabel detected in the chloroplasts may be a result of contamination from the microsomes. It is possible that the microsomes are less prone to "sticking" to *tgd* mutant chloroplasts because of the presence of trigalactosyldiacylglycerol

(TGDG) which is already known to cause differences in chloroplast properties (see Chapter 3). Alternatively, these lipids are actually transferred in this *in vitro* system, which may lack specificity.

Variations of the Radiolabeling Assay Did Not Produce Conclusive Results.

One caveat to using ER microsomes extracted from labeled plants as the donor for the transport assay with isolated chloroplasts was that the microsomes were always contaminated with labeled chloroplast lipids such as MGDG and DGDG. With several lipids being labeled and given the highly active metabolism of intact chloroplasts, it is difficult to distinguish direct transport of a specific lipid from its production from a transported precursor. Because of these experimental hurdles, several variations of the *in vitro* transport experiment were pursued (Table 1).

In one variation, commercially available ^{14}C-PA and ^{14}C-PC were simultaneously incorporated into ER microsomes extracted from pea or WT Arabidopsis plants. The two labeled lipids were incorporated simultaneously, so I could determine if one substrate was preferred over the other when in direct competition. These donor microsomes from pea or Arabidopsis were then incubated with pea or WT Arabidopsis isolated chloroplasts, respectively, in the presence or absence of ATP. Because the TGD1,2,3 complex is an ABC transporter with an ATPase subunit, it was expected the labeled transported lipid species would not be as abundant in the acceptor chloroplasts in the absence of ATP. However, the results showed no significant difference in the amounts of ^{14}C-PA or ^{14}C-PC found in the acceptor chloroplasts with or without ATP (Table 1). This was done with both pea and Arabidopsis chloroplasts because, while Arabidopsis has its TGD proteins well characterized, pea, as an 18:3 plant (29), is almost completely reliant on the ER pathway for the synthesis of chloroplast lipids. With this dependence on lipid flux coming into the chloroplast from the ER, pea chloroplasts were expected to show a more robust result than the Arabidopsis chloroplasts.

Label	Donor	Acceptor	Variable	Above Background?	Significant Difference?
^{14}C-Acetate	Pea Microsomes	Col-2 or *tgd1-1* chloroplasts	N/A	Yes	Yes
^{14}C-PA and ^{14}C-PC	WT Microsomes	Col-2 chloroplasts	+/- ATP	Yes	No
^{14}C-PA and ^{14}C-PC	Pea Microsomes	Pea chloroplasts	+/-ATP	Yes	No
^{14}C-PA or ^{14}C-PC	WT Microsomes	WT or *tgd3-1* chloroplasts	+/- UDP-Gal	Yes*	No
^{14}C-Acetate	Pea Microsomes	Pea chloroplasts	+/- cytosol	Yes*	No
^{32}P-H_3PO_4	Pea Microsomes	WT or *tgd1-1* chloroplasts	N/A	No	No
^{14}C-Acetate	Liposomes from Extracted Pea Lipids	WT, *tgd1-1*, *sfr2-3* or *sfr2-3 tgd1-1* chloroplasts	N/A	Yes*	No
^{14}C-Acetate	Liposomes from Extracted Pea Lipids	Pea chloroplasts	pH 7.3 or 8.0	Yes	No

Table 4.1 Summary of Radiolabeled Lipid Transport Assays. * Indicates only 50% of the repeats were above background levels. In order to determine if there was significant difference of $p < 0.05$, Student's t-test was performed between WT and mutant and/or the variable condition (e.g. +/-ATP) when applicable. WT, wild type Arabidopsis.

Another experimental variation used Arabidopsis ER microsomes as mentioned above, but the microsomes were either labeled with ^{14}C-PA or ^{14}C-PC, but not both concomitantly. This was done in order to simplify the interpretation of the experimental results. By using either ^{14}C-PA or ^{14}C-PC individually, I could distinguish which lipid is the donor of the label present in any other radiolabeled lipids. The acceptor chloroplasts were isolated from either WT or *tgd3-1* mutant chloroplasts with the expectation that the *tgd3-1* mutant chloroplasts would accept less of one of the lipids, therefore, identifying it as the transported lipid species. While *tgd3-1* is a weak mutant compared to other *tgd* mutants, it was chosen for this set of experiments due to availability of seeds at the time. The stronger *tgd* mutant allele, *tgd1-1*, was used for most of the other experiments. Different concentrations of UDP-galactose (UDP-Gal) were also added to the incubation buffer. The addition of UDP-Gal was expected to stimulate the synthesis of

galactolipids, MGDG or DGDG, from the transported precursor, and in turn, result in more total label above background being detected in the acceptor chloroplasts. The results with neither ^{14}C-PA nor ^{14}C-PC showed significant differences between amounts of label detected in WT or *tgd3-1* chloroplasts nor with varying amounts of UDP-Gal (Table 1).

Previously, it was shown that when fed with labeled PA, WT chloroplasts produced more labeled MGDG when a cytosolic protein fraction was included in the buffer (30). Two more studies also suggested cytosolic factors might be involved in either the transport or metabolism of the lipid precursor from the ER (31, 32). One paper described that a cytosolic phospholipid transport protein from spinach could facilitate transport of PC from liposomes to chloroplasts (32), although this protein was later shown to be a cell wall protein rather than cytosolic (33). The other paper concluded cytosolic phospholipases are needed for chloroplasts to produce MGDG from labeled PC transported from liposomes (31). Based on the evidence from the first and third paper mentioned (30, 31), I hypothesized adding cytosol to these radiolabeling assays might increase the transfer and hence, the amount of radiolabel detected in the acceptor chloroplasts. A cytosolic protein fraction was extracted from pea leaf tissue and either included or excluded from incubation buffer in an assay using ER microsomes from ^{14}C-acetate labeled pea as described above as donor and unlabeled isolated pea chloroplasts as acceptor. The results of this assay showed no difference in amount of labeled lipid received by chloroplasts with or without the cytosolic fraction (Table 1).

It was thought that one of the reasons no statistically significant differences between mutant and WT were obtained with these different experimental variations was the overall low levels of label being detected in the acceptor chloroplasts. Some repeats of the experiments yielded results less than background levels of radioactivity while others were barely above background. In order to circumvent this issue, ^{32}P-H_3PO_4 was used to label pea plants. Because ^{32}P is a high energy β emitter, it was expected to be more easily detectable compared to the low energy β emitter, ^{14}C. Another reason for using ^{32}P was to only detect phospholipids and

simplify the results. ER microsomes were extracted from the labeled pea plants and used as the donor with WT or *tgd1-1* mutant chloroplast acceptors. The level of ^{32}P label incorporated into lipids was much lower than expected possibly due to seedlings already having a large unlabeled phosphate pool, so label could not be detected in the acceptor chloroplasts above background levels (Table 1).

Because the commercially available labeled lipids and the ^{32}P labeling did not work as expected, another variation was attempted in order to get statistically significant results from a more simplified donor than the ER microsomes extracted from ^{14}C labeled pea plants. In this version of the experiment, total lipids were extracted from ^{14}C-acetate labeled pea plants and separated by thin layer chromatography (TLC). Just the phospholipids, which would be found in the ER (i.e. PA, PC, PI, and PE), were extracted from the TLC plate and used to make synthetic donor liposomes. Those donor liposomes were incubated with WT or *tgd1-1* chloroplasts. Chloroplasts from mutants unable to produce TGDG, *sfr2-3* and *sfr2-3 tgd1-1*, were also included as acceptors in this experiment in order to mitigate any effects caused by the stability differences between WT and *tgd1-1* (see Chapter 3). While some of the experimental repeats resulted in acceptor label levels above background, no statistically significant differences were seen between the different mutant and WT acceptor chloroplasts (Table 1).

The last variation of the labeling experiment was done in consideration of cytosolic pH. All of the previous assays were conducted in pH 8.0 incubation buffer as is standard for protein import assays with isolated chloroplasts. However, the pH of the cytosol has been reported to be close to 7.3 (34). For this assay, liposomes were produced from lipids extracted from ^{14}C-acetate labeled pea plants as described above. The liposomes were incubated with isolated pea chloroplasts in either pH 8.0 or pH 7.3 incubation buffer. Results showed no statistically significant difference in the amount of label received by chloroplasts in the two different pH buffers (Table 1).

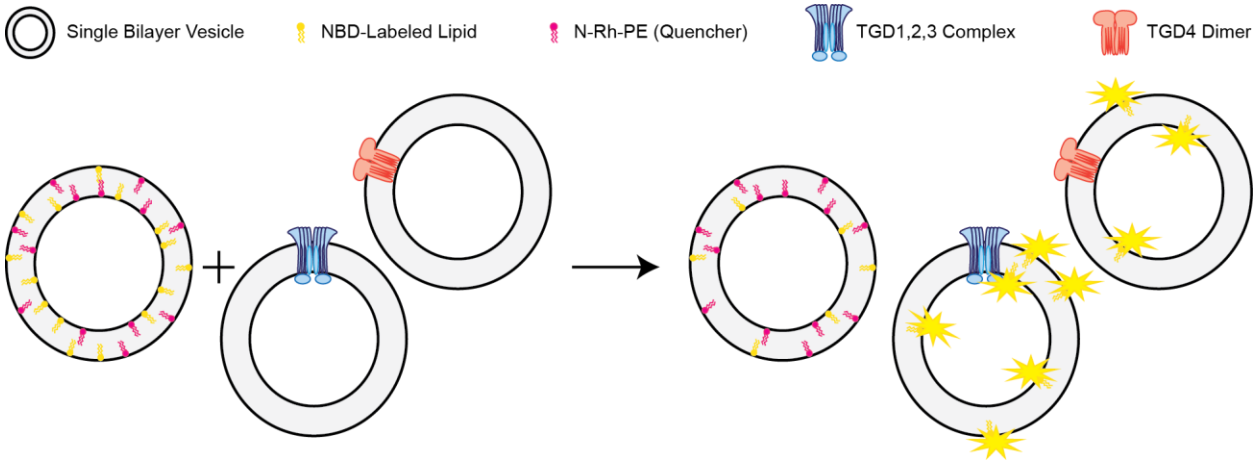

Figure 4.2 Fluorescent Lipid Transport Assay Model. A representative scheme is shown depicting how the transport of lipids tagged with an NBD fluorophore are transported from liposomes containing a quencher to isolated chloroplast envelopes which are the location of known lipid transporter proteins. When the NBD-labeled lipids are transported to the envelopes, an increase in fluorescence is expected due to removal from the quencher. NBD, 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid; N-Rh-PE, N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine.

Transport of Fluorescently Labeled Lipids Can Be Measured in Real-Time.

Because results for transport assays using radiolabel as the mode of detection were below or just above background levels and were inconclusive in identifying the transported lipid species between the ER and chloroplast, a different method was pursued. Commercially available lipids of interest (PA and PC) which were tagged with the fluorescent molecule, 6-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)hexanoic acid (NBD), were used in donor liposomes which included unlabeled phospholipids as well as a quencher, N-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine (N-Rh-PE). NBD-PS was also included as a control since PS is not found in chloroplasts and, therefore, should not be transported. The donor liposomes were always composed 25% each of some form of PA, PC, PS, and N-Rh-PE. The PA, PC, and PS were either dioleoyl or NBD-labeled; when one lipid was NBD-labeled, the other two would be dioleoyl. For this assay, the NBD fluorophore was quenched while in the donor liposomes, and lipid transport to an acceptor (away from the quencher) was detected as an increase in fluorescence over time (Figure 4.2).

Chloroplast envelope membranes were extracted from pea plants or WT, *tgd1-1*, and *tgd4-1* Arabidopsis plants and used as the acceptors in the assay. Envelope membranes were used rather than intact chloroplasts for two reasons. One, using envelopes prevents any issues with chlorophyll or other pigments found in thylakoids from interfering with excitation or detection during the experiment. Two, the use of envelopes makes it easier to separate the action of the TGD1,2,3 complex from that of TGD4 if they are isolated in such a way as to form separate inner and outer envelope vesicles. The TGD1,2,3 complex and TGD4 work in the same transport pathway, but TGD4 is located in the outer envelope membrane (12, 20), while TGD1,2,3 is located in the inner envelope membrane (8, 13). While the two complexes do not necessarily transport the same lipid species, the TGD1,2,3 complex presumably relies on TGD4 to transport its own substrate before TGD1,2,3 can access any substrate. This makes the use of intact chloroplasts for this type of transport assay complicated. Using envelopes eliminates this complexity by putting the TGD1,2,3 complex in direct contact with the donor membranes.

For experiments using pea chloroplasts as acceptors, the ATP and protein dependence of the lipid transport were tested. The ATP dependence is a measure of TGD1,2,3 complex function because of TGD3 being an ATPase, while the protein dependence is a measure of both TGD1,2,3 complex and TGD4 function in that it will distinguish between protein-mediated transport and some other nonspecific mechanism. The ATP dependence was tested by inclusion or omission of ATP in the incubation buffer as well as by inclusion or omission of apyrase, which depletes the ATP included in the incubation buffer. The transport of both NBD-PA and NBD-PC were reduced by omission of ATP while the transport of NBD-PS was unaffected by the absence of ATP (Figure 4.3A). The test of ATP dependence by inclusion of apyrase was only attempted with NBD-PA, and this experiment confirmed the results of the previous ATP dependence experiments (Figure 4.3B). While the NBD-PA result is consistent with what is known about the TGD1,2,3 complex, the NBD-PC result is unexpected. TGD1,2,3 is not expected to transport PC as PC is only found in the outer leaflet of the outer envelope of the

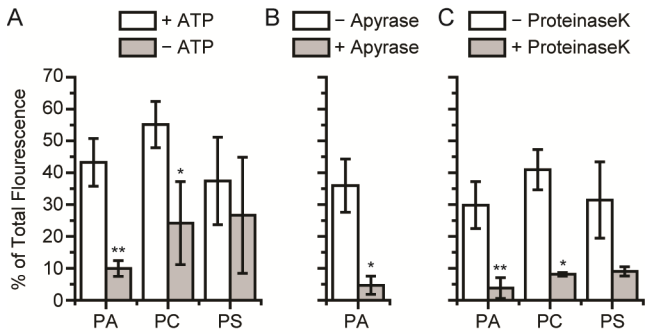

Figure 4.3 NBD-Lipid to Pea Envelope Transport. The percentage of the total fluorescence transported from donor liposomes to acceptor envelopes as represented by the increase in fluorescence after a 200 second incubation with envelopes. The percentage of increase in fluorescence was calculated using the amount of flourescence after addition of Triton X-100 as the total since the detergent separates the NBD from the quencher. The label on the x-axis indicates which lipid in the donor liposomes was labeled with NBD. (A) The percentage of total NBD-lipid transported is shown in the presence (white) and absence (grey) of ATP. PA, n = 9; PC and PS, n = 4. (B) The percentage of total transported NBD-PA is shown without (white) or with (grey) addition of apyrase. n = 3. (C) The percentage of total transported NBD-lipid is shown without (white) or with (grey) ProteinaseK. PA, n = 7; PC and PC, n = 3. Student's t-test comparing the conditions for each lipid tested. * $p < 0.05$, ** $p < 0.005$. Error bars denote SD. PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine.

chloroplast, and, therefore, the TGD1,2,3, complex would not be expected to have access to PC *in vivo* (23). The result of NBD-PS being transported at all is also unexpected because chloroplasts do not naturally contain any PS. Protein dependence of NBD-lipid transport was tested by inclusion or omission of a protease, ProteinaseK. The transport of all tested lipid species was decreased when donor liposomes were incubated with protease-treated envelopes (Figure 4.3C). This was an important control experiment to confirm that the measured transport was protein-mediated and not an artifact from using NBD-lipids which may possess altered biophysical properties from native lipid species.

For fluorescently labeled lipid transport assays using Arabidopsis chloroplast envelopes as acceptors, envelopes from WT, *tgd1-1*, and *tgd4-1* plants were used. The *tgd1-1* mutant allele was chosen to represent the TGD1,2,3 complex because it has the most severe of all of the *tgd* mutant phenotypes and may, therefore, yield the most obvious results. The *tgd4-1* mutant allele was chosen to represent the TGD4 complex because while it may not have the most severe phenotype of all the *tgd4* mutant alleles, it is possible to maintain a homozygous seed stock for this allele. Homozygous knock-out lines of TGD4 are sterile (11), and for the purposes of extracting chloroplast envelopes, the availability of homozygous seed for *tgd4-1*

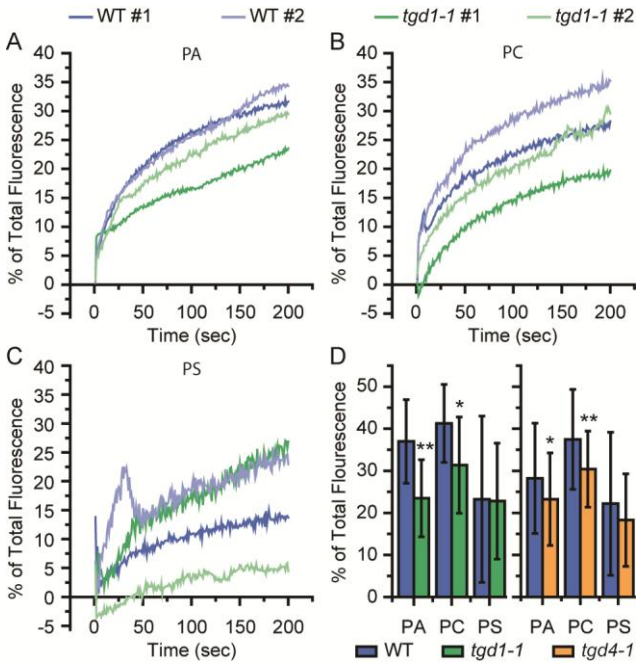

Figure 4.4 NBD-Lipid to Arabidopsis Envelope Transport. (A-C) The increase in fluorescence over time after addition of acceptor envelopes to donor liposomes is shown for three NBD-labeled lipids: (A) PA, (B) PC, and (C) PS. Each shows 2 replicates for wild-type (WT, blues) and *tgd1-1* mutant (greens) collected during the same experimental repeat. (D) The results of all experimental repeats taken as the % of total fluorescence after 200 seconds of incubation with envelopes are shown for WT, *tgd1-1*, and *tgd4-1*. Student's t-test was performed comparing mutant to wild-type results from the same experiments. $10 \leq n \leq 21$, * $p < 0.05$, ** $p < 0.005$. Error bars denote SD. PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine.

was crucial. Both *tgd1-1* and *tgd4-1* showed less increase in fluorescence over time than the wild-type for NBD-PA and NBD-PC while no differences were seen with NBD-PS (Figure 4.4). The difference with NBD-PA appeared to be greater and statistically significant for *tgd1-1*, and the difference with NBD-PC appeared to be greater and statistically significant for *tgd4-1* (Figure 4.4D). While this could suggest PA as the preferred substrate for TGD1,2,3 and PC for TGD4, the results were often too variable within even a single experiment (Figure 4.4A-C) to draw firm conclusions.

Discussion

As an approach to identify which lipid structure(s) is/are transported from the ER to the chloroplast both radiolabeling and fluorescent studies were used with isolated chloroplasts or chloroplast envelopes, respectivel,. A previous study used radiolabeled PC and isolated pea chloroplasts to show that PC can be transferred to chloroplasts in a time- and protein-dependent

manner (35). The results of the radiolabel assays with Arabidopsis chloroplasts for the current study corroborated these previous results. The methods used by the previous paper had similar issues as the current study with obtaining results above background levels when trying to look at multiple simultaneously labeled phospholipids. The previous study also recorded transport of PE as was observed in our results (Figure 4.1) (35). Neither the published results nor this study could show ATP dependent transport using radiolabeled lipids and isolated chloroplasts which would be indicative of transport by the TGD1,2,3 complex (35). Two older studies also looked specifically at the transfer of labeled PC to intact chloroplasts and isolated envelopes (31, 32). Miquel et al. concluded that while labeled PC can be transferred to both envelope vesicles and intact chloroplasts with help of a cytosolic factor, the label remains on the outer leaflet of the outer envelope membrane (32). Andersson et al. then proceeded to show cytosolic phospholipases are needed in order to produce MGDG from transported PC, but this paper also had problems with PI and PE being transported along with PC (31). Taken together, based on the work presented here and previous publications, the use of radiolabeled liposomes incubated with isolated chloroplasts may not be a sufficiently robust system for measuring details of the ER to chloroplast lipid transport. While I was able to show differences in PA and PC transport in WT and *tgd1-1* mutant chloroplasts, the amount of radiolabeled PE detected in chloroplasts was also different between WT and mutant (Figure 4.1). This result in combination with difficulties obtaining levels of radioactivity above background for transferred lipids make the interpretations of results of this assay difficult. Perhaps the background issues could be resolved by longer incubation times as commonly used in other lipid labeling studies, but isolated chloroplasts do not remain intact long enough during longer incubation periods and were, therefore avoided. It should also be noted that the results using intact isolated chloroplast are complicated by the presence of two different transport complexes in the inner and outer envelopes and the fact that these complexes may or may not transport the same lipid species. Using the *tgd4-1* mutant in conjunction with the *tgd1-1* mutant may have been able to help minimize this complication, but

the phenotype of *tgd4-1* is very weak. This weak phenotype and the problems with getting results above background levels did not allow me to draw solid conclusions using this approach.

The fluorescent lipid transport assays seemed to provide more reliable results than the radiolabeling experiments despite the lipid acyl chains being tagged with a bulky NBD fluorophore. Many studies have been done investigating the biological relevance of using NBD lipids. While many studies have found the NBD-lipids to act normally within the membrane and with known protein interactions, others have found the presence of NBD can affect the fluidity and architecture of membranes (36). For the assays performed here, the NBD-lipids were able to form stable donor liposomes and levels of transport from the liposomes to acceptor envelope membranes were low enough that the integrity of acceptor membranes should be maintained for the short duration of the experiment. For these fluorescent lipid transport assays, there were no problems with background levels, transport could be measured in real time rather than with end point analysis, and the two TGD complexes could be distinguished by use of different *tgd* mutants. The problem of the substrate for TGD1,2,3 having to first travel through the outer envelope membrane was also resolved by using envelopes which were isolated in such a way to get a mixture of vesicles made only of either inner or outer envelopes (Figure 4.2). While the results of this experiment point towards the transported lipid species being PC for TGD4 and PA for TGD1,2,3, the use of PS as a control complicates matters. The results show that while PS is not differentially transported by WT and mutant or influenced by the presence or absence of ATP, its transport is still protein-dependent (Figures 4.3 and 4.4). The PS transport could be due to promiscuity of the transport proteins *in vitro* and limits the interpretations of the rest of the results obtained by this method.

The studies described here yielded results that illustrate the challenges in identifying a transported lipid species for the TGD complexes., Clearly, additional methods will need to be explored for further investigation into the transported lipid species. A full reconstitution of the complex in the test tube or molecular structures of different forms of the complex may be

required to ultimately reveal the nature of the lipid molecules that are transported from the ER to chloroplasts.

Materials and Methods

Plant Growth

Arabidopsis (*Arabidopsis thaliana*) were grown on petri plates containing MS medium as described for organelle isolation in Chapter 2. Pea (*Pisum sativum* "Little Marvel") plants were grown in vermiculite at room temperature under a 16h light/8h dark cycle.

Organelle Isolation

Chloroplast and ER isolation from Arabidopsis were carried out as described in Chapter 2. For organelle isolation from pea the following adjustments were made. Two-week-old pea seedlings were harvested for both ER and chloroplast extraction, and the ER extraction was carried out as for Arabidopsis. For pea chloroplast isolation, the pelleting steps were carried out at 1500 x g, and the Percoll gradient step was carried out at 8000 x g. Chloroplast envelope extraction was carried out with the same procedures for both Arabidopsis and pea. The method for envelope extraction was developed combining techniques as described in (13, 37, 38), and all steps were done at 4°C. The chloroplasts were initially resuspended in hypertonic 0.6 M sucrose in TE buffer (10 mM tricine pH 7.5 KOH, 2 mM EDTA) in order to separate the outer and inner envelope membranes before breakage. The chloroplasts were then broken by 10 strokes with a Dounce homogenizer. This method was chosen over the common freeze-thaw method in order to ensure the integrity of protein complexes such as the TGD complex. The thylakoids were removed by three 5 min centrifugations at 1500 x g after each of which the supernatant was maintained. The supernatant was then subjected to a 30 min centrifugation at 100,000 x g in order to obtain a crude envelope membrane pellet. The pellet was resuspended in 100 µL 0.2 M sucrose in TE buffer and loaded onto a step sucrose gradient composed of 750 µL 0.45M

sucrose in TE buffer and 750 µL 1 M sucrose in TE buffer. The gradient was centrifuged at 100,000 x g for 2 hours, and the faint yellow interface was collected into a new tube and diluted with 1 mL TE buffer. The final envelope membrane pellet was obtained by centrifuging the suspension at 100,000 x g for 1 hour, and the pellet was resuspended in incubation buffer (330 mM sorbitol, 50 mM HEPES pH 8.0 KOH, 10 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP) for use in transport assays.

Isolation of Cytosol Fraction

The cytosol fraction was isolated from pea seedlings according to (39) using 10 mL of homogenization buffer instead of 50 mL.

Variations of Incubation Buffer for Both Radiolabeled and Flourescent Lipid Transport Assays

The standard incubation buffer used in the transport assays was 330 mM sorbitol, 50 mM HEPES pH 8.0 KOH, 10 mM KCl, 2.5 mM $MgCl_2$, 1 mM ATP. Some assays used pH 7.3 buffer, but all of the buffers with a variable ingredient were at pH 8.0. When cytosol fraction was used, 5 µL of the cytosol was added in place of 5 µL of water when preparing the buffer. When UDP-Gal was added, it was done so at either 2 mM, 6 mM, or 20 mM concentrations in order to determine the effect of increasing concentrations. Apyrase was used in the incubation buffer at 0.04 U/µL, and ProteinaseK was used at 200 µg/mL.

Donor Vesicle Preparation for Radiolabeled Lipid Transport Assays

American Radiolabeled Chemicals, Inc. was the supplier for all radioactive material. For half of the assays using ^{14}C-acetate labeled ER microsomes, ten 7-day-old pea seedlings were cut at the stem while under water, and each seedling was placed in 900 µL water with 100 µL of 57 mCi/mmol acetic acid [1,2-^{14}C] sodium salt in ethanol (10 µCi) for 8 h. The labeled seedlings

were then mixed with the leaves of 10-15 unlabeled pea seedling and used for ER microsome preparation as described above. The labeled seedlings were mixed with unlabeled seedlings for ER microsome preparation in order to increase total yield of ER. For the other half of assays using ^{14}C-acetate labeled ER microsomes, cut leaves from 7-day-old pea seedlings were incubated in 5 mL of 20 mM MES pH5.5, 0.01% Tween20 containing 250 µCi of 50mCi/mmol acetic acid sodium salt [1-^{14}C] for 1.75 hours with light and shaking. The leaf slices were then combined with leaves from 30 unlabeled seedlings and used for ER microsome preparation as described above.

To make ER microsomes labeled with commercially available ^{14}C-phospholipids, ER microsomes extracted from either pea or Arabidopsis seedlings were centrifuged at 100,000 x g for 10 min at 4°C. The pellet was resuspended in 1 mL of the desired incubation buffer, and 0.1 µCi of phosphatidic acid, L-α-dioleoyl [2-oleoyl-1-^{14}C] sodium salt and/or phosphatidylcholine, L-α-dioleoyl [dioleoyl-1-^{14}C] per µg of protein equivalents were added. The suspension was sonicated 3 x 10 seconds at power level 1.0 (3 W), and then centrifuged at 100,000 x g for 10 min at 4°C. The supernatant was removed and subjected to scintillation counting in order to determine the success of label incorporation. The pellet was resuspended in 1 mL of incubation buffer to wash and then centrifuged at 100,000 x g for 10 min at 4°C. This final pellet was resuspended in the desired variation of incubation buffer at 0.5 µg protein equivalents per µL of buffer.

To prepare microsomes with ^{32}P-labeled lipids, cut leaves from 7-day-old pea seedlings were incubated in 5 mL of 20 mM MES pH 5.5, 0.01% Tween20 containing 1000 µCi of 150mCi/mL orthophosphoric acid [^{32}P] for 1.75 hours with light and shaking. The leaf slices were then combined with leaves from 30 unlabeled seedlings and used for ER microsome preparation as described above.

For assays using liposomes prepared from lipids extracted from ^{14}C-acetate labeled pea seedlings, cut leaves from 7-day-old pea seedlings were incubated in 500 µL of 20mM MES pH

5.5, 0.01% Tween20 containing 250 µCi of 57 mCi/mmol acetic acid sodium salt [1-^{14}C] for 2 hours with light and shaking. All excess liquid was then removed from the leaf slices, and 900 µL of methanol:chloroform:formic acid (2:1:0.1 v/v) was added and vortexed in order to extract lipids. The lipid extract was moved to a fresh tube, and 450 µL of 0.2 M H_3PO_4, 1 M KCl was added. The mixture was vortexed and centrifuged at max speed in a microfuge for 1 min. The bottom organic layer was removed to a glass tube and dried under a nitrogen gas stream. The lipids were then resuspended in chloroform for loading on a TLC for polar lipid separation with acetone:toluene:water (91:30:7 v/v/v). PA, PE+PI, and PC were then scraped from the plate and extracted as described for the lipids used for positional analysis in Chapter 2. Once extracted, the lipids were dried under a nitrogen gas stream and placed under vacuum for 30-40 min in order to remove any residual solvent. Then 1 mL of the desired incubation buffer was added, the lipids were sonicated with a probe 3 x 10 seconds at power level 1.0 (3 W).

Radiolabeled Lipid Transport Assays

Each assay which included some form of labeled ER microsomes used 100 µL of labeled ER (50 µg protein equivalents) and 50 µL of chloroplasts (50 µg chlorophyll equivalents) all in incubation buffer. The samples were incubated at room temperature in the light for 30 min. After 30 min, the suspension was loaded onto a 1 mL 40% Percoll cushion in import buffer (330 mM sorbitol, 50 mM HEPES pH8.0 KOH) and centrifuged in a 4° microfuge at 3000 rpm for 5 min. Each pellet was extracted with 800 µL 2 methanol: 1 chloroform and vortexed. The samples were centrifuged in a microfuge at max speed for 1 min, and the extract was transferred to a cuvette for measuring absorbance at 652 nm. Chlorophyll equivalents were determined with the same equation used for the chloroplast extraction protocol, and equal chlorophyll equivalents for each sample were transferred to new tubes. The equivalent of the solvent volume of 0.2 M H_3PO_4, 1 M KCl was added to each tube. Samples of 100 µL of labeled ER (50 µg protein equivalents) were also extracted with 800 µL 2 methanol: 1 chloroform and then 600 µL 0.2 M

H_3PO_4, 1 M KCl to use as controls for total amounts of radiolabel available for transport. All samples were vortexed and centrifuged at max speed in a microfuge for 1 min. The bottom organic layer was moved to a fresh tube for each sample. The samples were dried down and resuspended in chloroform for loading onto a TLC plate. Polar lipids were separated as described above. PA, MGDG, PG, DGDG, SQDG, PE+PI, and PC bands were scraped into scintillation vials, 1 mL of scintillation fluid was added to each vial, and the levels of radioactivity for each lipid were counted.

Fluorescent Lipid Transport Assays

Donor liposomes were a mix of 25% each of N-Rh-PE, dioleoyl-PA or NBD-PA, dioleoyl-PC or NBD-PC, and dioleoyl-PS or NBD-PS. Only one type of lipid was labeled with NBD at a time. The liposomes were prepared by drying 10 nmol of total lipid under a nitrogen gas stream and placing them under vacuum for 30-45 min. The lipids were then solubilized in 30 µL of 100% ethanol and injected into 1 mL of cold incubation buffer. The liposomes as well as the acceptor chloroplast envelope membranes were kept on ice until measurement. The chloroplast envelope membranes were used at 25 µg protein equivalents per mL of incubation buffer. The fluorescent quality of the liposomes was tested by recording an emission spectra for 200µL of each set of quenched liposomes at excitation 450 nm and emission 485 - 585 nm followed by a second emission spectra with 20 µL of 10% Triton X-100 added to break the liposomes for maximum separation for quencher and, therefore, maximum fluorescence. Once quality of the liposomes was assured, time-based measurements were taken at excitation 450 nm and emission 530 nm. For each sample, 200 µL of liposomes were measured for 100 seconds to establish a baseline fluorescence value. Then 20 µL of envelope membranes were carefully added with mixing by pipetting up and down 5 times, and measurements were taken for another 200 seconds. 20 µL of 10% Triton X-100 were then added to break the liposomes and to provide a total fluorescence value after another 120 seconds.

REFERENCES

REFERENCES

1. Stroebel D, Choquet Y, Popot JL, & Picot D (2003) An atypical haem in the cytochrome b(6)f complex. *Nature* 426(6965):413-418.

2. Jordan P*, et al.* (2001) Three-dimensional structure of cyanobacterial photosystem I at 2.5 A resolution. *Nature* 411(6840):909-917.

3. Liu Z*, et al.* (2004) Crystal structure of spinach major light-harvesting complex at 2.72 A resolution. *Nature* 428(6980):287-292.

4. Loll B, Kern J, Saenger W, Zouni A, & Biesiadka J (2005) Towards complete cofactor arrangement in the 3.0 A resolution structure of photosystem II. *Nature* 438(7070):1040-1044.

5. Jones MR (2007) Lipids in photosynthetic reaction centres: structural roles and functional holes. *Prog Lipid Res* 46(1):56-87.

6. Holzl G*, et al.* (2009) The role of diglycosyl lipids in photosynthesis and membrane lipid homeostasis in Arabidopsis. *Plant Physiol* 150(3):1147-1159.

7. Prinz WA (2014) Bridging the gap: Membrane contact sites in signaling, metabolism, and organelle dynamics. *J Cell Biol* 205(6):759-769.

8. Awai K, Xu C, Tamot B, & Benning C (2006) A phosphatidic acid-binding protein of the chloroplast inner envelope membrane involved in lipid trafficking. *Proc Natl Acad Sci U S A* 103(28):10817-10822.

9. Fan J, Zhai Z, Yan C, & Xu C (2015) Arabidopsis TRIGALACTOSYLDIACYLGLYCEROL5 Interacts with TGD1, TGD2, and TGD4 to Facilitate Lipid Transfer from the Endoplasmic Reticulum to Plastids. *Plant Cell*.

10. Lu B, Xu C, Awai K, Jones AD, & Benning C (2007) A small ATPase protein of Arabidopsis, TGD3, involved in chloroplast lipid import. *J Biol Chem* 282(49):35945-35953.

11. Xu C, Fan J, Cornish AJ, & Benning C (2008) Lipid trafficking between the endoplasmic reticulum and the plastid in Arabidopsis requires the extraplastidic TGD4 protein. *Plant Cell* 20(8):2190-2204.

12. Xu CC, Moellering ER, Muthan B, Fan JL, & Benning C (2010) Lipid transport mediated by Arabidopsis TGD proteins is unidirectional from the endoplasmic reticulum to the plastid. *Plant Cell Physiol* 51(6):1019-1028.

13. Roston RL, Gao J, Murcha MW, Whelan J, & Benning C (2012) TGD1, -2, and -3 proteins involved in lipid trafficking form ATP-binding cassette (ABC) transporter with multiple substrate-binding proteins. *J Biol Chem* 287(25):21406-21415.

14. Xu C, Fan J, Riekhof W, Froehlich JE, & Benning C (2003) A permease-like protein involved in ER to thylakoid lipid transfer in Arabidopsis. *EMBO J* 22(10):2370-2379.

15. Roston R, Gao J, Xu C, & Benning C (2011) Arabidopsis chloroplast lipid transport protein TGD2 disrupts membranes and is part of a large complex. *Plant J* 66(5):759-769.

16. Frentzen M (1986) Biosynthesis and Desaturation of the Different Diacylglycerol Moieties in Higher-Plants. *J Plant Physiol* 124(3-4):193-209.

17. Frentzen M, Heinz E, McKeon TA, & Stumpf PK (1983) Specificities and selectivities of glycerol-3-phosphate acyltransferase and monoacylglycerol-3-phosphate acyltransferase from pea and spinach chloroplasts. *Eur J Biochem* 129(3):629-636.

18. Hurlock AK, Roston RL, Wang K, & Benning C (2014) Lipid trafficking in plant cells. *Traffic* 15(9):915-932.

19. Wang Z, Anderson NS, & Benning C (2013) The phosphatidic acid binding site of the Arabidopsis Trigalactosyldiacylglycerol 4 (TGD4) protein required for lipid import into chloroplasts. *J Biol Chem* 288(7):4763-4771.

20. 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(4):614-623.

21. Lu BB & Benning C (2009) A 25-Amino Acid Sequence of the Arabidopsis TGD2 Protein Is Sufficient for Specific Binding of Phosphatidic Acid. *J Biol Chem* 284(26):17420-17427.

22. Andersson MX & Dörmann P (2009) Chloroplast Membrane Lipid Biosynthesis and Transport. *The Chloroplast,* Plant Cell Monographs, eds Sandelius A & Aronsson H (Springer Berlin Heidelberg), Vol 13, pp 125-158.

23. Dorne AJ, Joyard J, Block MA, & Douce R (1985) Localization of phosphatidylcholine in outer envelope membrane of spinach chloroplasts. *J Cell Biol* 100(5):1690-1697.

24. Mongrand S, Bessoule JJ, & Cassagne C (1997) A re-examination in vivo of the phosphatidylcholine-galactolipid metabolic relationship during plant lipid biosynthesis. *Biochem J* 327 (Pt 3):853-858.

25. Mongrand S, Cassagne C, & Bessoule JJ (2000) Import of lyso-phosphatidylcholine into chloroplasts likely at the origin of eukaryotic plastidial lipids. *Plant Physiol* 122(3):845-852.

26. Bessoule JJ, Testet E, & Cassagne C (1995) Synthesis of phosphatidylcholine in the chloroplast envelope after import of lysophosphatidylcholine from endoplasmic reticulum membranes. *Eur J Biochem* 228(2):490-497.

27. Williams JP, Imperial V, Khan MU, & Hodson JN (2000) The role of phosphatidylcholine in fatty acid exchange and desaturation in Brassica napus L. leaves. *Biochem J* 349(Pt 1):127-133.

28. Hamilton JA, Bhamidipati SP, Kodali DR, & Small DM (1991) The interfacial conformation and transbilayer movement of diacylglycerols in phospholipid bilayers. *J Biol Chem* 266(2):1177-1186.

29. Mongrand S, Bessoule JJ, Cabantous F, & Cassagne C (1998) The C-16 : 3/C-18 : 3 fatty acid balance in photosynthetic tissues from 468 plant species. *Phytochemistry* 49(4):1049-1064.

30. Xu C, Fan J, Froehlich JE, Awai K, & Benning C (2005) Mutation of the TGD1 chloroplast envelope protein affects phosphatidate metabolism in Arabidopsis. *Plant Cell* 17(11):3094-3110.

31. Andersson MX, Kjellberg JM, & Sandelius AS (2004) The involvement of cytosolic lipases in converting phosphatidyl choline to substrate for galactolipid synthesis in the chloroplast envelope. *Biochim Biophys Acta* 1684(1-3):46-53.

32. Miquel M*, et al.* (1988) Protein-Mediated Transfer of Phosphatidylcholine from Liposomes to Spinach Chloroplast Envelope Membranes. *Biochim Biophys Acta* 937(2):219-228.

33. Thoma S, Kaneko Y, & Somerville C (1993) A non-specific lipid transfer protein from Arabidopsis is a cell wall protein. *Plant J* 3(3):427-436.

34. Shen J*, et al.* (2013) Organelle pH in the Arabidopsis Endomembrane System. *Mol Plant* 6(5):1419-1437.

35. Yin C, Andersson MX, Zhang H, & Aronsson H (2015) Phosphatidylcholine is transferred from chemically-defined liposomes to chloroplasts through proteins of the chloroplast outer envelope membrane. *FEBS Lett* 589(1):177-181.

36. Haldar S & Chattopadhyay A (2013) Application of NBD-Labeled Lipids in Membrane and Cell Biology. *Fluorescent Methods to Study Biological Membranes*, eds Mély Y & Duportail G (Springer Berlin Heidelberg, Berlin, Heidelberg), pp 37-50.

37. Cline K, Andrews J, Mersey B, Newcomb EH, & Keegstra K (1981) Separation and characterization of inner and outer envelope membranes of pea chloroplasts. *Proc Natl Acad Sci U S A* 78(6):3595-3599.

38. Keegstra K & Yousif AE (1986) Isolation and Characterization of Chloroplast Envelope Membranes. *Method Enzymol* 118:316-325.

39. Andersson MX, Kjellberg JM, & Sandelius AS (2001) Chloroplast biogenesis. Regulation of lipid transport to the thylakoid in chloroplasts isolated from expanding and fully expanded leaves of pea. *Plant Physiol* 127(1):184-193.

CHAPTER 5

Conclusions

Understanding plant lipid metabolism is not only important for gaining a comprehensive picture of plant biology and biochemistry ranging from cellular structure and signaling to energy storage and conversion, but it is also necessary in order to engineer plants for better nutrition and biofuel production. The effort to advance our understanding of such a complex process requires a combination of many approaches. The work presented here combines both *in vivo* and *in vitro* methods in order to investigate some of the areas of plant lipid metabolism in which our knowledge is incomplete, including lipid transport between the endoplasmic reticulum (ER) and the chloroplast, acyl editing, and how lipid composition affects membrane stability.

The *in vivo* studies made use of a unique type of desaturase, a Δ6 desaturase, which Arabidopsis is normally lacking. A Δ6 desaturase incorporates a double bond at the 6^{th} carbon position from the carboxyl end of the acyl group. Arabidopsis is known to have Δ9, Δ12, and Δ15 desaturases, so the addition of a Δ6 desaturase was expected to add a subtle but readily detectable tag to membrane lipids, which I anticipated would only minimally affect the natural composition or function of the respective membrane or the overall balance of lipid metabolism. A well-characterized Δ6 desaturase from *Physcomitrella patens* (Δ6D) was chosen because of its location within the cell, the ER, and its known substrate specificity for the *sn*-2 position of the glycerol backbone of phosphatidylcholine (PC). This location and specificity provided a unique starting position for a "tag and track" approach as most *in vivo* radiolabel approaches start labeling at fatty acid synthesis within the chloroplast or with pre-labeled fatty acids. With the goal of probing lipid transport and metabolism with increased sensitivity, several mutants of lipid metabolism were used for the "tag and track" approach described in Chapter 2. While the lipid phenotypes of these plants verified key findings of previous studies of plant lipid metabolism in both wild type (WT) and mutant plants validating the method, several of the results were unexpected and provided novel insights into the complexities of plant lipid metabolism. Since phosphatidylglycerol (PG) is thought to be made nearly exclusively from plastid precursors (1), it was interesting to detect Δ6 acyl groups, which had to have originated in the ER, in PG at much

higher levels than expected and in both the *sn*-1 and *sn*-2 positions (Figure 2.5A&B). Another striking observation was that the ratio of the Δ6 acyl groups in the *sn*-1 and *sn*-2 positions changed between PC in the ER and the envelope membranes and genuine thylakoid lipids (Figure 2.4B). This result combined with confirmation of an ER-derived Δ6 acyl group on the same lipid molecule as a plastid derived 16:3 (carbon #: double bond #) acyl group provided evidence for extensive acyl editing of chloroplast lipids (Figures 2.5C & 2.6). Knowing that acyl editing occurs both outside (2) and inside the chloroplast (Chapter 2 and (3)) enriches our view of lipid metabolism, and it can help us better explain results and formulate new hypotheses for other studies involving these pathways.

New discoveries related to lipid metabolism may add complexity to our current models as seen from the results described in Chapter 2, but they will get us closer to fully understanding the dynamic processes governing plant lipid metabolism. Currently, designing experiments for study of plant lipid metabolism can be difficult due to many unknown factors which can affect the results. Experiments involving isolated chloroplasts are designed in order to remove some of these unknown factors because there is only one organelle to consider. It provides the opportunity to study chloroplasts in a controlled environment and focus on how its metabolism functions under the chosen conditions. Unfortunately, even with this seemingly well-controlled system, some variability is still to be expected. Chapter 3 focused on how differences in lipid composition could affect these types of experiments and shows apparent pitfalls of studying lipid metabolism with chloroplasts isolated from different lipid mutants. Because fatty acid synthesis and one of the two major glycerolipid synthesis pathways occur in the chloroplast, it is not uncommon to do experiments, which compare isolated chloroplasts from WT and lipid metabolism mutant plants. If the distinct lipid composition of those mutant chloroplasts can alter the conditions of these experiments, the design of experiments and interpretation of the results must be adjusted accordingly. I found that both the presence of oligogalactolipids and the double bond index and/or 16C to 18C acyl chain length ratio seemed to have an effect on either

isolated chloroplasts' resistance to breakage or the ability to reseal the membrane (Figures 3.1A, 3.2C, 3.4A&B). Differences in chloroplast breakage or the ability to reseal the membrane could affect the composition of the buffer and substrate accessibility of chloroplast proteins. For example, a broken chloroplast could give a stromal protein access to the contents of the outer envelope or an outer envelope protein access to the contents of the thylakoid membrane. Therefore, with the stability differences shown between isolated chloroplasts of different lipid composition, these types of possibilities need to be taken into consideration when interpreting the results. Although some of these aspects of studies with isolated chloroplast are concerning, they provide a novel way to systematically analyze the role of membrane lipid composition on chloroplast stability that should be pursued further in future studies.

Although the results described in Chapter 3 point out potential issues with comparing isolated chloroplasts with different lipid compositions, an isolated chloroplast experimental system could still be useful in determining the transported lipid species from the ER to the chloroplast as long as the results are interpreted with careful consideration of the Chapter 3 findings. With this in mind, *in vitro* experiments using donor liposomes or ER microsomes and acceptor whole chloroplasts or envelopes from WT and lipid transport mutants were undertaken in order to identify the transported lipid species between the ER and the chloroplast. In experiments with whole chloroplasts as acceptors, I tested different variations of radiolabeled donor liposomes or ER microsomes. These experiments showed potential transport of both phosphatidic acid (PA) and PC (Figure 4.1A), but the amounts of radiolabel detected were barely above background questioning the efficacy of this assay under the different conditions tested. The results were further complicated by the fact that the phosphatidylethanolamine+ phosphitidylinositol (PE+PI) fraction also showed possible transport (Figure 4.1A). These are lipids typically absent from chloroplasts, thereby questioning the specificity of transport observed in this system.

The experiments which used fluorescently labeled donor liposomes with chloroplast envelope acceptors showed similar results to the radiolabeling experiments with PA and PC. In particular, reduced transport of these two lipids to the mutant envelopes compared to WT were promising (Figure 4.4D). However, these results were ultimately inconclusive because phosphatidylserine (PS), a lipid not known to move to the chloroplast, also showed protein-dependent transport to envelopes; although, there were no differences between mutant and wild-type chloroplasts (Figures 4.3C & 4.4D). Based on these data, I could not rule out that the lipid transport systems in the chloroplast may lack specificity *in vitro* resulting in experiments which do not reflect the situation in the cell, possibly, because other critical factors are missing. Summarizing the results of these experiments, my data are consistent with the hypothesis that PC is first transported to the outer envelope by the TGD4 dimer and that PA is then transported to the inner envelope by the TGD1,2,3 complex. This would require the conversion of PC to PA by a phospholipase D, which has not yet been identified. Thus, aside from further development of a robust *in vitro* transport assay, one key experiment to support this hypothesis would be to identify a phospholipase D in the outer envelope membrane of chloroplasts. Given the large number of predicted lipases in the plant genome (4), identifying such a lipase may not be an easy task, particularly if there are redundant enzymes .

All of the methods presented here have the potential for continued use in the field of plant lipid research. While the *in vitro* methods require further optimization, the results are promising. Perhaps with more study of the donor liposomes and better understanding of their biophysical properties over time, the transport assays would show solidly conclusive results. The *in vivo* "tag and track" method is ready to use as is. It will have value when used in other mutant backgrounds of interest, and it may be able to help researchers answer questions that cannot quite be addressed with other approaches such as radiolabeling.

To ultimately understand the nature of the lipid species transported by the TGD complex and its mechanistic function, structural analysis of the protein complex with trapped

intermediates might be necessary in the future. The TDG complexes are relatively stable during isolation (5, 6), and cryo-electron microscopy techniques combined with cross-linking mass spectrometry are particularly applicable to large complexes (7). These techniques have advanced to a level that they may allow us to address this problem in the near future (7).

REFERENCES

REFERENCES

1. Browse J, Warwick N, Somerville CR, & Slack CR (1986) Fluxes through the prokaryotic and eukaryotic pathways of lipid synthesis in the '16:3' plant Arabidopsis thaliana. *Biochem J* 235(1):25-31.

2. Bates PD, Ohlrogge JB, & Pollard M (2007) Incorporation of newly synthesized fatty acids into cytosolic glycerolipids in pea leaves occurs via acyl editing. *J Biol Chem* 282(43):31206-31216.

3. Tjellstrom H, Yang Z, Allen DK, & Ohlrogge JB (2012) Rapid kinetic labeling of Arabidopsis cell suspension cultures: implications for models of lipid export from plastids. *Plant Physiol* 158(2):601-611.

4. Wang K, Froehlich JE, Zienkiewicz A, Hersh HL, & Benning C (2017) A Plastid Phosphatidylglycerol Lipase Contributes to the Export of Acyl Groups from Plastids for Seed Oil Biosynthesis. *Plant Cell*.

5. Roston RL, Gao J, Murcha MW, Whelan J, & Benning C (2012) TGD1, -2, and -3 proteins involved in lipid trafficking form ATP-binding cassette (ABC) transporter with multiple substrate-binding proteins. *J Biol Chem* 287(25):21406-21415.

6. Wang Z, Anderson NS, & Benning C (2013) The phosphatidic acid binding site of the Arabidopsis Trigalactosyldiacylglycerol 4 (TGD4) protein required for lipid import into chloroplasts. *J Biol Chem* 288(7):4763-4771.

7. Schmidt C & Urlaub H (2017) Combining cryo-electron microscopy (cryo-EM) and cross-linking mass spectrometry (CX-MS) for structural elucidation of large protein assemblies. *Curr. Opin. Struct. Biol.* 46(Supplement C):157-168.