Mechanisms of Lipid Transport Involved in Organelle Biogenesis in Plant Cells

Christoph Benning

Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824; email: benning@msu.edu

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Key Words

chloroplast, photosynthetic membrane, endoplasmic reticulum, lipid assembly, lipid transport, galactolipid

Abstract

Chloroplasts are the defining organelle of photoautotrophic plant cells. Photosynthetic light reactions and electron transport are the functions of an elaborate thylakoid membrane system inside chloroplasts. The lipid composition of photosynthetic membranes is characterized by a substantial fraction of nonphosphorous galactoglycerolipids reflecting the need of sessile plants to conserve phosphorus. Lipid transport and assembly of glycerolipids play an essential role in the biogenesis of the photosynthetic apparatus in developing chloroplasts. During chloroplast biogenesis, fatty acids are synthesized in the plastid and are exported to the endoplasmic reticulum, where they are incorporated into membrane lipids. Alternatively, lipids can also be assembled de novo at the inner envelope membrane of plastids in many plants. A rich repertoire of lipid exchange mechanisms involving the thylakoid membranes, the chloroplast inner and outer envelope membranes, and the endoplasmic reticulum is emerging. Studies of thylakoid biogenesis provide new insights into the general mechanisms of intermembrane lipid transfer.

Contents

INTRODUCTION: LIPID DIVERSITY AND BIOGENIC MEMBRANES

Membranes of eukaryotic cells play many roles, from providing the boundaries of cells and organelles to the conversion of light into chemical energy during photosynthesis. Therefore, it is not surprising that different subcellular membranes have very different protein and lipid compositions that meet the functional requirements of the respective specialized cell membrane. Compared to typical cell membranes of animals, fungi, or nonphotosynthetic bacteria, the lipid composition of chloroplast membranes seems at first sight unusual with its abundance of nonphosphorous galactoglycerolipids, mono- and digalactosyldiacylglycerol (MGDG and DGDG, respectively), the presence of the sulfolipid sulfoquinovosyldiacylglycerol (SQDG), and the conspicuous underrepresentation of phospholipids with the exception of phosphatidylglycerol (PtdGro) and phosphatidylcholine (PtdCho) (Browse & Somerville 1994, Jouhet et al. 2007). The latter is present in the outer envelope membrane of chloroplasts but mostly absent from the thylakoid membranes, although this question is still not unambiguously resolved (Andersson et al. 2001, Dorne et al. 1990). Structures of these lipids are shown in **Figure 1**. To understand the combinatorial complexity of polar membrane lipids in plants, one has to keep in mind that the shown structures (**Figure 1*a*,*b***) are representatives of lipid classes. Each class consists of a number of possible molecular species characterized by their respective fatty acyl chain substituents, which can vary in length as well as number and position of double bonds (**Figure 1*c***). This diversity of molecular species within each lipid class is relevant to the discussion of alternate pathways for the synthesis of thylakoid lipids, and it provides an excellent diagnostic opportunity for monitoring fluxes through different pathways of lipid assembly in wild type and mutants (Browse et al. 1986b, Heinz & Roughan 1983, Kunst et al. 1988, Xu et al. 2003).

Even within plant cells, the chloroplast lipid composition is very different from the composition of membranes in other plant organelles (Browse & Somerville 1994, Jouhet et al. 2007). However, looking at a prominent group of photosynthetic bacteria, the cyanobacteria, their cell membrane lipid composition resembles that of plant chloroplast thylakoids, supporting the generally accepted hypothesis that plant chloroplasts evolved from endosymbiotic ancestral cyanobacteria (Reyes-Prieto et al. 2007). Moreover, considering that photosynthetic organisms—cyanobacteria, algae, and land plants—dominate the biosphere, the abundance of nonphosphorous glycoglycerolipids in biomembranes is not surprising. Rather, given the global abundance of these lipids, their importance for photosynthesis, their predominance in photosynthetic membranes, and their relative absence in nonphotosynthetic membranes have made the biogenesis of the photosynthetic membrane a fertile testing ground for new ideas regarding the general principles of fatty acid and polar lipid transfer through and between membranes (Benning 2008, Jouhet

MGDG: monogalactosyldiacylglycerol

DGDG: digalactosyldiacylglycerol

PtdGro: phosphatidylglycerol

PtdCho: phosphatidylcholine

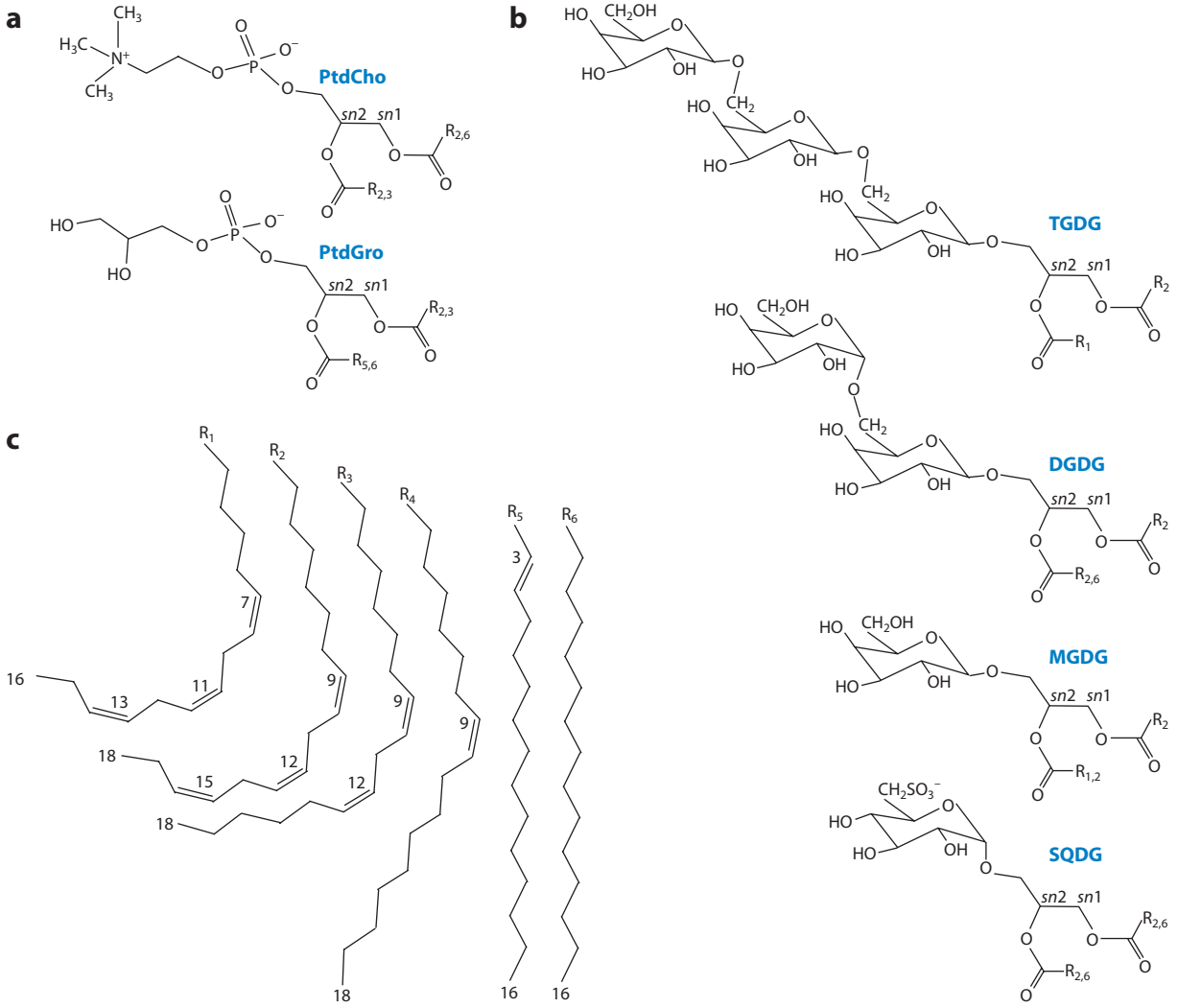

Figure 1

Glycerolipids associated with plastids and their prevalent molecular species. Glycerolipid classes typified by their respective polar head groups are divided into phosphoglycerolipids (*a*) and non-phosphorous glycoglycerolipids (*b*). The richness of molecular species diversity within each lipid class arises from combinations of acyl chains (R-groups) attached to the respective *sn*-1 or *sn*-2 positions of the glycerol backbone. Typical acyl chains depicted as R-groups in panel (*c*) specify the prevalent molecular species of each lipid class shown in (*a*) and (*b*). Only some of the most likely R-group combinations are shown in (*a*) and (*b*), and many others have been described. Indication of two R-groups in specific positions implies that one or the other can be present at high frequency. Note that certain acyl chains occur only in specific positions of the glycerol backbone of specific lipids. These differences can be diagnostic for the assembly pathway giving rise to a specific lipid molecular species. Lipid classes depicted in (*a*) are phosphatidylcholine (PtdCho) and phosphatidylglycerol (PtdGro); in (*b*) trigalactosyldiacylglycerol (TGDG), which accumulates in *Arabidopsis* lipid trafficking mutants, digalactosyldiacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG), and sulfoquinovosyldiacylglycerol (SQDG). The bulk of the thylakoid membrane is made up of PtdGro, DGDG, MGDG, and SQDG. The R-groups shown in (*c*) specify the following acyl chains [trivial names of the corresponding free acid–R-group plus the carboxyl carbon, which is not shown in (*c*)–from left to right]: hexadecatrienoic acid, α-linolenic acid, linoleic acid, oleic acid, Δ3-trans hexadecenoic acid, and palmitic acid. The chain length of the respective acyl group (shown R-group plus the carboxyl carbon) and the position of the double bonds (counting from the carboxyl end) are indicated.

Plastid: plant organelle including chloroplasts and plastids in nongreen tissues

ER: endoplasmic reticulum

et al. 2007). Contributing to our increasing understanding of chloroplast lipid assembly has been the progress in experimental approaches. Rapid pulse-chase labeling experiments have provided a refined view of the dynamics of lipid transfer processes and the initial precursor pools (Bates et al. 2007), and powerful genetic and genomic tools applied to the model plant *Arabidopsis* have identified components of a proposed lipid transporter involved in chloroplast biogenesis (Awai et al. 2006, Lu et al. 2007, Xu et al. 2003).

It is the chemical nature of the lipid building blocks of membranes that they are not freely soluble at higher concentrations in water but form aggregates such as micelles or bilayered vesicles (Webb & Green 1991). This inherent property has mechanistic consequences for the mode of transport of polar membrane lipids and the enzymes involved in their synthesis. In simplistic terms, when studying membrane lipids, solution biochemistry in a three-dimensional space gives way to chemistry in two dimensions represented by the plane of the membrane bilayer. A third, rather constrained dimension comes into play when lipids move between the two membrane leaflets or between different membranes. Most membranes do not have the protein machinery to assemble all polar membrane lipids of which they are composed, and consequently acquire their lipid building blocks by lipid transfer from biogenic membranes (those capable of assembling lipids). Among the membranes most active in lipid biosynthesis in plant cells are the plastid envelope membranes and the endoplasmic reticulum (ER).

The complexity of thylakoid lipid biosynthesis arises from the fact that four membrane systems participate: (*a*) the thylakoid membranes as the ultimate recipient of polar lipid building blocks, (*b*) the inner plastid envelope membrane, (*c*) the outer plastid envelope membrane, and (*d*) the ER. The latter three are biogenic membranes because different lipid assembly machineries are associated with these three membranes that give rise to thylakoid lipids. There is also some redundancy built into the system in many plants, with parallel lipid assembly pathways and lipid acyl group modification systems (fatty acid desaturases) associated with the inner plastid envelope membrane and the ER. One key advantage of *Arabidopsis* in the study of lipid transfer phenomena is that *Arabidopsis* belongs to a group of plants (Mongrand et al. 1998) that can assemble thylakoid glycoglycerolipids de novo at the chloroplast envelope membranes and that are also able to import thylakoid lipid precursors into the plastid that are first assembled at the ER. As a consequence, viable *Arabidopsis* mutants specifically affected in one or the other pathway are available for detailed phenotypic analysis.

THE DYNAMIC NATURE OF LIPID METABOLISM

Owing to their sessile nature, plants cope with a fluctuating growth environment primarily by invoking physiological and biochemical adaptations. The ability of plants and cyanobacteria to adjust their membrane lipid molecular species composition, that is, the desaturation level of the lipid acyl chains in response to temperature, has long been known (e.g., Matsuda et al. 2005, Suzuki et al. 2000). However, the extent of changes in lipid molecular species composition in response to temperature stress is dwarfed by the drastic alteration in lipid class composition in response to phosphate deprivation observed in *Arabidopsis* (Essigmann et al. 1998; Härtel et al. 2000, 2001; Li et al. 2006). This phenomenon is most strongly visible in roots, which have to increase their resorptive surface area under these conditions. Membrane remodeling in response to phosphate limitation might be ubiquitous to all land plants (Tjellstrom et al. 2008). It leads to a replacement of phosphoglycerolipids with nonphosphorous glycoglycerolipids and remobilizes phosphorus from plant membranes that typically bind one third of the organic phosphorus in *Arabidopsis* (Poirier et al. 1991).

What makes this phenomenon relevant to the discussion of lipid transport is the fact that glycoglycerolipids produced at the chloroplast

envelopes replace not only phosphoglycerolipids in the thylakoid membrane, but also in extraplastidic membranes as shown in **Figure 2**. The anionic sulfolipid SQDG assembled at the inner plastid envelope replaces PtdGro in thylakoid membranes (Essigmann et al. 1998, Yu et al. 2002, Yu & Benning 2003). The galactoglycerolipid DGDG substitutes PtdCho in extraplastidic membranes (Härtel et al. 2000, 2001), mitochondria (Jouhet et al. 2004), the tonoplast (Andersson et al. 2005), the plasma membrane (Andersson et al. 2003, Russo et al. 2007), and the peribacteroid membrane in legumes (Gaude et al. 2004). In general, this process reverses as phosphate deprivation is relieved (Tjellstrom et al. 2008). Interestingly, the monogalactosyl form, MGDG, is not observed in extraplastidic membranes, although this lipid is an excellent non-bilayer-forming lipid that can functionally replace the non-bilayer-forming lipid phosphatidylethanolamine (PtdEtn) in *Escherichia coli* (Wikstrom et al. 2008).

Isoforms of phospholipase D appear to be involved in phosphoglycerolipid turnover in roots (Cruz-Ramirez et al. 2006, Li et al. 2006), whereas phospholipase C isoforms have been implicated in leaves (Andersson et al. 2005, Gaude et al. 2008, Nakamura et al. 2005). Glycosyltransferases required for the biosynthesis of DGDG under phosphate limitation are associated with the outer plastid envelope (**Figure 2**), which in *Arabidopsis* are the MGDG synthases MGD2 and MGD3 (Awai et al. 2001; Kobayashi et al. 2006, 2008), and the DGDG synthases DGD1 and DGD2 (Härtel et al. 2000, Kelly & Dörmann 2002, Kelly et al. 2003). The SQDG synthase, SQD2, is associated with the inner plastid envelope membrane (Seifert & Heinz 1992, Yu et al. 2002), and consequently SQDG replaces anionic phospholipids in the thylakoids (**Figure 2**) but not in extraplastidic membranes as was recently confirmed by labeling experiments (Tjellstrom et al. 2008). The expression of the respective genes is coordinately and strongly induced under phosphate-limited conditions (Hammond et al. 2003, Misson et al. 2005, Morcuende et al. 2007, Wu et al. 2003). Recently, reactive oxygen species and growth factors such as auxin and cytokinin have been implicated in the regulation of lipid genes in response to phosphate deprivation (Kobayashi et al. 2006, Xu et al. 2008b). Mutant analysis in *Arabidopsis* has unambiguously established that the substitution of phosphoglycerolipids with glycoglycerolipids is an essential process under phosphate-limited growth conditions (Kelly & Dörmann 2002, Kelly et al. 2003, Kobayashi et al. 2008, Yu et al. 2002, Yu & Benning 2003). It is apparent that during phosphoglycerolipid turnover, lipid breakdown products have to move back from nonbiogenic membranes to the plastid envelope membranes, and that glycoglycerolipids produced at the envelopes have to move to their

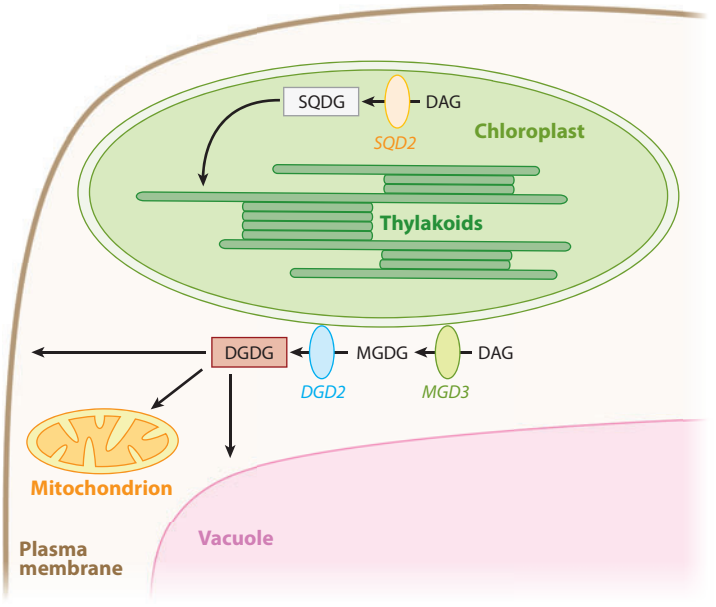

Figure 2

Transfer of glycoglycerolipids from the plastid envelopes following phosphate deprivation. Sulfolipid (SQDG) is produced at the inner plastid envelope from diacylglycerol (DAG) and UDP-sulfoquinovose (not shown) catalyzed by the SQDG synthase encoded in *Arabidopsis* by *SQD2* and transferred to the thylakoids. Digalactosyldiacylglycerol (DGDG) is produced under phosphate-limited conditions preferentially by the sequential action of galactosyltransferases at the outer plastid envelope and encoded in *Arabidopsis* by *MGD3* and *DGD2*. Lipid substrates are diacylglycerol (DAG) and monogalactosyldiacylglycerol (MGDG). The galactosyl group donor UDP-Gal is not shown. DGDG produced under these conditions is transferred to different subcellular membranes as indicated by arrows.

DGD1: glycosyltransferase involved in digalactosyldiacylglycerol synthesis

CoA: coenzyme A

PtdOH: phosphatidic acid

ACP: acyl carrier protein

ABC: ATP-binding cassette

respective nonbiogenic membrane destinations. However, the underlying mechanisms still remain unexplored.

FATTY ACID EXPORT FROM PLASTIDS

In plants, the activity of a prokaryotic type II multipartite fatty acid synthase complex (Harwood 1996, White et al. 2005) located in the plastid is the major source of acyl groups that ultimately are incorporated into cell membrane lipids (Ohlrogge et al. 1979). Accordingly, the *Arabidopsis mod1* mutant, which is deficient in a plastid fatty acid synthase component, is severely impaired in growth (Mou et al. 2000). The classical hypothesis for fatty acid export and subsequent reactions at the ER is shown in **Figure 3*a*** (pathway 1). Oleic or palmitic acids are exported from the plastid, activated to acyl-CoAs, assembled into phosphatidic acid (PtdOH) at the ER, converted to PtdCho, and then diversified by acyl desaturation into other molecular species (Browse & Somerville 1991, Ohlrogge & Browse 1995). Most ER-assembled PtdOH and a large fraction of PtdCho are metabolized to the building blocks for all extraplastidic glycerolipids. Flux of acyl chains through the plastid export pathway can be particularly substantial in developing oil seed embryos of plants such as *Brasscia napus*, which store up to 50% of their dry mass in triacylglycerols. However, to date no specific protein has been unambiguously implicated in the export mechanism for fatty acids from plastids in plants.

Permeability coefficients for long chain fatty acids tested on pure lipid membranes are relatively high, leading some to suggest that proteins are not necessarily required to facilitate fatty acid transfer through cell membranes (Hamilton 2007, Kamp & Hamilton 2006). Even if this were the case, enzymes converting activated acyl-ACP (acyl carrier protein) intermediates on the inside of plastids into acyl-CoAs present in the cytosol such as thioesterases and long chain acyl-CoA synthetases need to be involved. The formation of free fatty acids during this process has been confirmed by [$^{13}C_2{}^{18}O_2$]acetate labeling experiments with spinach leaves. A 50% reduction in ^{18}O-content of glycerolipids assembled outside the plastid was consistent with the formation of free fatty acids by hydrolysis during export (Pollard & Ohlrogge 1999). Furthermore, in kinetic labeling experiments with spinach and pea leaves using [^{14}C]acetate, a channeled pool of fatty acids closely linked to long chain acyl-CoA synthesis was observed (Koo et al. 2004). Whereas a role for long chain acyl-CoA synthetases in fatty acid export seems all too obvious, it has been difficult to identify a particular enzyme, possibly because of redundancy. For example, *Arabidopsis* contains a large number (67) of acyl-activating enzymes (Shockey et al. 2003) including nine long chain acyl CoA synthetases presumed to be involved in fatty acid and glycerolipid metabolism (Shockey et al. 2002). Fatty acid export from plastids must be essential in plants, but inactivation of the most prevalent plastid isoform in *Arabidopsis*, LACS9, did not affect growth of the plant (Schnurr et al. 2002). Whereas other isoforms could take over the function of LACS9 in the mutant, the result did not rule out that LACS9 is a major player in fatty acid export in vivo.

In *E. coli*, a fatty acid transport protein in the outer membrane, FadL, and an acyl-CoA synthetase, FatD, associated with the cytosolic side of the cell membrane, are proposed to act in concert in the uptake and activation of free long chain fatty acids, a mechanism described as vectorial catalysis (Black & DiRusso 2003). However, orthologs of FadL have so far not been identified in plant genomes. Precedence for a possible plant fatty acid transporter arises from a peroxisomal ABC transporter required for fatty acid breakdown (Footitt et al. 2002, Hayashi et al. 2002, Zolman et al. 2001). Plants contain a large number of putative ABC transporters awaiting functional assignment (Rea 2007), and a systematic analysis of ABC transporters predicted to be associated with the plastid envelope in *Arabidopsis* might be promising.

To probe the mechanism for the export of fatty acids from plastids, the initial fate of

nascent fatty acids destined for the cytosol has been determined. Kinetic labeling experiments with [^{14}C]carbon dioxide and molecular species analysis of lipids with *Brassica napus* leaf discs revealed extensive scrambling between labeled and unlabeled fatty acids in PtdCho, which was attributed to acyl exchange between different molecular species of PtdCho (Williams et al. 2000). This result did not agree with the classic view of a biosynthetic sequence in which oleic or palmitic acids are exported from the plastid and first assembled into PtdOH at the ER (**Figure 3*a***, pathway 1). More recently, rapid kinetic labeling experiments

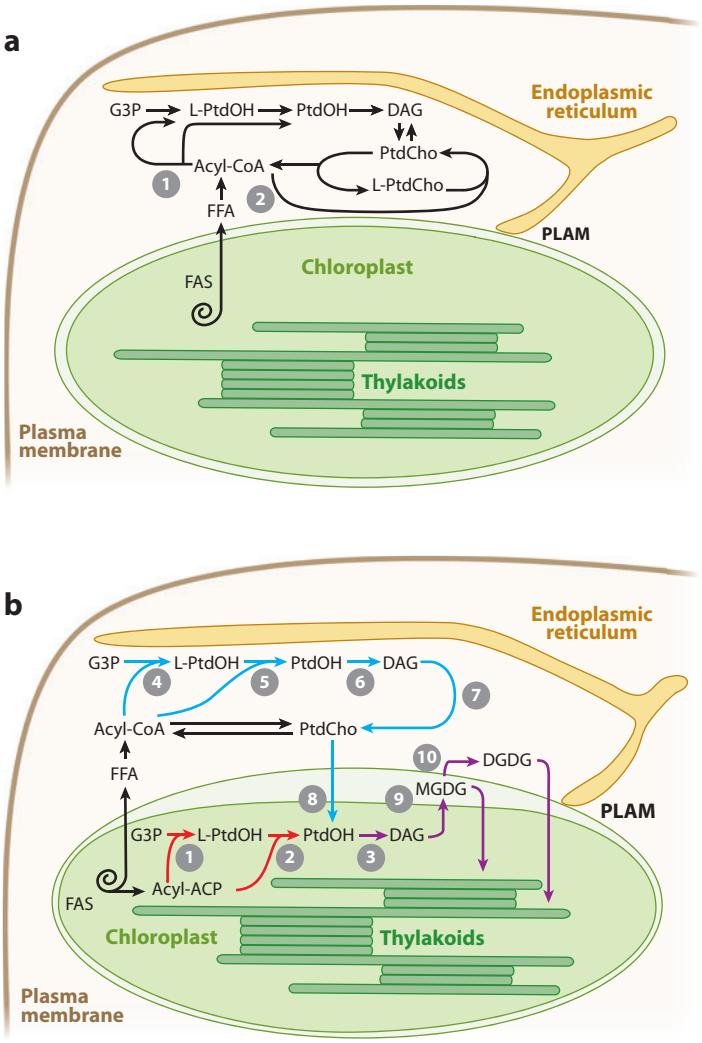

Figure 3

ER-plastid interaction in glycerolipid biosynthesis. (*a*) Export of fatty acids from plastids. (*b*) The two pathways of thylakoid lipid biosynthesis. The plastid envelope ER-contact site (PLAM) is shown in both panels. (*a*) Acyl-ACPs (not shown) are released from the fatty acid synthase complex (FAS) and hydrolyzed to free fatty acids (FFA), which are reactivated to acyl-CoAs on the cytosolic face of the outer envelope membrane. The classic hypothesis is that phospholipids are directly assembled from nascent fatty acids (pathway 1) by reacting glycerol 3-phosphate (G3P) with one acyl-CoA to give rise to lysophosphatidic acid (L-PtdOH) and a second acyl-CoA to produce phosphatidic acid (PtdOH). PtdOH is further converted to diacylglycerol (DAG), which is the precursor for phosphatidylcholine (PtdCho) biosynthesis along with CDP-choline. Recent studies (Bates et al. 2007) suggest the involvement of a PtdCho editing pathway (pathway 2) in which PtdCho is constantly turned over to lyso-phosphatidylcholine (L-PtdCho). Newly exported fatty acids are predominantly channeled to L-PtdCho and then exchanged between the PtdCho and the acyl-CoA pool followed by de novo synthesis (pathway 1). All these reactions could occur in the PLAMs in which ER and plastid outer envelope membranes are tightly associated. (*b*) Lipid assembly reactions of the plastid pathway are shown with red arrows, reactions of the ER pathway with blue arrows, and common reactions with purple arrows. The focus is on the biosynthesis of the major galactoglycerolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) starting from fatty acid synthesis (FAS) in the plastid. The biosynthesis of MGDG occurs at the interenvelope face of the inner envelope membrane, that of DGDG at the cytosolic face of the outer envelope membrane. In C16:3 plants such as *Arabidopsis*, both pathways contribute to galactoglycerolipids. The exact nature of the lipid precursor returned to the plastid is not known. Lipid precursor abbreviations are as defined for (*a*). Enzymes or enzyme complexes are indicated by numbers: 1, plastid G3P:acylACP acyltransferase (ATS1/ACT1 in *Arabidopsis*); 2, plastid L-PtdOH:acyl-ACP acyltransferase; 3, plastid PtdOH phosphatase; 4, ER G3P:acylACP acyltransferase; 5, ER L-PtdOH:acyl-ACP acyltransferase; 6, ER PtdOH phosphatase; 7, DAG:CDP-choline phosphotransferase; 8, PtdOH importer (TGD1,2,3 in *Arabidopsis*); 9, DAG:UDP-Gal galactosyltransferase (MGDG synthase, MGD1 in *Arabidopsis*); 10, MGDG:UDP-Gal galactosyltransferase (DGDG synthase, DGD1 in *Arabidopsis*).

MGD1: glycosyltransferase involved in monogalactosyldiacylglycerol synthesis

with pea leaves did not find a precursor-product relationship between nascent fatty acids and PtdOH (Bates et al. 2007). Instead, an acyl editing mechanism acting on the *sn*-1 and *sn*-2 positions of PtdCho was determined as one of the first processes in the assembly of fatty acids into membrane lipids. The presence of labeled acyl groups preferentially in the *sn*-2 position of the glycerol backbone of PtdCho, as was also observed for leek seedlings (Mongrand et al. 1997, 2000), also disagrees with an initial synthesis of PtdOH and its subsequent conversion to PtdCho. Rather, recycled acyl-groups exchanged between PtdCho and the cytosolic acyl-CoA pool are made available for de novo PtdOH and subsequent PtdCho biosyntheses at the ER in expanding leaves (**Figure 3*a***, pathway 2). Activities of PtdCho:CoA acyl exchange associated with plastids have been described (Bessoule et al. 1995, Kjellberg et al. 2000). Alternatively, phospholipases in combination with acyl-CoA synthetases could mediate the incorporation of nascent fatty acids into PtdCho. Determining the molecular identity and biochemical activity of the proteins catalyzing the initial incorporation of nascent fatty acids into PtdCho will provide an important breakthrough for our current understanding of fatty acid export from plastids.

TWO PATHWAYS FOR THE ASSEMBLY OF THYLAKOID LIPIDS

Up to this point the discussion has exclusively focused on the export of lipids and fatty acids from the plastid. Assuming that most lipid precursors originate in the plastid, these processes can be classified as anterograde lipid transport. However, plastids in most plants are capable of importing lipid precursors assembled at the ER and incorporating them into the different thylakoid lipids, a process that can be described as retrograde lipid transport. Many land plants use two pathways for the assembly of thylakoid lipid precursors as shown in **Figure 3*b***: a de novo lipid assembly pathway in the plastid (**Figure 3*b***, reactions 1–3) and a pathway operating at the ER (**Figure 3*b***, reactions 4–7). This two-pathway hypothesis was originally proposed by Roughan and coworkers (Roughan et al. 1980) following in vivo labeling experiments with leaves and in vitro labeling experiments with isolated plastids and microsomes done by a number of groups as summarized in two classic reviews (Frentzen 1986, Roughan & Slack 1982). A key finding was that glycerolipids produced by the ER pathway have a different molecular species composition (18-carbon fatty acids in the *sn*-2 position of the glycerol backbone) than those produced by the plastid pathway (16-carbon fatty acids in *sn*-2) (Heinz & Roughan 1983). Different substrate specificities of acyltransferases at the ER and the plastid envelope account for these differences (Frentzen et al. 1983).

Molecular lipid species analysis was developed as a tool to determine the relative flux contribution of the two pathways to the synthesis of the four thylakoid lipid classes. For example, in *Arabidopsis* approximately equal amounts of chloroplast lipids are produced by the two pathways (Browse et al. 1986b). All PtdGro in the plastid is derived from the plastid pathway, as is the largest fraction of MGDG and SQDG. All of the PtdCho associated with plastids is derived from the ER pathway, as is most of DGDG.

For the sake of simplicity, much of this discussion focuses on the assembly of the two galactoglycerolipids (Benning & Ohta 2005), which also dominate the thylakoid lipid composition. As shown in **Figure 3*b***, the bulk of MGDG in plastids of *Arabidopsis* is synthesized at the intermembrane facing leaflet of the inner plastid envelope membrane where the responsible galactosyltransferase, MGD1, is localized (Awai et al. 2001, Jarvis et al. 2000, Xu et al. 2005). The bulk of DGDG is synthesized at the cytosolic face of the outer envelope membrane catalyzed by a second galactosyltransferase DGD1 (Dörmann et al. 1999, Froehlich et al. 2001). The contribution of the two pathways is different depending on the plant species. Molecular species analysis of lipids divides plants into two groups: those with a large fraction of 16-carbon fatty acids (mostly

hexadecenoic acid, C16:3, in MGDG) in the *sn*-2 position of glycerol in thylakoid glycoglycerolipids (C16:3 plants) and those (C18:3 plants) with exclusively 18-carbon fatty acids in *sn*-2 (mostly linolenic acid, C18:3, in MGDGD and DGDG) (Heinz & Roughan 1983, Mongrand et al. 1998). Some of the most evolved plants have lost the plastid pathway for glycoglycerolipid biosynthesis and are C18:3 plants.

Based on molecular species composition and label experiments with isolated plastids, plastid PtdGro biosynthesis is an exception, because in all plants tested, its biosynthesis seems to depend on the plastid pathway. One explanation proposed early on was that C18:3 plants have reduced PtdOH phosphatase activity (**Figure 3*b***, enzyme 3) (Frentzen et al. 1983, Heinz & Roughan 1983), which is not required for PtdGro biosynthesis (Frentzen 2004) but is necessary for galactoglycerolipid biosynthesis (Benning & Ohta 2005). However, the recent discovery of a presumed PtdOH transporter involved in the import of lipids into the plastid as part of the ER-pathway in *Arabidopsis* (**Figure 3*b***, enzyme complex 8) as discussed in detail below appears to conflict with this idea, because in the current model lipids derived from the ER and the plastid pathways would have to be metabolized by a plastid PtdOH phosphatase (**Figure 3*b***, enzyme 3).

The two-pathway hypothesis shown in **Figure 3*b*** and the correlation of lipid molecular species with their respective origins from one of the two pathways was independently corroborated by Browse, Somerville, and coworkers, who studied a series of *Arabidopsis* mutants deficient in fatty acid biosynthesis and modification, and glycerolipid assembly (Browse & Somerville 1991, 1994; Wallis & Browse 2002). Of particular interest here is the *ats1* mutant (formerly *act1*), in which the plastid G3P:acylACP acyltransferase (**Figure 3*b***, enzyme 1) is affected, leading to a loss of the galactoglycerolipid species derived from the plastid pathway. This effectively turns *Arabidopsis* from a C16:3 plant into a C18:3 plant (Kunst et al. 1988). Surprisingly, all alleles of *ats1* still produce considerable amounts of PtdGro in the plastid despite a very strong reduction in enzyme activity (Xu et al. 2006). Either the residual activity is sufficient or a not-yet discovered alternative pathway for PtdGro biosynthesis is active in plastids.

Fatty acid desaturase mutants also provided information on the acyl composition of the possible lipid intermediate returning from the ER to the plastid. The *fad2* mutant of *Arabidopsis* lacking the ER-associated oleoyl-PtdCho desaturase showed a strong reduction of molecular MGDG species derived from the ER-pathway, suggesting that the ER-to-plastid lipid transfer machinery prefers lipid molecular species containing acyl chains with more than one double bond (Miquel & Browse 1992). These genetic data confirmed earlier results of labeling experiments suggesting that C18:2-containing molecular species of PtdCho are likely precursors for C18:3-containing molecular species of MGDG (Slack et al. 1977). The already mentioned uneven labeling of fatty acids in the two positions of the glycerol backbone (Bates et al. 2007, Williams et al. 2000) has been interpreted as the re-import of lyso-PtdCho from the ER (Mongrand et al. 1997, 2000). However, because it is not clear where acyl editing occurs, at the envelopes or the ER, the interpretation of these results is ambiguous. One clue that PtdCho plays a critical role in the retrograde transfer of lipids from the ER to the plastid comes from the green alga *Chlamydomonas reinhardtii*, which lacks PtdCho and accordingly ER-derived thylakoid lipids (Giroud et al. 1988).

Genetic analysis in *Arabidopsis* has provided surprising evidence that highly unsaturated lipid precursors can leave the plastid, even under phosphate-replete conditions. In mutants in which ER-associated fatty acid desaturases are deficient, unsaturated fatty acids are still found in extraplastidic lipids, suggesting that a significant amount of unsaturated molecular species can be exported from the plastid (Browse et al. 1993, Miquel & Browse 1992). Conversely, evidence for this export of highly unsaturated lipid molecular species is also visible in a mutant deficient in a plastid fatty acid desaturase,

Plastid–associated microsomes (PLAM): derived from ER-plastid contact sites

***tgd1,2,3,4*:** mutants of *Arabidopsis* accumulating trigalactosyldiacylglycerol

DAG: diacylglycerol

in which extraplastidic lipids are less saturated as well (Browse et al. 1986a). Thus, the interaction between the ER and plastid pathways of lipid assembly is complex—involving intricate lipid transfer reactions. Indeed, distinguishing lipid assembly and transport processes at the ER and the plastid outer envelope membrane have become conceptually more difficult with the discovery of ER-plastid contact sites (**Figure 3*a*,*b***) that give rise to a plastid-associated microsome fraction (PLAM) with classically ER-associated enzymatic activities (Kjellberg et al. 2000). These contact sites have recently been visualized, and the strength of interaction between the two membrane systems has been determined using optical tweezers (Andersson et al. 2007).

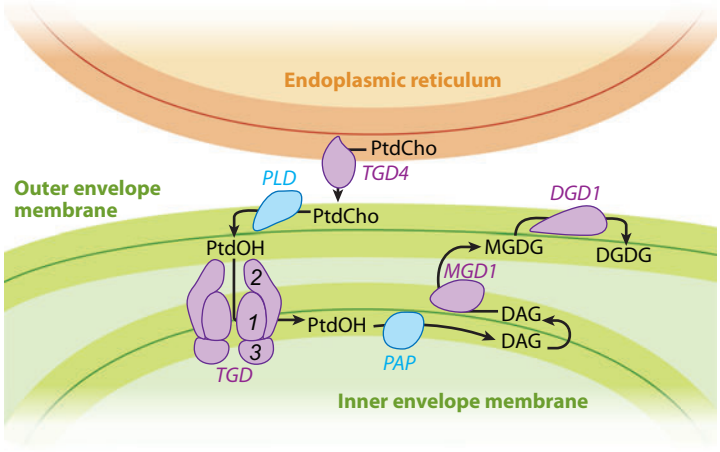

Figure 4

Interaction of components involved in ER-to-plastid lipid transport and galactoglycerolipid assembly in *Arabidopsis*. The endoplasmic reticulum (ER) and the outer and inner envelope membranes of the plastid are shown. The two layers of the membranes are indicated. Proteins for which the identity is known are shaded purple; those for which the identity is unclear are shown in blue. TGD4 is associated with the ER but its exact role is not yet known. A phospholipase D (PLD) is proposed to convert phosphatidylcholine (PtdCho) to phosphatidic acid (PtdOH), which is the proposed substrate of the TGD1,2,3 transporter complex in the inner envelope membrane. TDG2 is proposed to accept PtdOH from the outer envelope. Phosphatidic acid phosphatase (PAP) at the inside of the inner envelope converts PtdOH to diacylglycerol (DAG). DAG is made available for the biosynthesis of monogalactosyldiacylglycerol (MGDG) by MGD1 located at the intermembrane face of the inner plastid envelope membrane. MGDG is converted to digalactosyldiacylglycerol (DGDG) by DGD1 located at the cytosolic face of the outer envelope membrane. All these proteins could be organized in a supercomplex involving the three membranes and allowing substrate channeling between the components.

LIPID TRANSFER BETWEEN THE ER AND PLASTID ENVELOPES

The complexity and redundancy of thylakoid lipid biosynthesis as depicted in **Figure 3*b*** clearly invokes lipid transport processes that must shuttle lipid precursors and products between the three involved biogenic membranes and the thylakoids. In recent years, *Arabidopsis* genetics provided identification of some of the genes and proteins involved in the process, and a current model depicting the location and possible function of these proteins is shown in **Figure 4**. In addition to the already mentioned *ats1* mutant disrupted in the plastid pathway of galactolipid biosynthesis, mutants of *Arabidopsis* were identified that clearly meet phenotypic criteria for a disruption in the ER pathway of thylakoid lipid biosynthesis. These are the *tgd* mutants named after oligogalactoglycerolipids, for example, trigalactosyldiacylglycerol (TGDG; see **Figure 1**), accumulating in their tissues (Awai et al. 2006; Lu et al. 2007; Xu et al. 2003, 2005, 2008a). The oligogalactoglycerolipids produced in the *tgd* mutants are structurally different from the typical galactoglycerolipids found in leaves, such that they are not likely the product of the nonprocessive UDP-Gal-dependent MGD1 or DGD1 galactosyltransferases or their MGD2/3 and DGD2 paralogs (Xu et al. 2003). Instead, these oligogalactoglycerolipids appear to be produced by a processive UDP-Gal-independent galactosyltransferase associated with the outer envelope membrane. This activity was also previously observed in plastid preparations by Wintermans and colleagues (Heemskerk et al. 1988, van Besow & Wintermans 1978). The respective enzyme transfers galactosyl residues repeatedly from MGDG onto an acceptor lipid, for example a second MGDG, thereby releasing diacylglycerol (DAG) (Benning & Ohta 2005).

The function of this enzyme in the wild type is not clear at this time. However, a candidate

gene and a mutant of *Arabidopsis* deficient in this activity were recently isolated and provide the basis for a functional analysis in the near future (B. Muthan, E.R. Moellering, C. Xu, and C. Benning, unpublished work). It is possible that this enzyme is activated by PtdOH, which according to the current model shown in **Figure 4** is produced at the outer plastid envelope membrane. Incidentally, PtdOH levels were found to be elevated in the *tgd1* mutant (Xu et al. 2005), but the extent of its increase was dependent on the growth conditions with older plants grown on agar plates showing the highest PtdOH levels (C. Xu and C. Benning, unpublished work). The mutants also accumulate triacylglycerols in their leaf tissue, and triacylglycerol biosynthesis could be stimulated at the ER to remove PtdOH backing up in *tgd* mutants. Therefore, the buildup of unusual lipids, although highly diagnostic for this mutant class, appears to be a secondary phenotype.

The primary biochemical phenotype of the *tgd* mutants is a disruption of lipid transfer from the ER to the plastid apparent in an altered molecular species composition—increased 16-carbon fatty acids in the *sn*-2 position of the glycerol backbone—of the thylakoid lipids consistent with a relatively higher fraction of lipids derived from the plastid pathway and a decreased flux through the ER pathway. The molecular species composition of the accumulating oligogalactolipids suggests that they are derived from the plastid pathway, whereas that of leaf triacylglycerols is consistent with their synthesis at the ER. Molecular species of PtdCho in *tgd1* were very similar to that of accumulating PtdOH, suggesting a possible precursor-product relationship (Xu et al. 2005). Contrary to molecular species analysis of lipids, pulse-chase labeling experiments with acetate or oleic acid provide a direct way of examining lipid dynamics in vivo (Benning 2008). Pulse-chase labeling clearly showed altered kinetics in the *tgd* mutants consistent with a reduction in import of lipids from the ER into the plastid (Xu et al. 2003, 2008a).

The TGD proteins are essential to plant growth, because as the severity of the mutant alleles increases, the mutations either become embryo-lethal as for *tgd1* (Xu et al. 2005) or cause severe growth reduction as for *tgd4* (Xu et al. 2008a). Compared to the plastid pathway, the ER pathway of thylakoid lipid biosynthesis is prevalent during *Arabidopsis* embryo development and seems to be required for the transition of proplastids into chloroplasts (Xu et al. 2005). The TGD1, 2, and 3 proteins are similar to the permease, substrate-binding, and ATPase proteins, respectively, of a multipartite ABC transporter (**Figure 4**). Many gram-negative bacteria have orthologs of the *Arabidopsis tgd1,2,3* genes organized in operons. These orthologs are not essential for growth in *E. coli* (Gerdes et al. 2003), but in *Pseudomonas putida* this complex was shown to be required for solvent tolerance and has been proposed to constitute a toluene efflux pump (Kieboom et al. 1998a,b; Kim et al. 1998). However, toluene as a substrate has not yet been confirmed. It seems just as likely that the bacterial orthologs are involved in lipid remodeling of the cell membrane in response to solvent or osmotic stress.

Although there was initial evidence based on protease protection experiments that TGD1 was associated with the outer envelope membrane of plastids (Xu et al. 2003), later experiments found the TGD1,2,3 proteins more likely to be associated with the inner envelope membrane as shown in **Figure 4** (Awai et al. 2006, Lu et al. 2007, Xu et al. 2005). However, it cannot be ruled out that the complex is located in a contact site between the two envelope membranes in which TGD1 would also appear to be associated with the outer envelope membrane. A direct determination of the substrate for this putative plastid transporter has not been accomplished. However, the TGD2 protein, which is the presumed substrate-binding protein of the proposed complex, specifically binds PtdOH (Awai et al. 2006). Furthermore, the incorporation of label from PtdOH into galactoglycerolipids by isolated *tgd1* mutant plastids was reduced (Xu et al. 2005). In **Figure 4** the TGD2 protein while anchored in the inner envelope is shown to touch the outer envelope. Whereas this interaction may be

transient and difficult to prove, it seems possible that TGD2 extracts PtdOH formed at the outer envelope and makes it available to the TGD1,3 core transporter. Two lines of evidence are consistent with a possible role of TGD2 in a two-membrane contact site: (*a*) TGD2 contains a mycobacterial cell entry domain typically found in cell surface proteins that are needed by virulent mycobacteria to enter the host cells (Chitale et al. 2001). (*b*) In experiments to localize TGD2 in isolated plastids, it was observed that the bulky green fluorescent protein fused to the TGD2 wild-type protein and the *tgd2-1* mutant protein by itself were accessible to Trypsin, whereas the functional wild-type TGD2 protein by itself was inaccessible (Awai et al. 2006). Current efforts to dissect the PtdOH binding site of TGD2 and to crystallize portions of TGD2 are under way (C. Lu and C. Benning, unpublished).

Whereas the discovery of the TGD1, 2, and 3 proteins have begun to shed light on lipid transport from the outer to the inner plastid envelope membrane, TGD4 is a recently discovered candidate for a protein involved in transfer of lipids from the ER to the outer envelope (Xu et al. 2008a). The *tgd4* mutant of *Arabidopsis* shows all the phenotypes observed for the other *tgd* mutants described above. A possible paralog is present in *Arabidopsis*, but its inactivation did not cause any *tgd* phenotypes (Xu et al. 2008a). Interestingly, only N-terminally green fluorescent protein–tagged versions of TGD4 restore the wild-type phenotype in the mutant background, whereas C-terminally tagged versions do not. The N-terminally tagged TGD4 protein was localized to the ER-network, but it cannot be ruled out that TGD4 in native form in vivo is present in the plastid outer envelope or in ER-plastid contact sites. The number of ER-plastid contact sites was not affected in the mutant (Xu et al. 2008a), making it unlikely that TGD4 promotes their formation. Possible orthologs of TGD4 are present in other plants and green algae, but do not provide any clues to the function of TGD4. The protein has a potential membrane-spanning domain that is not clearly predicted by all available algorithms but could anchor TGD4 in the ER (**Figure 4**). One possible function of TGD4 is that it recruits proteins, for example, a specific phospholipase D, to an ER-plastid contact site.

The current hypothesis about the synthesis of the galactolipids by the ER pathway in *Arabidopsis* is summarized in **Figure 4**: TGD4 mediates either directly or indirectly through interacting proteins the transfer of PtdCho assembled at the ER to the outer plastid envelope. At the outer envelope PtdCho has to be converted to PtdOH to provide the substrate for the TGD1,2,3 complex. A to-date unspecified phospholipase D is predicted by the model and should be localized at the outer envelope or in ER-plastid contact zones. The TGD1,2,3 complex mediates the transfer of PtdOH from the outer envelope to the inside of the inner envelope membrane where the plastid PtdOH phosphatase is located. The PtdOH phosphatase as part of the import process has not yet been unambiguously identified, but a promising candidate was recently described (Nakamura et al. 2007). Substrate channeling between this transporter and the respective PtdOH phosphatase is likely because in the wild type, ER-derived PtdOH is apparently not available to the competing first enzyme of PtdGro biosynthesis (CDP-DAG synthase). On the other hand, a plastid-targeted *E. coli* DAG kinase had access to the ER-derived DAG pool in the transgenic plants leading to the biosynthesis of unusual ER-derived PtdOH and PtdGro molecular species (Fritz et al. 2007).

The DAG present in the inner envelope regardless of its origin is available as substrate to the MGDG synthase MGD1 located at the intermembrane surface of the inner envelope. This enzyme in turn provides MGDG to the DGDG synthase on the cytosolic side of the outer envelope membrane. A conflicting observation is the already mentioned low PtdOH phosphatase activity observed in C18:3 plant chloroplasts (Frentzen et al. 1983, Heinz & Roughan 1983), because these plants entirely rely on ER-derived lipids for the biosynthesis of galactoglycerolipids. One could argue that a PtdOH phosphatase activity that channels

ER-derived PtdOH from the proposed TGD1,2,3 complex to the DAG pool in the inner envelope is not accurately measurable in isolated plastids. It has also been reported that the major MGDG synthase activity in the C18:3 plant pea is associated with the outer envelope membrane based on assays with chloroplast fractions (Cline & Keegstra 1983). If this in vitro activity represents the in vivo situation in pea, the proposed TGD1,2,3 complex would not be necessary for galactoglycerolipid biosynthesis in C18:3 plants. However, the TGD2 protein was identified in plastid envelope preparations of pea by proteomics (Brautigam et al. 2008), and rice, which is another C18:3 plant, has a set of predicted TGD1, 2, 3, and 4 orthologs. Determining the localization and membrane topology of the involved proteins in a C18:3 model plant in comparison to the C16:3 model plant *Arabidopsis* should provide clues that explain the differences in the utilization of the ER and plastid pathways for thylakoid lipid biosynthesis in these plant groups.

LIPID TRANSFER FROM THE ENVELOPES TO THE THYLAKOIDS

Lipids, whether assembled at the outer and inner envelope membranes of the plastid or imported from the ER, ultimately have to be delivered to the thylakoids. A vesicular mechanism has been proposed based on ultrastructural observations (von Wettstein 2001), specific inhibitors (Westphal et al. 2001b), or arrest of vesicle transfer under low temperatures (Morre et al. 1991). Ultrastructural examination of photosynthetic organisms in lineages derived from the first photosynthetic eukaryote suggests that the presence of an intraplastidic vesicular transport system may be limited to embryophytes (Westphal et al. 2003). Potential plastid-targeted protein orthologs of the yeast secretory pathway are present in plants but have not yet been investigated in detail (Andersson & Sandelius 2004). However, genetic analysis has identified at least one protein candidate that is proposed to be involved in vesicle formation in plastids. Loss of VIPP1 (vesicle-inducing protein in plastids 1) in *Arabidopsis* causes an absence of thylakoids (Kroll et al. 2001). This protein is present in bacteria as well, and mutation in cyanobacteria affects thylakoid formation (Westphal et al. 2001a). The fact that bacteria generally lack the machinery for vesicular transport casts doubts on a direct role of VIPP1 in intraplastidic vesicular transport. The VIPP1 protein interacts with chaperone proteins and forms ring-like structures in vitro (Aseeva et al. 2004, 2007; Liu et al. 2005, 2007). It also interacts with other proteins essential for the assembly of the photosynthetic apparatus (Gohre et al. 2006). However, direct involvement of VIPP1 in vesicle initiation or its mechanism of action has not been demonstrated at this time.

VIPP1: protein required for thylakoid formation

There are other examples of proteins apparently involved in thylakoid formation based on the respective phenotype of the mutant in *Arabidopsis*, for example, THF1 (thylakoid formation 1) (Wang et al. 2004). However, this protein seems to be involved in signaling rather than in vesicular transport (Huang et al. 2006). Thus, although multiple lines of evidence suggest a vesicular mechanism for the transfer of membrane lipids from the inner envelope to the thylakoids, the exact mechanism and the involved proteins remain to be identified.

Lipid transfer from the envelopes to the thylakoids also needs to be intricately integrated with overall chloroplast development. Breakdown of the coordination between envelope membrane formation and lipid transfer was recently observed when an inner membrane protein (TIC40) was overproduced in tobacco plastids by expression from a transgene inserted into the plastid genome (Singh et al. 2008). A striking proliferation of the inner envelope membrane was observed in these transgenic plastids. Apparently, increased insertion of an inner membrane protein led to an increase in membrane lipid synthesis, but vesicle transfer from the envelope to the thylakoids was not upregulated in this transgenic line.

ANSWERS TO EMERGING QUESTIONS

The recent breakthroughs in our understanding of thylakoid lipid biosynthesis and the underlying lipid transport processes, in particular, the discovery of proteins and genes required for the process, provide new opportunities for filling the remaining gaps of knowledge. Proteins involved in fatty acid export from plastids may be identified by systematic analysis of *Arabidopsis* mutants disrupted in plastid targeted proteins (e.g., Lu et al. 2008). It may also be possible to identify fatty acid auxotrophic mutants in the unicellular algae *Chlamydomonas* that are disrupted in the export machinery. The intricate differences in lipid metabolism between C16:3 and C18:3 plants can be explored at the molecular and cellular level by targeting genes, which are orthologs of those known to be involved in the C16:3 plant *Arabidopsis*, in an appropriate C18:3 plant, for example, rice. The TGD1, 2, and 3 proteins of *Arabidopsis* that are involved in lipid transfer from the outer to the inner envelope membrane need to be reconstituted, and the complex needs to be studied for transport activity and substrate specificity. Whether this complex is present in contact zones between the outer and inner envelopes remains an interesting hypothesis that will require creative means to test.

Likewise, a possible function of TGD4 in ER–outer plastid envelope membrane contact sites can now be investigated by studying the interaction of this protein with other proteins, by reconstitution and activity assays in vitro, and by studying its subcellular localization dynamics during leaf development. Finding proteins specifically recruited to these contact sites would provide markers that would also be helpful in studying the possible role of these contact sites in the export of glycerolipids from plastids under phosphate-limited conditions. The study of plastid-targeted orthologs of proteins involved in vesicular transport as part of the secretory pathway should provide insights into vesicular lipid trafficking mechanisms between the inner plastid envelope membrane and the thylakoids.

As more components of interorganelle lipid transport in plants are discovered and as more plant species are compared at the molecular and cell biological level, the pace of discovery will increase. As we gain a detailed understanding of the complex lipid transport process between the plastid and the extraplastidic membranes in different plants, we may discover that lipid transfer processes provide a vivid picture of the evolution of the interaction between the past endosymbiont and the host cell.

SUMMARY POINTS

1. Under phosphate-limiting growth conditions, plants remodel their membranes to reduce phosphate bound in phosphoglycerolipids. Chloroplasts export digalactosyldiacylglycerol to substitute phosphoglycerolipids in extraplastidic membranes. Phosphatidylglycerol is substituted with sulfoquinovosyldiacylglycerol in thylakoid membranes.
2. Fatty acids are synthesized in the plastid and are exported from the chloroplast for the biosynthesis of extraplastidic membranes. Nascent fatty acids appear to be first incorporated into phosphatidylcholine by acyl editing. There is no direct precursor-product relationship between phosphatidic acid and phosphatidylcholine.
3. With some variation depending on the plant species, thylakoid lipids are either derived from precursors assembled de novo in the plastid or imported from the ER. The ER pathway is generally present in plants, whereas the plastid pathway is absent in many, typically more recently evolved, plants. The TGD proteins of *Arabidopsis* are implicated

in the ER-to-plastid lipid transfer based on mutant analysis. TGD1, 2, and 3 constitute a proposed phosphatidic acid transporter at the inner plastid envelope; TGD4 is a unique plant ER protein with currently unknown molecular function.

4. Vesicular transport between the inner plastid envelope and the thylakoids is proposed. Predicted plastid-targeted orthologs of proteins involved in the secretory pathway are present in plants. VIPP1 of *Arabidopsis* has been implicated in the process, but its role in vesicle formation at the inner envelope could be indirect.

FUTURE ISSUES

1. Components of the plastid fatty acid export machinery remain elusive, and the mechanism is still under debate. Because all fatty acids for extraplastidic membranes and for triacylglycerol biosynthesis in oil crops have to be exported from the plastid, this represents a critical lack of knowledge for an essential cell function. Knowledge of this process could provide new leads for the engineering of oil crops for the production of fuels and specialized fatty acid–derived chemicals.
2. Understanding the molecular differences between C16:3 and C18:3 plants, the latter of which lack the plastid pathway for galactoglycerolipid biosynthesis, could provide us with an opportunity to observe evolution in progress. The reasons for the exclusive synthesis of plastid phosphatidylglycerol by the plastid pathway in all plants remain unclear.
3. How is TGD4 involved in lipid transfer from the ER to the plastid? Is it present in ER-plastid outer envelope membrane contact sites, and is its location dynamic? What role do ER-plastid outer envelope membrane contact sites play in the transfer of lipids from the ER to the plastid, and how is the lipid transfer and lipid synthesis machinery organized in ER-plastid outer envelope and inner envelope membrane contact sites?

DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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Contents

Annual Review of Cell and Developmental Biology

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Indexes

Errata

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