Chloroplast Membrane Lipid Biosynthesis and Transport

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Abstract The photosynthetic membranes of chloroplasts are characterized by a high abundance of glycolipids. The two galactolipids monogalactosyldiacylglycerol and digalactosyldiacylglycerol (DGDG) are the predominant constituents of thylakoid membranes, while phospholipids (phosphatidylcholine, phosphatidylglycerol) and a sulfolipid (sulfoquinovosyldiacylglycerol) are minor components. Galactolipids are synthesized in the envelope membranes from precursors originating from the chloroplast or from the endoplasmic reticulum (ER). Direct contact sites ("plastid-associated membranes") between the ER and the chloroplast may be involved in the transport of lipid precursors to the envelope membranes. During chloroplast development, thylakoids are established from invaginations of the inner envelope, whereas in mature chloroplasts, a vesicle-based transport system was suggested to supply galactolipids to the thylakoids. During phosphate limitation, phospholipids are replaced with glycolipids in plastidial and extraplastidial membranes. DGDG produced in the chloroplast was suggested to be transferred to the mitochondria via direct contact sites. The transport of DGDG to the plasma membrane and tonoplast is believed to be mediated via the ER and Golgi vesicle trafficking system.

1 Introduction

Oxygenic photosynthesis in its very essence is a membrane-bound process. The primary light absorption is mediated by membrane-bound protein–pigment complexes, the electron transport chain consists of membrane-bound components, and the proton gradient that drives ATP synthase is built across the thylakoid membrane. It is thus not surprising that the higher-plant chloroplast is a very membrane rich organelle.

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The chloroplast internal thylakoid membranes are the site of the photosynthetic electron transport chain and constitute an extremely large surface area in green plant tissues. In fact, the bulk of the membrane lipids in a green leaf are situated in the thylakoid membranes. The chloroplast is delimited from the cytosol by the double-envelope membrane, and all molecules exchanged between the chloroplast and other cellular compartments must at some point pass across the envelope. The envelope is also the site for the final assembly of the major chloroplast lipids.

A consensus on the overall lipid composition of the different chloroplast membranes was reached in the mid-1980s. At the same time, many of the major membrane lipid biosynthetic pathways were worked out. The 1990s and the beginning of the postgenomic era marked the cloning of a number of important chloroplast lipid biosynthesis genes from the model plant *Arabidopsis thaliana* and other species. Membrane lipid biosynthesis mutants generated by forward or reverse genetic approaches shed more light on the in vivo function(s) of individual membrane lipid classes. In the last decade it has become clear that a particular chloroplast galactolipid, digalactosyldiacylglycerol (DGDG), can play a major role in extraplastidial membranes during phosphate-limited growth conditions. Thus, galactolipid synthesis under normal and phosphate-limited conditions requires the involvement of several cellular compartments. The remaining questions are concerned mainly with the regulation of the synthesis pathways and transport of chloroplast lipids and precursors. In the present chapter, we will first give a brief overview of the chloroplast lipids and their particular functions, and then summarize the current understanding of biosynthesis and transport of chloroplast membrane lipids. We have tried to include as many as possible of the primary literature sources, but owing to space limitations, it was impossible to cover all relevant aspects. We apologize to all whose excellent contributions we have not been able to refer to.

2 The Chloroplast Lipidome

The lipid composition of the chloroplast membranes differs in several ways from that of other membranes in the plant cell and seems to largely reflect the cyanobacterial origin of the organelle. Phosphoglycerolipids constitute only a minor proportion of the chloroplast membranes; instead the chloroplast membranes are highly enriched in galactoglycerolipids. Sterols and sphingolipids, which are important constituents of the plant plasma membrane, Golgi apparatus and tonoplast are completely absent from the chloroplast membranes. The chloroplasts are the only plant organelles that contain the anionic sulfur-containing lipid sulfoquinovosyldiacylglycerol (SQDG). The thylakoids, which constitute the bulk of the membrane surface in a mature chloroplast (approximately 90 mol% of the membrane lipids), are, in principle, composed of four different lipid classes, the galactolipids monogalactosyldiacylglycerol (MGDG) and DGDG and the two anionic lipids phosphatidylglycerol (PG) and SQDG. The structures of the most common molecular species of the chloroplast membrane lipids are shown in Fig. 1 and the membrane lipid composition of chloroplasts and chloroplast subfractions is shown in Table 1.

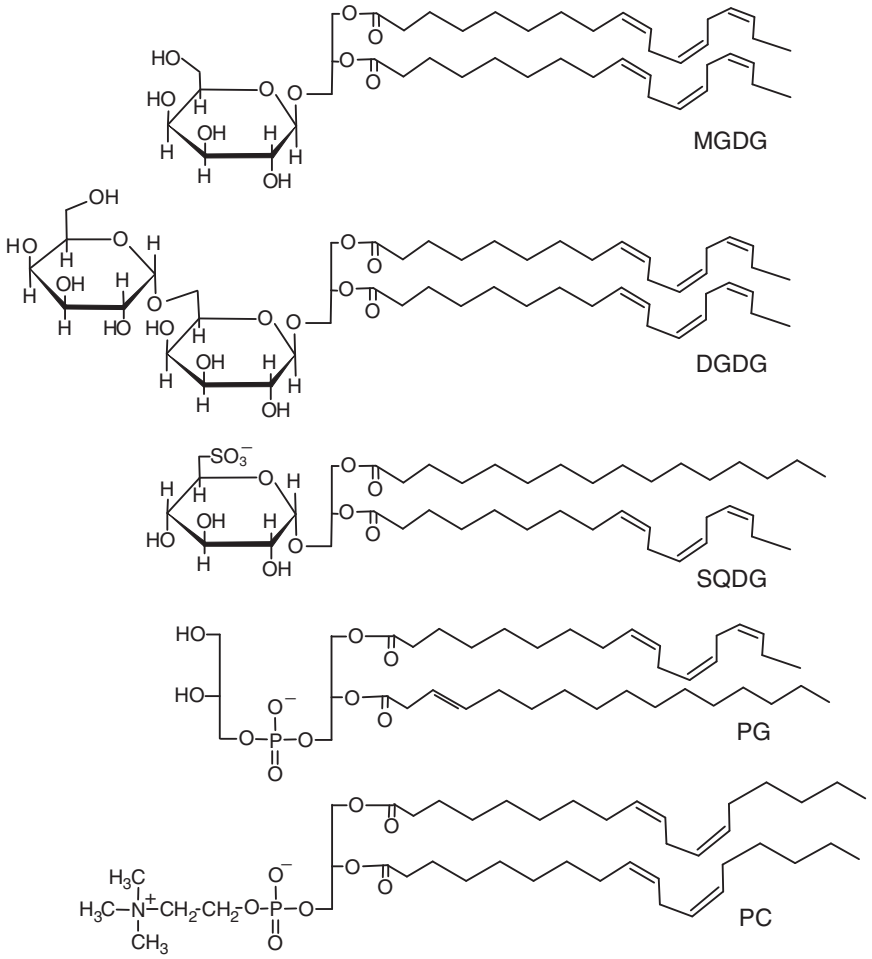

Fig. 1 Glycerolipids found in chloroplast membranes. Chloroplasts contain large amounts of the two galactolipids monogalactosyldiacylglycerol (*MGDG*) and digalactosyldiacylglycerol (*DGDG*). Furthermore, two anionic lipids are found in chloroplasts, the sulfolipid sulfoquinovosyldiacylglycerol (*SQDG*) and the phospholipid phosphatidylglycerol (*PG*). The zwitterionic phospholipid phosphatidylcholine (*PC*) is found primarily in the outer-envelope membrane

The galactose head group in MGDG and DGDG is directly linked through a β-glycosidic bond to the glycerol backbone, and the second galactose in the DGDG head group is linked by an α-glycosidic bond to the first galactose (Fig. 1). The fatty acids found in the chloroplast membrane lipids are highly unsaturated. Trienoic acids usually constitute more than 80–90% of the acyl groups found in MGDG and DGDG. SQDG and PG, on the other hand, contain a relatively larger proportion of more saturated fatty acids. The most common trienoic acid found in plants is linolenic acid

Table 1 Lipid composition (mol%) of chloroplast membranes

Plant	Membrane	MGDG	DGDG	SQDG	PG	PC	PI	PE
Spinach (Block et al., 1983b)	Total envelope	36	29	6	9	18	2	ND
	Outer envelope	17	29	6	10	32	5	ND
	Inner envelope	49	30	5	8	6	1	ND
	Thylakoid	52	26	6.5	9.5	4.5	1.5	ND
Pea (Andersson et al., 2001)	Intact chloroplasts	46	32	7	6	7	1	ND
	Thylakoid	51	33	8	5	2	0	ND
Pea (Cline et al., 1981)	Outer envelope	6	33	3	6	44	5	2
	Inner envelope	45	31	2	7	10	2	1
Wheat (Bahl et al., 1976)	Total envelope	22	44	10	9	14	0	0
	Lamellar thylakoid	42	37	9	10	2	0	0
	Grana thylakoid	47	36	7	9	1	0	0
Broad bean (Mackender and Leech, 1974)	Total envelope	29	32	ND	9	30	ND	0
	Thylakoid	65	26	ND	6	3	ND	0
Acer hippocastanum (Chapman et al., 1986)	Thylakoid	43	31	5	15	–[a]	–[a]	–[a]
Pea (Chapman et al., 1986)	Thylakoid	42	29	8	11	–[a]	–[a]	–[a]
Dark grown wheat (Selstam and Sandelius, 1984)	Envelope	44	37	6	6	5	2	ND
	Prothylakoid	45	40	8	7	–	–	ND
	Prolamellar body	52	32	8	8	–	–	ND
Consensus	Outer envelope	6–17	30	3–6	6–10	32–44	5	0
	Inner envelope	45–49	30	2–5	6–8	6–10	1–2	0
	Thylakoid	42–65	26–33	5–8	5–15	2–4	0–1	0

MGDG monogalactosyldiacylglycerol, *DGDG* digalactosyldiacylglycerol, *SQDG* sulfoquinovosyldiacylglycerol, *PG* phosphatidylglycerol, *PC* phosphatidylcholine, *PI* phosphatidylinositol, *PE* phosphatidylethanolamine

ND not detectable, – not reported

[a]Not reported, but "minor phospholipids" present in low amounts of 6–8 mol%

(18:3). Hexadecatrienoic acid (16:3) is restricted to the *sn*-2 position of MGDG and occurs in significant amounts only in some plant species, the so-called 16:3 plants (for example *Arabidopsis* and spinach). The ratio of 18:3 to 16:3 fatty acids esterified to chloroplast galactolipids varies considerably among different plant species (Mongrand et al., 1998). The majority of plant species are devoid of 16:3 and are referred to as "18:3 plants". Chloroplast PG in both 16:3 and 18:3 plants contains only molecular lipid species synthesized by the prokaryotic pathway (see Sect. 5) (Roughan, 1985; Dorne and Heinz, 1989). Chloroplast PG contains approximately 20% of the unusual

monene fatty acid *trans*-3-hexadecenoic acid, which is strictly associated with the *sn*-2 position (Dubacq and Tremolieres, 1983; Browse et al., 1986).

Methods to isolate the outer and inner chloroplast envelopes in purity and quantity sufficient for reliable biochemical characterization were developed in the early 1980s (Cline et al., 1981; Block et al., 1983a,b). The inner envelope is in terms of lipid composition closely related to the thylakoids, whereas the outer-envelope lipid composition is more related to that of other extraplastidial membranes (Table 1). Notably, the outer-envelope membrane contains a substantial amount of the zwitterionic phospholipid phosphatidylcholine (PC; Fig. 1). The amount of PC in the inner envelope and the thylakoids is very low. A few reports indeed suggest that the occurrence of PC in all other chloroplast compartments except the outer leaflet of the outer envelope is entirely artefactual (Dorne et al., 1985; Dorne et al., 1990). Nevertheless, the majority of studies report a PC content in thylakoids and inner envelope of 1–4 mol%. The ratio of DGDG to MGDG is much higher in the outer envelope than in the inner envelope and the thylakoids. The lipid-to-protein ratio changes from the outer envelope to the thylakoid. The outer envelope is lipid-rich, with a ratio of lipid to protein of about 2.5, whereas the inner envelope and the thylakoids have lipid-to-protein ratios of about 1 and 0.4, respectively (Block et al., 1983b).

3 Chloroplast Membrane Lipid Function

The primary function of the chloroplast membrane lipids is to provide a structural environment for the photosynthetic membrane protein complexes and a barrier for the different solutes present in the thylakoid lumen and chloroplast stroma, which is also a prerequisite for the establishment of the photosynthetic proton gradient. In addition, particular lipids are also found in very close association with or embedded into the photosynthetic membrane protein complexes and linked to specific biochemical functions. The major thylakoid lipid, polyunsaturated MGDG, has a small polar head group but at the same time bulky fatty acid chains. The other major chloroplast lipid, DGDG, has a much larger head group and thus an almost cylindrical geometry. DGDG by itself in excess water forms a stable bilayer, whereas MGDG favours the formation of inverted hexagonal or cubic phases. Pure lipid mixtures resembling the composition of the thylakoid membrane do not form stable bilayers on their own, but rather complex mixtures of inverted hexagonal and cubic phases (Brentel et al., 1985). The non-bilayer-forming tendency is highly dependent on the degree of desaturation of the membrane lipids (Gounaris et al., 1983). The reason why the non-bilayer lipid mixture still forms a stable thylakoid membrane is probably the very high content of bilayer-spanning proteins. In addition to the bilayer-spanning proteins, carotenoids may also contribute to the stabilization of the inner envelope and the thylakoid membrane. Several studies indicated that carotenoids contribute to membrane stability of lipid bilayers (Gruszecki and Sielewiesiuk, 1991; Gabrielska and Gruszecki, 1996; Wisniewska and Subczynski, 1998; Berglund et al., 1999; Munné-Bosch and Alegre, 2002; Szilágyi et al., 2008). The exact amount of carotenoids freely dissolved in the thylakoid lipid bilayer is not known

and the contribution of the carotenoids to the thylakoid membrane stability remains an open question. It has, however, been suggested that the xanthophyll cycle participates in regulating thylakoid membrane stability (Gruszecki and Strzalka, 1991; Szilágyi et al., 2008).

Mutations which cause a substantial loss of MGDG or DGDG lead to severely compromised chloroplast function in *Arabidopsis* (Dörmann et al., 1995; Härtel et al., 1997; Jarvis et al., 2000; Kelly et al., 2003; Kobayashi et al., 2007); however, the chonsequences of a loss of DGDG are not as strong as a loss of MGDG. DGDG deficiency has severe effects on photosynthetic performence and growth (Kelly et al., 2003), whereas the loss of MGDG in the *mgd1* mutant leads to albino plants with a strong decrease in thylakoid membrane abundance and photosynthetic capacity (Kobayashi et al., 2007). A complete loss of chloroplast DGDG has severe effects on photosynthetic performance and growth (Kelly et al., 2003), whereas the loss of MGDG in the *mgd1* mutant leads to albino plants with a strong decrease in thylakoid membranes and photosynthetic capacity (Kobayashi et al., 2007). *Arabidopsis* is able to cope with a total loss of SQDG, at least under standard conditions (Essigmann et al., 1998; Yu et al., 2002). Although it seems that the two anionic thylakoid lipids can compensate for each other to some extent, it is clear that any substantial loss of chloroplast PG strongly affects photosynthetic activity, growth and thylakoid development (Hagio et al., 2002; Xu et al., 2002; Babiychuk et al., 2003).

An important question is what the function of the very high content of trienoic acids in the chloroplast membrane lipids might be? In fact, an *Arabidopsis* fatty acid desaturase tripple mutant which completely lacks trienoic fatty acids has a very mild phenotype with regard to growth, photosynthesis and chloroplast ultrastructure under standard growth conditions (Routaboul et al., 2000); however, the mutant reveals severe defects in growth and photosynthesis at low temperatures. Thus, the high amount of trienoic fatty acids in chloroplast membrane lipids appears to be important for maintaining membrane functionality at low temperatures. It was recently shown that the loss of trienoic fatty acids from chloroplast membrane lipids had a detrimental effect on one of the pathways for translocation of proteins across the thylakoid membrane to the thylakoid lumen (Ma and Browse, 2006). Certain lipids also appear to have other more specific roles beside their general structural contribution to the chloroplast membranes. Several chloroplast lipids have been shown to be intrinsic components of the photosystems (Jordan et al., 2001; Loll et al., 2005, 2007). MGDG in the outer envelope was suggested to be required for protein recognition and targeting to the chloroplast (Bruce, 1998). The trienoic acids in the chloroplast lipids serve as substrates for the chloroplast lipoxygenase pathway (Feussner and Wasternack, 2002). This pathway provides several potent signalling compounds to the plant; the best characterized is jasmonic acid. The jasmonic acid which is responsible for anther dehiscence in *Arabidopsis* is synthesized from 18:3 released from PC by an envelope-localized acyl hydrolase (Ishiguro et al., 2001), whereas wound-induced jasmonic acid is predominantly produced from 18:3 released by a galactolipase (Hyun et al., 2008).

4 Minor Chloroplast Lipids

In addition to the major lipid classes found in the chloroplast membranes, a number of minor lipid species have also been reported. The chloroplast membranes contain a small proportion of intermediates of the biosynthetic pathways for chloroplast lipids; these will be discussed in the context of the respective biosynthesis pathways. Other minor lipid classes reported in chloroplasts or chloroplast subfractions include inositol phospholipids (Siegenthaler et al., 1997; Bovet et al., 2001), oligogalactolipids, phosphorylated galactolipids (Müller et al., 2000) and acylated galactolipids (Heinz, 1967; Heinz and Tulloch, 1969; Heinz et al., 1978). In addition, a number of oxygenated species of chloroplast galactolipids have been described in plant tissues, but never actually isolated from a purified chloroplast fraction. Trigalactosyldiacylglycerols (TGDG) and tetragalactosyldiacylglycerols are not normally found in plant lipid extracts, but have been described as minor constituents in isolated chloroplasts (Cline et al., 1981; Wintermans et al., 1981).

In general, a small proportion of phosphatidylinositol (PI; Table 1) was reported to be present in chloroplast envelopes. In other cellular membranes, the mono-, di- and triphosphorylated analogues of PI provide important functions in intracellular signalling and cytoskeletal organization (Mueller-Roeber and Pical, 2002). An ATP-dependent, wortmannin-sensitive PI kinase activity has been reported to occur in outer envelope from spinach chloroplasts (Siegenthaler et al., 1997; Bovet et al., 2001). Thus, it is possible that phosphatidylinositide-dependent signalling or cytoskeletal reorganization can also emanate from the chloroplast. In addition to phosphatidylinositolphosphates, Bovet and colleagues (Müller et al., 2000; Bovet et al., 2001) reported CTP-dependent phosphorylation of MGDG and lyso-MGDG in spinach chloroplast envelopes. These phosphorylated lipids have so far only been reported as radiolabelled products after in vitro feeding with nucleotides. Steady-state concentrations and the physiological relevance of the phosphorylated chloroplast lipids remain open questions.

The chloroplast lipids are rich in polyunsaturated fatty acids and thus sensitive to chemical or enzymatic oxidation. The higher-plant chloroplast contains lipoxygenases with specificity for the 13-position of C_{18} fatty acids as well as several other enzymes which catalyse downstream reaction of oxygenated fatty acids (Feussner and Wasternack, 2002). Several different chloroplast lipid species containing oxygenated fatty acids have been described in extracts from *Arabidopsis*. Information on other plant species, however, remains very scarce. The oxygenated galactolipids described so far include MGDG and DGDG containing keto fatty acids (Buseman et al., 2006) or oxo-phytodienoic acid at the *sn*-1 and/or the *sn*-2 position (Stelmach et al., 2001; Hisamatsu et al., 2003; Stenzel et al., 2003; Hisamatsu et al., 2005; Ohashi et al., 2005; Buseman et al., 2006; Nakajyo et al., 2006; Böttcher and Weiler, 2007). MGDG esterified with oxo-phytodienoic acid has been demonstrated to be present in thylakoid and envelope fractions as well as detergent-solubilized thylakoid pigment protein complexes (Böttcher and Weiler, 2007). In addition, MGDG carrying an extra oxo-phytodienoic acid on the glycerol backbone as well as on the C-6 position of the galactose head group has been found

in *Arabidopsis* (Andersson et al., 2006; Kourtchenko et al., 2007). The latter substances were found to accumulate in response to wounding and in the hypersensitive response induced by avirulence peptides. The other oxo-phytodienoic acid containing galactolipids were also induced by wounding of *Arabidopsis* tissues (Buseman et al., 2006; Böttcher and Weiler, 2007; Kourtchenko et al., 2007). Oxo-phytodienoic acid containing galactolipids have so far only been found in extracts from *Arabidopsis thaliana*, *Arabidopsis arenosa* (Böttcher and Weiler, 2007) and *Ipomoea tricolor* (Ohashi et al., 2005). The evidence for a physiological function of these particular oxylipin-containing lipids remains rather scarce, but senescence-promoting effects, antipathogenic properties, stomatal closing and a role as precursors for free oxylipins have been reported. In addition to these particular molecular lipid species, it seems likely that the everyday wear and tear of the photosynthesis machinery would cause some damage to unsaturated galactolipids. Thus, it seems likely that a steady-state concentration of oxygenated fatty acids is present in the thylakoid lipid pool and that these fatty acids are continuously removed from the complex lipid pool. However, this remains poorly explored territory.

5 Origin of Chloroplast Membrane Lipid Acyl Groups

The bulk of the fatty acid synthesis in plant cells takes place in the chloroplast stroma. Plant mitochondria also contribute to fatty acid synthesis, but only in a very minor way. The fatty acid synthesis machinery inside the plastid is related to that of bacteria rather than to cytosolic fatty acid synthesis in other eukaryotic organisms. The substrate for fatty acid synthesis in the stroma was traditionally thought to be acetate, but this has been questioned and the topic is not completely settled (Bao et al., 2000; Rawsthorne, 2002). Regardless of the actual identity of the fatty acid synthesis substrate, exogenous acetate is efficiently channelled into the pathway. This has been instrumental in many acyl labelling studies where radiolabelled acetate has been fed to isolated chloroplast or intact plant tissue. During the fatty acid synthesis cycle, the growing acyl chain is attached via a thioester bond to the small (approximately 9 kDa) acidic acyl carrier protein (ACP). For each turn of the cycle, the fatty acid grows by two carbon atoms. Two steps in the cycle require reducing power which is derived from NAD(P)H. A special stroma-localized desaturase accepts 18:0-ACP as a substrate and introduces a *cis* double bond at the Δ9-position, yielding oleic acid (18:1). Thus, 18:1 and hexadecanoic acid (16:0) are the major fatty acids synthesized in the stroma (Ohlrogge and Browse, 1995; Rawsthorne, 2002). The fatty acids are then either used directly in the chloroplast for glycerolipid synthesis (prokaryotic pathway), or exported to the endoplasmic reticulum (ER) for lipid synthesis (eukaryotic pathway). In 18:3 plants all the fatty acids found in the chloroplast glycolipids take the detour through the ER, whereas in 16:3 plants a portion of the fatty acids are assembled into galactolipids without ever having to leave the chloroplast (Fig. 2). The reason for the difference between the fatty acid composition of chloroplast lipids in 18:3 and 16:3 plants is the different specifici-

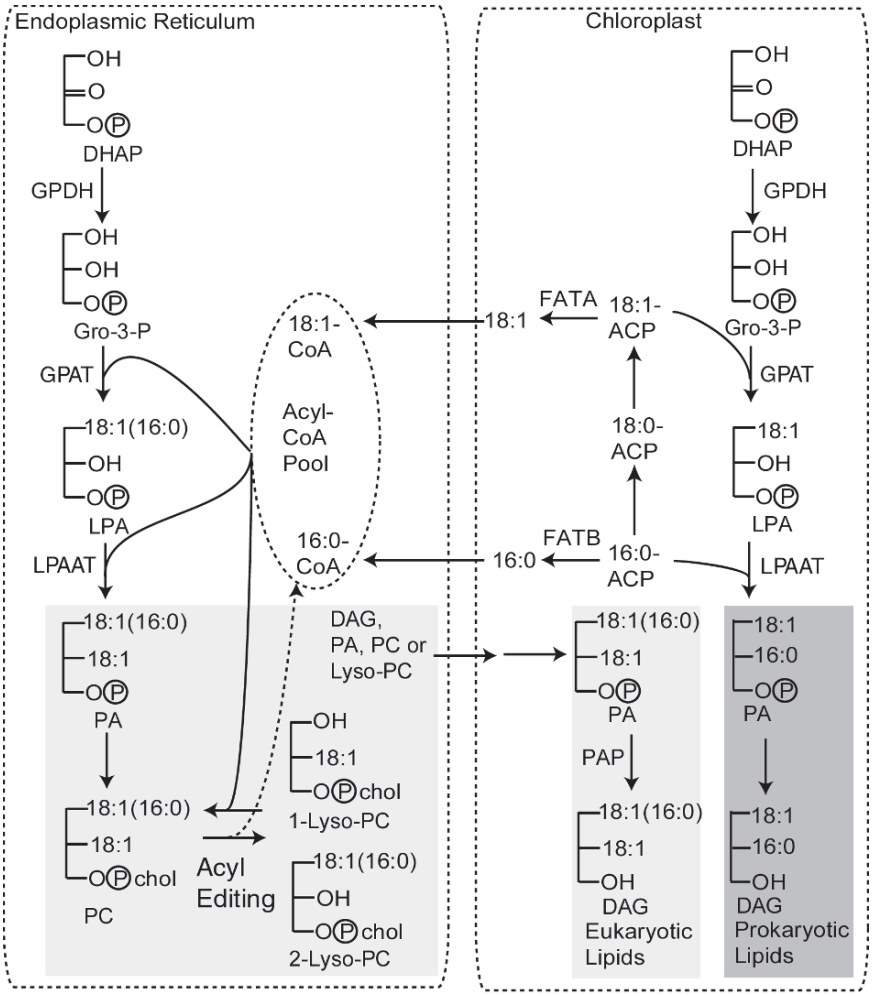

Fig. 2 Dihydroxyacetonephosphate serves as precursor for the synthesis of glycerol-3-phosphate, which is sequentially acylated leading to lyso-phosphatidic acid and phosphatidic acid (*PA*) production in chloroplasts and at the endoplasmic reticulum (ER). Acyl editing represents an alternative pathway for the incorporation of plastid-derived acyl groups into the cytosolic PC pool. PA (or another PC-derived lipid metabolite, DAG, PC or lyso-PC) is transported from the ER to the chloroplast. While dephosphorylation of ER-derived PA results in the synthesis of glycerolipids with eukaryotic structure (C_{16} and C_{18} at *sn*-1, C_{18} at *sn*-2, *light grey*), plastidial PA is the precursor for glycerolipids with prokaryotic structure (C_{18} at *sn*-1, C_{16} and C_{18} at *sn*-2, *dark grey*). The distinct distribution of acyl groups to the glycerol backbone is based on the substrate specificity of ER-localized, acylcoenzyme A (acyl-CoA) dependent acyltransferases, and plastidial, acyl carrier protein (*ACP*) dependent acyltransferases

ties of the acyltransferases which assemble phosphatidic acid (PA) in the ER and the chloroplast (Roughan and Slack, 1982; Heinz and Roughan, 1983; Browse et al., 1986). The basis for the loss of the prokaryotic pathway in 18:3 plants is not well understood, but the fact that several independent groups of plants are devoid of the prokaryotic pathway suggests that the loss of the capability to synthesize prokaryotic lipids has occurred several times during evolution (Mongrand et al., 1998). Fatty acids that are exported from the chloroplast are cleaved from ACP by stromal ACP thioesterases. The fatty acids are transported by some unknown mechanism to the outer envelope, where acylcoenzyme A (acyl-CoA) synthases reactivate the fatty acids and render them soluble in the cytosol. There is experimental evidence for a direct channelling from the stromal fatty acid synthesis to the acyl-CoA synthesis (Koo et al., 2004). The acyl-CoAs are then utilized by acyltransferases for phospholipid synthesis in the ER.

6 Lipid Transport from the ER to the Chloroplast

All plants rely on the import of diacylglycerol (DAG) backbones assembled in the ER to the chloroplast for galactolipid synthesis (Fig. 2). DAG units derived from the ER ("eukaryotic lipids") are devoid of 16:3 and contain mostly C_{18} fatty acids at the *sn*-1 and *sn*-2 positions (minor amounts of C_{16} are also found at the *sn*-1 position). A large fraction of the lipid precursors assembled at the ER has to be transported back to the chloroplast for incorporation into galactolipids. In situ radiolabelling pulse chase studies on 18:3 plants have demonstrated that the radiolabel becomes transiently associated with PC prior to incorporation into MGDG (Slack et al., 1977; Hellgren et al., 1995; Hellgren and Sandelius, 2001). Thus, it seems likely that ER-localized PC is an important precursor for chloroplast lipids derived from the eukaryotic biosynthesis pathway. However, the exact identity of the lipid moiety that is transported from the ER to the chloroplast is still unclear. PC might seem to represent a likely candidate since it is present in the ER and the outer chloroplast envelope. In fact, since the chloroplast lacks the capacity for assembling the phosphorylcholine head group, some kind of transport mechanism for PC between the ER and the chloroplast must exist. In addition to PC (Oursel et al., 1987; Andersson et al., 2004), lyso-PC (Mongrand et al., 1997; Mongrand et al., 2000), DAG (Williams et al., 2000) and most recently PA (Xu et al., 2005; Awai et al., 2006; Lu et al., 2007) have been suggested to represent the lipid molecules transferred from the ER to the chloroplast.

Possible modes of transport include the diffusion of water-soluble molecules (e.g. lyso-PC), protein-mediated transport, vesicle transport or lipid transfer via ER–chloroplast contact sites (Moreau et al., 1998). Transport of proteins and membrane material synthesized at the ER and destined for the secretory pathway is known to be mediated via vesicles. The vesicles are derived from ER membranes and after budding off the ER system they fuse with the *cis* Golgi membranes,

and their cargo is subsequently sorted to the vacuole or plasma membrane. A detailed overview of the vesicle-mediated lipid transport system in plants was recently included in Jouhet et al. (2007). However, the existence of an analogous, vesicle-based transport system for the transfer of proteins or lipids to the plastids is unclear. Specific contact sites of the ER with other cellular membranes have previously been described (Staehelin, 1997). In yeast, special regions of the ER are believed to be involved in lipid and protein transfer to the mitochondria (mitochondria-associated membranes, MAM) (Gaigg et al., 1995; Achleitner et al., 1999) or the plasma membrane (plasma-membrane-associated membranes, PAM) (Pichler et al., 2001). These specialized regions were found to be closely associated with their respective organelles and could be co-isolated with these. Contact sites between the ER and the plastid outer envelopes have previously been observed by electron microscopy of various tissues (Wooding and Northcote, 1965; Schlötz, 1975; Whatley et al., 1991; Kaneko and Keegstra, 1996). Apparent contacts between the ER and chloroplasts were also observed by confocal microscopy in *Arabidopsis* expressing green fluorescent protein (GFP) targeted to the ER lumen (Hanson and Kohler, 2001; Andersson et al., 2007). Fluorescent pieces of ER remained associated with chloroplasts isolated from GFP-expressing protoplasts (Andersson et al., 2007). Furthermore, use of optical tweezers demonstrated that chloroplast-associated ER was firmly attached to the chloroplast surface, indicating that tight connections, possibly based on protein–protein interactions, exist between the outer envelope of chloroplasts and the ER (Andersson et al., 2007). An ER-derived fraction could also be isolated from intact pea chloroplast (Andersson et al., 2007). This fraction was by analogy to MAM and PAM dubbed PLAM for plastid-associated membranes. Although direct evidence for lipid transfer between the PLAM and the chloroplast has not yet been obtained, further characterization of this fraction seems promising. The contact sites between ER and chloroplast envelopes could also be related to the so-called stromules, tubular stroma-containing extensions of the envelope membranes that interconnect plastids in plants (Kwok and Hanson, 2003). Interestingly, such tubular connections of plastid envelopes were also observed with nuclear and cell membranes, suggesting that they could in general be involved in connecting the plastids with the cellular membrane system (Kwok and Hanson, 2004). In conclusion, the current understanding points towards a scenario where the transfer of eukaryotic lipid precursors to the chloroplasts is mediated via ER–outer envelope contact sites. Similarly, such contact sites could be involved in the export of galactolipids observed during phosphate deprivation (see below).

Protein factors involved in ER to chloroplast lipid transfer have for a long time remained enigmatic. Analysis of *Arabidopsis* mutants affected in galactolipid metabolism suggested that lipid transport is mediated via an ATP-binding cassette (ABC) transporter complex in the envelope membranes (Xu et al., 2003). ABC transporters are membrane proteins that in general transport small molecules, i.e. phytohormones, peptides or sugars, across membranes, accompanied by the hydrolysis of ATP. The *Arabidopsis* genome contains more than 100 genes

with sequence similarities to ABC transporters (Sánchez-Fernández et al., 2001). In addition to the nuclear binding fold domain, ABC transporters are characterized by the presence of one or more transmembrane domains. ABC transporters can be encoded by one multifunctional protein, or by several genes encoding polypeptides that assemble into a functional transporter complex. With employment of a genetic screening strategy, *Arabidopsis* mutants were isolated with alterations in regulation and transport of galactolipids (Xu et al., 2003). The *tgd1* mutant (trigalactosyldiacylglycerol 1) is characterized by the accumulation of triacylglycerol and the unusual oligogalactolipid TGDG. *TGD1* encodes a permease-like protein which constitutes one of the subunits of an ABC transporter. The TGD transporter represented the first ABC transport complex for lipid molecules discovered in higher plants. Biochemical and radioactive labelling experiments suggest that PA is the lipid molecule that is transported through the TGD transporter complex (Xu et al., 2005). *TGD1* localizes to the inner chloroplast envelope membrane. The *tgd2* (Awai et al., 2006) and *tgd3* (Lu et al., 2007) mutants of *Arabidopsis* show the same biochemical phenotype as *tgd1*, e.g. they also accumulate triacylglycerol and TGDG. *TGD2* encodes a substrate binding domain subunit of the lipid ABC transporter which binds PA with high affinity, and *TGD3* encodes a small ATPase associated with the transporter complex. Taken together, this suggests that the three TGD proteins form a lipid transport complex in the envelope membrane (Lu et al., 2007). These data imply that the TGD lipid transporter is involved in the transfer of PA from the ER to the envelopes, where it is hydrolyzed by a PA phosphatase (PAP), yielding DAG, the substrate for galactolipid synthesis. However, there is clearly much less PAP activity in the chloroplast envelope in 18:3 plants than 16:3 plants (see further below). Thus, PA transfer from the ER to the chloroplast might be favoured in 16:3 plants. However, there must be additional ER–chloroplast transport mechanisms at least for PC given the fact that PC is present in both the chloroplast envelope and the ER, and the chloroplast envelope is devoid of PC synthases. The published data regarding 18:3 plants do seem to favour the hypothesis that PC and/or lyso-PC are the chloroplast galactolipid precursors that are transported from the ER to the chloroplast envelope (Oursel et al., 1987; Andersson et al., 2004; Mongrand et al., 1997; Mongrand et al., 2000). On the other hand, sequences with high similarity to *TGD* genes from *Arabidopsis* are present in the genome of rice, suggesting that a TGD/PA-mediated transport mechanism is also operating in 18:3 plants. Further research is clearly needed to completely resolve the issue of which lipid(s) is transported from the ER to the chloroplast. The *Arabidopsis* inventory of putative lipid ABC transporters (Jouhet et al., 2007) is certainly large enough to provide more than one chloroplast-localized transporter. Still, the TGD complex must be of high importance for chloroplast biogenesis, since effective silencing of the *TGD1* gene leads to embryo lethality (Xu et al., 2005). One interesting feature of the TGD proteins is that GFP fusions of all three proteins seem to be localized in dense patches on the chloroplast surface (Xu et al., 2005; Awai et al., 2006; Lu et al., 2007). It is extremely tempting to speculate that these patches might correspond to the sites on the chloroplast surface interacting with the PLAM.

7 Phospholipid Metabolism in the Chloroplast Envelope

The higher-plant chloroplast contains a complete glycerolipid synthesis machinery, the prokaryotic pathway; however, only in the so-called 16:3 plants does this pathway contribute DAG backbones for galactolipid synthesis (Fig. 2). In all plants, the plastidial pathway contributes to PG synthesis in the chloroplast. A soluble, stroma-localized enzyme transfers fatty acids from ACP to glycerol-3-phosphate to yield inner-envelope-localized lyso-PA (Douce and Joyard, 1977). The lyso-PA is then acylated at the *sn*-2 position by an inner-envelope-localized enzyme to yield PA (Andrews et al., 1985). The chloroplast acyltransferases specifically transfer C_{18} fatty acids to the *sn*-1 position of the glycerol; the *sn*-2 acyltransferase is less specific and also accepts C_{16} fatty acids (Frentzen et al., 1983). This pathway seems to be essentially conserved between cyanobacteria and higher-plant chloroplasts (Murata and Nishida, 1987). The genes encoding the two acyltransferases required for PA synthesis in the inner chloroplast envelope have been identified in *Arabidopsis* and knockout mutations have been investigated. Surprisingly, the two different mutations were reported to cause rather different phenotypes. Mutations in the first acyltransferase gene cause a rather subtle phenotype (Kunst et al., 1988, 1989), whereas a knockout mutation of the other enzyme is embryo-lethal (Bin et al., 2004; Kim and Huang, 2004). These conflicting data were recently resolved by the demonstration that the previously described mutants in the first acyltransferase were leaky alleles and therefore could sustain a low rate of prokaryotic lipid synthesis in the chloroplast (Xu et al., 2006). The important products of the prokaryotic lipid pathway are probably not the galactolipids, but rather PG. Apparently the small amount of plastidially produced PA is more efficiently directed into PG synthesis than into galactolipid synthesis (Xu et al., 2006). The recently described transgenic approach of channelling ER-derived lipid backbones into plastidial PG (Fritz et al., 2007) might be a suitable way to rescue a complete knockout mutation of the prokaryotic pathway.

PA produced by the plastidial pathway is converted to PG by three inner-envelope-localized enzymes (Fig. 2) (Andrews and Mudd, 1985). PA is activated to CDP-DAG by CDP-DAG synthase, and a PG-phosphate synthase transfers glycerol-3-phosphate to the lipid. The resulting PG-phosphate is finally dephosphorylated to PG by PG-phosphate phosphatase. Interestingly, the PG-phosphate synthase protein is targeted to both the plastid and the mitochondria (Muller and Frentzen, 2001; Babiychuk et al., 2003). The protein was found to be essential for chloroplast function, but dispensable for mitochondrial function, indicating that mitochondria but not chloroplasts can import PG from the ER (Babiychuk et al., 2003). There are three candidate genes in the *Arabidopsis* genome for the plastid CDP-DAG synthase, but experimental data regarding this activity are missing. No genes have been identified for the plastidial PG-phosphate phosphatase, although candidate genes have been suggested on the basis of sequence similarity and phylogeny (Lykidis, 2007).

PA of eukaryotic fatty acid composition in the inner envelope seems to be capable of entering the PG biosynthesis pathway (Fritz et al., 2007). Therefore, there

is currently no good explanation for the question how eukaryotic lipid species are kept out of the chloroplast PG pool, given the finding that PA might be a major lipid precursor transported from the ER to the inner envelope (Xu et al., 2005; Awai et al., 2006; Lu et al., 2007). A possible solution for this dilemma would be a very tight channelling of different PA pools in the inner envelope. One suggestion is that the TGD transporter complex binds PA in the inner envelope and shields the imported PA from the CDP-DAG synthase activity (Fritz et al., 2007).

In 16:3 plants, the PA produced in the chloroplast envelope is utilized for galactolipid synthesis. This requires PAP activity to form DAG. This activity was demonstrated in envelope preparations from spinach more than 30 years ago (Douce and Joyard, 1977). The PAP activity in pea chloroplasts is localized to the inner envelope (Andrews et al., 1985). Only very recently, a small gene family that might encode the chloroplast-localized PAPs was identified in *Arabidopsis* (Nakamura et al., 2007). A knockout mutation of one of the particular isoforms in *Arabidopsis* caused reduced pollen fertility, but no definite link could be made to plastidial galactolipid synthesis. The envelope PAP activity has been proposed to be the metabolic point of divergence between 16:3 and 18:3 plants (Heinz and Roughan, 1983; Gardiner et al., 1984). This is also supported by the observation that chloroplast PAP activity is much lower (more than 10 times lower) in 18:3 than in 16:3 plants (Gardiner et al., 1984). However, this suggestion fits poorly with the hypothesis that direct import of PA from the ER to the inner envelope is the basis for formation of eukaryotic chloroplast lipids (Xu et al., 2005; Awai et al., 2006). Again, channelling and/or regulation of chloroplast PAP activity might resolve this apparent discrepancy.

A chloroplast-envelope-localized activity which transfers fatty acids from acyl-CoA to lyso-PC has been described in leek and pea seedlings (Bessoule et al., 1995; Kjellberg et al., 2000; Mongrand et al., 2000). This has led to the suggestion that lyso-PC might be the precursor translocated from the ER to the chloroplast (Bessoule et al., 1995; Mongrand et al., 1997; Mongrand et al., 2000). The chloroplast-localized lyso-PC acyltransferase was found to be protected from the protease thermolysine and co-fractionated with the inner envelope in pea (Kjellberg et al., 2000). In general, very little is known about the roles of similar acyltransferase activities in plant membranes. It was, however, recently reported that acyl editing of PC in the ER might be more important than previously recognized for de novo synthesis of phospholipids in the ER (Bates et al., 2007).

If PC delivered directly from the ER or assembled from lyso-PC in the envelope represents a precursor for galactolipid synthesis in the chloroplast, the phosphorylcholine head group needs first to be cleaved off to generate DAG required for galactolipid synthesis. This could be accomplished in two ways. The phosphorylcholine could be cleaved in one piece by the action of a phospholipase C (PLC). Alternatively, the head group could be cleaved off in two steps. First a phospholipase D (PLD) could cleave choline, and then PAP activity could remove the phosphate group. *In vitro* experiments clearly demonstrated that eukaryotic PC in the chloroplast envelope can serve as a precursor for galactolipid synthesis, provided that the PC is

degraded by exogenously added PLC (Miquel et al., 1988). However, no PLC or PLD activity has ever been demonstrated to be present in an isolated chloroplast fraction. Thus, if PC in the envelope is a precursor for galactolipid synthesis in the chloroplast, the required phospholipase(s) must be a soluble cytosolic enzyme which only transiently associates with the chloroplast. *In vitro* experiments with isolated pea chloroplasts and envelopes demonstrated that PC synthesized in the chloroplast envelope from lyso-PC and acyl-CoA could be used as a substrate for galactolipid synthesis provided that cytosolic proteins were added to the reaction mixture (Andersson et al., 2004). Furthermore, the sensitivity to specific inhibitors suggested that the cytosolic enzymes required were PLD and PAP. The same set of enzyme activities could also be involved if PA is the major eukaryotic precursor transported from the ER to the chloroplast. In this case, the PLD activity could produce PA in the ER and a soluble PAP would provide the activity required for DAG production in the envelope. This was also supported by *in vitro* transfer experiments of PA from liposomes to chloroplasts isolated from wild-type *Arabidopsis* and the *tgd1* mutant (Xu et al., 2005). Information on soluble PAP activities in plants is rather scarce and most of the attention has focused on the envelope-localized PAP and the plasma-membrane-bound PAPs most probably involved in signalling rather than bulk lipid metabolism. Soluble PAP activity has been described in *Vicia faba* leaves (Königs and Heinz, 1974) and developing seeds of *Brassica napus* (Kocsis et al., 1996; Furukawa-Stoffer et al., 1998). No candidate genes have been identified, but the data presented by Andersson et al. (2004) suggest that the sought-after activity is a soluble PAP that is insensitive to *N*-ethylmaleimide and has a native size exceeding 100 kDa.

8 Glycolipid Biosynthesis in the Chloroplast Envelope

Glycolipids are assembled from DAG and UDP-sugars in the envelope membranes of chloroplasts (Fig. 3) (Neufeld and Hall, 1964). *Arabidopsis* contains three MGDG synthases: MGD1, which localizes to the inner envelope, is involved in the synthesis of the largest proportion of MGDG. MGD2 and MGD3, which are found in the outer envelope, are only active in some tissues or under specific conditions, e.g. phosphate deprivation (Awai et al., 2001). The MGDG synthase transfers a galactose moiety from UDP-galactose onto DAG under inversion of the anomeric configuration. Therefore, the galactose is linked in β-anomeric configuration to DAG. Two DGDG synthases are present in *Arabidopsis*, DGD1 and DGD2 that galactosylate the C-6 position of the galactose moiety in MGDG (Dörmann et al., 1999; Kelly et al., 2003). Since the reaction follows a retaining mechanism, the outermost galactose residue in DGDG has α-configuration. DGD1 is responsible for the production of the largest proportion of DGDG in leaves, while both enzymes contribute to DGDG production under phosphate deprivation (Kelly et al., 2003). The two DGDG synthases localize to the outer envelope of chloroplasts (Froehlich et al., 2001; Kelly et al., 2003).

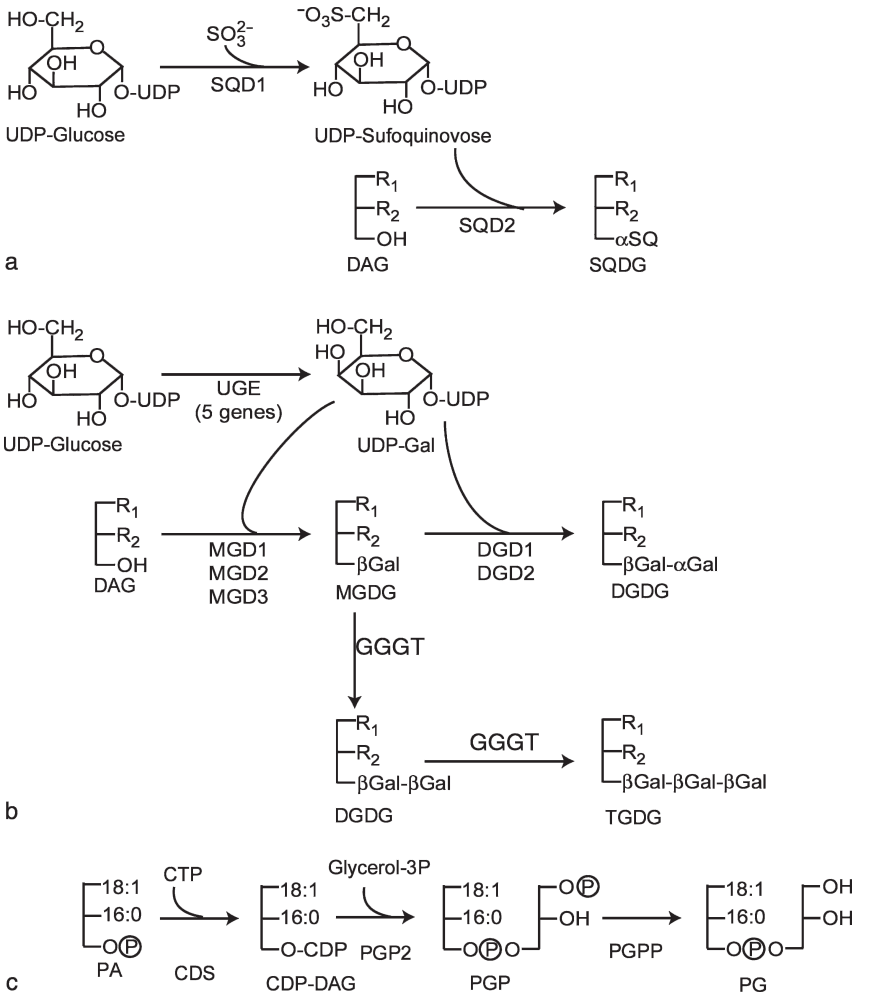

Fig. 3 Synthesis of glycoglycerolipids in plants. UDP-sulfoquinovose, the donor for the head group of sulfolipid (SQDG), is synthesized from UDP-glucose and sulfite by SQD1 (*top part*). Subsequently, SQD2 produces SQDG from diacylglycerol and UDP-sulfoquinovose. The bottom part shows galactolipid synthesis. UDP-glucose is converted into UDP-galactose by one of the five UDP-glucose epimerases present in *Arabidopsis*. UDP-galactose is the head group donor for MGDG and DGDG synthesis by one of the MGDG synthases (MGD1, MGD2 and MGD3) or DGDG synthases (DGD1 and DGD2). The galactolipid:galactolipid galactosyltransferase (*GGGT*), a processive oligogalactolipid synthase with unknown identity and unknown function, produces small amounts of trigalactosyldiacylglycerol (*TGDG*). Note that the glycosidic linkages derived from the MGDG synthase reaction and from the GGGT reaction are β, while DGDG synthases produce α-glycosidic bonds

In addition to the two DGDG synthases described above, *Arabidopsis* contains a third activity, the galactolipid:galactolipid galactosyltransferase (GGGT), capable of synthesizing DGDG and oligogalactolipids with three (TGDG) or more galactose units in the head group (van Besouw and Wintermans, 1978; Heemskerk et al., 1990). The GGGT activity localizes to the outer chloroplast envelope (Douce, 1974; Cline and Keegstra, 1983). The amount of oligogalactolipids, including TGDG, in leaves is usually very low, but it can increase to high amounts in stressed leaves (Sakaki et al., 1990). The GGGT activity was described as an enzyme transferring a galactose unit from one MGDG to the other, thereby producing DAG and DGDG (van Besouw and Wintermans, 1978). GGGT is independent of the DGDG synthases DGD1 and DGD2 since GGGT activity is still detectable in the *dgd1 dgd2* double mutant (Kelly et al., 2003). As indicated above, TGDG accumulates in the mutants *tgd1*, *tgd2* and *tgd3* (Xu et al., 2003). The reason why mutations in the TGD lipid transporter complex stimulate TGDG synthesis in *Arabidopsis* remains unknown. It is possible that the TGDG-synthesizing activity from *tgd1* is identical or related to the GGGT activity described above. The fact that the galactose moieties in TGDG of the *tgd1* mutant are all in β-linkage indicates that this enzyme is specific for β-anomeric bonds, in contrast to the two DGDG synthases DGD1 and DGD2, which are specific for α-glycosidic bonds (Xu et al., 2003). However, the identity of GGGT or the TGDG-synthesizing activity in the *tgd* mutants, the function of TGDG synthesis and the relation to the known enzymatic steps of galactolipid synthesis remain enigmatic.

The sulfolipid SQDG contains a modified glucose head group carrying a sulfonic acid moiety at the C-6 position (Benson et al., 1959). The head group for SQDG synthesis, UDP-sulfoquinovose, is produced from UDP-glucose and sulfite (SO_3^{2-}) in the stroma by the SQD1 gene product in *Arabidopsis*. SQD1 shows sequence similarity to UDP-sugar epimerases (Essigmann et al., 1998). Crystallization of the protein revealed that SQD1 binds NADH as a cofactor (Mulichak et al., 1999). The reaction mechanism for the conversion of UDP-glucose into UDP-sulfoquinovose proceeds through a reduced "glucosene" form which serves as the acceptor for sulfite addition. Subsequently, the sulfoquinovose is transferred onto DAG in the outer-envelope membrane of chloroplasts by SQDG synthase (SQD2) (Yu et al., 2002). The reaction follows a retaining mechanism resulting in α-glycosidic linkage between the sugar and the glycerol backbone.

9 Chloroplast Lipid Fatty Acid Desaturation

The introduction of further double bonds beyond the Δ9-double bond in 18:1 in chloroplast membrane lipids is dependent on membrane-bound desaturases which accept intact glycerolipids as substrates (Schmidt and Heinz, 1990a,b, 1993; Sperling et al., 1993). Two ER-localized membrane lipid desaturases were cloned by forward genetic screens for *Arabidopsis* mutants with deficiencies in unsaturated fatty acids (Arondel et al., 1992; Yadav et al., 1993; Okuley et al., 1994). The sequences

for the two ER desaturases FAD2 and FAD3 were then used to identify three chloroplast-localized lipid desaturases FAD6, FAD7 and FAD8 (Iba et al., 1993; Falcone et al., 1994; Gibson et al., 1994; Hitz et al., 1994). The ER-localized desaturases FAD2 and FAD3 produce linoleic acid (18:2) and 18:3, which are esterified to phospholipids in the ER. Thus, these two enzymes contribute to desaturation of chloroplast lipid species assembled through the eukaryotic pathway. A specific envelope-localized desaturase introduces the Δ7-double bond in 16:0 esterified to MGDG assembled by the prokaryote pathway. This activity is encoded by a single nuclear gene, *FAD5*, in *Arabidopsis* (Mekhedov et al., 2000; Heilmann et al., 2004). No candidate gene for the FAD4 desaturase which introduces the *trans*-3 double bond in palmitic acid esterified to chloroplast PG has yet been found. In *Arabidopsis*, three chloroplast-localized desaturases catalyse the production of dienoic and trienoic acids on galactolipids. FAD6 introduces a second double bond in palmitoleic acid (16:1) and 18:1 and FAD7 and FAD8 introduce the third. The ER-localized lipid desaturases use cytochrome b_5 as an electron donor, whereas the chloroplast-localized desaturases use reduced ferredoxin.

10 Lipid Transport from the Envelope to the Thylakoids

Glycolipids (MGDG, DGDG and SQDG) are synthesized in the envelope membranes, but accumulate in the thylakoid membranes in high amounts. Similarly, PG and other lipids of the photosynthetic membranes (phylloquinone, plastoquinone, tocopherol, carotenoids) are also derived from the envelope membranes. Thus, massive transport of membrane material from the envelope to the thylakoids is required for the establishment of thylakoid membranes during chloroplast development (Fig. 4). Contact sites between the inner envelope and the thylakoid are quite frequently observed in developing chloroplasts (Carde et al., 1982). It has thus been proposed that the thylakoid membranes originate from invaginations of the inner-envelope membrane (Mühlethaler and Frey-Wyssling, 1959; Vothknecht and Westhoff, 2001). Recent data obtained from the analysis of an *Arabidopsis mgd1* mutant support this hypothesis (Kobayashi et al., 2007). This mutant, which is completely devoid of MGDG, develops large invaginations from the chloroplast envelope, suggesting that the loss of MGDG prevents the separation of regular thylakoids derived from the inner envelope.

In mature chloroplasts, contacts between the inner envelope and the thylakoid are only very rarely observed. The inner envelope and the thylakoids are usually separated by at least 50–100 nm of aqueous stroma (Morré et al., 1991b; Ryberg et al., 1993). Even though mature chloroplasts in comparison to developing chloroplasts do not require extensive transport of lipids from the envelope to the thylakoids, there is still a certain turnover of thylakoid lipids in a mature chloroplast. Thus, there is always a need for a certain flow of membrane material from the inner envelope to the thylakoid. A vesicle transport system has been proposed as a model for lipid transfer from the inner envelope to the thylakoids. Electron microscopy of leaves exposed

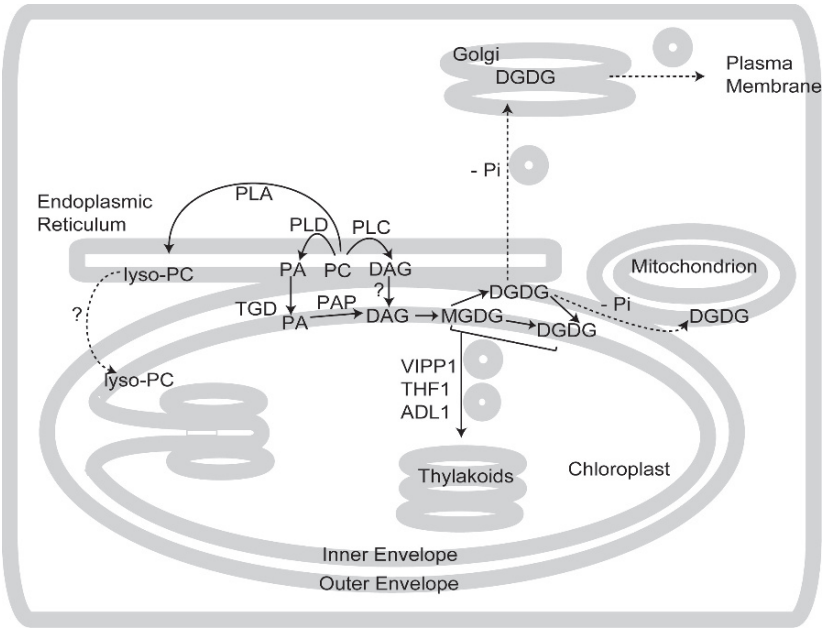

Fig. 4 Intraplastidial and extraplastidial lipid transport in *Arabidopsis.* Thylakoid lipids are derived from the inner-envelope membrane by invagination or from vesicle transport. Eukaryotic lipid precursors assembled at the ER are transported to the chloroplast, presumably via the TGD transporter complex. During phosphate deprivation, DGDG is exported from chloroplasts to extraplastidial membranes, i.e. plasma membrane and mitochondria

to low temperature revealed the accumulation of vesicles in the stroma close to the envelope membranes (Morré et al., 1991b). While vesicles are usually absent from *Arabidopsis* chloroplasts raised at normal growth conditions, vesicle formation and fusion can be blocked by inhibitors that affect cytosolic vesicle transport systems (Westphal et al., 2001b). Furthermore, contact sites between the two envelope membranes, and vesicles originating from the envelope have been observed in osmotically stressed chloroplasts, indicating that lipids and proteins might move between the two envelope membranes via contact sites, and that vesicles might be involved in the transfer to the thylakoids (Cremers et al., 1988). Finally, *in organello* assays also demonstrate that the transport of newly formed galactolipids to the thylakoid in intact chloroplasts is inhibited at the same temperature that results in the accumulation of vesicles in the stroma (Andersson et al., 2001).

The *Arabidopsis* genome codes for a number of proteins with putative chloroplast localization signals and that show sequence similarity to factors of the yeast cytosolic/ER-localized vesicle transport system, e.g. COPII coat proteins and small GTPases (Andersson and Sandelius, 2004). This suggests that a transport mechanism related to ER–Golgi transport in the cytosol might also operate within the plastid. It is known that galactolipids can be released from isolated envelopes in an

ATP- and stroma-dependent fashion (Räntfors et al., 2000). In vitro transfer of galactolipids from envelope to thylakoid seems to depend on similar factors (Morré et al., 1991a). A stromal protein, plastid fusion/transformation factor (Pftf), showing sequence similarity to yeast and mammalian NSF and bacterial FtsH proteins is required for fusion of vesicles derived from chromoplasts of pepper (Hugueney et al., 1995). The *Arabidopsis* dynamin-like 1 (ADL1) protein shows sequence similarity to mammalian dynamin proteins (Park et al., 1998). *Arabidopsis* lines deficient in ADL1 expression show yellowish leaves and contain fewer chloroplasts with strongly reduced thylakoids. Dynamin-like proteins harbour an N-terminal GTPase activity and are known to be involved in vesicle trafficking in the eukaryotic cells. The fact that ADL1 localizes to the chloroplasts suggests that this protein might participate in vesicle trafficking in the plastid.

A vesicle-based mechanism for lipid transport from the envelope to the thylakoids is also supported by data obtained from additional *Arabidopsis* mutant analysis. The thylakoid formation 1 (*THF1*) gene controls thylakoid development in *Arabidopsis* (Wang et al., 2004). THF1 localizes to the chloroplast, and the corresponding mutant shows disturbed thylakoid development and the accumulation of vesicles in the stroma. The gene is present in all organisms performing oxidative photosynthesis, but shows no sequence similarity to other genes in the databases. A mutant in vesicle inducing in plastid protein 1 (VIPP1) shows the accumulation of vesicles and a strongly reduced thylakoid system in the chloroplasts of *Arabidopsis* and also in cyanobacteria (Kroll et al., 2001; Westphal et al., 2001a). VIPP1 was previously discovered as a protein associated with thylakoids and the inner envelope (Li et al., 1994). VIPP1 shows sequence similarity with the phage shock protein A (PspA) from bacteria (Westphal et al., 2001a). VIPP1, similar to its ancestor protein PspA, forms large homo-oligomeric rings which localize to the inner envelope of chloroplasts (Aseeva et al., 2004). VIPP1 rings assemble into larger, rod-like filaments which could be the basis for the microtubule-like structures observed previously in chloroplasts (Liu et al., 2007). Plastidial chaperons and co-chaperons (CDJ2, CGE2 and the heat shock protein HSP70B) are involved in the ATP-dependent assembly and disassembly of VIPP1-containing complexes, thereby modulating thylakoid development (Liu et al., 2007). Thus, while thylakoid biogenesis in developing chloroplasts seems to be based on invaginations of the inner envelope, lipid transfer to thylakoids in mature leaves might be mediated via vesicle transport. This vesicle transport system appears to have features and components derived from both the endosymbiont cyanobacteria and the host eukaryote.

11 Chloroplast Lipid Metabolism and Galactolipid Export During Phosphate-Limited Growth

Approximately one third of all organically bound phosphate in an *Arabidopsis* leaf is bound to phospholipids (Poirier et al., 1991). Since galactolipids are phosphate-free, they largely contribute to phosphate homeostasis in the plant. Under normal

growth conditions, galactolipids accumulate predominately in chloroplast membranes. The amount of galactolipids detected in extraplastidial membranes is in general very low, and it is has been a matter of debate as to whether they are authentic components or mere contaminations derived from plastidial membranes during isolation. The first indication that growth under phosphate limitation results in the accumulation of glycolipids as surrogates for phospholipids came from the analysis of bacterial lipid metabolism (Minnikin et al., 1974). In *Rhodobacter* and in cyanobacteria, the amount of sulfolipid increases strongly at the expense of phospholipids during phosphate-limited conditions (Benning et al., 1993; Güler et al., 1996). Similarly, the sulfolipid content in chloroplasts of *Arabidopsis* increases upon phosphate deprivation at the expense of phospholipids (Essigmann et al., 1998). Furthermore, the non-ionic galactolipid DGDG also accumulates in cyanobacteria and in *Arabidopsis* under phosphate limitation (Güler et al., 1996; Härtel et al., 2000). In contrast to SQDG, which remains localized to the chloroplast membranes, DGDG accumulates in the chloroplast as well as in extraplastidial membranes (Härtel et al., 2000).

The increase in SQDG and DGDG observed during phosphate deprivation represents an active process involving the induction of gene expression. Under phosphate limitation, the expression of the *SQD1* and *SQD2* messenger RNAs is induced, resulting in increased SQDG production (Essigmann et al., 1998; Yu et al., 2002). Furthermore, the expression of MGD2 and MGD3, but not of MGD1, is induced upon phosphate deprivation (Awai et al., 2001). Therefore, it is believed that MGD2 and MGD3 provide the substrate for the extra amount of DGDG synthesized during phosphate deprivation. Induction of DGD1 and DGD2 expression during phosphate limitation provides the means for increased DGDG production (Kelly and Dörmann, 2002; Kelly et al., 2003). While DGD1 is believed to synthesize DGDG for the chloroplasts, DGD2, which is only induced during phosphate deprivation, produces DGDG exported to extraplastidial membranes.

The first indication that DGDG might substitute for phospholipids in extraplastidial membranes during phosphate-limited growth was provided by Härtel et al. (2000). Phosphate limitation was shown to cause accumulation of DGDG in microsome fractions isolated from *Arabidopsis*. The first unambiguous demonstration of extraplastidial phospholipid replacement by DGDG was based on the isolation of plasma membranes in very high purity from roots and shoots of phosphate-starved oat (Andersson et al., 2003). Subsequently, the same phenomenon was shown to occur in oat root tonoplasts (Andersson et al., 2005) and in mitochondria in *Arabidopsis* cell suspension (Jouhet et al., 2004). The degree of phospholipid replacement by DGDG is quite impressive. In plasma membranes isolated from oat roots cultivated without phosphate for 4 weeks, the phospholipid content decreased by about 70% (Andersson et al., 2003; Andersson et al., 2005). Most of the phospholipids were replaced by DGDG, albeit there was also a minor contribution from glucosylceramide and sterolglucosides (Andersson et al., 2005). The phospholipid to DGDG replacement has been shown to occur in *Arabidopsis* (Härtel et al., 2000, 2001), oat (Andersson et al., 2003), sycamore maple (Jouhet et al., 2003), soybean (Gaude et al., 2004), rice, maize, radish, garden nasturtium and sunflower (H. Tjellström, M.A. Andersson, K.E. Larsson and A.S. Sandelius, unpublished results).

Since the phosphate-starvation-induced DGDG synthase machinery is clearly localized to the chloroplast envelope, a mechanism for the export of DGDG from the chloroplast must exist. Jouhet et al. (2004) suggested that DGDG is directly transferred from the chloroplast to the mitochondria. This is supported by electron micrographs showing that phosphate limitation causes an increase in close contacts between chloroplasts and mitochondria. For export of DGDG to the plasma membrane and the tonoplast, it seems reasonable to assume that the lipid is exported to the ER and from there sorted into the secretory pathway towards the tonoplast or the plasma membrane. In the *Arabidopsis fad2* mutant, which lacks ER localized oleoyl-PC desaturase, 18:2 and 18:3 fatty acids are still found in the extraplastidial phospholipids (Miquel and Browse, 1992); thus, lipid-bound fatty acids are exported from the chloroplast under other conditions as well. However, the export of DGDG so far is the only certain example for the export of intact glycerolipids from the chloroplast. The molecular details of the export of DGDG to mitochondria or the ER are at this point obscure.

If the replacement of phospholipids with DGDG takes place in fully expanded cells, the phospholipids first have to be degraded. In rapidly expanding tissues, on the other hand, phospholipid degradation would be less important than de novo synthesis of DGDG, which would quickly dilute the pre-existing phospholipids. In either case, DAG must be made available for galactolipid synthesis in the chloroplast envelope. *Arabidopsis* and *Acer pseudoplatanus* suspension cells respond to removal of phosphate from the medium with a transient increase in PC prior to accumulation of DGDG (Jouhet et al., 2003). While the PC content increased, there was a sharp decline in the content of other phospholipids. This underlines the role of PC as a precursor for galactolipid synthesis. It was suggested that hydrolysis of phospholipids plays a major role for the liberation of phosphate and the rerouting of DAG backbones from phospholipids to galactolipids. The current knowledge of phospholipase activities involved in these processes is very limited. A small family of genes with similarities to bacterial PC-specific PLC was identified in the *Arabidopsis* genome. One of the phospholipase genes, *NPC4*, was also found to be upregulated by phosphate starvation and the protein accumulated in the plasma membrane (Nakamura et al., 2005). The recombinant NPC4 protein exhibits a calcium-independent PLC activity towards PC, phosphatidylethanolamine and PA. However, inactivation of the *NPC4* gene in the *npc4* mutant had no effect on the phospholipid to DGDG exchange during phosphate starvation. The NPC4 protein was also found to accumulate in plasma membranes isolated from phosphate-starved oat roots (Andersson et al., 2005); however, in the latter case the major lipase activity detected in the isolated plasma membranes was PLD rather than PLC. The two *Arabidopsis* PLD isoforms PLDζ1 and PLDζ2 were also found to be induced by phosphate starvation (Cruz-Ramirez et al., 2006; Li et al., 2006a). A knockout mutation of PLDζ2 caused a reduction in phospholipid replacement in phosphate-starved roots, and the simultaneous knockout of both isoforms caused a change in the morphological response of the roots to low phosphate levels (Li et al., 2006b). It is thus clear that the phospholipid to DGDG replacement is accompanied by induction of phospholipase genes. However, an alternative function of these

phospholipases could be the production of intracellular signals for phosphate homeostasis or for bulk phospholipid degradation as a prerequisite for DGDG production.

12 Chloroplast Galactolipid Degradation Pathways

Chloroplast lipids are subject to constant degradation and resynthesis. Thus, glycerolipids in the membranes are hydrolysed by phospholipases, glycolipases and glycosidases, and replaced with newly assembled lipids. The identity of the enzymes involved in this process, however, is to a large extent unknown.

Two galactosidase activities were described with specificities for a and b glycosidic bonds (Sastry and Kates, 1964; Helmsing, 1967). While α-galactosidase activity is required for the hydrolysis of the outermost galactose residue in DGDG, the inner galactose of DGDG and that of MGDG are cleaved by β-galactolipase activity. Furthermore, an α-glycosidase activity is involved in sulfolipid breakdown. The identities of the genes involved remain unknown. *Arabidopsis* contains almost 400 glycosylhydrolase sequences (Henrissat et al., 2001) which can be organized into α and β specific enzymes. However, the large number of candidate genes prevents a straightforward sequence-homology-based approach of identifying lipid glycosylhydrolase genes. Furthermore, it is still unclear whether galactolipids are first cleaved by galactolipases (i.e. removing the acyl groups), and the remaining digalactosylglycerol or galactosylglycerol moieties cleaved by galactosidases, or whether galactosidases directly act on the two galactolipids MGDG and DGDG.

Fatty acids are cleaved from glycolipids by the action of glycolipases, in analogy to the phospholipases A1 and A2 which are well known from the animal and yeast field. Galactolipases have been characterized in plant extracts (Sastry and Kates, 1964; Helmsing, 1967). The enzymes are specific for hydrolysis of acyl groups from *sn*-1 and *sn*-2 positions of MGDG and DGDG. Two genes encoding chloroplast-localized galactolipases and phospholipases involved in releasing 18:3 destined for the oxylipin pathway (i.e. jasmonate production) have been identified in *Arabidopsis* (Ishiguro et al., 2001; Hyun et al., 2008). A sulfolipase activity was described for *Scenedesmus*, but similar to the galactolipases, the identity of the corresponding gene remains unknown (Yagi and Benson, 1962). Labelling experiments with $H_2{}^{18}O$ suggested that the rate of lipid deacylation/acylation ("retailoring") reactions in non-stressed leaves is quite high, similar to the de novo fatty acid synthesis rate (Pollard and Ohlrogge, 1999). Bao et al. (2000) showed that the rate of fatty acid breakdown amounts to about 4% of total leaf fatty acid per day; therefore, the entire acyl matrix in membranes is turned over in about 25 days. It is possible that acyl retailoring is involved in channelling different fatty acids to specific membrane lipids. For example, some of the ER-derived fatty acids bound to PC seem to be the precursors for galactolipids in the chloroplast (Roughan, 1970). Removal of polyunsaturated fatty acids damaged by reactive oxygen species might be essential for maintaining a functional thylakoid lipid matrix.

The amount of free fatty acids in leaves is generally very low. During senescence or abiotic stress, large amounts of lipids are broken down, resulting in the release of free fatty acids (Conconi et al., 1996). A certain proportion of the fatty acids derived from galactolipid breakdown are exported from the plastid. Free fatty acids in *Arabidopsis* are converted into their acyl-CoA derivatives by one of the nine long-chain acyl-CoA synthetases (Fulda et al., 2002; Hayashi et al., 2002; Schnurr et al., 2002; Shockey et al., 2002). Acyl-CoA esters serve as substrates for the further degradation via β-oxidation in the peroxisome. Furthermore, ACP synthases were identified in *Arabidopsis* which are possibly involved in plastidial fatty acid remodelling (Koo et al., 2005).

In addition to galactolipid-derived free fatty acids, free phytol and chlorophyllide from chlorophyll are released by action of chlorophyllases in photosynthetic membranes during stress or senescence. While the pathway of chlorophyllide degradation has been studied in detail, not much is known about the fate of phytol in plant metabolism. Phytol represents a C_{20} alcohol derived from the isoprenoid pathway. It is known that phytol and free fatty acids have detergent-like characteristics and, therefore, their accumulation is toxic for the membranes. During senescence or nitrogen deprivation, large amounts of fatty acid phytyl esters accumulate in the chloroplasts (Ischebeck et al., 2006; Gaude et al., 2007). The phytyl esters are mostly localized to plastoglobules, small lipid protein particles in the chloroplasts. Phytyl esters have previously been identified in algae, mosses and in higher plants subjected to abiotic stress or senescence (Csupor, 1971; Gellerman et al., 1975; Cranwell et al., 1985; Patterson et al., 1993). The identity of the protein involved in fatty acid phytyl ester synthesis and its role during senescence remains enigmatic. It is possible that phytyl esters represent a transient sink for free fatty acids and phytol released from galactolipids and chlorophyll, respectively, and that they are finally channelled into degradation by β-oxidation in the peroxisome. In this regard it is interesting to note that plastoglobules contain a number of additional non-polar lipids that accumulate during stress or senescence, including phylloquinone, fatty acid phytyl ester and tocopherol (Lohmann et al., 2006; Vidi et al., 2006).

13 Outlook

The last 30 years has seen tremendous advances in our understanding of the biosynthesis and function of the chloroplast membrane lipids. Nearly all the important genes involved in synthesis of the major chloroplast lipids have been cloned from *Arabidopsis*. Taking advantage of the emerging plant genome projects, one can now employ these sequences to identify orthologues in other plants, including the major crop species. Figures 2–4 show tentative models for the different biochemical pathways presented here. Glycerolipids are synthesized in only a few membranes of the plant cells, in particular the chloroplast envelopes, the ER and, to a lesser extent, the mitochondrion. Therefore, lipid trafficking is required to transfer precursors or entire lipid molecules to other organelles. Direct contact sites seem to be involved

in the transport of membrane material between the ER and the envelope. The first genes encoding an ABC transporter presumably involved in the transfer of PA from the ER to the chloroplast have been cloned. The molecular basis for DGDG export from the plastids during phosphate deprivation is not understood. Within young chloroplasts, thylakoids seem to be derived from inner-envelope invaginations, while a vesicle-based system seems to be operating in older chloroplasts. However, the molecular basis for intraplastidial lipid trafficking is unclear. Future research will have to focus on the missing factors involved in intra- and extraplastidial lipid homeostastis and lipid trafficking.

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