The Digalactosyldiacylglycerol (DGDG) Synthase DGD1 Is Inserted into the Outer Envelope Membrane of Chloroplasts in a Manner Independent of the General Import Pathway and Does Not Depend on Direct Interaction with Monogalactosyldiacylglycerol Synthase for DGDG Biosynthesis

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John E. Froehlich‡, Christoph Benning§, and Peter Dörmann¶

From the ‡Department of Energy Plant Research Laboratory and the §Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48823 and the ¶Max-Planck-Institute of Molecular Plant Physiology, 14476 Golm, Germany

Galactolipids make up the bulk of chloroplast lipids. Therefore, the genes involved in the synthesis of the galactolipids monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) play a critical role in chloroplast development. In this study, we analyzed the subcellular localization of the Arabidopsis DGDG synthase DGD1, which was recently identified by complementation of the Arabidopsis dgd1 mutant. In vitro import experiments demonstrated that DGD1 was targeted to the chloroplast outer envelope in an ATP-independent manner. DGD1 could not be extracted from the membranes by high salt or alkali, suggesting that it is an integral membrane protein. Uptake experiments with truncated versions of DGD1 indicated that the information for targeting and insertion into the outer envelope resides in the N-terminal half of DGD1, but not in the first 33 amino acids. DGD1 apparently does not contain a cleavable signal peptide. Antibodies to Arabidopsis DGD1 detected a 90-kDa protein localized to the chloroplast envelopes of both pea and Arabidopsis. Transformation of DGD1 constructs into cyanobacteria resulted in the expression of active DGDG synthase and demonstrated that DGDG synthesis depends on MGDG lipid, but does not require direct interaction with the plant MGDG synthase.

Chloroplasts are the site of numerous biological processes and contain a variety of metabolic pathways critical for plant survival. A unique characteristic of chloroplasts is their high content of the two galactolipids monogalactosyldiacylglycerol (MGDG)1 and digalactosyldiacylglycerol (DGDG), which constitute up to 80% of total chloroplast lipids. Galactolipids serve numerous physiological functions (1). For example, the unusual chemical structures of MGDG and DGDG result in distinct packing properties that are critical for the formation of thylakoid membrane stacks (2). Consequently, galactolipids play an essential role in chloroplast membrane biogenesis and in photosynthesis (3–5). Furthermore, MGDG and DGDG are indispensable for correct protein import into chloroplasts (6). For instance, the DGDG-deficient dgd1 mutant of Arabidopsis, while normal for importing chloroplast outer membrane proteins, is defective in importing precursor proteins targeted to the interior of chloroplasts (6).

The first committed step in galactolipid biosynthesis is the transfer of galactose from UDP-galactose to diacylglycerol by the MGDG synthase to generate MGDG. MGDG is subsequently converted to DGDG by the action of DGDG synthase (for recent reviews, see Refs. 7 and 8). A critical step toward advancing our understanding of galactolipid biosynthesis and function was achieved with the isolation of the cDNAs coding for MGDG and DGDG synthases (9, 10). The DGDG synthase DGD1 was isolated by chromosome walking based on the availability of the Arabidopsis dgd1 mutant. From detailed analyses of dgd1, it was inferred that DGD1 is required for the synthesis of >90% of DGDG in chloroplasts (10). Expression of DGD1 in Escherichia coli resulted in the accumulation of DGDG only after simultaneous expression of an MGDG synthase from cucumber. Therefore, it was concluded that MGDG is required as a substrate for DGD1 enzymatic activity.

Membrane fractionation analyses demonstrated that both MGDG and DGDG synthase activities are localized to the chloroplast membrane (11). Dorne et al. (12) as well as Cline and Keegstra (13) also showed that DGDG synthase is associated with the outer envelope. With the isolation of the DGDG synthase DGD1 (10), the subcellular localization of this important enzyme can now be investigated in greater detail.

The vast majority of proteins targeted to plastids are nuclear encoded and synthesized in the cytosol as precursors containing an N-terminal extension (transit peptide) that is essential for correct targeting (14). A large number of proteins that are imported according to this so-called general import pathway are destined for the interior of chloroplasts. In general, the transit peptide is removed during protein translocation. However, since many of the enzymes involved in galactolipid synthesis are associated with the envelope membranes, targeting of these proteins may follow one of two different routes that were described for outer envelope proteins (for reviews, see Refs. 14 and 15). The first route does not require a functional transit peptide, ATP, or surface-exposed receptors for insertion...
into the envelope membrane. This pathway is commonly referred to as the "OM14 pathway" (16–20). The second route does, however, require the presence of an N-terminal transit peptide as well as components of the general import apparatus (21). Interestingly, the sequence of DGD1 was predicted to contain a putative transit peptide that may target DGD1 to chloroplasts (10). However, no experimental data were presented demonstrating that this sequence actually contains targeting information for the chloroplasts.

In this study, using in vitro import assays in isolated pea chloroplasts, we examined the requirements for targeting and subcellular localization of DGD1. Western blot analysis supported the localization of endogenous DGD1 to the envelopes of both pea and Arabidopsis chloroplasts. Finally, by heterologous expression in cyanobacteria, we analyzed whether DGD1 activity is dependent on protein-protein interactions with the MGDG synthase and whether the extreme N terminus of DGD1 is critical for enzymatic activity.

**EXPERIMENTAL PROCEDURES**

**Plant Material—**Pea seeds (cultivar little marvel) were obtained from F. Heide Co. (Madison, WI). Arabidopsis plants (wild-type Col-2 or dgd1 mutant) were grown in tissue culture or in soil as previously described (4).

**Origin of DGD1 cDNA Constructs—**The DGD1 cDNA (clone 22-1) (10) was the template for PCR amplification of different parts of the coding region. Oligonucleotides were used for PCR with BamHI sites (primers Ben271, PD1, and PD2) or PstI sites (primers Ben241 and PD5) at their 5′-ends for subcloning into pBluescript II KS+. In addition, primers Ben271, PD1, and PD2 introduced a new in-frame start codon into the amplified PCR product, whereas primers PD3 and Ben241 carried an in-frame stop codon. Amplification with Ben271 (GGG GAT CCA TGT TAT CGT TTT TGT CGA AA) and Ben241 (TTC CAA AAT CAG T) resulted in a cDNA encoding the N-terminal part of (GCG GAT CCA TGT TAT CGT TTT TGT CGA AA) and Ben241. The C-terminal half of DGD1 was amplified by PCR using primers (ATG GAT CCG GAG ACA CCG GAA AAC AAA) and Ben241. Primers PD1, PD2, and Ben293 (ATG GAT CCG GAG ACA CCG GAA AAC AAA) were used for PCR with the C-terminal part of DGD1. The fragment amplified with primers PD1 (GGG GAT CCA TGT TAT CGT TTT TGT CGA AA) and Ben241 resulted in a cDNA encoding the N-terminal part of DGD1 up to Pro337 (DGD1-N). PCR of clone 22-1 with primers PD2 (TTC CAA AAT CAG T) and Ben241 yielded a cDNA encoding the C-terminal part of DGD1 (DGD1-C).

For heterologous expression in E. coli, the polymerase of the C-terminal part of DGD1 was amplified by PCR using primers Ben293 (ATG GAT CCG GAG ACA CCG GAA AAC AAA) and Ben241. This fragment was ligated into the BamHI and PstI sites of pQE31 (QIAGEN Inc., Hilden, Germany), resulting in the construct pQE31-293, and transformed into E. coli M15 (DE3P4) for protein expression and analysis by SDS-PAGE and fluorography.

**Expression of DGD1 in Synechocystis sp.** PCC6803 and **Lipid Analysis—**The ft cassette was released from pHP45 (22) with HindIII and ligated into the broad host range expression vector pLR59.1. The full-length DGD1 cDNA of clone 22-1 (10) or a truncated version lacking the signal peptide (DGD1-noSP; see above) was digested with BamHI and Xhol and ligated into the BamHI and SalI sites of plasmid pRL59.1. Synechocystis sp. PCC6803 was transformed by conjugation (25) and grown in chemically defined medium containing 0.5 mM MgCl₂ as the sole source of Mg. Mutant strains containing the DGD1 construct were selected on plates containing 0.5 mM MgCl₂. All fractions were analyzed by SDS-PAGE and fluorography.

**Antibodies and Western Blotting—**All antibodies were polyclonal and raised in rabbits. Antisera to pea Tic110 (translocon of the inner envelope of chloroplasts) and Toe75 (translocon of the outer envelope of chloroplasts) were prepared as described (39). The C-terminal part of DGD1 was expressed in E. coli harboring the construct pQE31-293. The fragment was solubilized with urea-containing buffers and purified by Nt-recognizing chromatography and affinity chromatography following the protocol of QIAGEN Inc. The purified DGD1 C-terminal polypeptide was used to raise polyclonal antiserum in rabbits. Pea and Arabidopsis chloroplasts were isolated and fractionated as previously described (31). Fractions were separated by SDS-PAGE, and proteins transferred onto polyvinylidene difluoride membranes (Immobilon P, Millipore Corp., Bedford, MA). Western blots were performed as described (36). Primary antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD).

**RESULTS**

**Import of DGD1 into Chloroplasts Is ATP-independent and Does Not Require Surface-exposed Receptors—**Import experiments of in vitro expressed proteins using isolated chloroplasts have been widely employed to determine the subcellular localization of proteins within chloroplasts (31). We therefore used the entire DGD1 open reading frame for in vitro expression and purification of the N-terminal part of pea chloroplasts (Fig. 1A). In vitro translation of DGD1 generated a 35S-labeled protein of ~90 kDa, which correlates well with the predicted size of the DGD1 open reading frame. Additional smaller radioactive polypeptides were detected in the translation product lane derived from translation of the entire DGD1 open reading frame as well as shorter constructs (see below). These additional bands are...
DGD1 Targeting to the Chloroplast Outer Envelope

**Fig. 1. Targeting of DGD1 to pea chloroplasts is ATP-independent.** 35S-Labeled DGD1 (A), luciferase (B), OM14 (C), and pSS (D) proteins were incubated with pea chloroplasts in the presence of 0, 0.1, or 4 mM MgATP. All assays were treated with (+) or without (−) thermolysin. Intact chloroplasts were recovered by centrifugation, lysed, and separated into total membrane (M) and soluble (S) fractions. All fractions were analyzed by SDS-PAGE and fluorography. Luciferase, which is not targeted to chloroplasts, was used to assess the amount of nonspecific binding during import. The translation product (TP) lane represents 10% of the radiolabeled protein added to each assay. Arrows indicate the locations of pSS and the mature form of the small subunit of Rubisco (mSS).

supposedly derived from truncated translation products. 35S-Labeled DGD1 was incubated with isolated chloroplasts and fractionated into a total membrane (M) and soluble (S) fraction (Fig. 1). DGD1 consistently fractionated to the total membrane fraction (Fig. 1A, lanes 1, 5, and 9), which contains inner and outer envelope membranes as well as thylakoids. DGD1 association with the chloroplast membrane was ATP-independent (Fig. 1A, compare lanes 1–4 with lanes 5–8 and 9–12), and the protein did not undergo any detectable processing. Furthermore, the association of DGD1 with chloroplast membranes was specific because under similar conditions, luciferase, which was used as a control protein not localized to chloroplasts, did not associate with the chloroplast membrane (Fig. 1B, lanes 1–12). Hence, the ATP-independent association of DGD1 with chloroplast membranes presumably utilizes the OM14 targeting pathway (16, 19).

Following a DGD1 uptake experiment, chloroplasts were treated with thermolysin to remove all surface-exposed proteins facing the cytosol. DGD1 was susceptible to digestion by thermolysin (Fig. 1A, lanes 3, 7, and 11), indicating that DGD1 has considerable thermolysin-sensitive domains oriented toward the cytosol. The protease sensitivity of DGD1 was similar to that observed for OM14, which is shown as a control for an authentic outer envelope protein (Fig. 1C, lanes 1–4). In contrast, the small subunit of Rubisco (pSS), which is imported into the stroma and processed during uptake, was protected from thermolysin degradation (Fig. 1D, lanes 1–4). Other proteinases such as trypsin and proteinase K likewise completely digested DGD1 after uptake (data not shown), providing additional support for the finding that the predominant part of the DGD1 polypeptide faces the cytosol.

Since DGD1 did not require ATP for import, we addressed the question of whether its membrane association depends on the presence of surface-exposed receptors on the chloroplast envelope. Chloroplasts were pretreated with thermolysin to degrade surface-exposed receptor proteins prior to targeting experiments with DGD1. As shown in Fig. 2, the association of DGD1 and OM14 was not affected by pretreatment with thermolysin (Fig. 2, A and B, compare lanes 1 and 3), whereas import of pSS, a protein requiring receptor proteins at the outer envelope, was essentially abolished (Fig. 2C, compare lanes 2 and 4). We conclude from these observations that the association of DGD1 with chloroplasts is ATP-independent and does not require surface-exposed receptors.

**DGD1 Is Localized to the Outer Envelope Membrane of Chloroplasts**—To investigate the exact suborganellar localization of DGD1, a detailed fractionation analysis was performed in which chloroplasts derived from a large-scale import reaction were separated into outer (OM) and inner (IM) envelopes, stroma (Str), and thylakoids (Thy) (Fig. 3). The DGD1 protein was localized to the outer envelope fraction (Fig. 3A, lane 3), similar to OM14, which is known to be localized to the outer envelope (Fig. 3B, lane 3). In contrast, no DGD1 was observed that co-fractionated with tp110-110N, a marker protein for the inner envelope (Fig. 3C, lanes 4 and 5), or the small subunit of Rubisco (pSS), a protein targeted to the stroma (Fig. 3D, lane 6). Therefore, DGD1 behaves as a chloroplast outer envelope membrane protein.

**Integration of DGD1 into the Envelope Membrane Is Independent of a Putative N-terminal Transit Sequence**—The vast majority of proteins taken up by chloroplasts are synthesized as precursors with an N-terminal transit peptide that is removed after import (14, 40, 41). DGD1 processing could not be detected upon insertion into the chloroplast membrane (Fig. 1A). Nevertheless, a non-cleavable targeting signal that directs DGD1 to the chloroplast membrane may be located at the N terminus. However, identifying chloroplast targeting sequences embedded within a membrane protein has proven difficult. We therefore attempted to find putative targeting sequences using various software tools (ChloroP Version 1.0 and

**Fig. 2. Insertion of DGD1 into chloroplast envelope membranes does not require surface-exposed receptors.** Pea chloroplasts were pretreated with (+) or without (−) thermolysin to degrade surface-exposed receptors. Thermolysin was quenched with 5 mM EDTA, and intact chloroplasts were recovered by centrifugation. After washing and resuspension in import buffer, chloroplasts were incubated with 35S-labeled DGD1 (A), OM14 (B), or pSS (C) for 30 min. MgATP (4.0 mM) was added to support import of pSS, but was omitted for DGD1 and OM14. After import, chloroplasts were again recovered by centrifugation, lysed, and separated into total membrane (M) and soluble (S) fractions. All fractions were analyzed by SDS-PAGE and fluorography. The translation product (TP) lane represents 10% of the radiolabeled protein added to each assay. Arrows indicate the locations of pSS and the mature form of the small subunit of Rubisco (mSS).
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SignalP Version 1.1 (42) and SPSCAN of the GCG software, Genetics Computer Group, Inc., Madison, WI). The outcome of these computer analyses suggested the following. (i) Amino acids Met1–Ala32 might represent a putative targeting sequence (Fig. 4A). This N-terminal region of DGD1 is rich in hydroxylated amino acid residues (54% serine/threonine content), which is a common feature of transit peptides (40, 41). (ii) Hydrophobicity analysis predicted the presence of five hydrophobic regions stretching from amino acids 22 to 37, 136 to 150, 248 to 259, 347 to 372, and 644 to 670 (Fig. 4B). Although only the regions of amino acids 347–372 and 644–670 are sufficient in length to represent transmembrane domains, it is unknown which of these regions might be essential for targeting of DGD1 to and insertion into the chloroplast envelope. (iii) Sequence comparison with other plant genes deposited in the GenBankTM/EBI Data Bank revealed that DGD1 is composed of two domains, a C-terminal domain with sequence similarity to glycosyltransferases and an N-terminal domain of unknown function (10). To analyze the role of all of these domains for targeting properties, truncated versions of the DGD1 cDNA were prepared for in vitro import assays (Fig. 4C). These constructs encompassed the entire DGD1 open reading frame, a cDNA lacking the N-terminal 33 amino acids (DGD1-noSP), the N-terminal part of DGD1 up to Pro357 (DGD1-N), and the C-terminal part of DGD1 starting with Glu338 (DGD1-C).

DGD1 and truncated versions of DGD1 were incubated with chloroplasts and subsequently fractionated into a mixed envelope fraction (containing outer and inner envelopes) using a sucrose step gradient centrifugation (Fig. 5, A–D, lanes 1 and 2). Isolated mixed envelopes were extracted with buffer (Fig. 5, A–F, lanes 1 and 2), NaCl (lanes 3 and 4), or Na2CO3 (lanes 5 and 6). The proteins allene-oxide synthase (extractable with Na2CO3 solution) and OM14 (not extractable with Na2CO3) served as controls (Fig. 5, E and F). DGD1, DGD1-noSP, and DGD1-N all associated with the envelope fraction and could not be extracted by treatment with NaCl or Na2CO3. Although DGD1-C contains two predicted transmembrane domains (residues 347–372 and 644–670), it did not associate with or insert into the chloroplast envelope. Hence, these domains may serve as components of the active site of the predicted glycosyltransferase rather than being involved in targeting (Fig. 5D). Therefore, DGD1, DGD1-noSP, and DGD1-N behaved as integral membrane proteins and contain the targeting determinants of DGD1 that are predominantly located at the N terminus of the protein. Furthermore, the association of DGD1 with the envelope membrane does not require an N-terminal transit peptide. However, the first 33 amino acids do appear to improve the affinity of DGD1 for the chloroplast envelope (Fig. 5, A and B, lanes 1–6, compare insertions of DGD1 and DGD1-noSP).

**Western Blot Analysis of Pea and Arabidopsis Chloroplasts**

**Supports the Localization of Endogenous DGD1 to the Envelope Membrane**—Pea chloroplasts derived from a large-scale isolation experiment were separated into outer (OM) and inner (IM) envelopes, stroma (Str), and thylakoids (Thy) (Fig. 6A). Since obtaining high amounts of Arabidopsis chloroplasts for detailed fractionation studies proved very difficult, Arabidopsis chloroplasts were separated only into mixed envelopes (Euv), stroma, and thylakoids (Fig. 6B). Chloroplast fractions were analyzed by Western blotting and probed with antibodies raised against Arabidopsis DGD1 (C-terminal half) or, as controls, pea Tic110 (inner envelope marker) and pea Toc75 (outer envelope marker). Anti-DGD1 antibodies detected a 90-kDa pro-
brane.

**FIG. 5.** Truncated forms of DGD1 containing N-terminal domains specifically insert into the chloroplast envelope membrane. 

**A** 35S-Labeled DGD1 (A), DGD1-noSP (B), DGD1-N (C), DGD1-C (D), allene-oxide synthase (AOS; E), and OM14 (F) were used for uptake experiments with pea chloroplasts. After a 30-min incubation, intact chloroplasts were recovered by centrifugation. Chloroplasts were lysed, and a mixed envelope membrane fraction (containing outer and inner envelopes) was isolated by sucrose step gradient and ultracentrifugation. The mixed envelopes (Env) were extracted with control buffer or with buffer containing NaCl or Na2CO3. Extracted envelopes were recovered by centrifugation at 100,000 × g, and the soluble (S) material was precipitated with trichloroacetic acid. All fractions were analyzed by SDS-PAGE and fluorography. Allene-oxide synthase is a control for an extractable envelope protein. OM14 is a control for a non-extractable outer envelope protein. The translation product (TP) lane represents 10% of the radiolabeled protein added to each assay.

**FIG. 6.** Endogenous DGD1 is localized to the envelopes of pea and *Arabidopsis* chloroplasts. Pea (A) and *Arabidopsis* (B) chloroplasts were isolated, fractionated, and analyzed by Western blotting. For pea chloroplasts, a detailed fractionation analysis was performed in which a large amount of chloroplasts was separated into outer (OM) and inner (IM) envelopes, stromata (Str), and thylakoids (Thy). A small-scale chloroplast preparation of *Arabidopsis* was separated into mixed envelopes (Env), stromata, and thylakoids. All fractions were separated by SDS-PAGE and analyzed by Western blotting with antibodies raised against *Arabidopsis* DGD1, pea Tic110 (inner envelope marker), and pea Toc75 (outer envelope marker).

**TABLE I**

| Control | DGD1 | DGD1-noSP |
|---------|------|-----------|
| MGDG    | 41.4 ± 0.5 | 22.8 ± 1.2 | 26.1 ± 3.5 |
| Phosphatidylglycerol | 5.2 ± 0.1 | 7.9 ± 0.6 | 9.1 ± 0.9 |
| DGDG    | 17.3 ± 0.3 | 41.4 ± 1.1 | 38.6 ± 2.1 |
| Sulfoquinovosyldiacylglycerol | 36.2 ± 0.7 | 27.9 ± 0.7 | 26.5 ± 2.4 |

lines and in the control stayed the same, with 16:0, 16:1ω9, 18:2ω6, 18:3ω3ω6, and 18:3ω9ω6ω3 representing the most abundant fatty acids (data not shown). Therefore, the N-terminal 33 amino acids of DGD1 do not appear to be essential for DGDG synthase activity.

**DISCUSSION**

Investigations by many laboratories have revealed that the synthesis of chloroplast galactolipids in certain higher plants, *e.g.* *Arabidopsis*, proceeds by two different pathways. The enzymes of one pathway, which is commonly referred to as “prokaryotic,” are exclusively localized to the plastid. The enzymes of the second, eukaryotic pathway are derived from both the plastid and the endoplasmic reticulum. The lipids originating from the different pathways can be distinguished based on their fatty acid composition. In *Arabidopsis*, the prevalent fraction of MGDG is made via the prokaryotic pathway, whereas the eukaryotic pathway contributes to a large extent to the...
synthesis of DGDG (43, 44). The enzymes involved in galactolipid biosynthesis presumably play a critical role in determining the relative contribution of the two different pathways to synthesis of MGDG and DGDG. Consequently, the exact localization and mechanism of MGDG and DGDG formation raise many interesting questions.

Biochemical fractionation studies (11, 12, 34, 46, 47) have shown that a number of enzymes involved in galactolipid biosynthesis are located in the chloroplast envelope. However, this exclusive localization is still a matter of much controversy. We investigated the targeting and localization of the Arabidopsis DGDG synthase DGD1 (10) to chloroplasts. In vitro import assays confirmed that DGD1 was targeted to chloroplasts (Fig. 1A), and subsequent fractionation studies provided additional evidence that DGD1 was localized to the outer envelope membrane (Fig. 3A). These findings support previous biochemical experiments showing that DGDG synthase activity is localized to the outer envelope of spinach and pea chloroplasts (12, 13). Our import assays also demonstrated that newly inserted DGD1 was completely susceptible to digestion with thermolysin (Fig. 1A). Interestingly, DGDG synthase activity was also shown to be susceptible to protease degradation and therefore is orientated toward the cytosolic side of the envelope membranes (50).

The targeting of DGD1 to the chloroplast outer envelope raises many interesting questions concerning DGDG formation. For instance, how are the enzymes involved in the final reactions of galactolipid biosynthesis organized within the envelope of chloroplasts? A picture of the cellular organization of the galactolipid biosynthetic pathway is slowly emerging. Recently, it was shown that the spinach MGDG synthase MGDA is located in the chloroplast inner envelope (48). However, with pea chloroplasts, MGDG synthase activity was exclusively detected at the outer envelope (49). In Arabidopsis, three MGDG synthases have been described (MGDA/MGDB, MGDB/MGD2, and MGDC/MGD3) (3, 9, 48, 50). MGDA/MGD1 from Arabidopsis is most similar to spinach MGDA and therefore presumably is localized to the inner envelope membrane (48). The exact localization of MGDB/MGD2 and MGDC/MGD3 from Arabidopsis is not yet clear. As mentioned above, the predominant fraction of DGDG in Arabidopsis is derived from eukaryotic diacylglycerol moieties assembled at the endoplasmic reticulum, which are incorporated into MGDG in the chloroplast envelope by one of the MGDG synthases (43). This eukaryotic type MGDG is then converted to DGDG by DGD1. Hence, the localization of DGD1 to the chloroplast outer envelope may be a prerequisite for its role in synthesizing DGDG via the eukaryotic pathway. At this stage, we do not know which of the MGDG synthases generates the precursor for DGDG biosynthesis.

In Fig. 1A, we demonstrated that DGD1 was targeted to the chloroplast envelope via a route similar to OM14 (16, 19). Surprisingly, however, computer-based analyses of the DGD1 amino acid sequence predicted that Met<sup>1</sup>–Ala<sup>33</sup> may represent a putative transit peptide. To examine the function of different domains of DGD1, truncated versions of DGD1 were constructed and used for import (Figs. 4 and 5) and enzymatic assays (Table I). All truncated versions of DGD1 containing an N-terminal domain (Fig. 4C) were found to associate with the chloroplast membrane (Fig. 5, A–D), whereas the C-terminal part of the DGD1 protein (DGD1-C) containing the glycosyltransferases domain did not associate with the chloroplast membrane. Likewise, removal of the N-terminal 33 amino acids did not abolish DGD1 activity when expressed in cyanobacteria. No difference in lipid or fatty acid composition between Synechocystis cells expressing the full-length clone of DGD1 or the truncated version (DGD1-noSP) was observed (see Table I). From this study, we conclude that the N-terminal portion of DGD1 between Leu<sup>34</sup> and Pro<sup>337</sup> contains information sufficient to target DGD1 to the chloroplast envelope. Likewise, we propose that DGD1 utilizes a non-cleavable targeting signal that directs it to the outer envelope in a similar way to COM70, a spinach HSP70 protein (51, 52). However, more experiments will be required to exactly identify the outer envelope-targeting determinants of DGD1.

Finally, with evidence accumulating that both MGDG and DGDG synthases are localized to the envelope membranes, we investigated whether these two enzymes need to associate with each other to functionally synthesize DGDG. By coexpression in E. coli with MGDG synthase, we initially demonstrated that DGD1 encodes a functionally active DGDG synthase (10). However, the enzymatic activity in this recombinant system was very low and did not clarify the question of whether an MGDG-DGDG synthase complex was necessary for DGDG formation. We therefore resorted to utilizing cyanobacteria for expression of DGD1 because these cells contain high amounts of the galactolipid MGDG. In addition, cyanobacteria employ a different pathway for galactolipid biosynthesis compared with higher plants (53), and although the complete genome of Synechocystis sp. PPC6803 has been sequenced, the genes involved in galactolipid synthesis are still unknown. There are no sequences present in Synechocystis with clear similarities to the plant-derived galactolipid synthases (9, 10). Our results show that DGD1 is highly active in cyanobacteria since a large fraction of MGDG was converted to DGDG (see Table I). This clearly demonstrates that the Arabidopsis DGDG synthase can utilize the cyanobacterial MGDG lipid as a substrate. It has been proposed that in complex biosynthetic pathways, substrates are oftentimes not released from the enzymes, but rather stay closely bound and are passed on to the next active site for subsequent catalysis. This process, which is referred to as “substrate channeling,” requires a close association of the participating enzymes in a protein complex (e.g. Ref. 54). The finding that both MGDG and DGDG biosynthesis occurs within the envelope membrane in Arabidopsis (46) led to the hypothesis that a substrate channeling mechanism may be functioning in DGDG formation. Hence, intimate protein-protein interactions between MGDG and DGDG synthases would be an essential requirement in this scenario. However, because there are no genes similar to higher plant MGDG synthases in Synechocystis, the requirement for protein-protein interactions between DGD1 and a plant MGDG synthase may not necessarily be a prerequisite for DGDG synthesis. With the ability to manipulate DGD1 and other galactolipid biosynthetic enzymes in transgenic Arabidopsis, we now have the tools to dissect both the prokaryotic and eukaryotic pathways of galactolipid biosynthesis. Questions concerning how these enzymes may interact with each other and how they are regulated can now be addressed. Furthermore, the transport of the DGDG lipid produced by DGD1 from the outer to the inner envelope and to the thylakoids remains yet another unresolved problem that will stimulate future research.

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John E. Froehlich, Christoph Benning and Peter Dörmann

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