

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*

Received for publication, May 22, 2001, and in revised form, June 27, 2001
Published, JBC Papers in Press, June 27, 2001, DOI 10.1074/jbc.M104652200

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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 un-

usual 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

* This work was supported in part by Grant MCB93-03693 from the Cell Biology Program of the National Science Foundation (to Ken Keegstra), by the Division of Energy Biosciences of the United States Department of Energy, and by Grant DE-FG-02-98ER20305 from the United States Department of Energy (to C. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; Rubisco, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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 Olds Seed 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 *Bam*HI sites (primers Ben271, PD1, and PD2) or *Pst*I sites (primers Ben241 and PD3) 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 (GCG GAT CCA TGT TAT CGT TTT TGT CGA AA) and Ben241 (TTC TGCA GTC TAC CAG CCG AAG ATT GG) yielded a cDNA encoding a truncated DGD1 protein lacking the N-terminal 33 amino acids (DGD1-noSP). The fragment amplified with primers PD1 (CCG GAT CCC ATG GTA AAG GAA ACT CTA) and PD3 (GGC TGC AGC TAA GGC TTCA CAA AAT CAG T) resulted in a cDNA encoding the N-terminal part of DGD1 up to Pro³³⁷ (DGD1-N). PCR of clone 22-1 with primers PD2 (TCG GAT CCA TGG ACA CAC CCG AAA ACA AA) and Ben241 produced a fragment encoding the C-terminal half of DGD1 starting with Glu³³⁸ (DGD1-C).

For heterologous expression in *E. coli*, the part of clone 22-1 encoding the C-terminal half 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 *Bam*HI and *Pst*I sites of pQE31 (QIAGEN Inc., Hilden, Germany), resulting in the construct pQE31-293, and transformed into *E. coli* M15(pREP4) for protein expression and antibody generation.

Expression of DGD1 in *Synechocystis* sp. PCC6803 and Lipid Analysis—The Ω cassette was released from pHP45 (22) with *Hind*III and ligated into the broad host range expression vector pRL59EH (23, 24), resulting in plasmid pRL59- Ω . 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 *Bam*HI and *Xho*I and ligated into the *Bam*HI and *Sal*I sites of pRL59- Ω . *Synechocystis* sp. PCC6803 was transformed by conjugation (25) and grown photoautotrophically at 28 °C as described (26). Lipids were isolated from *Synechocystis* sp. PCC6803 cultures harboring the different constructs (pRL59- Ω control or DGD1 or DGD1-noSP in pRL59- Ω), separated by thin-layer chromatography, and quantified by gas chromatography after derivatization to fatty acid methyl esters (27).

In Vitro Translation of Protein Precursors—Plasmids containing cDNA clones of the following protein precursors were linearized as indicated: pSS (precursor to the small subunit of Rubisco) with *Pst*I (28), OM14 (outer chloroplast envelope membrane protein of 14 kDa) with *Eco*RI (16), tp110-110N with *Xho*I (29), allene-oxide synthase with *Xho*I (30), and DGD1 and truncated DGD1 constructs with *Xho*I. After linearization, the cDNAs for pSS and OM14 were transcribed with SP6 RNA polymerase; tp110-110N and the truncated DGD1 constructs were transcribed with T7 RNA polymerase; and allene-oxide synthase and the full-length DGD1 cDNA were transcribed with T3 RNA polymerase. The mRNAs of pSS, tp110-110N, allene-oxide synthase, and the full-length and truncated constructs of DGD1 were translated using the nuclease-treated rabbit reticulocyte lysate translation system (Pro-

mega, Madison, WI) following the manufacturer's protocol. OM14 was translated using a wheat germ translation system as previously described (31). All proteins were radiolabeled by translation in the presence of [³⁵S]methionine.

Isolation of Pea Chloroplasts—Intact chloroplasts were isolated from 8–12-day-old pea seedlings (*Pisum sativum* cultivar little marvel) as previously described (31). *Arabidopsis* chloroplasts were isolated according to Fitzpatrick and Keegstra (32). Chloroplasts were washed twice with import buffer (50 mM HEPES-KOH (pH 7.5) and 0.33 M sorbitol) and resuspended in the same buffer to a concentration of 1 mg/ml chlorophyll.

Binding and Import Assays—Binding and import experiments with ³⁵S-labeled DGD1 protein were performed as previously described (31). Briefly, prior to binding or import reactions, chloroplasts were incubated with 6 mM nigericin for 10 min in the dark to deplete internal ATP levels. In each reaction, ³⁵S-labeled DGD1, nigericin-treated chloroplasts, and 0, 0.1, or 4.0 mM MgATP were incubated in the dark for 30 min at room temperature. Intact chloroplasts were recovered by sedimentation through a 40% (v/v) Percoll cushion. The pellets were resuspended in lysis buffer (25 mM HEPES-KOH (pH 8.0) and 4 mM MgCl₂) and incubated on ice for 15 min. The total membranes were separated from soluble fractions by ultracentrifugation at 100,000 × g. All fractions were analyzed by SDS-PAGE (33) and fluorography. Treatment of binding and import reactions with thermolysin was performed for 30 min at 4 °C in the dark essentially as described (34).

Extraction of Mixed Envelope Membranes Isolated from Pea Chloroplasts—From a large-scale import reaction with ³⁵S-labeled protein, intact chloroplasts were recovered by sedimentation through a 40% (v/v) Percoll cushion. Purified chloroplasts were hypotonically lysed in lysis buffer for 20 min on ice. Mixed envelope membranes were recovered by a sucrose step gradient and ultracentrifugation as described (35). After two extractions with NaCl (36) or Na₂CO₃ (37, 38) buffer, mixed envelope membranes were re-isolated by ultracentrifugation, and the supernatant was precipitated with trichloroacetic acid. Both mixed envelope membranes and supernatant fractions were analyzed by SDS-PAGE (33) and fluorography.

Isolation of Chloroplast Outer and Inner Envelope Membranes from Pea—Following a large-scale import reaction, chloroplasts were hypotonically lysed in 0.6 M sucrose, 25 mM HEPES-KOH (pH 8.0), and 4 mM MgCl₂ for 20 min on ice. After one freeze-thaw cycle (−20 °C for 1 h), the solution was adjusted to 0.3 M sucrose by dilution with lysis buffer. The ruptured chloroplasts were then fractionated into outer envelope membranes, inner envelope membranes, stromata, and thylakoids using a sucrose step gradient and ultracentrifugation as described (36). 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 Toc75 (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. Protein was solubilized with urea-containing buffers and purified by Ni²⁺-nitrilotriacetic acid 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 uptake experiments into 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

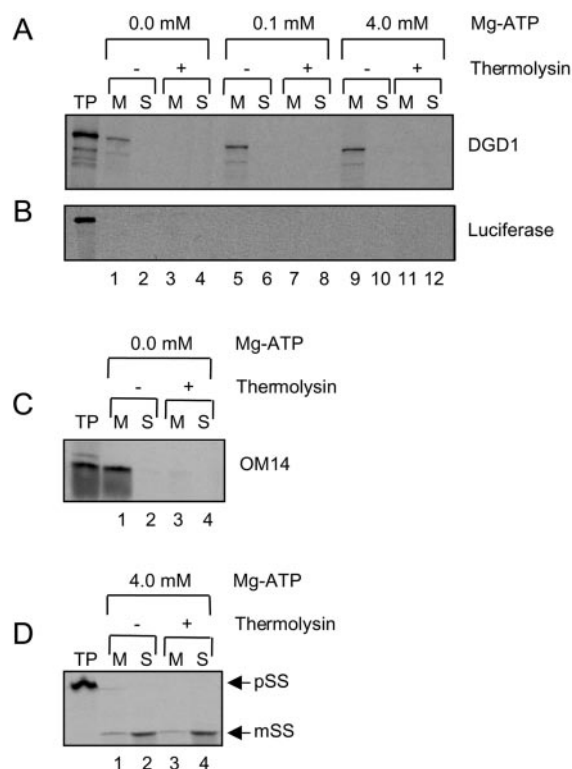


FIG. 1. Targeting of DGD1 to pea chloroplasts is ATP-independent. ³⁵S-Labeled DGD1 (A), luciferase (B), OM14 (C), and pSS (D) proteins were incubated with pea chloroplasts in the presence of 0, 0.1, or 4 mM MgATP. All assays were treated with (+) or without (–) thermolysin. Intact chloroplasts were recovered 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 orientated 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

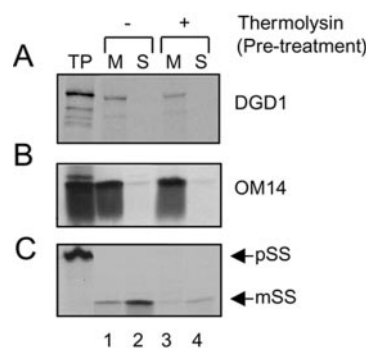


FIG. 2. Insertion of DGD1 into chloroplast envelope membranes does not require surface-exposed receptors. Pea chloroplasts were pretreated with (+) or without (–) thermolysin to degrade surface-exposed receptors. Thermolysin was quenched with 5 mM EDTA, and intact chloroplasts were recovered by centrifugation. 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).

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, stromata (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 imbedded within a membrane protein has proven difficult. We therefore attempted to find putative targeting sequences using various software tools (ChloroP Version 1.0 and

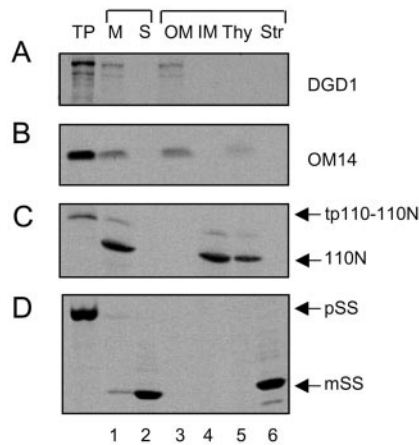


FIG. 3. DGD1 is localized to the outer envelope membranes of chloroplasts. 35 S-labeled DGD1 (A), OM14 (a marker for the outer envelope marker; B), tp100-110N (an inner envelope marker; C), and pSS (a stroma-targeted protein; D) were incubated with isolated pea chloroplasts in separate import assays. Intact chloroplasts from an aliquot of each assay were recovered by centrifugation through a Percoll cushion, lysed, and separated into total membrane (M) and soluble (S) fractions (lanes 1 and 2). Chloroplasts from the remaining portion of the import reaction were likewise recovered, lysed, and fractionated by sucrose step gradient centrifugation into outer envelope membranes (OM), inner envelope membranes (IM), thylakoids (Thy), and stromata (Str) (lanes 3–6). 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). In C, arrows indicate the precursor and mature forms of 110N.

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–Ala³³ 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, black boxes). 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 Pro³³⁷ (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 and ultracentrifugation (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

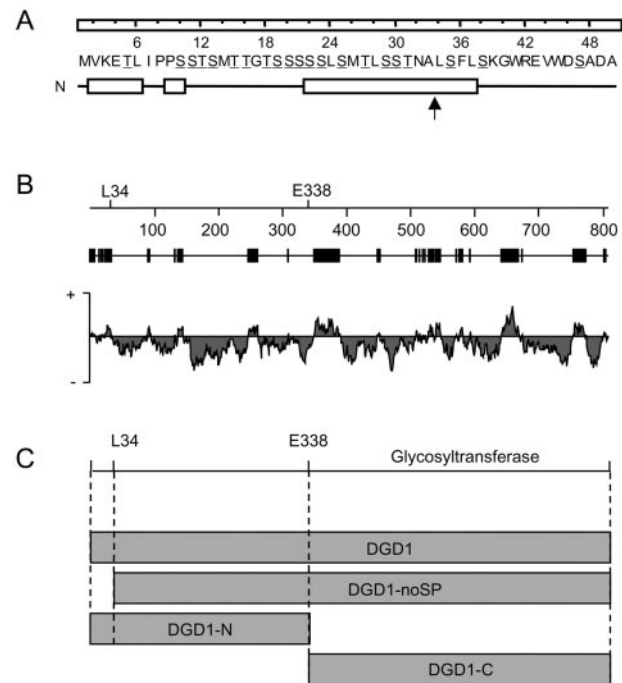


FIG. 4. Sequence characteristics of DGD1. A, characteristics of the putative N-terminal transit peptide sequence of DGD1 (amino acids 1–50). Hydrophobic regions are indicated by open boxes. Serine and threonine residues are underlined. The end of the putative targeting sequence is indicated with an arrow. B, hydrophobicity plot of DGD1. The method of Kyte and Doolittle (45) was used with an average residue window of 19 to determine hydrophobic regions in DGD1. Amino acid numbering is shown on top, including Leu³⁴ and Glu³³⁸, which indicate the end of the putative targeting sequence and the start of the glycosyltransferase domain, respectively. Hydrophobic regions of DGD1 are shown as black boxes, and their localization is shown in a Kyte and Doolittle plot. +, hydrophobic amino acid residues; –, hydrophilic amino acid residues. C, truncated constructs of DGD1. Partial cDNA clones were generated from DGD1 lacking the apparent N-terminal targeting sequence (DGD1-noSP) or encoding the glycosyltransferase domain (DGD1-C) or the N-terminal domain (DGD1-N).

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, stromata (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 (Env), stromata, 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-

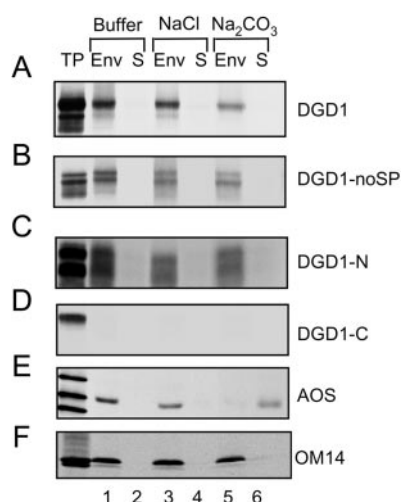


FIG. 5. Truncated forms of DGD1 containing N-terminal domains specifically insert into the chloroplast envelope membrane. ³⁵S-labeled DGD1 (A), DGD1-noSP (B), DGD1-N (C), DGD1-C (D), allene-oxide synthase (AOS; E), and OM14 (F) were used for uptake experiments with pea chloroplasts. After a 30-min incubation, 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.

tein that localized to both the pea outer envelope (Fig. 6A, lane 1) and thylakoid (lane 4) fractions. The fractionation pattern of DGD1 was identical to that of Toc75, a protein known to be localized to the outer envelope of pea chloroplasts (36). The presence of Tic110, DGD1, and Toc75 in the thylakoid fraction is not surprising because thylakoids are usually contaminated with outer and inner envelopes. Interestingly, DGD1 was not detected in the pea inner envelope fraction, whereas Tic110 was readily detected (Fig. 6A, compare lanes 1 and 2). Likewise, DGD1 was detected in the total envelope fraction of *Arabidopsis* (Fig. 6B, lane 1). However, further separation into outer and inner envelopes could not be achieved due to limitations in the availability of *Arabidopsis* chloroplasts.

The N-terminal 33 Amino Acids in DGD1 Are Not Required for Functional DGDG Synthase Activity—Whereas amino acids 22–38 of DGD1 contain a stretch of hydrophobic residues, which could be important for protein-membrane interactions, the N-terminal 33 amino acids of DGD1 were not required for targeting to chloroplasts (Figs. 4 and 5). Hence, we considered the possibility that this hydrophobic portion of the N terminus may play a role in the assembly of a functionally active enzyme within the envelope. We therefore expressed the entire open reading frame of DGD1 as well as a truncated version of DGD1 missing the N-terminal 33 amino acids (DGD1-noSP) in a recombinant expression system. We chose *Synechocystis* sp. PCC6803 since cyanobacteria contain high amounts of MGDG, which has been shown to be a required substrate for DGD1 (10). Lipids were isolated and quantified from a control culture and from DGD1- and DGD1-noSP-expressing cyanobacterial cells. The two lines expressing DGD1 and DGD1-noSP were both capable of converting MGDG to DGDG because they contain strongly reduced amounts of MGDG and a 2-fold increase in DGDG (Table I). We did not find any difference in lipid or fatty acid composition between these two lines. The fatty acid composition of the two galactolipids in the two overexpressing

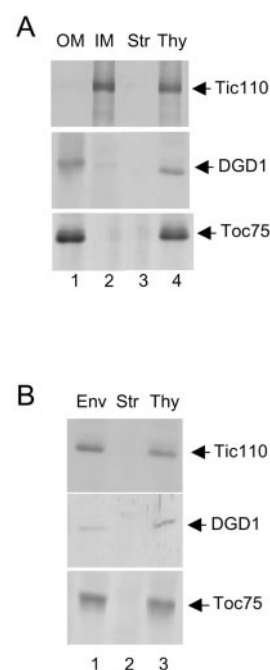


FIG. 6. Endogenous DGD1 is localized to the envelopes of pea and *Arabidopsis* chloroplasts. Pea (A) and *Arabidopsis* (B) chloroplasts were isolated, fractionated, and analyzed by Western blotting. For pea chloroplasts, a detailed fractionation analysis was performed in which a large amount of chloroplasts 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
*Lipid composition of *Synechocystis* sp. PCC6803 overexpressing *Arabidopsis* DGD1*

Lipids were extracted from *Synechocystis* control cells and from cells overexpressing the entire DGD1 open reading frame or a truncated form of DGD1 lacking the N-terminal 33 amino acids (DGD1-noSP). Lipid classes were separated by thin-layer chromatography and quantified by gas chromatography of fatty acid methyl esters. The values indicate the means ± S.D. of three measurements.

	Control	DGD1	DGD1-noSP
	mol %	mol %	mol %
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, 16:1^{Δ9}, 18:2^{Δ9,12}, and 18:3^{Δ6,9,12} 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/MGD1, 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 Met¹–Ala³³ 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 glycosyltransferase 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 Leu³⁴ and Pro³³⁷ 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.

Acknowledgments—We thank Dr. K. Keegstra for critical reading of the manuscript and for graciously allowing part of this work to be carried out in his laboratory. We thank Marlene Cameron and Kurt Stepnitz for preparation of figures.

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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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J. Biol. Chem. 2001, 276:31806-31812.

doi: 10.1074/jbc.M104652200 originally published online June 27, 2001

Access the most updated version of this article at doi: [10.1074/jbc.M104652200](https://doi.org/10.1074/jbc.M104652200)

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