The galactolipid digalactosyldiacylglycerol (DGDG), one of the main chloroplast lipids in higher plants, is believed to be synthesized by the galactolipid:galactolipid galactosyltransferase, which transfers a galactose moiety from one molecule of monogalactosyldiacylglycerol (MGDG) to another. Here, we report that Arabidopsis as well as other plant species contain two genes, DGD1 and DGD2, encoding enzymes with DGDG synthase activity. Using MGDG and UDP-galactose as substrates for in vitro assays with DGD2 we could for the first time measure DGDG synthase activity of a heterologously expressed plant cDNA. UDP-galactose, but not MGDG, serves as the galactose donor for DGDG synthesis catalyzed by DGD2, providing clear evidence for the existence of a UDP-galactose-dependent DGDG synthase in higher plants. In in vitro assays, DGD2 was capable of galactosylating DGDG, resulting in the synthesis of an oligogalactolipid tentatively identified as trigalactosyldiacylglycerol. DGD2 mRNA expression in leaves was very low but was strongly induced during growth under phosphate-limiting conditions. This induction correlates with the previously described increase in DGDG during phosphate deprivation. Therefore, in contrast to DGD1, which is responsible for the synthesis of the bulk of DGDG found in chloroplasts, DGD2 apparently is involved in the synthesis of DGDG under specific growth conditions.

In contrast to animals, yeast, and many bacteria, plant membranes contain high amounts of the two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) (1–3). These galactolipids are highly abundant in thylakoids, intricate membrane systems in chloroplasts containing the complexes of photosystem I and II (4–17). Biochemical studies with spinach envelope membranes suggested that another MGDG molecule, but not UDP-galactose, is used as the galactose donor for DGDG synthesis. The isolation of Arabidopsis mutants deficient in MGDG or DGDG has advanced our current understanding of the biosynthesis and function of galactolipids in higher plants (14, 15). MGDG and DGDG were found to be critical for functional intactness of the complexes of photosystems I and II (14–17). By complementation of the DGDG-deficient dgd1 mutant, the first gene encoding a DGDG synthase was isolated (18). This gene, DGD1, is critical for synthesis of the bulk amount of DGDG found in chloroplasts (14). Several lines of evidence point to the direction that in Arabidopsis, besides DGD1, at least one additional enzyme is involved in the synthesis of DGDG as follows. (i) The dgd1 mutant, which presumably represents a null mutation caused by the introduction of a premature stop codon into the coding region of the DGD1 gene, still contains residual amounts of DGDG (18). (ii) Chloroplasts isolated from dgd1 plants were capable of synthesizing DGDG in similar amounts as compared with wild type (14). (iii) Upon phosphate deprivation, wild type and dgd1 mutant plants accumulate high amounts of DGDG (20). The additional amount of DGDG produced during phosphate deprivation was suggested to be associated with the chloroplast as well as with extraplastidic membranes, where it presumably substitutes for phospholipids. In Arabidopsis as well as in many other plants (so-called 16:3 plants), the two galactolipids MGDG and DGDG differ with regard to their fatty acid composition. MGDG, which is mostly derived from diacylglycerol assembled inside the chloroplast (“prokaryotic-type lipid”), is rich in hexadecatrienoic acid (16:3), whereas DGDG contains fatty acids originating from the endoplasmic reticulum and, thus, is enriched in α-linolenic acid (18:3) (“eukaryotic type lipid”) but devoid of 16:3 (3, 21). In contrast, only the eukaryotic lipid synthesis pathway seems to be operational in the group of 18:3 plants. The difference be-

MGDG and DGDG are synthesized by sequential galactosylation of diacylglycerol (e.g. Refs. 4 and 5). Only in the recent past, the first genes encoding MGDG synthase and DGDG synthase were isolated (for reviews see Ref. 6 and Footnote 2). In Arabidopsis thaliana, three genes (MGD1, MGD2, MGD3) are known encoding MGDG synthases. The corresponding enzymes transfer a galactose moiety from UDP-galactose to diacylglycerol (8–11). The synthesis of DGDG in plants is assumed to involve the galactosylation of MGDG by the galactolipid:galactolipid galactosyltransferase (EC 2.4.1.184; Refs. 12 and 13). Biochemical studies with spinach envelope membranes suggested that another MGDG molecule, but not UDP-galactose, is used as the galactose donor for DGDG synthesis.

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between these two pathways may be explained by distinct substrate specificities or by different locations of the enzymes involved in galactolipid synthesis in the envelope membranes. In fact, MGD1 from spinach and Arabidopsis was not specific for different diacylglycerol substrates and was localized to the inner chloroplast envelope (9), whereas MGD2 and MGD3 from Arabidopsis were highly active with eukaryotic type substrate and may be localized to the outer envelope (11). The DGD1 protein as well as the galactolipid:galactolipid galactosyltransferase activity was localized to the outer chloroplast envelope (22–24).

On chromosome 4 of the Arabidopsis genome, a second putative DGDG synthase gene tentatively designated DGD2 was identified based on high sequence similarity to DGD1 (18). We isolated the corresponding cDNA from Arabidopsis for functional analysis of enzyme activity and substrate specificity after heterologous expression in Escherichia coli to test whether this gene encodes a second DGDG synthase and to unravel its relation to the galactolipid:galactolipid galactosyltransferase activity. Furthermore, we analyzed DGD2 expression during phosphate deprivation to address the question of whether DGD2 might be involved in regulation of DGDG synthesis during growth under phosphate-limiting conditions.

EXPERIMENTAL PROCEDURES

Isolation of DGD2 cDNAs from Arabidopsis—The expressed sequence tag (EST) 176K12T7 (Arabidopsis Biological Resource Center, Columbus, OH) corresponding to the DGD1-like gene P6N23.24 (Gen-Bank™ accession number AF058919) on chromosome 4 of Arabidopsis was used for screening different cDNA libraries. The cDNA clone 4-1-1 was isolated from a Zpllox cDNA library derived from mixed Arabidopsis tissues (25). Clone 16 originates from a cotyledon cDNA Azap1I library (26). Sequencing of the two cDNA clones revealed that clone 4-1-1 was derived from the same transcribing start site as clone 16 but was different in the first intron-splicing site. In contrast to clone 16, an extra 34 nucleotides containing the start codon were deleted in clone 4-1-1 during splicing. Because the longest open reading frame of clone 4-1-1 encodes a truncated, presumably inactive enzyme, only the clone 16 harboring a full-length open reading frame was used for further analysis.

Plant Growth Conditions and Origin of Mutants—A. thaliana wild type plants (Col-2) were grown according to Dörmann et al. (14). Arabidopsis mutants used in this study were described previously (14, 28).—Plant Growth Conditions and Origin of Mutants—A. thaliana wild type plants (Col-2) were grown according to Dörmann et al. (14). Arabidopsis mutants used in this study were described previously (14, 28).

Expression of DGD2 in E. coli—The entire coding region of the DGD2 cDNA clone 16 was amplified by PCR using the oligonucleotides PD25 (ATG GAT CCG ACG CAT CAG GAG CCA) and PD26 (GCG GUA CCA ACT CGT TGC TGC). The PCR product was ligated into the BamHI, KpnI sites of pACYC31, a derivative of pACYC184 (29) containing the XhoI, PvuII fragment with the expression cassette of pQE31 (Qiagen Inc. (18)). This construct was transferred into XL1-Blue cells harboring the cucumber MGD1 cDNA on pGEX-3X (8) for co-expression of DGD2 with MGD1. Likewise, the fragment amplified from the DGD2 cDNA clone 16 with the oligonucleotides PD25 and PD26 was ligated into the BamHI, KpnI sites of pQE31 (Qiagen Inc.) and transferred into E. coli M15(pREP4). For in vitro enzyme assays, E. coli cells expressing DGD2 on pQE31 were lysed by sonication. After centrifugation, the E. coli cell pellet containing membrane proteins was resuspended in 10 mM Tris, pH 7.2, 20% (v/v) glycerol and stored at –70 °C. The protein was purified after solubilization with 30 mM Triton X-100 using Ni2+ affinity chromatography according to the manufacturer’s protocol (Qiagen Inc.).

Substrates and Standards for in Vitro Assays—Nonradioactive lipids were isolated from Arabidopsis Col-2 wild type or act1 mutant plants (28) after extraction with chloroform/methanol according to Bligh and Dyer (30). Lipids were separated by thin-layer chromatography and, after isolation from the plate, were eluted into chloroform/methanol (2:1, v/v). Lipids were quantified by gas chromatography of fatty acid methyl esters according to Browse et al. (31). The radioabeled substrates UDP-[U-14C]galactose (10.286 GBq/mmole), UDP-[U-14C]glucosamine (11.211 GBq/mmole), and [1-14C]acetate (74 MBq/mmole) were obtained from PerkinElmer Life Sciences. Lipids were radioabeled by incubating 14-day-old Arabidopsis wild-type seedlings overnight in 20 mM MES-KOH, pH 6.0, with 50 μM of [1-14C]acetate. Radioabeled lipids were extracted, and [14C]MGDG and [14C]DIGDG were isolated by thin-layer chromatography as described above. The specific radioactivity of the 14C-labeled lipid was calculated after quantifying radioactivity by scintillation counting, and lipid was quantified by gas chromatography of fatty acid methyl esters.

MGDG synthases were isolated from Arabidopsis protoplasts according to Fitzpatrick and Keegstra (32). Lipid extracts from chloroplasts were used as standards for oligogalactolipids, because they were found to be enriched in TriMGDG and TetraMGDG.3

DGDG Synthase Assay—DGDG synthase assays with different substrates were done with recombinant E. coli membrane proteins according to Heemskerk et al. (10) with some alterations. In vitro assays used the following reaction mix: 10 mM Tris, pH 7.2, 30 mM MgCl2, 3.0 mM dithiothreitol, 7.5 mM sodium deoxycholate. E. coli protein extract (50 μl) was added, and the reaction was started with 50 μl of UDP-[U-14C]galactose (288 pmol) or 50 μl of nonradioactive UDP-galactose (2 μmol). The reaction was stopped by extracting lipids with chloroform/methanol (2:1, v/v). Nonradioactive DGDG was added to the extract before separation by thin-layer chromatography for the exact identification of the DGDG band. After staining with iodine vapor, radioactive lipids were visualized by autoradiography, or the silica matrix containing DGDG was further analyzed.

RESULTS

Sequence Characteristics of DGD2 from A. thaliana—In addition to the DGDG synthase DGD1 on chromosome 3, a second, DGD1-like gene (P6N23.24, designated DGD2 (18)) was identified on chromosome 4 of Arabidopsis. The corresponding DGD2 cDNA was isolated from an Arabidopsis cotyledon library to determine if this gene also encodes a functional DGDG synthase.

The DGD2 cDNA contains an open reading frame coding for a 473-amino acid protein (Fig. 1A). The protein sequence of DGD2 is very similar to the C-terminal, glycosyltransferase-like part of DGD1 (50.8% identity) but is not related to the N-terminal part of DGD1 (Fig. 1B). Data base searches with DGD1 and DGD2 revealed that there is an abundance of EST sequences from different plant species in the data bases. Amino acid sequences derived from ESTs of several plant species (e.g. tomato, rice, Medicago) clustered in two classes based on their degree of sequence similarity to either Arabidopsis DGD1 or DGD2 (Fig. 1C). The presence of at least two genes, DGD1 and DGD2, for DGDG synthesis seems to be common to many species, including 16:3 plants (Arabidopsis, tomato, rice), 18:3 plants (Medicago, monocotyledons (rice), and dicotyledons (Arabidopsis, tomato, Medicago).

Co-expression of DGD2 with Cucumber MGD1 Results in Accumulation of DGDG in E. coli—Based on biochemical studies, DGDG synthase activity was found to be dependent on the presence of MGDG as a substrate (13). We therefore expressed the DGD2 cDNA using an E. coli strain that already harbored the MGDG synthase MGD1 from cucumber (8). This E. coli strain contains high amounts of MGDG and was already employed to analyze the DGDG synthase activity of DGD1 (18). When both cucumber MGD1 and Arabidopsis DGD2 were expressed in E. coli, a large fraction of MGDG was converted to a...
new lipid band co-migrating with a plant DGDG standard (Fig. 2, lane 5). This lipid was stained positive with α-naphthol indicating the presence of sugars in its head group (data not shown). Therefore, DGD2 encodes an enzyme with DGDG synthase activity. The amount of DGDG accumulating in E. coli cells expressing MGD1 and DGD2 was much larger than in cells expressing MGD1 and Arabidopsis DGD1 (Fig. 2, lanes 4 and 5). This may be caused by different specific activities or by different expression levels of the recombinant proteins. Expression of DGD2 without MGD1 did not result in the production of DGDG (Fig. 2, lane 2). Therefore, similar to DGD1 (14), the DGD2 reaction depends on the presence of MGDG, but diacylglycerol cannot serve as an acceptor molecule for galactosylation. This result is in accordance with biochemical data obtained for the DGDG synthase of spinach chloroplasts (13).

Whereas the first galactose moiety in DGDG of higher plants is linked in β-glycosidic bond to diacylglycerol, the second galactose is bound in α-glycosidic linkage (37). To elucidate the anomic configuration of the second galactose moiety of DGDG synthesized by DGD2, we isolated DGDG from a large culture of E. coli cells co-expressing cucumber MGD1 and Medicago truncatula DGD2 (cf. Fig. 2, lane 5) for 1H NMR analysis (Fig. 3). Doublet signals at high chemical shift (around 5.0 ppm) and low chemical shift (around 4.0 ppm) are characteristic for glycosidic carbon atoms in α and β-glycosidic configuration, respectively (38, 39). As depicted in Fig. 3, peak doublets at 4.7 and 4.0 ppm were detected in the 1H NMR spectra of authentic plant DGDG as well as of DGDG isolated from recombinant E. coli cells. Therefore, DGDG produced by recombinant MGD1 and DGD2 contains one galactose linked in β-glycosidic linkage to diacylglycerol and a second one in α-glycosidic linkage, suggesting that it is structurally identical to the authentic plant lipid, 1,2-diacyl-3-O-(α-D-galactopyranosyl-(1→6)-O-β-D-galactopyranosyl)-sn-glycerol.

UDP-galactose, but not MGDG, Is the Galactose Donor for DGD2-mediated Galactosylation of MGDG—DGD2 was heterologously expressed in E. coli without the cucumber MGD1 cDNA to analyze its substrate specificity. A large fraction of the recombinant DGD2 protein was found to be associated with an insoluble inclusion body/membrane fraction (data not shown). After solubilization with deoxycholate, this protein extract was used for in vitro DGDG synthase assays. To elucidate the nature of the galactose donor for the DGDG synthase reaction, recombinant DGD2 was incubated with MGDG in the presence or absence of UDP-galactose. As shown in Fig. 4, the presence of radioactive MGDG alone was not sufficient for DGDG synthesis, as would have been expected if MGDG would serve both as donor and acceptor of galactose (Fig. 4, lane 2). Only after the addition of nonradioactive UDP-galactose, DGDG was produced by recombinant DGD2 (Fig. 4, lane 1). In assays with nonradioactive MGDG and 14C-labeled UDP-galactose, radioactive DGDG was synthesized, again indicating that the galactose moiety of UDP-galactose was used for galactosylation of MGDG (Fig. 4, lane 3). No DGDG synthesis was observed in reactions without DGD2 protein or without MGDG (Fig. 4, lanes 4 and 5). In all assays containing radioactive UDP-galactose, an extra lipid was synthesized that was attributed to another protein of the E. coli extract (Fig. 4, lanes 3–6 and 8–9, Lipid X). Taken together, these results clearly demonstrate

**Fig. 1. Structural characteristics of DGD1 and DGD2 sequences.** A, structural alignment of DGD1 and DGD2 proteins. The region of amino acid sequence similarity to glycosyltransferases is depicted in gray. B, amino acid sequence comparison of the C-terminal part of DGD1 (top row) and DGD2 (lower row). The amino acid residues of DGD1 and those of DGD2 identical to DGD1 are highlighted in black. C, phylogenetic tree of amino acid sequence alignment of A. thaliana (At) DGD1 and DGD2 and ESTs from tomato (Le), rice (Os), and Medicago truncatula (Mt). Amino acid sequences derived from EST clones are indicated by their GenBankTM accession numbers. The alignment was done with the Clustal Method of MegAlign (DNASTAR, Lasergene Inc.).
that DGD2 encodes a UDP-galactose-dependent DGDG synthase.

DGD2 was isolated from recombinant E. coli cells to demonstrate that the DGDG synthase activity is indeed associated with this protein. After solubilization with Triton X-100, DGD2 was purified by nickel affinity chromatography and used for a DGDG synthase assay with nonradioactive MGDG and 14C-labeled UDP-galactose (Fig. 4, lane 7). Although the purified protein was also capable of synthesizing DGDG, its activity was much lower as compared with total E. coli membrane fractions and deteriorated during storage. For this reason, all further experiments were done with protein obtained from solubilized E. coli membranes.

The DGDG assay with MGDG and radioactive UDP-galactose consistently resulted in the synthesis of an additional lipid, co-migrating with a TriGDG standard obtained from isolated chloroplasts (Fig. 4, lanes 3, 6, and 10). If this lipid indeed was TriGDG, one would expect that it could also be synthesized by direct galactosylation of DGDG. Indeed, using nonradioactive MGDG and 14C-labeled UDP-galactose, a radioactive lipid was synthesized that co-migrated with TriGDG (Fig. 4, lane 8). The synthesis of this lipid was dependent on the presence of DGD2 protein (Fig. 4, lane 9) and UDP-galactose, because in DGD2 assays with 14C-labeled DGDG but without UDP-galactose no TriGDG formation was observed (data not shown). Therefore, the additional radioactive lipid band co-migrating with TriGDG was synthesized from MGDG or DGDG, and 14C-UDP-galactose and was tentatively identified as TriGDG.

**Enzymatic Characteristics of DGD2—**Enzyme assays of membrane proteins from E. coli expressing DGD2 with nonradioactive MGDG and 14C-UDP-galactose showed that the reaction was linear up to the addition of about 1.0 mg of protein/assay (Fig. 5A). We therefore used 0.66 mg of protein in all further assays. A broad pH optimum was found for DGD2, with a plateau between pH 6.0 and 7.5 (Fig. 5B). Fig. 5C shows the effect of substituting or adding different reagents to the standard DGD2 assay. DGD2 activity was dependent on the presence of UDP-galactose no TriGDG formation was observed (data not shown). Therefore, the additional radioactive lipid band co-migrating with TriGDG was synthesized from MGDG or DGDG, and 14C-UDP-galactose and was tentatively identified as TriGDG.

**FIG. 4.** Substrate specificity of the DGDG synthase DGD2. Membrane proteins were solubilized from E. coli cells expressing recombinant DGD2 with 3.75 mM sodium deoxycholate and incubated with different 14C-labeled and nonradioactive substrates to measure DGDG synthase activity. After incubation, lipids were extracted and separated by thin-layer chromatography, and products were visualized by autoradiography (lanes 1–9). In addition to the radioactive lipid bands co-migrating with MGDG, DGDG, and TriGDG, in some labeling experiments a further unknown lipid (Lipid X) was radioactively labeled by an enzyme present in the E. coli protein extract. 1, [14C]MGDG + nonradioactive UDP-galactose; 2, [14C]MGDG without addition of UDP-galactose; 3, nonradioactive MGDG + UDP-[14C]galactose; 4, UDP-[14C]galactose without addition of MGDG; 5, nonradioactive MGDG + UDP-[14C]galactose, empty vector control; 6, nonradioactive MGDG + UDP-[14C]galactose, E. coli protein solubilized with Triton X-100 instead of sodium deoxycholate; 7, nonradioactive MGDG + UDP-[14C]galactose, DGD2 protein purified by Ni2+ affinity chromatography; 8, nonradioactive DGDG + UDP-[14C]galactose; 9, nonradioactive DGDG + UDP-[14C]galactose, empty vector control; 10, total lipid extract from isolated chloroplasts, stained with a-naphthol.
UDP-galactose-dependent Digalactosyldiacylglycerol Synthase

**Fig. 5. Enzymatic characteristics of DGD2.** DGDG synthase activity of recombinant DGD2 protein was determined in a standard assay containing 7.5 mM Tricine-KOH, pH 7.2, 15 mM MgCl₂, 1.5 mM dithiothreitol, 3.75 mM sodium deoxycholate, 0.66 mg of E. coli protein, 50 nmoL of nonradioactive MGDG, and 288 pmol of UDP-[U-14C]galactose in 200 μL of total volume (cf. “Experimental Procedures”). Lipids were extracted and separated by thin-layer chromatography. After staining with iodine, DGDG was isolated from the plate, and the radioactivity was determined by scintillation counting. Values are presented as the average and S.E. of three measurements. A, DGDG synthase activity assayed with different amounts of protein extracted from E. coli cells expressing DGD2. For all further assays (B and C), 0.66 mg of protein was used. B, pH optimum of DGD2. The DGDG synthase assay was done in 7.5 mM of MES-KOH (pH 5.5, 6.0, 6.5, 7.0) and 7.5 mM Tricine-KOH (pH 7.2, 7.5, 8.0, 8.5, 9.0). C, effect of different reagents on DGDG synthase activity of DGD2. The standard assay (control, lane 1) was set to 100%. The enzyme activities after the addition of different detergents (lanes 2–9), MGDG substrate with an altered molecular species composition, or after replacing UDP-galactose with UDP-glucose (lanes 10 and 11) as well as after the addition of extra salt or extra lipid (lanes 13–15) are presented in % of control activity. 1, control (3.75 mM sodium deoxycholate); 2, 0.375 mM sodium deoxycholate; 3, 3.75 mM Triton X-100; 4, 0.375 mM Triton X-100; 5, 3.75 mM β-decylmaltoide; 6, 0.375 mM β-decylmaltoide; 7, 3.75 mM CHAPS; 8, 0.375 mM CHAPS; 9, without detergent; 10, MGDG isolated from act1 mutant; 11, 288 pmol UDP-[14C]galactose; 12, without MgCl₂; 13, 100 mM NaCl; 14, 25 mM phosphatidylcholine; 15, 25 nmoL of DGDG.

DGDG synthase activity of DGD2. We did not find a large effect on enzyme activity after the addition of salt (NaCl, Fig. 5C, lane 13) or extra lipids normally present in plastid envelopes (phosphatidylethanolamine and DGDG; Fig. 5C, lanes 14 and 15).

The fatty acid compositions of DGDG and its precursor MGDG in *Arabidopsis* are very different because MGDG contains high amounts of 16:3, a fatty acid that is largely absent from DGDG. To test if the difference in fatty acid composition of DGDG and its precursor, MGDG, may be caused by substrate specificity of DGDG synthases, recombinant DGD2 protein was incubated with MGDG isolated from *Arabidopsis* wild-type plants or from the *Arabidopsis* act1 mutant. In *Arabidopsis* wild type, MGDG contains high amounts of 16:3 in the sn-2 position and, thus is predominantly (about 72%) of prokaryotic origin (21). However, MGDG from the acyl-ACP:glycerol-3-phosphate acyltransferase-deficient mutant act1 is almost exclusively of eukaryotic origin and therefore devoid of 16:3 (28). As shown in Fig. 5C (lanes 1 and 10), DGDG synthesis by DGD2 with either of these two forms of MGDG lipid was very similar. Time course experiments also demonstrated that DGD2 does not show a strong preference for MGDG derived from wild type or act1 (data not shown).

**Induction of DGD2 Expression by Phosphate Deprivation**—A weak band at 1.8 kilobases corresponding to the full-length DGD2 mRNA was found to hybridize to DGD2 in Northern experiments (Fig. 6A). The mRNA signal was barely detectable in leaves, flowers, and roots and was basically absent from stems and siliques. There was no increase in DGD2 expression in *dgd1* mutant seedlings, indicating that there was no compensatory induction of DGD2 expression caused by the block in DGDG synthesis in this mutant. To elucidate the role of DGD2 during DGDG synthesis under phosphate limiting growth conditions (cf. Ref. 20), DGD2 expression was measured in seedlings of the *pha1* mutant of *Arabidopsis*. The *pha1* mutant carries a block in a phosphate translocator resulting in permanent phosphate starvation of the shoot (27). In agreement with the increased DGDG synthesis previously observed in *pha1* (20, 35), expression of DGD2 was strongly induced (Fig. 6B). To obtain direct evidence for the influence of phosphate availabil-
UDP-galactose-dependent Digalactosyldiacylglycerol Synthase

**Fig. 7. Galactosylation reactions catalyzed by DGD2.** MGDG contains one galactose bound in β-glycosidic linkage to diacylglycerol. DGD2 transfers a second galactose group from UDP-α-D-galactose onto MGDG in α-glycosidic linkage (solid arrow). MGDG cannot be used as galactose donor (dotted arrow). Therefore, DGD2 catalyzes the UDP-galactose-dependent galactosylation of MGDG with retention of the anomeric configuration of the second galactose group. Furthermore, in *in vitro* assays, a third galactose group can be attached to diacylglycerol resulting in the synthesis of TriDGG.

Several lines of evidence suggest that in addition to DGD1, at least one further DGDG synthase activity exists in *Arabidopsis* (14, 20). Indeed, in this study a second cDNA (*DGD2*) was isolated from *Arabidopsis* encoding a functionally active DGDG synthase. The amino acid sequence of DGD2 was very similar to the C-terminal, glycosyltransferase-like part of DGD1 (Fig. 1, A and B). The N-terminal part of DGD1, which is missing in DGD2, was recently shown to be involved in the targeting of the DGD1 protein to the outer envelope of chloroplasts (24). Because this part of the DGD1 sequence is not present in DGD2, it apparently is dispensable for DGDG synthase activity. The fact that no sequences similar to DGD1 or DGD2 are present in the cyanobacterial genome (40) suggests that DGDG synthase genes in higher plants have evolved independently from lipid biosynthesis of the endosymbiont. However, the close similarity of the two plant DGDG syntheses to glycosyltransferases of primary metabolism (e.g., sucrose synthase, sucrose-phosphate synthase) might indicate that the two genes were derived from primary metabolism of the plant host or the endosymbiont. For many plant species, both a DGD1-like and a DGD2-like EST sequence were found in GenBank™ (Fig. 1C). It has been speculated that the different lipid synthesis pathways present in 16:3 and 18:3 plants (eukaryotic/prokaryotic pathway) might be related to the presence of different isoforms of enzymes of galactolipid synthesis (e.g., Refs. 21 and 41). However, because ESTs similar to DGD1 as well as DGD2 were present in both 16:3 and 18:3 plants, it is unlikely that the DGDG synthase genes are responsible for differences in galactolipid synthesis between these groups of plants. Similarly, Awai *et al.* (11) showed that the two major types of MGDG synthase genes found in *Arabidopsis* (type A, MGD1; type B, MGD2/MGD3) are found in both 16:3 and 18:3 plants.

By measuring DGDG synthesis with recombinant DGD2 using MGDG and UDP-galactose as substrates, we could for the first time determine *in vitro* DGDG synthase activity of a heterologously expressed plant cDNA (Figs. 4 and 5). In addition to the detergent and lipid substrates employed (MGDG or DGDG), the DGDG synthase assays contained proteins and lipids normally found in *E. coli* membranes. If, similar to DGD1 (24), DGD2 was also localized to the chloroplast envelope, the DGD2 activity might depend on the presence of additional lipids of the envelope membranes. In contrast to *E. coli*, which is rich in phospholipids (phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin), chloroplast envelopes contain the glycolipids MGDG, DGDG, and sulfoquinovosyldiacylglycerol as well as the phospholipids PG and phosphatidylcholine (2). However, no stimulating effect was observed after the addition of phosphatidylcholine or DGDG, suggesting that DGD2 does not require interaction with these lipids for activation of enzyme activity.

Because DGDG synthase activity with MGDG isolated from wild type (mostly prokaryotic type MGDG) was very similar as compared with MGDG from the *act1* mutant (eukaryotic type MGDG), DGD2 does not exhibit strong preference for prokaryotic or eukaryotic substrates. Siebertz and Heinz (42) showed that the galactosylation of different MGDG forms by protein extracts from pea leaves was dependent on the total numbers of double bonds in the two fatty acid chains of MGDG. It was concluded that differences in molecular species composition of MGDG and DGDG in mature plants are not related to substrate specificity of the DGDG synthase. Similarly, no specificity for eukaryotic or prokaryotic diacylglycerol was observed for MGD1 of spinach or *Arabidopsis* (9, 15). MGD2 and MGD3, however, showed higher activity with eukaryotic type diacylglycerol. The eukaryotic form of MGDG synthesized by MGD2 and MGD3 supposedly accumulates in the outer chloroplast envelope (11), where it might serve as the substrate for DGDG synthesis. Therefore, the accumulation of eukaryotic type DGDG in *Arabidopsis* apparently is determined on the level of the synthesis of its precursor, MGDG.

Labeling studies with isolated spinach chloroplasts indicated that DGDG synthesis catalyzed by the galactolipid:galactolipid galactosyltransferase includes the transfer of one galactose moiety from one MGDG molecule to another (Fig. 7, dotted arrow; Refs. 12, 13, and 43). In contrast, many other glycosylation reactions in plants, including MGDG synthesis, were shown to be dependent on UDP sugars. In pea chloroplasts, Siebertz and Heinz (42) measured DGDG synthesis with MGDG and UDP-galactose. However, analyzing a variety of plant species including 16:3 and 18:3 plants (e.g., spinach, tobacco, corn, pea), Heemskerk *et al.* (13) could not find biochemical evidence for this alternative UDP-galactose-dependent galactosylation reaction (Fig. 7, solid arrow). In Fig. 4, we demonstrated that DGD2 was dependent on UDP-galactose,
because DGDG synthesis could not be established with MGDG as the sole donor and acceptor for galactosylase. Therefore, in contrast to previous studies (e.g., Refs. 12, 13, and 43), this result clearly demonstrates that Arabidopsis contains a UDP-galactose-dependent DGDG synthase. Furthermore, other enzymes of galactolipid synthesis (e.g., DGD1) might be responsible for the galactolipid:galactolipid galactosyltransferase activity detectable in isolated chloroplasts. The transfer of one galactose from one MGDG molecule to another (Fig. 7, dotted line) would require the inversion of the anomeric carbon of the second galactose moiety, because its configuration would have to be changed from $\beta$ in MGDG to $\alpha$ in DGDG. However, as demonstrated in this study, the transfer of galactose from UDP-galactose to MGDG as catalyzed by DGD2 (Fig. 7, solid arrow) involves retention of the $\alpha$-configuration of the second galactose group (cf. Fig. 3). The retention of the anomeric configuration is in accordance with classification systems of plant glycosyltransferases developed by and Campbell et al. (44) and Henrisat and Davies (45). Enzymes that fall into the same family not only share a high degree of sequence similarity but exhibit similar structural characteristics and follow the same glycosyltransferase mechanism. The DGDG synthases DGD1 and DGD2 can be assigned to the family GT4 containing glycosyltransferases of primary metabolism (45). Enzymes of this class were shown to transfer sugar moieties with retention of the anomeric configuration. The sequence similarity of DGD2 to these glycosyltransferases is in accordance with the finding that DGD2 encodes a UDP-galactose-dependent DGDG synthase that transfers galactose to MGDG with retention of the $\alpha$-anomeric configuration.

After incubation of DGD2 with MGDG or DGDG and radioactive UDP-galactose, we observed the synthesis of a radioactively labeled DGDG (data not shown), indicating that DGDG was not hydrolyzed to MGDG by a galactosidase activity derived from the E. coli protein extract. Incorporation of radioactivity into DGDG might be explained by a head group exchange reaction, where one galactose group of DGDG is released in a reverse DGD2 reaction, resulting in synthesis of MGDG, which might rapidly be re-galactosylated with radioactive UDP-galactose. Previously, glycosyltransferases were shown to be capable of catalyzing the sugar transfer in two directions depending on the relative concentrations of substrates and products (7).

The identification of DGD2 as a second, DGD1-independent DGDG synthase in Arabidopsis and presumably also in many higher plants leaves us with the question of the exact function of this additional enzyme in plant lipid metabolism. Based on the very low DGD2 expression level in plants grown under normal conditions (Fig. 6) and on the fact that a mutation in DGD1 leads to a 90% reduction of DGDG in chloroplasts (14), one has to conclude that DGD1 and not DGD2 represents the enzyme responsible for synthesizing the predominant fraction of DGDG in chloroplasts. The strong DGD2 mRNA induction and increase of DGDG synthesis after phosphate deprivation (20) suggests that DGD2 is involved in synthesizing DGDG during phosphate deprivation. Recently, Awai et al. (11) demonstrated that the MGDG synthase genes MGD2 and MGD3 of Arabidopsis are induced by phosphate deprivation. Therefore, during growth under phosphate-limiting conditions, several enzymes of galactolipid biosynthesis are induced that finally results in massive DGDG production. The finding that DGD2 represents a UDP-galactose-dependent DGDG synthase induced by phosphate starvation raises the question of the exact reaction mechanism of DGD1 and its role during growth under phosphate-limiting conditions. The additional N-terminal part present in DGD1 might not only be critical for protein targeting (cf. Ref. 24) but may as well be involved in modifying enzyme activity or substrate specificity. Further experiments are therefore required to elucidate the exact contribution of the two different DGDG synthase pathways as well as the intracellular location and transport of galactolipids in higher plants.

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