Digalactosyldiacylglycerol Synthesis in Chloroplasts of the Arabidopsis *dgd1* Mutant[1]

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Galactolipid biosynthesis in plants is highly complex. It involves multiple pathways giving rise to different molecular species. To assess the contribution of different routes of galactolipid synthesis and the role of molecular species for growth and photosynthesis, we initiated a genetic approach of analyzing double mutants of the digalactosyldiacylglycerol (DGDG) synthase mutant *dgd1* with the acyltransferase mutant, *act1*, and the two desaturase mutants, *fad2* and *fad3*. The double mutants showed different degrees of growth retardation: *act1,dgd1* was most severely affected and growth of *fad2,dgd1* was slightly reduced, whereas *fad3,dgd1* plants were very similar to *dgd1*. In *act1,dgd1*, lipid and chlorophyll content were reduced and photosynthetic capacity was affected. Molecular analysis of galactolipid content, fatty acid composition, and positional distribution suggested that the growth deficiency is not caused by changes in galactolipid composition per se. Chloroplasts of *dgd1* were capable of synthesizing monogalactosyldiacylglycerol, DGDG, and tri- and tetragalactosyldiacylglycerol. Therefore, the reduced growth of *act1,dgd1* and *fad2,dgd1* cannot be explained by the absence of DGDG synthase activity from chloroplasts. Molecular analysis of DGDG accumulating in the mutants during phosphate deprivation suggested that similarly to the residual DGDG of *dgd1*, this additional lipid is synthesized in association with chloroplast membranes through a pathway independent of the mutations, *act1*, *dgd1*, *fad2*, and *fad3*. Our data imply that the severe growth defect of *act1,dgd1* is caused by a reduced metabolic flux of chloroplast lipid synthesis through the eukaryotic and prokaryotic pathway as well as by the reduction of photosynthetic capacity caused by the destabilization of photosynthetic complexes.

The two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), are the major lipids of photosynthetic membranes (Browse and Somerville, 1991; Joyard et al., 1998). Their biosynthesis is complex. Two parallel pathways for the production of diacylglycerol, the precursor for galactolipid biosynthesis, are present in the plastid and the endoplasmic reticulum (ER) of plants such as Arabidopsis (Heinz and Roughan, 1983; Browse et al., 1986). The prokaryotic pathway is restricted to the plastid, whereas the eukaryotic pathway involves reactions at the plastid and the ER. This requires bidirectional transport of lipid moieties between the ER and the plastid with the actual mechanisms remaining obscure. Thylakoid lipids derived from each of the two pathways can be distinguished by their fatty acid composition such that certain molecular species are diagnostic for the responsible pathway (Heinz and Roughan, 1983). Their specific occurrence and high abundance in chloroplasts implies that galactolipids are important for the integrity of the phototsynthetic complexes in the thylakoids. However, not much is known about the role of the individual molecular species of galactolipids for the functionality of light absorption and electron transfer during photosynthesis or about their localization in different suborganellar membranes.

Mutants of Arabidopsis deficient in different aspects of lipid biosynthesis are available (Browse and Somerville, 1994; Vijayan et al., 1998) and can be used to study the genetic interaction between different loci encoding enzymes of thylakoid lipid biosynthesis. In the *dgd1* mutant, the biosynthesis of DGDG is impaired, resulting in a reduction in DGDG content by 90% (Dörmann et al., 1995). Because the molecular analysis of the *dgd1* mutant allele suggested complete inactivation of the *DGD1* gene (Dörmann et al., 1999), another reaction must be responsible for the biosynthesis of the residual DGDG in the *dgd1* mutant. Clear evidence for a DGD1-independent pathway of DGDG synthesis came from the analysis of Arabidopsis plants raised under phosphate-limiting conditions (Härtel et al., 2000). The fact that DGDG synthesis was increased under phosphate deficiency in the *dgd1* mutant strongly suggested the existence

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of a second, DGD1-independent pathway. Membrane fractionation experiments indicated that under phosphate starvation, DGDG also accumulated in extraplastidic membranes.

The *act1* mutant of Arabidopsis is deficient in the plastidic acyl-ACP:glycerol-3-phosphate acyltransferase leading to the inactivation of the prokaryotic pathway (Kunst et al., 1988). We recently constructed an Arabidopsis *act1,dgd1* double mutant as part of our effort to understand the function of the *DGD1* gene (Dörmann et al., 1999). The severe growth phenotype of the *act1,dgd1* double mutant was suggested to be caused by a strong reduction in overall membrane biosynthesis.

The aim of this study was: (a) to analyze the contribution of the different pathways to the overall metabolic flux of galactolipid biosynthesis under normal growth conditions, (b) to characterize the contribution of the different pathways to the synthesis of galactolipids in plants raised under phosphate-limiting growth conditions, and (c) to unravel the function of the molecular species of galactolipids derived from the different pathways for plant growth and photosynthesis. For this purpose, we initiated a series of experiments including further studies to address the possible causes for the severe growth phenotype observed for *act1,dgd1*. Changes of chloroplast lipid biosynthesis might lead to a decreased photosynthetic capacity of the double mutant as compared with *dgd1*. Therefore, we measured total lipids and photosynthetic pigments, analyzed chloroplast ultrastructure, and recorded chlorophyll fluorescence for *act1,dgd1* plants. Additional double mutant lines (*fad2,dgd1* and *fad3,dgd1*) were constructed to address the question of whether metabolic flux through different lipid biosynthesis pathways, the amounts of the lipids, or fatty acid composition of the residual DGDG are changed in mutant lines homozygous for *dgd1*. *FAD2* and *FAD3* encode 18:1 and 18:2 desaturases, respectively, which are both localized at the ER (Miquel and Browse, 1992; Browse et al., 1993). In the *fad2* mutant, thylakoid lipids derived from the eukaryotic pathway as well as extraplastidic lipids are affected and show an altered fatty acid composition (Miquel and Browse, 1992), whereas in the *fad3* mutant, only extraplastidic lipids but none of the thyalkoid lipids are altered (Browse et al., 1993). Furthermore, chloroplasts isolated from wild type and *dgd1* were compared for their capability of galactolipid synthesis to find out if the residual DGDG found in *dgd1* homozygous lines might still be synthesized in the chloroplast. Finally, we compared the fatty acid composition and positional distribution of the residual amount of DGDG of the double mutants with that of plants raised under phosphate-limiting conditions to address the question of whether these functionally important pools of DGDG are synthesized by related pathways.

RESULTS

Double Mutants of *dgd1* with Mutants in the Prokaryotic (*act1*) or Eukaryotic Pathway (*fad2,fad3*) Are to Different Extents Affected in Growth

The *act1,dgd1* double mutant carrying a block in the plastid acyl-ACP:glycerol-3-phosphate acyltransferase and in the DGDG synthase DGD1 was severely affected in growth (Dörmann et al., 1999; Fig. 1). The *act1,dgd1* plants could be propagated on Suc-supplemented medium, but were virtually unable to survive on soil (Fig. 1), suggesting that this mutant is incapable of photoautotrophic growth. To further dissect residual DGDG synthesis in *dgd1*, we generated double mutants between *dgd1* and the mutants *fad2* and *fad3* affected in the activity of ER-localized desaturases. The *fad2,dgd1* mutant was impaired in growth as compared with *dgd1* and produced only very few seeds. The plants grew better on Suc-supplemented medium, and only a small fraction survived on soil (Fig. 1). In contrast to *fad2,dgd1*, the *fad3,dgd1* mutant was very similar in growth to the *dgd1* single mutant, could be maintained on soil, and was fertile (Fig. 1).

The Amount of Total Lipids and Photosynthetic Membranes Is Drastically Reduced in *act1,dgd1*

To estimate the capacity of overall lipid biosynthesis of the most severely affected line, *act1,dgd1*, the total amount of fatty acids per gram leaf fresh weight was determined (Fig. 2). Total fatty acids were slightly reduced in *dgd1* as compared with wild type

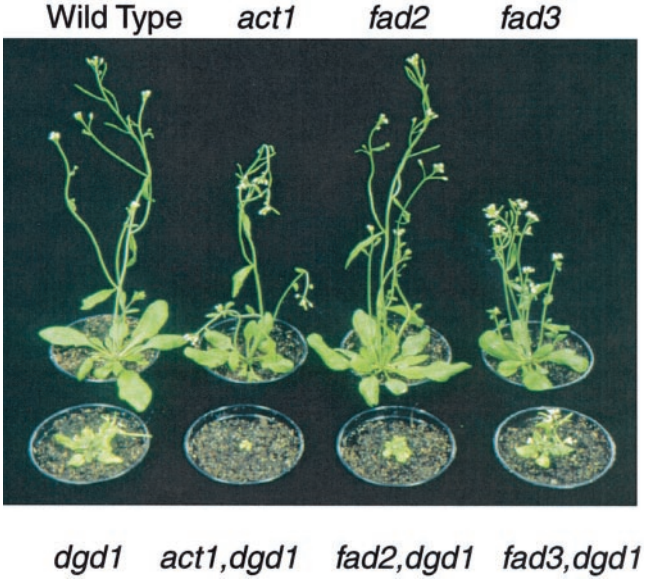

Figure 1. Visible phenotype of *dgd1* and double mutants. Six-week-old plants raised on soil are shown for Arabidopsis wild type, *act1*, *fad2*, and *fad3* (top, from left to right) as well as *dgd1* and the double mutants *act1,dgd1*, *fad2,dgd1*, and *fad3,dgd1* (bottom, from left to right).

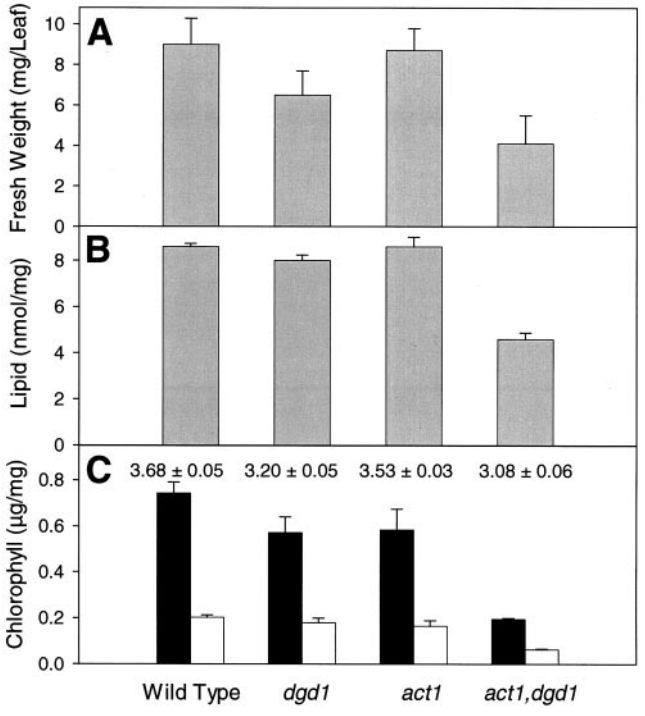

Figure 2. Amounts of lipid and photosynthetic pigments of the *act1,dgd1* double mutant. A, Fresh weight per rosette leaf. B, Lipid measured as nanomoles fatty acid per milligram leaf fresh weight. C, Chlorophyll a (black bars) and chlorophyll b (white bars) in leaves in micrograms per milligram fresh weight The numbers indicate the chlorophyll a to b ratio in each line. All values represent averages ± SE of four measurements. Plants were grown on solidified Murashige and Skoog medium supplemented with Suc.

and *act1*, but were reduced to about 50% of wild-type amounts in *act1,dgd1*. Also taking into consideration the reduced fresh weight per leaf in the double mutant (Fig. 2), one can conclude that overall lipid biosynthesis is severely affected far beyond what is observed for *dgd1*. We quantified photosynthetic pigments in wild type, *dgd1*, *act1*, and *act1,dgd1* (Fig. 2) to estimate the effect of the reduction in overall lipid biosynthesis on the amount of photosynthetic membranes. Total chlorophyll, which was reduced in *dgd1* and in *act1*, was even further decreased in the double mutant to approximately 25% of wild type. The chlorophyll a/b ratio was also decreased in *act1,dgd1*, but was similar to *dgd1*. A decrease of total chlorophyll content without changes in the chlorophyll a/b ratio in *act1,dgd1* indicates that the ratio of light-harvesting antenna to the reaction center/core complexes remains constant; therefore, all pigment-protein complexes, including photosystem (PS) I and II, are decreased in parallel. To study the reduction of photosynthetic membranes in *act1,dgd1* at the level of chloroplasts, light and electron microscopic analysis was done with leaf thin sections of all four lines. Chloroplast numbers per cell cross section were reduced in the *act1,dgd1* mutant to approximately 50% of wild type (7.6 ± 0.4, 8.3 ± 0.5, 7.3 ± 0.3, and 3.9 ± 0.4 for WT, *dgd1*, *act1*, and *act1,dgd1*, respectively; n = 10 cell cross sections). As observed for *dgd1* (Dörmann et al., 1995), thylakoid membranes of the *act1,dgd1* mutant were curved and stroma areas were increased (Fig. 3). Apparently, the combinations of two blocks in lipid biosynthesis of *act1* (prokaryotic pathway) and *dgd1* (eukaryotic pathway) drastically affect overall lipid biosynthesis and as a result the total amount of photosynthetic membranes is reduced in the *act1,dgd1* double mutant.

The Photosynthetic Capacity of the Residual PSs Is Compromised in *act1,dgd1* as Compared with *dgd1*

The reduction in lipid biosynthesis and the drastic impairment of photosynthetic membranes observed for *act1,dgd1* prompted us to investigate the photosynthetic competence of this mutant in greater detail. Pulse amplitude-modulated chlorophyll fluorescence analysis is a sensitive, noninvasive method that gives information about changes in electron-transport reactions within thylakoids and the overall photosynthetic capability of leaves under in vivo conditions (for review, see Krause and Weis, 1991; Horton et al., 1996). Figure 4A shows light response curves for the fluorescence parameter 1-qP, an estimate for the reduction state of the primary electron acceptor of PSII

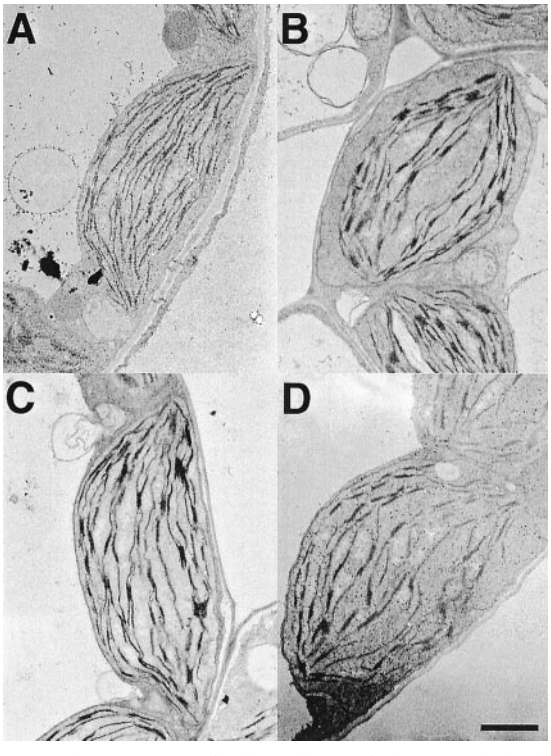

Figure 3. Ultrastructure of chloroplasts of the *act1,dgd1* double mutant. Representative chloroplasts are shown for four-week-old plants of Arabidopsis wild type (A), *dgd1* (B), and *act1* (C) single mutants and the *act1,dgd1* double mutant (D). Bar = 1 μm. Plants were grown on solidified Murashige and Skoog medium supplemented with Suc.

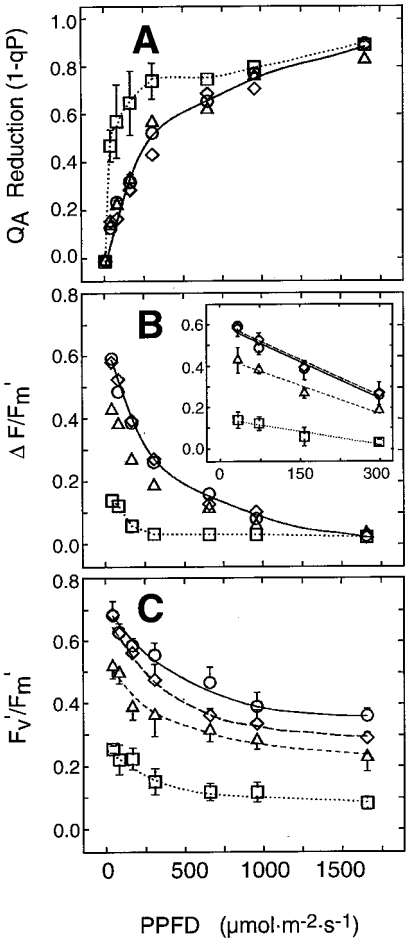

Figure 4. Photosynthtetic capability in *act1,dgd1*. Chlorophyll fluorescence of Arabidopsis wild type (circle), *act1* (diamond), *dgd1* (triangle), and *act1,dgd1* (square) was measured in whole leaves exposed to different light intensities. The parameters 1-qP (A), $\Delta F/F_m'$ (B), and F_v'/F_m' (C) were obtained from fluorescence measurements. The inset in B shows $\Delta F/F_m'$ on a different scale. Values represent the means ± SE of five measurements.

(Q_A). The Q_A reduction increases more strongly with increase in the photosynthetic photon flux density (PPFD) in leaves of *act1,dgd1* than in the wild type and the other mutant plants, and is almost saturated at 75 μmol photons m^{-2} s^{-1}, the PPFD employed for plant growth in this study. The quantum yield of linear electron flux through the photosynthetic electron transport chain ($\Delta F/F_m'$) is strongly reduced in *act1,dgd1* (Fig. 4B). As previously shown for plants grown on soil, $\Delta F/F_m'$ is also reduced in *dgd1* (Dörmann et al., 1995; Härtel et al., 1998), whereas virtually no differences are observed between *act1* and wild type. The intrinsic efficiency of open PSII reaction centers in the light-adapted state (F_v'/F_m') was drastically decreased in *act1,dgd1* as compared with wild type, *dgd1*, and *act1* (Fig. 4C), which indicates a reduced photochemical efficiency of PSII. Taken together, in vivo chlorophyll fluorescence data indicate a severe impact on the utilization of light energy by the residual photosynthetic complexes of *act1,dgd1*.

The Total Amount and Molecular Species Distribution of Galactolipids Are Similar in the Double Mutants and *dgd1*

The growth defects observed for *act1,dgd1* and *fad2,dgd1* might be caused by changes in the total amounts of DGDG, alterations of its fatty acid composition, or distribution at the *sn*-1 and *sn*-2 positions of the glycerol backbone. Therefore, molecular species composition of *act1,dgd1*, *fad2,dgd1*, *fad3,dgd1*, and *dgd1* was analyzed in greater detail. The relative amount of DGDG in all four lines was very similar (2.0%–2.8%; Table I). Therefore, the additional growth defect of *act1,dgd1* and *fad2,dgd1* cannot be explained by a further reduction in the total amount of DGDG. Fatty acid composition of the residual DGDG in *act1,dgd1* was very similar to that of *dgd1* (Table I; Dörmann et al., 1999), excluding the possibility that the severe growth retardation of this line is because of changes in the DGDG fatty acid composition. The *fad2,dgd1* mutant showed a shift in fatty acid composition, i.e. an increase of 18:1 in DGDG with a further decrease in 18:3 when compared with either parent (Table I). However, because the shift of 18:3 to 18:1 caused by the *fad2* mutation can already be observed in the *fad2* single mutant that shows normal growth, it is unlikely that this small change might affect growth of *fad2,dgd1* plants.

The DGDG fatty acid composition in the double mutants *act1,dgd1* and *fad2,dgd1* was not very differ-

Table I. *DGDG lipid content and fatty acid composition of DGDG in fad2,dgd1, fad3,dgd1, and act1,dgd1*

Values are given as mol % and represent averages of three measurements. SE was less than 2% for all measurements. Plants were grown on soil except *act1* and *act1,dgd1*, which were grown on solidified Murashige and Skoog medium supplemented with Suc. n.d., Not detected.

Lipid	Wild Type	*dgd1*	*fad2*	*fad2,dgd1*	*fad3*	*fad3,dgd1*	*act1*	*act1,dgd1*
DGDG	16.5	2.0	15.4	2.8	17.0	2.8	15.9	2.1
16:0	10.1	21.3	13.3	24.1	10.2	21.9	7.2	23.0
16:1	1.1	3.8	1.8	6.1	1.3	3.7	1.1	4.7
16:2	0.6	2.8	1.4	2.7	1.0	2.6	0.3	1.9
16:3	2.0	2.0	3.3	2.6	1.8	2.0	n.d.	n.d.
18:0	0.9	4.2	1.2	3.7	1.1	2.6	1.1	6.7
18:1	1.7	10.9	8.0	19.5	3.1	6.7	2.2	10.1
18:2	3.2	7.3	5.1	5.3	6.2	8.4	5.2	9.0
18:3	80.4	47.7	65.9	36.2	75.4	52.0	83.0	44.6

ent from *dgd1* (Table I), still these two lines showed a stronger growth retardation than the *dgd1* single mutant. We considered the possibility that the molecular species composition of the galactolipids produced by the prokaryotic or eukaryotic pathways might be altered by the respective mutations and thus add to the growth phenotype. Because the two double mutants *act1,dgd1* and *fad2,dgd1* were not fertile and had to be maintained on Suc-supplemented medium, it was difficult to obtain sufficient amounts of tissue for positional analysis of galactolipids. Therefore, we analyzed the positional fatty acid distribution in the respective parental lines *dgd1*, *act1*, and *fad2* (Fig. 5). The amount of C16 fatty acids in the *sn-2* position of DGDG of *dgd1* and *act1* is extremely low (Fig. 5F), which confirms previous studies showing that in these two mutants, only very little DGDG is made via the prokaryotic pathway (Kunst et al., 1988; Härtel et al., 2000). Therefore, in DGDG of *act1,dgd1*, C16 fatty acids supposedly are also excluded from the *sn-2* position, which in turn suggests that similarly to the *dgd1* single mutant, the high amount of C16 fatty acids in DGDG of *act1,dgd1* (Table I) must be localized at *sn-1*. The total amounts of C16 and C18 fatty acids in DGDG (Fig. 5E) as well as the positional distribution at the *sn-2* position (Fig. 5F) are very similar for wild type and *fad2*. In *dgd1*, an increase in total C16 of DGDG was observed that must be located at the *sn-1* position, because C16 fatty acids are largely excluded from *sn-2* (compare with Härtel et al., 2000). Because the positional distribution of fatty acids in DGDG of wild type and *fad2* is very similar, this mutation apparently has no large effect on the fatty acids at *sn-2*, and therefore positional distribution in DGDG of *fad2,dgd1* should be very similar to that of *dgd1*. For these reasons, the growth differences observed between *dgd1*, *act1,dgd1*, and *fad2,dgd1* cannot be explained by alterations in total amount of DGDG, its fatty acid composition, or positional distribution.

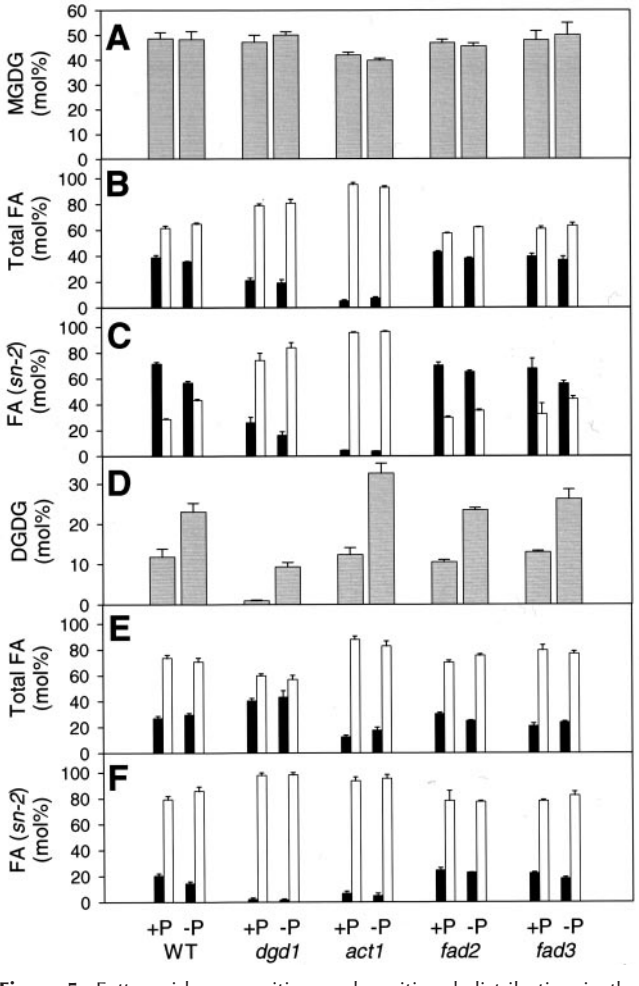

Figure 5. Fatty acid composition and positional distribution in the galactolipids of *act1*, *fad2*, and *fad3* grown on phosphate-supplied and -deficient medium. Plants were raised on Murashige and Skoog medium for 12 to 14 d and further propagated for an additional 10 d on phosphate-containing or -deficient medium (Estelle and Somerville, 1987). A, Amount of MGDG; B, total fatty acids in MGDG; C, fatty acids in lyso-MGDG (*sn-2* position); D, amount of DGDG; E, total fatty acids in DGDG; F, fatty acids in lyso-DGDG (*sn-2* position). Black bars, C16 fatty acids; white bars, C18 fatty acids. Values represent mean and SE of three experiments.

DGDG in *dgd1* Homozygous Lines Is Synthesized at the Chloroplast

Härtel et al. (2000) demonstrated that the additional amount of DGDG synthesized during phosphate deprivation was at least in part localized in extraplastidic membranes. Therefore, we wondered whether the residual DGDG in *dgd1* was actually produced in the chloroplast, because the integrity of the photosynthetic apparatus might be affected if it were associated with extraplastidic membranes (Härtel et al., 2000, 2001). This question could be addressed in part by analyzing the fatty acid composition of the *fad3,dgd1* double mutant. No changes in the fatty acid composition of leaf DGDG were found in *fad3,dgd1* as compared with *dgd1*. In particular, the amount of 18:3 in DGDG of *fad3,dgd1* and *dgd1* was very similar (52.0 and 47.7 mol %, respectively; Table I). The *fad3* mutation preferentially affects extraplastidic lipids (Browse et al., 1993). We detected large amounts of 18:3 in DGDG of *fad3,dgd1* that must be derived from the plastid FAD7 desaturase, because the FAD3 enzyme localized at the ER is not active in this line. These findings suggest that DGDG in *dgd1* is accessible to chloroplast desaturases.

We performed galactolipid synthesis assays with isolated chloroplasts as a direct way to determine whether *dgd1* chloroplasts harbor the enzyme(s) of a second DGDG synthesis pathway. In a previous study (Dörmann et al., 1995), assays with chloroplasts directly isolated from homogenized leaves resulted in low DGDG synthase activity. In this study, we isolated chloroplasts from protoplasts, which resulted in plastid preparations with much higher

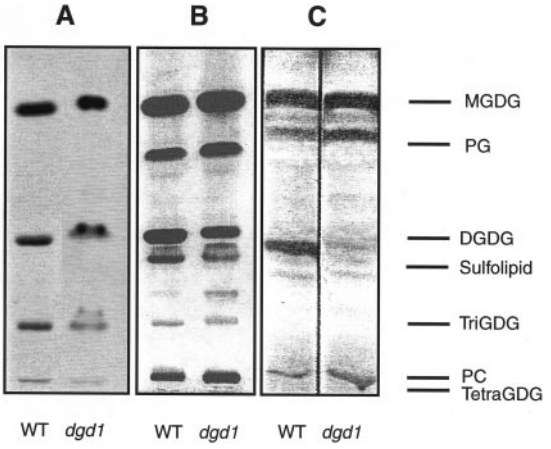

Figure 6. DGDG synthesis in chloroplasts of Arabidopsis wild type and *dgd1* mutant. A, Chloroplasts of Arabidopsis wild type and *dgd1* mutant were isolated from protoplasts and incubated with MGDG and UDP-[^{14}C]Gal. After extraction, lipids were separated by thin-layer chromatography and radiolabeled lipids visualized by autoradiography. B, Chloroplasts of wild type and *dgd1* were isolated from protoplasts, lipids extracted, and stained with iodine. C, Chloroplasts of wild type and *dgd1* were isolated by homogenization of leaves (Price et al., 1994), lipids extracted, and stained with iodine. Galactolipids and sulfolipid were identified by staining with α-naphthol (not shown).

DGDG synthase activity. We observed very similar incorporation of radiolabel from UDP-[^{14}C]Gal into the galactolipids MGDG, DGDG, and oligogalactolipids comigrating with trigalactosyldiacylglycerol (TriGDG) and tetragalactosyldiacylglycerol (TetraGDG) for wild type and *dgd1* (Fig. 6A). The chloroplasts isolated from wild-type and *dgd1* protoplasts contained high amounts of DGDG and oligogalactolipids that could easily be detected by staining with iodine (Fig. 6B) and the sugar-specific reagent α-naphthol (not shown). However, in chloroplasts isolated directly from fresh leaves, only low amounts of DGDG were found in *dgd1* and oligogalactolipids were absent (Fig. 6C). Apparently, during protoplastation, a galactosyltransferase activity was induced in wild-type and *dgd1* chloroplasts that synthesized DGDG and the oligogalactolipids TriGDG and TetraGDG. This clearly demonstrates that a second DGDG synthase activity is present in *dgd1* chloroplasts.

Additional DGDG Synthesized during Phosphate Deprivation Has Similar Characteristics as the Residual DGDG of *dgd1* Homozygous Lines

During phosphate limitation, DGDG was found to accumulate in membranes of wild-type and *dgd1* mutant leaves (Härtel et al., 2000). This DGD1-independent pathway of DGDG biosynthesis was induced by phosphate deprivation, but might also be active to a minor extent under phosphate-sufficient conditions and thus be involved in the synthesis of the residual amount of DGDG in *dgd1*. We measured total amounts, fatty acid composition, and positional distribution of galactolipids in wild type and different mutant lines raised under normal and phosphate-limiting conditions to investigate the relationship between DGDG synthesized during phosphate deprivation and the residual amount of DGDG in *dgd1*. As shown in Figure 5 (A–C), the total amount of MGDG and its fatty acid composition and positional distribution was not drastically changed in any of the mutants, *dgd1*, *act1*, *fad2*, or *fad3*, during growth on phosphate-deficient medium. The amount of DGDG increased to a similar extent in all lines when propagated on phosphate-deficient medium (Fig. 6D). Therefore, the synthesis of this extra amount of DGDG was not affected by any of these mutations. The fatty acid composition of DGDG was not drastically altered during phosphate deprivation (Fig. 6E). The amount of C16 fatty acids in the *sn-2* position of DGDG slightly decreased under phosphate deficiency in all lines analyzed (Fig. 6F; compare with Härtel et al., 2000), indicating a redirection of fatty acids in DGDG derived from the prokaryotic to the eukaryotic pathway. During phosphate deficiency, DGDG increased in leaves of the *fad3,dgd1* double mutant from 1.1 to 9.5 mol % (Table II), whereas the amount of 18:3 in DGDG remained unchanged (53.2

Table II. *Changes in DGDG lipid content and fatty acid composition of DGDG in fad3,dgd1 double mutants after phosphate deprivation*

Values are given as mol % and represent averages of three measurements. SE error was less than 2% for all measurements. Plants were grown on solidified medium supplemented with Suc containing 1 mM (+) or no phosphate (−). n.d., Not detected.

Lipid	WT		*dgd1*		*fad3*		*fad3,dgd1*	
	+	−	+	−	+	−	+	−
DGDG	11.8	23.0	0.9	9.3	12.9	26.2	1.1	9.5
16:0	15.4	21.8	26.0	29.8	15.3	16.0	22.7	22.5
16:1	2.1	1.6	4.2	2.3	2.2	1.4	2.4	0.7
16:2	0.9	n.d.	1.6	0.3	0.7	0.2	3.1	n.d.
16:3	2.6	1.9	3.4	1.1	3.4	2.5	1.9	1.8
18:0	1.2	2.5	3.7	5.3	4.5	2.4	2.1	3.1
18:1	2.0	2.9	6.4	4.9	3.2	2.3	5.4	2.3
18:2	4.9	10.8	9.2	12.5	7.1	12.2	9.2	15.6
18:3	70.9	58.6	45.2	44.0	63.7	63.0	53.2	53.4

and 53.4 mol %, respectively). As already pointed out for the residual DGDG lipid in *fad3,dgd1*, the high amount of 18:3 in DGDG isolated from this line after induction by phosphate deprivation is derived from desaturation by the chloroplast FAD7 enzyme. Therefore, DGDG biosynthesis induced by phosphate deprivation involves desaturases associated with the chloroplast.

DISCUSSION

The *act1,dgd1* double mutant has one of the most extreme phenotypes of all known Arabidopsis lipid mutants. In a previous study (Dörmann et al., 1999), the drastically impaired growth of *act1,dgd1* led us to hypothesize that an overall reduction in membrane lipid synthesis might be one of the reasons for the severe phenotype and suggested that DGD1 plays a critical role in chloroplast lipid production. This idea was based on the proposed reaction mechanism of the DGDG synthase, which releases one diacylglycerol molecule per molecule of DGDG synthesized (van Besouw and Wintermans, 1978; Heemskerk et al., 1990). Diacylglycerol produced in this reaction could be used for the biosynthesis of other eukaryotic lipids in the plastid (Williams and Khan, 1996; Dörmann et al., 1999) and may be limiting in all plants homozygous for *dgd1*. In the present study, we observed a strong reduction in the amounts of total fatty acids and of photosynthetic pigments as well as a reduction in the number of chloroplasts per cross section in *act1,dgd1*. This clearly points toward a reduction in overall thylakoid membrane lipid biosynthesis as one of the causes for the drastic growth retardation of *act1,dgd1*. The strong phenotype caused by the combination of blocks in two parallel pathways of lipid biosynthesis emphasizes the importance of the prokaryotic and eukaryotic pathways for overall chloroplast lipid biosynthesis in Arabidopsis.

Two additional double mutants of *dgd1* and the ER-localized deasturases *fad2* and *fad3* were generated during this study. Whereas *fad2,dgd1* plants were affected in growth, *fad3,dgd1* plants were very similar to *dgd1*. As part of the eukaryotic pathway of lipid synthesis, diacylglycerol moieties, which are enriched in 18:2, are transported from the ER back to the chloroplast (Miquel and Browse, 1992). Because *fad2* is critical for synthesis of 18:2 at the ER, a block in *fad2*, but not in *fad3*, would be expected to affect the metabolic flux through the eukaryotic pathway. The finding that the *fad2,dgd1* double mutant, but not the *fad2* single mutant, showed a reduction as compared with the respective parental lines (*dgd1* and wild type, respectively), suggests that the *fad2* mutation affects lipid synthesis to a higher extent, when the flux through the eukaryotic pathway in the *dgd1* mutant background is already compromised. Therefore, the combinations of two mutations in the eukaryotic pathway, *dgd1* and *fad2*, results in strong reduction of growth caused by additive effects of two blocks in a linear pathway of lipid synthesis.

Figure 4 demonstrates that the utilization of light energy by the residual PSs of the *act1,dgd1* double mutant is impaired as compared with the parental mutants *act1* and *dgd1* (Kunst et al., 1989; Härtel et al., 1997). One possibility for this might be the specific association of the galactolipids with different complexes of photosynthesis. It has been shown that the lumen-exposed water oxidation complex is affected in *dgd1* (Reifarth et al., 1997). Consistent with this, the oxygen evolution rate in in vitro preparations of PS II was dependent on the amount of DGDG added (Gounaris et al., 1983). Furthermore, DGDG was found to be bound to the light-harvesting complex II, and one MGDG molecule was recently discovered in the crystal structure of PS I (Nuβberger et al., 1993; Jordan et al., 2001). Whereas in *dgd1*, the prokaryotic lipid pathway is still active (as indicated by the molecular species composition of MGDG; Fig. 5C), the additional block in *act1* in the double mutant may result in a specific loss of prokaryotic-type MGDG and DGDG in the chloroplast, giving rise to a destabilization and dysfunction of complexes of the photosynthetic apparatus which in turn could explain why the *act1,dgd1* double mutant was unable to grow photoautotrophically.

To investigate the amount and origin of DGDG synthesized in *dgd1*, we analyzed lipid and fatty acid composition in double mutants of *dgd1* with *act1* and the desaturase mutants *fad2* and *fad3*. The fatty acid composition of DGDG in lines with *dgd1* mutant background was found to be constant for plants analyzed within one set of experiments but somewhat variable between different sets (e.g. Dörmann et al., 1995, 1999; Härtel et al., 2000; this study). This variability might be caused by slight differences in growth conditions (e.g. propagation on soil or Murashige and Skoog medium) because it was shown that the amount of phosphate in the growth medium has a strong impact on fatty acid composition of DGDG (Härtel et al., 2000). The fact that the total amount of DGDG in the double mutants, the fatty acid composition, and positional distribution was not drastically altered as compared with the *dgd1* single mutant suggests that the growth deficiencies of *act1,dgd1* and *fad2,dgd1* are not caused by alterations in galactolipid composition per se.

The fatty acid composition of the *act1,dgd1* and *fad2,dgd1* double mutants suggested that a major portion of the residual DGDG in lines with *dgd1* genetic background is of eukaryotic origin: In *act1,dgd1*, we found a very similar fatty acid composition for DGDG as in *dgd1*, i.e. increased amounts of 16:0 and 18:1 at the expense of 18:3 (Table I). Palmitic acid is particularly enriched at the *sn-1* position of DGDG, indicating that the prevalent fraction of DGDG in *dgd1* is of eukaryotic structure (Härtel et al., 2001; Fig.

5F). Furthermore, the *act1* mutation did not eliminate residual amounts of DGDG in *act1,dgd1* as would be expected if the residual DGDG were completely synthesized via the prokaryotic pathway. Because the block in desaturation of 18:1 to 18:2 caused by the *fad2* mutation is reflected in the DGDG fatty acid composition in the *fad2,dgd1* plants, the residual amount of DGDG still contains molecular species derived from the eukaryotic pathway. Apparently, the block in the ER-localized desaturation in *fad2* cannot entirely be bypassed by plastid desaturases (i.e. FAD6).

Chloroplasts directly isolated from leaves of wild type and *dgd1* mutant were found to synthesize all galactolipids normally present in plants (MGDG and DGDG; Dörmann et al., 1995). This result again suggests that a second DGDG synthase is localize in chloroplasts. No oligogalactolipids (TriGDG and TetraGDG) were detected in these preparations (Dörmann et al., 1995; Fig. 6C). However, chloroplasts isolated from leaf protoplasts of wild type and *dgd1* were capable of producing DGDG, TriGDG, and TetraGDG from radioactive UDP-Gal (Fig. 6A). High amounts of DGDG and oligogalactolipids accumulated in chloroplasts of wild type and *dgd1* during protoplastation, diminishing the differences in the amounts of DGDG in these two lines (Fig. 6B). We concluded that during protoplastation, a DGDG synthase activity is induced leading to the formation of DGDG, TriGDG, and TetraGDG. These oligogalactolipids are normally absent from leaves and are only found in low amounts in non-photosynthetic tissues (e.g. Fujino and Miyazawa, 1979). Dorne et al. (1982) and Heemskerk et al. (1986) demonstrated that in isolated chloroplasts, DGDG, TriGDG, and TetraGDG accumulate at the expense of MGDG. The repeated glycosylation of glycolipids that was also described for bacterial genes was referred to as "processive" (Jorasch et al., 1998, 2000). There has been some debate on whether or not the enzyme responsible for producing DGDG, TriGDG, and TetraGDG, the galactolipid: galactolipid galactosyltransferase, represents the main activity for DGDG synthesis in chloroplasts (Heemskerk et al., 1988, 1990), or whether it merely is an "artificial" activity detectable in vitro only (Dorne et al., 1982). The result for the *dgd1* mutant clearly shows that the formation of TriGDG and TetraGDG are independent of the main pathway of DGDG synthesis through DGD1.

In addition to DGD1, which was shown to be responsible for the production of the predominant fraction of DGDG in chloroplasts (Dörmann et al., 1995), Härtel et al. (2000) and Härtel and Benning (2000) demonstrated that a second, DGD1-independent pathway is induced in Arabidopsis after phosphate deprivation, giving rise to accumulation of extraplastidic DGDG. Analysis of the fatty acid composition of *fad3,dgd1* plants revealed that DGDG produced after phosphate deprivation still contains fatty acids desaturated at the chloroplast. However, in roots of *fad3* single mutants, DGDG produced during phosphate deficiency showed a reduction in 18:3 (Härtel et al., 2000). This apparent discrepancy can be explained by the reduced activity of plastid desaturases in roots (Miquel and Browse, 1992). For this reason, the *fad3* mutation in roots cannot efficiently be circumvented by the plastid FAD7 desaturase as in leaves. Taken together, fatty acid data obtained for *fad3,dgd1* plants grown in the presence or absence of phosphate suggest that DGDG in this line is desaturated at chloroplast membranes, which in turn points to a second DGDG synthase activity localized at the chloroplast membranes. During phosphate deficiency, the additional amount of DGDG may be assembled in the plastid and then transported to the ER depending on lipid demand.

It will be interesting to elucidate how the DGD1-independent DGDG synthase induced by phosphate deprivation (Härtel et al., 2000) is related to the processive galactosyltransferase detected in isolated chloroplasts of *dgd1* (this study). In chloroplasts isolated from protoplasts as well as in plants raised under phosphate deficiency, a DGDG synthase activity is induced, resulting in massive production of DGDG (Dorne et al., 1982; Heemskerk et al., 1986; Härtel et al., 2000; this study). The processive galactosyltransferase is localized in the outer chloroplast envelope, where it presumably accepts eukaryotic MGDG as a precursor for DGDG synthesis. Similarly, DGDG produced by the phosphate-dependent DGDG synthase is mostly eukaryotic. Contrary to the processive enzyme, no oligogalactolipids were synthesized under phosphate-limiting conditions. However, this apparent discrepancy might be explained by the fact that DGDG produced during phosphate deprivation can be transported to extraplastidic membranes to substitute for their deficiency in phospholipids (Härtel et al., 2000). Under these conditions, DGDG may not be available for further galactosylation by plastid-localized enzymes. Because of the absence of extraplastidic membranes in the isolated chloroplast system, DGDG lipid cannot be removed from the outer envelope. Furthermore, because of the deficiency of diacylglycerol supplied by the ER, MGDG may become limiting in isolated chloroplasts and the processive galactosyltransferase may use DGDG for further galactosylation. Recently, a second DGDG synthase (DGD2) was described in Arabidopsis that was induced by phosphate deprivation and showed processive galactosylation activity after heterologous expression in *Escherichia coli* (Kelly and Dörmann, 2002). Therefore, DGD2 represents a candidate enzyme for both the DGD1-independent DGDG synthase induced by phosphate deficiency as well as for the processive galactosyltransferase detectable in *dgd1*. Further studies will be required to reveal which additional factors are involved in the

induction of DGD1-independent galactolipid synthesis in Arabidopsis.

MATERIALS AND METHODS

Plant Growth Conditions and Generation of Double Mutants

Arabidopsis wild type (Arabidopsis, ecotype Columbia-2) and different mutants were grown at light conditions of 60 to 70 μmol photons m^{-2} s^{-1} on soil or solidified Murashige and Skoog medium as described below (Murashige and Skoog, 1962). For phosphate deprivation experiments, plants raised on Murashige and Skoog medium for 11 to 14 d were transferred to Arabidopsis medium at one-half strength, where they were grown for an additional 10 d as described (Estelle and Somerville, 1987; Härtel et al., 2000). Double mutants were obtained by crossing *dgd1* plants (Dörmann et al., 1995) with *act1* (Nottingham Arabidopsis Stock Center, Nottingham University, Loughborough, UK; Kunst et al., 1988), *fad2-1* (Lemieux et al., 1990; Miquel and Browse, 1992), or *fad3* (Nottingham Arabidopsis Stock Center; Lemieux et al., 1990; Browse et al., 1993). Because all mutations analyzed are recessive, double homozygous plants were searched for in the F_2 generation, where they were expected to occur in a ratio of 1:16.

The *act1,dgd1* double mutant was obtained as described by Dörmann et al. (1999). The *act1,dgd1* plants were grown on solidified 1× Murashige and Skoog medium containing 1% (w/v) Suc, if not otherwise stated. F_2 plants derived from a cross of *dgd1* and *fad2* were screened for double-mutant plants by analyzing lipid and fatty acid patterns. No double homozygous plant was found in 405 plants of the F_2 generation, but only one line homozygous for *fad2* and heterozygous for *dgd1*. This segregation pattern indicates close linkage of the two genes with a calculated genetic distance of 1.4 cM, which is in good agreement with the published genetic locations of *dgd1* (about 16 cM; Dörmann et al., 1999) and *fad2* (12.5 cM; Okuley et al., 1994) on chromosome 3 of Arabidopsis. After germination on Murashige and Skoog medium with 1% (w/v) Suc, plants were transferred to soil. Double mutant plants of *fad3,dgd1* were found in an F_2 population of a cross between *dgd1* and *fad3* by screening for the respective lipid and fatty acid patterns. After germination on solidified Murashige and Skoog medium with 1% (w/v) Suc, these plants were transferred to soil.

Upon request, all seed stocks of double mutants described in this publication will be made available for noncommercial research purposes. No restrictions or conditions will be placed on the use of any materials described in this paper that would limit their use in noncommercial research purposes.

Analysis of Lipids and Chlorophyll and Chlorophyll Fluorescence

Lipids were extracted from leaves, separated by thin-layer chromatography, and quantified by gas chromatography as described by Dörmann et al. (1999). Chlorophyll was measured photometrically (Lichtenthaler, 1987). Positional analysis of fatty acids was done according to Miquel et al. (1998) and Siebertz and Heinz (1977).

In vivo chlorophyll fluorescence at room temperature was registered as described previously (Härtel et al., 1998). All plants were dark adapted for 1 h before fluorescence measurements. Fluorescence parameters used are as defined (Genty et al., 1989; van Kooten and Snel, 1990). PPFDs were measured with a quantum sensor (LI-189A; LI-COR, Lincoln, NE).

Electron Microscopy

Leaf tissue was cut into 1-mm-wide slices and immersed in a primary fixative of 2.5% (v/v) glutaraldehyde in 0.05 M sodium cacadylate, pH 6.8. The samples were fixed under vacuum until exhausted then further fixed for a total of 2 h at room temperature. After washing in three 20-min changes of 0.05 M cacadylate buffer, pH 6.8, the samples were post fixed in 1% (w/v) aqueous osmium tetroxide for 2 h. This was followed by three washes with deionized water, then a serial dehydration with 25%, 50%, 75%, and 100% (v/v) of acetone in water. The specimens were infiltrated with a series of 33%, 66%, and 100% (w/v) of epoxy resin in acetone (Equiequivalent vinyl cyclohexane dioxide and Quetol 651, with nonenyl succinic anhydride and dimethylaminoethanol). After three changes of pure resin over a 24-h period, the samples were cast into blocks and polymerized at 65°C for 12 h. Sections, 70 to 90 nm thick, were cut with an RMC MT-X ultramicrotome (RMC-Boeckeler Instruments Inc., Tucson, AZ) and mounted on plain 300-μm mesh copper grids. These were stained in aqueous uranyl acetate and lead citrate prior to viewing in a Philips CM-10 Transmission Electron Microscope (Eindhoven, The Netherlands) operating at 100 kV.

Chloroplast Isolation and Galactolipid Biosynthesis Assay

Chloroplasts were isolated from 3-week-old wild-type and *dgd1* plants grown in tissue culture using a protoplast method as described (Fitzpatrick and Keegstra, 2001). In brief, leaves were cut from the plants and digested with cellulase and macerozyme in 400 mM sorbitol; 20 mM MES-KOH, pH 5.2; and 0.5 mM $CaCl_2$. After 3 h, the protoplasts were purified by filtration through 200-μm mesh nylon. Chloroplasts were obtained by resuspending in 300 mM sorbitol, 20 mM EDTA, 5 mM EGTA, 10 mM $NaHCO_3$, and 0.1% (w/v) bovine serum albumin by passage through a 10-μM mesh nylon membrane. Intact chloroplasts were obtained by centrifugation through a Percoll gradient as described Bruce et al. (1994). Chloroplasts were directly isolated from fresh leaves without protoplastation according to Price et al. (1994).

Intact chloroplasts of wild type and *dgd1* mutant corresponding to 36 μg of total chlorophyll each were incubated in assay buffer (0.3 M sorbitol; 20 mM Tricine-KOH, pH 7.6; 5 mM $MgCl_2$; and 2.5 mM EDTA) in a total reaction volume of 250 μL containing 130 nmol of MGDG (isolated from

wild-type leaves), 100 nmol sodium deoxycholate, and 61.5 pmol UDP-[U-^{14}C]Gal (325 mCi/mmol) for 1 h at room temperature. Lipids were extracted and separated by thin-layer chromatography as previously described (Dörmann et al., 1999). Radioactive lipids were visualized by autoradiography.

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