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Isolation and Characterization of an Arabidopsis Mutant Deficient in the Thylakoid Lipid Digalactosyl Diacylglycerol

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The galactolipids monogalactosyl and digalactosyl diacylglycerol occur in all higher plants and are the predominant lipid components of chloroplast membranes. They are thought to be of major importance to chloroplast morphology and physiology, although direct experimental evidence is still lacking. The enzymes responsible for final assembly of galactolipids are associated with the envelope membranes of plastids, and their biochemical analysis has been notoriously difficult. Therefore, we have chosen a genetic approach to study the biosynthesis and function of galactolipids in higher plants. We isolated a mutant of Arabidopsis that is deficient in digalactosyl diacylglycerol by directly screening a mutagenized M_2 population for individuals with altered leaf lipid composition. This mutant carries a recessive nuclear mutation at a single locus designated *dgd1*. Backcrossed mutants show stunted growth, pale green leaf color, reduced photosynthetic capability, and altered thylakoid membrane ultrastructure.

INTRODUCTION

Almost 40 years ago, galactose-containing glycerolipids were discovered in wheat flour (Carter et al., 1956). Since that time, they have become firmly established as the predominant lipid components of photosynthetic membranes in plants, algae, and various bacteria (Heinz, 1977; Joyard et al., 1993). The two most common galactolipids are monogalactosyl diacylglycerol (1,2-diacyl-3-*O*-[β-D-galactopyranosyl]-*sn*-glycerol; MGD) and digalactosyl diacylglycerol (1,2-diacyl-3-*O*-[α-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl]-*sn*-glycerol; DGD). In higher plants, the occurrence of galactolipids is restricted to plastid membranes. Their abundance is particularly high in thylakoids, the predominant chloroplast membranes, in which MGD represents up to 50 mol% and DGD ~20 mol% of polar lipids (Douce and Joyard, 1980).

Based on the exclusive localization of MGD and DGD to plastids, with a particularly high amount found in chloroplasts, it has been suggested repeatedly that galactolipids may play an important role in photosynthesis. Indirect experimental evidence was provided by the observation that MGD is preferentially associated with photosystem II reaction center complexes (Murata et al., 1990) and DGD with photosystem II light-harvesting complexes (Nussberger et al., 1993). Due to the small size of the monogalactosyl head group and the high degree of fatty acid unsaturation, an overall wedge-shaped molecule structure can be calculated for MGD. This geometry of the MGD molecule is generally accepted as an explanation for the observation that the lipid molecules organize into a hexagonal phase (H_{II}) but not into a lamellar phase (L_α) in a pure MGD water mixture (Webb and Green, 1991). This feature sets MGD apart from DGD and other chloroplast lipids, which are proposed to adopt a cylindrical shape and form a lamellar phase (L_α) in mixtures with water. Therefore, it is expected that the ratio of the non-bilayer–forming lipid MGD to the other bilayer-forming thylakoid lipids is of critical importance for the chloroplast ultrastructure.

The pathway for the de novo biosynthesis of galactolipids is complex because the sugar nucleotide as well as fatty acid metabolism converge to give rise to a large number of different molecular species of MGD and DGD. In addition, two groups of plants can be distinguished by the fatty acid composition of their chloroplast galactolipids, particularly that of MGD. In 16:3 plants (e.g., spinach or Arabidopsis), the *sn*-1 position of the glycerol backbone of MGD is preferentially esterified to α-linolenic acid ($18:3\Delta^{9,12,15}$), whereas 7,10,13-hexadecatrienoic acid ($16:3\Delta^{7,10,13}$) is the most abundant fatty acid at the *sn*-2 position (Heinz, 1977). However, in 18:3 plants (e.g., pea), $18:3\Delta^{9,12,15}$ is the predominant fatty acid found in the *sn*-1 and *sn*-2 positions of MGD. The diacylglycerol moiety of MGD containing $16:3\Delta^{7,10,13}$ at the *sn*-2 position is assembled inside the chloroplast and is called "prokaryotic." In contrast, the "eukaryotic" diacylglycerol containing $18:3\Delta^{9,12,15}$ in both positions presumably originates in the endoplasmic reticulum. In plants, such as pea, only the eukaryotic pathway seems to be operational; in spinach and Arabidopsis, both pathways contribute to the biosynthesis of MGD (for a review, see Browse and Somerville, 1991).

The enzymes involved in the final assembly of galactolipids are associated with the chloroplast envelope membranes. Galactose is transferred from UDP-galactose to diacylglycerol, thereby forming MGD. The enzyme catalyzing this reaction,

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the UDP-galactose:diacylglycerol galactosyltransferase (MGD synthase; EC 2.4.1.46), has been localized to the inner envelope membrane of spinach (Block et al., 1983) but to the outer envelope of chloroplasts of pea plants (Cline and Keegstra, 1983). The final assembly of DGD is catalyzed by the galactolipid:galactolipid galactosyltransferase (DGD synthase; van Besouw and Wintermans, 1978). The DGD synthase has been localized to the outer envelope membrane of all plants analyzed (Dorne et al., 1982).

We decided to take a genetic approach to the analysis of the function and biosynthesis of galactolipids as well as other thylakoid lipids in higher plants. We performed a screen for Arabidopsis mutants lacking a complete class of complex lipids. That the expected drastic alterations in membrane lipid composition can be tolerated to a large extent by photosynthetic organisms had been demonstrated through the isolation of sulfolipid- and phosphatidylcholine-deficient mutants of the purple bacterium *Rhodobacter sphaeroides* (Arondel et al., 1993; Benning et al., 1993). In plants, mutants of Arabidopsis with changes in the fatty acid composition of complex lipids have been isolated, but no mutants with defects in lipid head group biosynthesis have been described to date (Browse and Somerville, 1991). The isolation of mutants of Arabidopsis deficient in the final assembly of thylakoid lipids represents the next step toward a more complete understanding of the biosynthesis and the specific role of plant lipids. Here, we describe the isolation and characterization of a novel mutant of higher plants deficient in an entire polar lipid class.

RESULTS

Isolation of a Mutant with Altered Polar Lipid Composition

To isolate mutants of Arabidopsis with altered leaf polar lipid composition, a screening procedure previously developed for the isolation of lipid mutants from the photosynthetic bacterium *R. sphaeroides* was adopted (Benning and Somerville, 1992). In the initial screen, 3000 leaf lipid extracts from individual M_2 plants were tested by thin-layer chromatography, and one mutant line, which showed a dramatic reduction in the content of DGD, was identified. This phenotype was inherited in the next generation. The F_1 plants derived from a cross between the wild type, ecotype Columbia (Col-2), and the mutant line showed no mutant phenotype. Of 422 F_2 plants tested, 84 (19.9%) were DGD deficient. Assuming a reduced penetrance of the mutant phenotype, this result is in agreement with a recessive mutation affecting a nuclear gene. The mutated locus was designated *dgd1*. To remove background mutations, the *dgd1* mutant was repeatedly crossed to Col-2 wild type, and further experiments were performed with plants that were the result of at least three backcrosses.

Table 1. Lipid Composition of Leaves of the Col-2 Wild Type and the *dgd1* Mutant

Lipid Class[a]	Wild Type[b]	*dgd1*[b]
MGD	51.3 ± 4.0	54.3 ± 2.7
PG	8.1 ± 1.2	8.6 ± 0.3
DGD	16.0 ± 2.1	1.2 ± 0.2
SL	2.4 ± 0.5	2.2 ± 0.2
PE	9.3 ± 0.9	12.0 ± 1.1
PC	12.8 ± 0.8	19.8 ± 1.4
MGD/(PG + DGD + SL)[c]	1.9	4.5

[a] PG, phosphatidylglycerol; SL, sulfolipid; PE, phosphatidylethanolamine; PC, phosphatidylcholine.
[b] Values are given as moles percent and represent the mean ± SE of three plants.
[c] Ratio of non-bilayer–to–bilayer-forming lipids in thylakoids.

Table 2. Fatty Acid Composition of Galactolipids from Leaves of the Col-2 Wild Type and the *dgd1* Mutant

Fatty Acid	MGD[a]		DGD[a]	
	Wild Type	*dgd1*	Wild Type	*dgd1*
16:0	0.7 ± 0.2	2.5 ± 0.7	11.3 ± 0.5	12.5 ± 1.7
16:1	1.1 ± 0.3	0.9 ± 0.1	0.5 ± 0.3	2.8 ± 0.4
16:2	1.4 ± 0.3	0.5 ± 0.2	0.8 ± 0.3	1.5 ± 2.2
16:3	32.1 ± 2.0	10.3 ± 0.9	2.8 ± 0.5	4.9 ± 0.4
18:0	0.2 ± 0.0	0.1 ± 0.0	0.9 ± 0.5	1.9 ± 0.6
18:1	0.0 ± 0.0	0.0 ± 0.0	1.1 ± 0.2	2.0 ± 0.3
18:2	1.8 ± 0.4	1.2 ± 0.2	4.1 ± 1.3	2.9 ± 0.1
18:3	62.8 ± 1.3	84.5 ± 0.4	78.4 ± 2.4	71.5 ± 3.8

[a] Values are given as moles percent and represent the mean ± SE of three independent plant batches.

The Biochemical Phenotype of the *dgd1* Mutant

The result of a quantitative comparison of the polar lipid composition of the wild type and the *dgd1* mutant is shown in Table 1. A strong reduction of the relative amount of DGD from 16.0 to 1.2 mol% was observed for the *dgd1* mutant. The total fatty acid content of the leaves was identical (3.3 mg g^{-1} fresh weight) in extracts from the wild type and the *dgd1* mutant. The relative amounts of the other lipids were increased in the mutant such that no specific lipid compensated for the loss of DGD. However, the ratio of the non-bilayer–forming thylakoid lipid MGD to the bilayer-forming thylakoid lipids phosphatidylglycerol, sulfolipid, and DGD was increased from 1.9 to 4.5 in the *dgd1* mutant (Table 1).

The fatty acid content of phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, and sulfolipid was not altered (data not shown), but there was a strong reduction in 16:3$\Delta^{7,10,13}$ and a concomitant increase in the content of 18:3$\Delta^{9,12,15}$ in MGD of the *dgd1* mutant as shown in Table 2. In contrast to MGD, the remaining DGD in the mutant showed

the same fatty acid pattern as in the wild type, with 18:3$\Delta^{9,12,15}$ as the predominant fatty acid (Table 2).

The *dgd1* Locus Maps to a Small Interval on Chromosome 3

Genetic mapping in Arabidopsis has recently been facilitated by the introduction of polymerase chain reaction (PCR)-based markers such as simple sequence length polymorphisms (Bell and Ecker, 1994) and cleaved amplified polymorphic sequences (Konieczny and Ausubel, 1993). In preparation for experiments to determine the genetic map position of *dgd1*, we scored different PCR-based DNA markers in the ecotypes Col-2 and Landsberg *erecta* (Ler) as well as in the original selfed *dgd1* mutant line and a *dgd1* mutant line following three backcrosses to Col-2. The results for the original *dgd1* mutant indicated that the genetic background was not pure Col-2, because Col-2 or Ler scoring markers were found interspersed throughout the genome. Following three backcrosses to the Col-2 wild type, all markers that scored Ler in the original *dgd1* mutant were converted to Col-2, except those located in a small region on the upper arm of chromosome 3 (nga162 and nga172). We crossed this particular *dgd1* mutant line to Col-2 and scored 70 *dgd1* F_2 plants. Linkage to markers nga162 and nga172 was observed, and the approximate map position for the *dgd1* locus was determined (Figure 1). A second F_2 mapping population was derived from a cross between the Col-2 backcrossed *dgd1* mutant line and ecotype Ler. Scoring 100 *dgd1* F_2 plants, no linkage to markers outside the upper arm of chromosome 3 was detected. Although the Col-2/Ler genetic background represents a complication for future fine mapping experiments, it nevertheless gave us a tool to monitor the exchange of mutagenized DNA regions with wild-type Col-2 DNA following backcrosses. Proper selection of backcrossed mutant lines for further experiments allowed us to minimize the risk that an observed phenotype was not attributable to a defect at the *DGD1* locus but to a secondary mutation.

Biochemical and Morphological Traits of the *dgd1* Mutant Cosegregate

Figure 2 shows the appearance of backcrossed *dgd1* mutant plants exposed to different light intensities over a period of 8 days. When grown under optimal light irradiation (90 μmol m^{-2} sec^{-1}), *dgd1* plants were reduced in size and had a bushy shape. The leaves were pale green and curly in appearance. With increasing light intensities, this phenotype became more severe, and leaves of the *dgd1* mutant started to deteriorate.

Because no additional independent mutant allele has been isolated to date, cosegregation analysis was performed to test whether the morphological phenotype of the *dgd1* mutant was caused by a secondary background mutation. For this purpose, the F_2 progeny from a cross between the *dgd1* mutant and wild-type Col-2 was scored. Of 422 plants analyzed, 84 plants (19.9%) were DGD deficient, stunted, and pale green, whereas the appearance of 388 plants (80.1%) with normal DGD content was indistinguishable from that of wild-type plants. In addition, 170 *dgd1* F_2 plants derived from different backcrosses to Col-2 or Ler ecotypes were all stunted and showed pale green leaf color. During the genetic mapping experiments described above, replacement of mutagenized DNA from the original *dgd1* line with Col-2 wild-type DNA up to an unknown position within the interval between the DNA markers nga162 and nga172 flanking *dgd1* (Figure 1) could be confirmed for 28 of these plants. Because these 28 plants, which were all stunted and pale green and showed reduced DGD content, carry a crossover at presumably random locations within the interval, the probability that there is a second mutation in the *dgd1* background causing stunted growth and pale green leaf color is very low. These results strongly suggest that the altered polar lipid composition of the *dgd1* mutant, the stunted growth, and pale green leaf color are caused by the same mutation.

Chloroplast Ultrastructure Is Altered in the *dgd1* Mutant

Cross-sections of Col-2 wild-type and *dgd1* mesophyll cell chloroplasts were analyzed by transmission electron microscopy to compare the ultrastructure of the thylakoid membrane system. Representative chloroplasts of both lines are shown in Figures 3A and 3B. In chloroplasts of the *dgd1* mutant, the thylakoid membranes were highly curved and displaced from the central stroma area toward the envelopes, resulting in a demixing of the stroma and the thylakoid membrane system. An overwhelming number of wild-type chloroplasts (99 of 100 examined in two plants) but only one-third of the *dgd1* chloroplasts (66 of 200 examined in four plants) showed a normal ultrastructure. To obtain a more quantitative measure of these ultrastructural changes, a morphometric analysis was performed. As shown in Table 3, slightly fewer chloroplasts per cell cross-section were observed in the *dgd1* mutant. The number of grana per chloroplast and the number of thylakoids per granum were found to be increased in the *dgd1* mutant. The values were significantly different as determined by a Student's

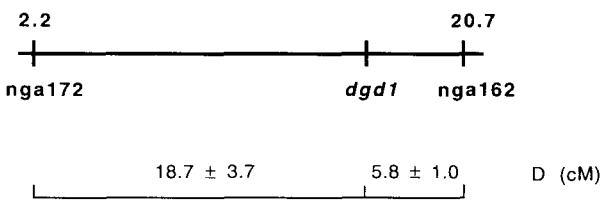

Figure 1. Map Position for the *dgd1* Locus on Chromosome 3.

The map is based on the analysis of 70 F_2 plants. The positions for the DNA markers are derived from the Arabidopsis integrated map published by Lister (1995). Distances are given in centimorgans (cM) and were calculated from recombination fractions.

t test ($P < 0.05$). The measurements revealed an increase in both stromal and granal thylakoid length in the mutant. In summary, these results imply a dramatic increase in the total length of membranes per plastid and a slight reduction in the ratio of appressed to nonappressed membranes in the *dgd1* mutant.

The *dgd1* Mutant Has Reduced Photosynthetic Capabilities

Because DGD is one of the major lipids of photosynthetic membranes, the possibility was considered that a significant reduction in the amount of this lipid might affect photosynthesis. To compare the photosynthetic quantum yield, the in vivo chlorophyll fluorescence of Col-2 wild-type and *dgd1* leaves was determined, using plants exposed to different light intensities, as indicated in Figure 2. In addition, the total chlorophyll content in these plants was measured and the chlorophyll *a*–to–chlorophyll *b* ratio was calculated. The results of these experiments are shown in Figure 4. A reduction in the quantum yield (Figure 4A) and in total chlorophyll content (Figure 4B) was observed in *dgd1* plants under all light conditions. Because there was a greater reduction in chlorophyll *a* compared with chlorophyll *b*, the chlorophyll *a*–to–chlorophyll *b* ratio was also lower in the *dgd1* mutant leaves (Figure 4C). In addition, there was a slight reduction in the total carotenoid content of the *dgd1* plants (data not shown). With increasing light intensities, an increase in the chlorophyll *a*–to–chlorophyll *b* ratio was found for both wild-type and *dgd1* plants. However, the *dgd1* mutant was apparently unable to respond to the same extent as the wild type to higher light intensities by increasing the chlorophyll *a*–to–chlorophyll *b* ratio.

To identify a possible change in the composition of photosystem I or II complexes in the *dgd1* mutant, thylakoids were isolated from wild-type and *dgd1* leaves and solubilized with detergent mixtures containing either octylglucoside or

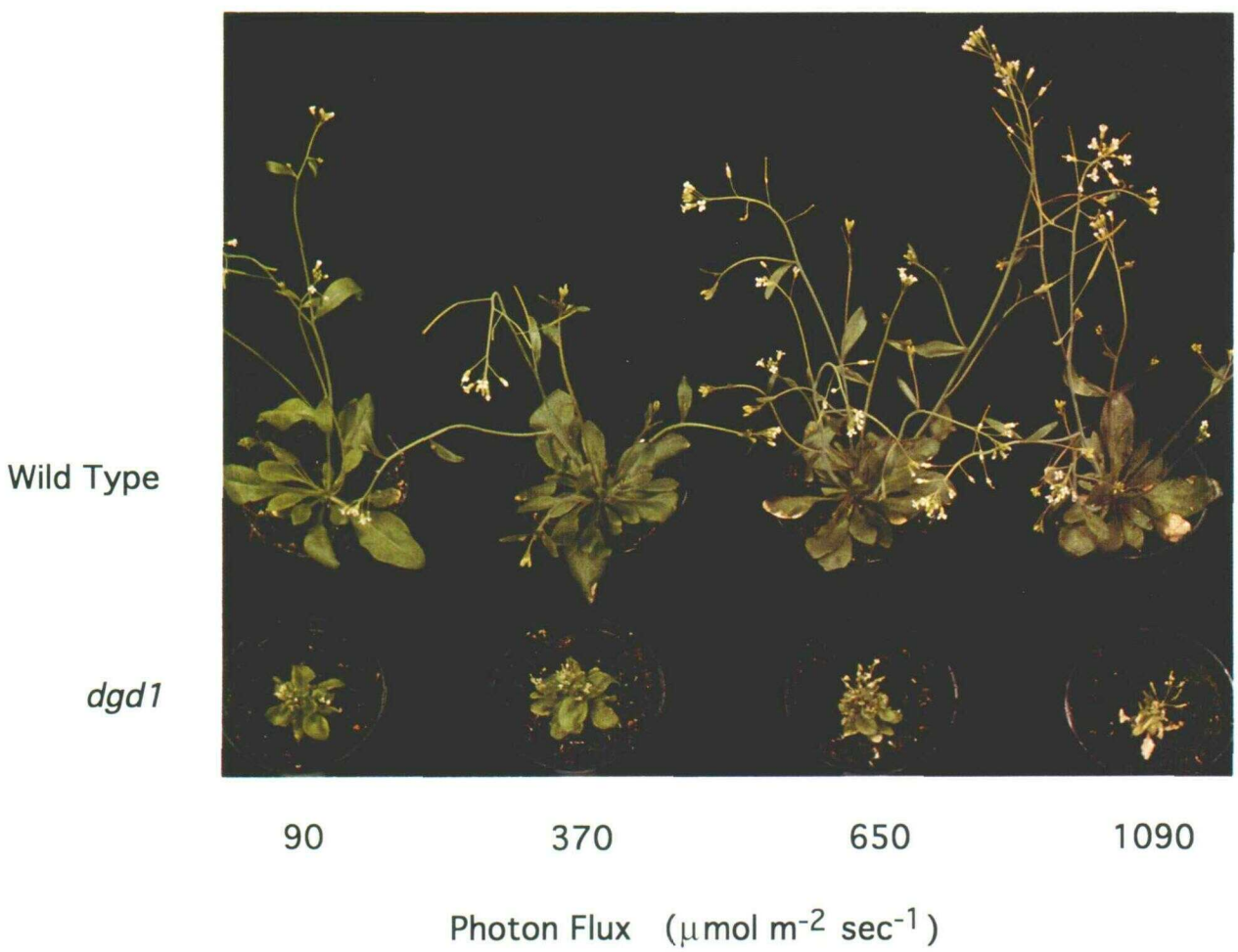

Figure 2. Visible Phenotype of Wild-Type Plants and *dgd1* Mutants.

Col-2 wild-type plants and *dgd1* mutants (24 days old) were adapted to different light irradiations as indicated.

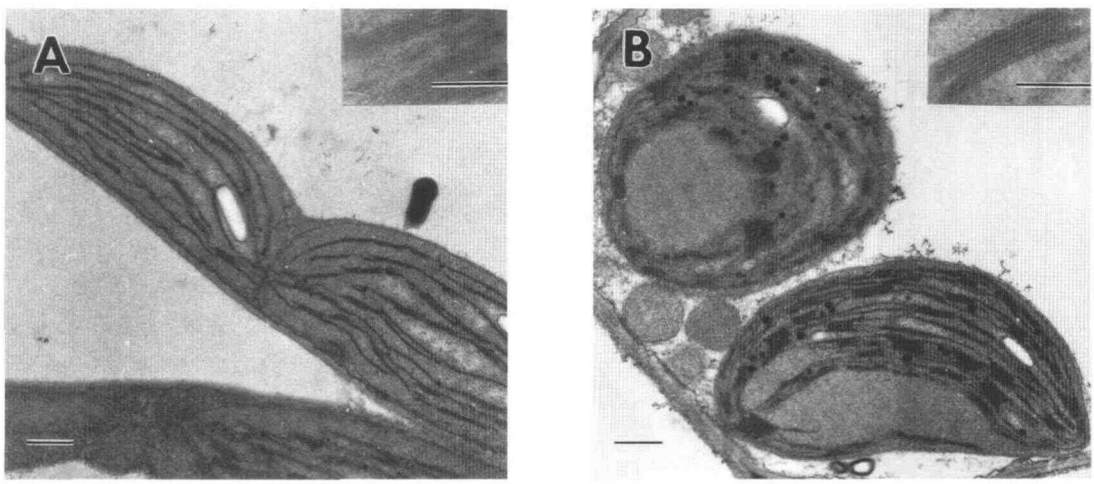

Figure 3. Chloroplast Ultrastructure of the Wild Type and the *dgd1* Mutant.

(A) Typical leaf chloroplast of the Col-2 wild type.
(B) Typical leaf chloroplast of *dgd1*.
Representative grana from wild-type or mutant chloroplasts are presented in the insets. Bars = 500 nm; bars in the insets = 200 nm.

decylmaltoside in combination with SDS. The solubilized pigment protein complexes were analyzed by partially denaturing gel electrophoresis. A representative result from a series of experiments is shown in Figure 5. Visual examination of decylmaltoside-treated samples revealed no differences between the wild type and mutant. However, following treatment with octylglucoside, a slight reduction of the trimeric form of light-harvesting complex II (LHCII) and a concomitant increase in the monomeric form were visible in the mutant sample. To obtain a more quantitative measure for these apparent differences, the relative abundance of pigment protein complexes was determined by scanning densitometry. Treatment with octylglucoside was found to change the ratio of the monomeric LHCII to trimeric LHCII from 1.7 to 3.7 in *dgd1* mutant samples. In contrast, this ratio was not increased in samples of the mutant treated with decylmaltoside (1.1 for wild type and 1.0 for *dgd1* mutant). No differences between the wild type and the mutant were observed with respect to the relative amounts of photosystem I components and the photosystem II core complex in samples treated with either of the detergent mixtures.

The Search for the Biochemical Defect in the *dgd1* Mutant

In an attempt to elucidate the primary biochemical defect causing the reduction in DGD content in the *dgd1* mutant, chloroplasts were isolated from Col-2 wild-type and *dgd1* leaves, and the incorporation of UDP-U-^{14}C-galactose into galactolipids by chloroplast membranes was monitored. The result of a representative experiment is given in Table 4. In all our experiments with Arabidopsis, the rate of incorporation of

Table 3. Ultrastructural Analysis of Chloroplasts of the Col-2 Wild Type and the *dgd1* Mutant

	Wild Type[a]	*dgd1*[a]
Plastid/cell cross-section (n = 30)	11.5 ± 0.5[b]	9.6 ± 0.5[b]
Plastid cross-sectional area (n = 15; μm^2)	9.0 ± 1.7	10.6 ± 1.9
Grana/plastid (n = 25)	49.6 ± 2.2[b]	55.6 ± 2.2[b]
Thylakoids/granum (n = 230)	4.8 ± 0.2[b]	5.5 ± 0.3[b]
Grana thylakoid length (n = 100; μm)	0.39 ± 0.01[b]	0.46 ± 0.01[b]
Stroma thylakoids/plastid (n = 15)	75.1 ± 7.5	90.6 ± 7.2
Stroma thylakoid length (n = 100; μm)	0.24 ± 0.02[b]	0.40 ± 0.02[b]
Appressed membranes (μm/plastid)	73.6[c]	115.3[c]
Nonappressed membranes (μm/plastid)	37.4[c]	61.8[c]
Total thylakoid membranes (μm/plastid)	111.0[c]	177.1[c]
Appressed/nonappressed thylakoids	2.0[c]	1.9[c]

[a] Values are given as means ± SE.
[b] Values are significantly different based on the Student's t test ($P < 0.05$).
[c] These values were calculated from measured parameters in the table.

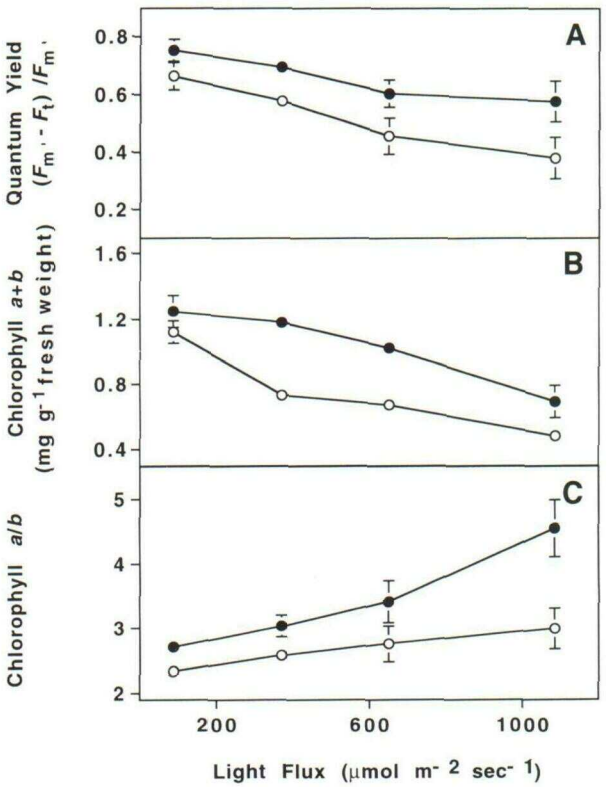

Figure 4. Photosynthetic Parameters of the Wild Type and the *dgd1* Mutant.

Col-2 wild-type (●) and *dgd1* plants (○) were adapted to different light irradiations as indicated.

(A) Quantum yield $(F_m-F_t)/F_{m'}$.

(B) Total chlorophyll.

(C) Chlorophyll *a*–to–chlorophyll *b* ratio.

The values represent the means and SEs of measurements taken on at least six different plants.

label from UDP-U-^{14}C-galactose into MGD was generally <10% of the rates determined for other plants. For example, in a control experiment, a rate of 546 ± 105 pmol mg^{-1} chlorophyll min^{-1} was determined for tobacco chloroplasts. Comparing the rates for MGD and DGD labeling from UDP-U-^{14}C-galactose in the Arabidopsis wild type and the *dgd1* mutant, no difference was detected (Table 4).

Because we could not exclude that the relatively low overall MGD and DGD labeling rates determined for Arabidopsis chloroplasts in vitro may not reflect the biosynthesis rates for galactolipids in vivo, we performed alternative labeling experiments using intact seedlings that were kept in liquid medium. For a substrate we employed U-^{14}C-glucose because it is readily taken up by seedlings and can be expected to label general lipid biosynthetic precursors such as acetyl-CoA or glycerol-3-phosphate as well as the specific galactolipid biosynthetic precursor UDP-galactose. In Table 4, the rates of MGD and DGD labeling in vitro and in vivo are compared as determined for the wild type and the mutant. Contrary to the labeling rates of MGD and other lipids (data not shown), the initial rate of in vivo DGD labeling was reduced to ~50% of wild-type levels in the *dgd1* mutant.

DISCUSSION

We isolated a mutant of Arabidopsis with a >90% reduction in the content of the galactolipid DGD following a direct screen. The mutation responsible for this biochemical phenotype is recessively inherited and maps to a single locus on chromosome 3. This locus was designated *dgd1*. The presence of residual DGD suggests that either the respective mutation is leaky or only one of a number of pathways for the biosynthesis of DGD is rendered inactive. Previously, many mutants of Arabidopsis affected in the fatty acid composition of leaf lipids were isolated (Browse and Somerville, 1991). However, the *dgd1* mutant is the first plant mutant with a reduction in the amount of a complete thylakoid lipid class. Therefore, it provides a novel opportunity to test current hypotheses about galactolipid biosynthesis and function in higher plants. Here, we discuss the results of our morphological, genetical, physiological, and biochemical analysis of the *dgd1* mutant.

In addition to their abnormal thylakoid lipid composition, *dgd1* plants show other phenotypic traits, of which the most visible are stunted growth and pale green leaf color. To draw a causal connection between the lipid phenotype and other phenotypic traits of the mutant, we had to be certain that these could be traced to the same genetic defect. Because no independent

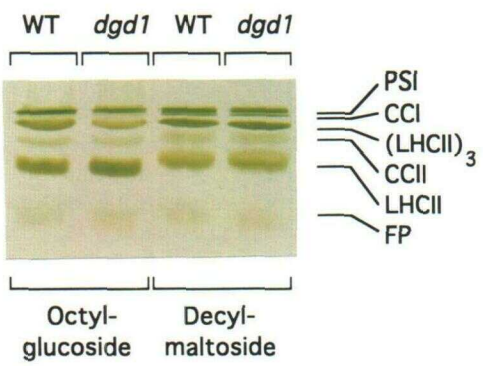

Figure 5. Separation of Pigment Protein Complexes Solubilized from Thylakoids of the Wild Type and the *dgd1* Mutant.

Pigment protein complexes were solubilized with mixtures of SDS and either octylglucoside or decylmaltoside and separated by partially denaturing gel electrophoresis. The green bands were visible without staining. CCI, core complex I; CCII, core complex II; FP, free pigment; LHCII, light-harvesting complex II; (LHCII)$_3$, trimeric form of LHCII; PSI, photosystem I; WT, wild type.

Table 4. Radioactive Labeling of Galactolipids in Chloroplast Membranes and Intact Seedlings

Lipid Class	Chloroplasts (pmol UDP-Galactose mg^{-1} chlorophyll min^{-1})		Seedlings[a] (pmol Glucose g^{-1} fresh weight hr^{-1})	
	Wild Type	*dgd1*	Wild Type	*dgd1*
MGD	20.2 ± 2.7	19.5 ± 3.3	13.5 ± 0.6	14.2 ± 0.6
DGD	5.2 ± 0.3	5.3 ± 0.2	4.2 ± 0.3	2.1 ± 0.3

[a] Values representing mean ± SE were obtained by linear regression during the linear phase of a time course experiment.

mutant carrying a second allele of *dgd1* has been isolated to date, we employed cosegregation analysis to address this issue. Extensive probing of F_2 populations from different backcrosses between *dgd1* and wild-type plants confirmed that the morphological and biochemical traits are caused by the same genetic mutation. Therefore, the analysis of the *dgd1* mutant provides conclusive experimental evidence that DGD is important for normal growth in higher plants; it is possible that a further reduction in the DGD content may be lethal.

In addition to stunted growth and pale green leaf color, we observed a dramatic alteration in overall chloroplast ultrastructure in leaves that can best be described as having a "warped" thylakoid membrane architecture. In view of the unique biophysical properties of MGD with respect to the formation of a hexagonal phase (H_{II}) in vitro, it is tempting to conclude that the increase in the curvature of thylakoid membranes in the *dgd1* mutant may be the direct consequence of the relative increase in the ratio of the non-bilayer–forming MGD to bilayer-forming thylakoid lipids. However, we observed similar changes in thylakoid ultrastructure in Arabidopsis chloroplasts with normal lipid composition which had been challenged by stress factors such as reduced light (data not shown). In addition, mutants with altered fatty acid composition of thylakoid lipids (e.g., Hugly et al., 1989) or mutants lacking different components of the photosynthetic apparatus (e.g., Simpson and von Wettstein, 1980; Allen et al., 1988) show similar ultrastructural changes of the chloroplast. In view of these observations, we conclude that a sufficient amount of DGD is only one of many factors required for the proper formation and maintenance of thylakoid membranes in vivo.

The pale green color of *dgd1* mutant leaves is reflected by the reduction in total chlorophyll content. A corresponding decrease in the photosynthetic quantum yield in *dgd1* plants was determined by in vivo fluorescence measurements. The reduced photosynthetic quantum yield may be a direct consequence of the reduction in chlorophyll content, and the decrease in the photosynthetic efficiency seems to be a likely cause for the stunted growth of the mutant. The *dgd1* and wild-type plants respond to higher light intensities with an increase in the chlorophyll *a*–to–chlorophyll *b* ratio, a well-known adaptation to changing light conditions (Anderson, 1986). However, the response of the wild type is much more pronounced. This difference between the two lines reveals an apparent limitation of the mutant to adjust the photosynthetic apparatus, which may explain the more severe visible phenotype under high light conditions.

The decreased stability of the most abundant pigment protein complex, LHCII, during electrophoresis suggests that the remaining lipids in the *dgd1* mutant can only partially substitute for the interaction of DGD with the pigment protein complexes of the photosynthetic apparatus. However, this effect is only visible following solubilization of the membranes with octylglucoside–SDS but not decylmaltoside–SDS mixtures. This result suggests that in mutant samples, decylmaltoside, which is a milder surfactant containing a dihexosyl head group resembling DGD (Peter and Thornber, 1991), may substitute for DGD in stabilizing LHCII. Typically, a stoichiometric number of DGD and phosphatidylglycerol molecules is found associated with isolated trimeric LHCII (Nussberger et al., 1993). In contrast to phosphatidylglycerol, DGD can easily be removed and is not required for trimerization in vitro. In addition, the removal of DGD renders trimeric LHCII unable to form two- or three-dimensional crystals in vitro. Thus, it appears that DGD is specifically bound to the periphery of LHCII and may prevent destabilization of the trimeric LHCII in the same way as the surfactant decylmaltoside during the analysis of the pigment protein complexes by partially denaturing gel electrophoresis. Although this experiment provides evidence that the complex isolated from the *dgd1* mutant is more unstable in vitro, it is not sufficient to conclude that trimeric LHCII, which represents the in vivo form of LHCII (Kühlbrandt and Wang, 1991), is more unstable in vivo due to the reduced DGD content in the mutant. An even more drastic effect on the stability of trimeric LHCII during partially denaturing gel electrophoresis was found for an Arabidopsis mutant lacking *trans*-3-hexadecenoic acid, a fatty acid specific for plastidic phosphatidylglycerol (McCourt et al., 1985). However, contrary to the *dgd1* mutant, the *trans*-3-hexadecenoic deficient mutant did not show an altered photosynthetic efficiency.

The current hypothesis about DGD biosynthesis implies that DGD is formed in the outer envelope of the chloroplast by transfer of a galactose moiety from one MGD molecule onto a second MGD molecule (van Besouw and Wintermans, 1978). In agreement with the typical fatty acid composition of DGD in Arabidopsis, one would expect that the eukaryotic MGD molecular species containing the fatty acid $18{:}3\Delta^{9,12,15}$ in both positions of the diacylglycerol moiety is the preferred acceptor. A deficiency in this reaction in the *dgd1* mutant should result in an accumulation of eukaryotic MGD species. Indeed, we observed an increase in the ratio of $18{:}3\Delta^{9,12,15}$ to $16{:}3\Delta^{7,10,13}$ in MGD of the *dgd1* mutant (Table 2). However, the total amount of MGD is only slightly increased. The reduction in the relative abundance of $16{:}3\Delta^{7,10,13}$ of MGD in the *dgd1* mutant suggests a decrease in the amount of MGD molecular species derived from the prokaryotic pathway. In comparison, a mutant of Arabidopsis, *act1*, for which a direct metabolic block in the prokaryotic pathway has been identified, contains very little $16{:}3\Delta^{7,10,13}$ in thylakoid lipids but slightly increased

amounts of DGD (Kunst et al., 1988). Therefore, it seems likely that the prokaryotic pathway is only indirectly affected in the *dgd1* mutant.

To understand the molecular basis for the reduced amount of DGD and the altered fatty acid composition of MGD in the *dgd1* mutant, it is necessary to identify the defective enzyme. The DGD synthase is the only enzyme proposed to be specifically involved in DGD biosynthesis and is therefore the most likely protein to be affected in the *dgd1* mutant. We determined the incorporation of radioactive label from UDP-U-^{14}C-galactose into MGD and DGD by isolated chloroplast membranes. Surprisingly, we observed nearly identical rates of incorporation for the wild type and the mutant, suggesting that enzymes directly involved in galactolipid head group biosynthesis may not be affected in the *dgd1* mutant. However, the overall incorporation of label is very low in comparison to chloroplasts from other species, such as tobacco. It is known that different plants exhibit different incorporation rates using this assay (Heemskerk et al., 1990). Particularly, isolated chloroplasts of white mustard, a plant closely related to Arabidopsis, show low galactolipid biosynthetic activity. Currently, we cannot exclude the possibility that the rates we observed for galactolipid labeling with isolated chloroplasts of Arabidopsis do not reflect the activities of the MGD and DGD synthases in vivo. Comparing the incorporation of U-^{14}C-glucose into galactolipids by intact seedlings of wild type and *dgd1* mutant, the DGD labeling rate was found to be reduced to only ~50% in the mutant, although the steady state level of DGD is decreased to <10%. The interpretation of this result is difficult, given that glucose is not a specific precursor for galactolipid biosynthesis. However, considering the results of the in vitro and in vivo experiments together, it is possible that the mutation at the *dgd1* locus may affect the DGD turnover rate rather than just the unidirectional synthesis.

Galactolipid degrading enzymes, such as galactolipases, α-, and β-galactosidases, are known to be present in leaf tissues of different plants (e.g., Sastry and Kates, 1964; Helmsing, 1969) but have not been characterized for Arabidopsis. In addition, breakdown of galactolipids in response to ozone fumigation has recently been reported for spinach leaves (Sakaki et al., 1990). However, galactolipid turnover rates in fully expanded leaves, which are not stressed, seem to be very low (Roughan, 1970). Further analysis of Arabidopsis wild-type and *dgd1* mutant plants may shed some light on the extent and role of galactolipid turnover in higher plants.

The changes in fatty acid composition in the *dgd1* mutant reveal the complexity of DGD biosynthesis. In addition, the difficulties encountered during the search for the enzymatic defect in the *dgd1* mutant are a further example of the problems inherent in a biochemical analysis of galactolipid biosynthesis. We hope to overcome these difficulties in the future by isolating the gene affected in the *dgd1* mutant. Analysis of the respective gene product as well as further characterization of the mutant should allow us to gain a deeper insight into the biosynthesis and function of the galactolipid DGD.

METHODS

Plant Material

All lines of *Arabidopsis thaliana* were of ecotypes Columbia (Col-2) or Landsberg *erecta* (Ler). Plants were grown in soil (Einheitserde Type P/Einheitserde Type T/sand [2:1:1]; Gebrüder Patzer, Sinntal-Jossa, Germany) under a 16-hr-light/8-hr-dark regime. The light intensity was set at 100 μmol m^{-2} sec^{-1} unless otherwise stated in the text.

To increase the mutation rate, Arabidopsis seeds (Col-2) were treated with ethyl methanesulfonate, giving rise to M_1 plants (Haughn and Somerville, 1986). Following self-pollination, 20 independent batches of M_2 seeds were collected, each representing ~1000 M_1 plants. To monitor the extent of mutagenesis, M_2 seeds were plated on 2,6-diaminopurine (Moffat and Somerville, 1988). Approximately 1 in 5000 seedlings was resistant, as expected for a well-mutagenized M_2 population, a prerequisite for successful screening.

Genetic Procedures

Segregation analysis was performed using F_2 populations originating from crosses as indicated, to map the *dgd1* locus relative to polymerase chain reaction (PCR) markers. Genomic DNA for PCR was isolated from the leaves of F_2 plants, as described by Rogers and Bendich (1994). Cleaved amplified polymorphic sequence markers were scored according to Konieczny and Ausubel (1993) and simple sequence-length polymorphism markers according to Bell and Ecker (1994). *Taq* polymerase and restriction enzymes were obtained from Gibco BRL (Gaithersburg, MD) and New England Biolabs (Beverly, MA), respectively, and DNA fragments were separated by agarose gel electrophoresis (Seakem LE Agarose and MetaPhor Agarose; FMC BioProducts, Rockland, ME). The map locations for the respective PCR markers were taken from the most recent integrated map generated for Ler/Col-0 recombinant inbred lines (Lister, 1995). Recombination fractions were obtained as number of crossovers divided by number of chromosomes analyzed, and map distances in centimorgans were calculated according to Kosambi (1944).

Lipid Analysis

During the screening procedure, single rosette leaves from M_2 plants were frozen in liquid nitrogen and lipids were extracted by homogenization in 50 μL chloroform/methanol/formic acid (1:1:0.1 [v/v]) and 25 μL of 1 M KCl-0.2 M H_3PO_4. Lipid extracts were separated on activated ammonium sulfate–impregnated silica gel thin-layer chromatography plates (Si 250 PA; J.T. Baker, Phillipsburg, NJ) as described by Benning and Somerville (1992), except that the solvent system was replaced by acetone/toluene/water (91:30:8 [v/v]). The plates were stained with iodine vapor and visually screened for alterations in polar lipid content.

For quantitative analysis, lipids were extracted from leaves and separated by thin-layer chromatography, as described above. Individual lipids were isolated from thin-layer chromatography plates and used to prepare fatty acid methyl esters with 1 N HCl in methanol at 80°C for 30 min. The methyl esters were quantified by gas chromatography, using myristic acid as internal standard (Benning and Somerville, 1992).

Transmission Electron Microscopy

Rosette leaves of 4-week-old plants were fixed in 4% (v/v) glutaraldehyde, 0.1 M sodium phosphate, pH 7.2, on ice followed by a secondary fixation in 1% (w/v) OsO_4 in the same buffer (Hoffmann-Benning et al., 1994). The specimens were dehydrated in a graded series of acetone and embedded in Spurr's epoxy resin. Ultrathin sections (80 nm) were stained with uranyl acetate and viewed in a Philips 400 transmission electron microscope. Dimensions were determined from photographs of two to three independent preparations of three (wild-type) to five (*dgd1*) plants.

Chlorophyll Fluorescence and Chlorophyll Quantification

A pulse amplitude modulation fluorometer (PAM-2000; Heinz Walz, Effeltrich, Germany) was used to measure chlorophyll fluorescence of Col-2 wild-type and *dgd1* mutant plants. The quantum yield was calculated according to the equation $(F_{m'}-F_t)/F_{m'}$, where F_t and $F_{m'}$ are the fluorescence emissions of a light-adapted plant under measuring light or after application of a saturating light pulse, respectively (Schreiber et al., 1986). Chlorophyll concentrations were calculated from the absorbances at 646 and 663 nm of an 80% (v/v) acetone extract according to Lichtenthaler (1987).

Chloroplast Isolation and Pigment Protein Electrophoresis

Chloroplasts were isolated from Col-2 wild-type and *dgd1* plants according to Price et al. (1994) and resuspended in 6.2 mM Tris, 48 mM glycine, 10% (v/v) glycerol, pH 8.3. Pigment protein complexes were solubilized with mixtures of SDS and one of the two non-ionic surfactants octylglucoside (*n*-octyl-β-D-glucopyranoside) or decylmaltoside (*n*-decyl-β-D-maltopyranoside; Sigma, Deisenhofen, Germany) in a ratio of chlorophyll/SDS/non-ionic surfactant of 1:1:9 (w/w). Solubilized pigment protein complexes representing 8 μg of total chlorophyll were separated by partially denaturing polyacrylamide gel electrophoresis (Andersson et al., 1982). The gel was not stained. Three independent thylakoid preparations from Col-2 wild-type and *dgd1* plants were analyzed in different gels, and the lanes scanned at 633 nm with an Ultroscan XL Laser Densitometer (Pharmacia LKB, Uppsala, Sweden).

Radioactive Labeling of Galactolipids

Chloroplasts (250 μg chlorophyll) for labeling experiments were prepared as described above, resuspended in 0.3 M sorbitol, 20 mM Tricine-KOH, pH 7.6, 5 mM $MgCl_2$, 2.5 mM EDTA, and incubated with 0.5 μCi UDP-U-^{14}C-galactose (329 mCi mmol^{-1}; Amersham Buchler, Braunschweig, Germany) in a total volume of 130 μL. After various times, 25 μL aliquots were removed, and polar lipids were extracted and separated by thin-layer chromatography as described above. The bands containing monogalactosyl diacylglycerol (MGD) and digalactosyl diacylglycerol (DGD) were isolated from the plates and used to determine the radioactivity by liquid scintillation counting. The reaction was found to be linear for at least 20 min, and the activity of UDP-U-^{14}C-galactose incorporation into galactolipids was calculated by linear regression. For the labeling of galactolipids in intact seedlings, ~100 Col-2 wild-type and *dgd1* plantlets (9 days old) were placed in 12.5 mL liquid medium, pH 6.0 (Murashige and Skoog, 1962), containing 10 μCi U-^{14}C-glucose (298 mCi mmol^{-1}; Amersham) under continuous illumination (30 μmol m^{-2} sec^{-1}). At various times, ~20 seedlings were removed, frozen in liquid nitrogen, and used to extract polar lipids. The incorporation of radioactivity into galactolipids was determined as described above and was found to be linear for at least 5 hr.

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REFERENCES

Allen, K.D., Duysen, M.E., and Staehelin, L.A. (1988). Biogenesis of thylakoid membranes is controlled by light intensity in the conditional chlorophyll *b*-deficient CD3 mutant of wheat. J. Cell Biol. **107**, 907–919.

Anderson, J.M. (1986). Photoregulation of the composition, function and structure of thylakoid membranes. Annu. Rev. Plant Physiol. **37**, 93–136.

Andersson, B., Anderson, J.M., and Ryrie, I.J. (1982). Transbilayer organization of the chlorophyll-proteins of spinach thylakoids. Eur. J. Biochem. **123**, 465–472.

Arondel, V., Benning, C., and Somerville, C.R. (1993). Isolation and functional expression in *Escherichia coli* of a gene encoding phosphatidylethanolamine methyltransferase (EC 2.1.1.17) from *Rhodobacter sphaeroides*. J. Biol. Chem. **268**, 16002–16008.

Bell, C.J., and Ecker, J.R. (1994). Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. Genomics **19**, 137–144.

Benning, C., and Somerville, C.R. (1992). Isolation and genetic complementation of a sulfolipid-deficient mutant of *Rhodobacter sphaeroides*. J. Bacteriol. **174**, 2352–2360.

Benning, C., Beatty, J.T., Prince, C.R., and Somerville, C.R. (1993). The sulfolipid sulfoquinovosyldiacylglycerol is not required for photosynthetic electron transport in *Rhodobacter sphaeroides* but enhances growth under phosphate limitation. Proc. Natl. Acad. Sci. USA **90**, 1561–1565.

Block, M.A., Dorne, A.-J., Joyard, J., and Douce, R. (1983). Preparation and characterization of membrane fractions enriched in outer and inner envelope membranes from spinach chloroplasts. II. Biochemical characterization. J. Biol. Chem. **258**, 13281–13286.

Browse, J., and Somerville, C.R. (1991). Glycerolipid synthesis: Biochemistry and regulation. Annu. Rev. Plant Physiol. Plant Mol. Biol. **42**, 467–506.

Carter, H.E., McCluer, R.H., and Slifer, E.D. (1956). Lipids of wheat flour. I. Characterization of galactosylglycerol components. J. Am. Chem. Soc. **78,** 3735–3738.

Cline, K., and Keegstra, K. (1983). Galactosyltransferases involved in galactolipid biosynthesis are located in the outer membrane of pea chloroplast envelopes. Plant Physiol. **71,** 366–372.

Dorne, A.-J., Block, M.A., Joyard, J., and Douce, R. (1982). The galactolipid:galactolipid galactosyltransferase is located on the outer surface of the outer membrane of the chloroplast envelope. FEBS Lett. **145,** 30–34.

Douce, R., and Joyard, J. (1980). Plant galactolipids. In The Biochemistry of Plants, Vol. 4, Lipids: Structure and Function, P.K. Stumpf, ed (New York: Academic Press), pp. 321–362.

Haughn, G.W., and Somerville, C.R. (1986). Sulfonyl-resistant mutants of *Arabidopsis thaliana*. Mol. Gen. Genet. **204,** 430–434.

Heemskerk, J.W.M., Strorz, T., Schmidt, R.R., and Heinz, E. (1990). Biosynthesis of digalactosyl diacylglycerol in plastids from 16:3 and 18:3 plants. Plant Physiol. **93,** 1286–1294.

Heinz, E. (1977). Enzymatic reactions in galactolipid biosynthesis. In Lipids and Lipid Polymers in Higher Plants, M. Tevini and H.K. Lichtenthaler, eds (Berlin: Springer-Verlag), pp. 102–120.

Helmsing, P.J. (1969). Purification and properties of galactolipase. Biochim. Biophys. Acta **189,** 95–105.

Hoffmann-Benning, S., Klomparens, K.L., and Kende, H. (1994). Characterization of growth-related osmiophilic particles in corn coleoptiles and deepwater rice internodes. Ann. Bot. **74,** 563–572.

Hugly, S., Kunst, L., Browse, J., and Somerville, C.R. (1989). Enhanced thermal tolerance of photosynthesis and altered chloroplast ultrastructure in a mutant of *Arabidopsis* deficient in lipid desaturation. Plant Physiol. **90,** 1134–1142.

Joyard, J., Block, M.A., Malherbe, A., Maréchal, E., and Douce, R. (1993). Origin and synthesis of galactolipid and sulfolipid head groups. In Lipid Metabolism in Plants, T.S. Moore, Jr., ed (Boca Raton, FL: CRC Press), pp. 231–258.

Konieczny, A., and Ausubel, F.M. (1993). A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J. **4,** 403–410.

Kosambi, D.D. (1944). The estimation of map distances from recombination values. Ann. Eugen. **12,** 172–175.

Kühlbrandt, W., and Wang, D.N. (1991). Three-dimensional structure of plant light-harvesting complex determined by electron crystallography. Nature **350,** 130–134.

Kunst, L., Browse, J., and Somerville, C.R. (1988). Altered regulation of lipid biosynthesis in a mutant of *Arabidopsis* deficient in chloroplast glycerol-3-phosphate acyltransferase activity. Proc. Natl. Acad. Sci. USA **85,** 4143–4147.

Lichtenthaler, H.K. (1987). Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. Methods Enzymol. **148,** 350–382.

Lister, C. (1995). Latest Lister and Dean RI map. Weeds World **2,** 23–30.

McCourt, P., Browse, J., Watson, J., Arntzen, C.J., and Somerville, C.R. (1985). Analysis of photosynthetic antenna function in a mutant of *Arabidopsis thaliana* (L.) lacking *trans*-hexadecenoic acid. Plant Physiol. **78,** 853–858.

Moffat, B., and Somerville, C.R. (1988). Positive selection for male-sterile mutants of *Arabidopsis* lacking adenine phosphoribosyl transferase activity. Plant Physiol. **86,** 1150–1154.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. **15,** 473–497.

Murata, N., Fujimura, Y., and Higashi, S. (1990). Glycerolipids in various preparations of photosystem II from spinach chloroplasts. Biochim. Biophys. Acta **1019,** 261–268.

Nussberger, S., Dörr, K., Wang, D.N., and Kühlbrandt, W. (1993). Lipid–protein interactions in crystals of plant light-harvesting complex. J. Mol. Biol. **234,** 347–356.

Peter, G.F., and Thornber, J.P. (1991). Electrophoretic procedures for fractionation of photosystems I and II pigment-proteins of higher plants and for determination of their subunit composition. Methods Plant Biochem. **5,** 195–210.

Price, C.A., Hadjeb, N., Newman, L., and Reardon, E.M. (1994). Isolation of chloroplasts and chloroplast DNA. In Plant Molecular Biology Manual, S.B. Gelvin and R.A. Schilperoort, eds (Dordrecht, The Netherlands: Kluwer Academic Press), Sect. D4, pp. 1–15.

Rogers, S.O., and Bendich, A.J. (1994). Extraction of total cellular DNA from plants, algae and fungi. In Plant Molecular Biology Manual, S.B. Gelvin and R.A. Schilperoort, eds (Dordrecht, The Netherlands: Kluwer Academic Press), Sect. D1, pp. 1–8.

Roughan, P.G. (1970). Turnover of glycerolipids of pumpkin leaves. The importance of phosphatidylcholine. Biochem J. **117,** 1–8.

Sakaki, T., Kondo, N., and Yamada, M. (1990). Pathway for the synthesis of triacylglycerols from monogalctosyldiacylglycerol in ozone-fumigated spinach leaves. Plant Physiol. **94,** 773–780.

Sastry, P.S., and Kates, M. (1964). Hydrolysis of monogalactosyl and digalactosyl diglycerides by specific enzymes in runner-bean leaves. Biochemistry **3,** 1280–1287.

Schreiber, U., Schliwa, U., and Bilger, W. (1986). Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. Photosynth. Res. **10,** 51–62.

Simpson, D.J., and von Wettstein, D. (1980). Macromolecular physiology of plastids. XIV. *Viridis* mutants of barley: Genetic, fluoroscopic and ultrastructural characterization. Carlsberg Res. Commun. **45,** 283–314.

van Besouw, A., and Wintermans, J.F.G.M. (1978). Galactolipid formation in chloroplast envelopes. I. Evidence for two mechanisms in galactosylation. Biochim. Biophys. Acta **529,** 44–53.

Webb, M.S., and Green, B.R. (1991). Biochemical and biophysical properties of thylakoid acyl lipids. Biochim. Biophys. Acta **1060,** 133–158.