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Changes in the Composition of the Photosynthetic Apparatus in the Galactolipid-Deficient *dgd1* Mutant of *Arabidopsis thaliana*[1]

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The glycerolipid digalactosyl diacylglycerol (DGDG) is exclusively associated with photosynthetic membranes and thus may play a role in the proper assembly and maintenance of the photosynthetic apparatus. Here we employ a genetic approach based on the *dgd1* mutant of *Arabidopsis thaliana* to investigate the function of DGDG in thylakoid membranes. The primary defect in the genetically well-characterized *dgd1* mutant resulted in a 90% reduction of the DGDG content. The mutant showed a decreased photosystem II (PSII) to photosystem I ratio. In vivo room- and low-temperature (77 K) chlorophyll fluorescence measurements with thylakoid preparations are in agreement with a drastically altered excitation energy allocation to the reaction centers. Quantification of pigment-binding apoproteins and pigments supports an altered stoichiometry of individual pigment-protein complexes in the mutant. Most strikingly, an increase in the amount of peripheral light-harvesting complexes of PSII relative to the inner antenna complexes and the PSII reaction center/core complexes was observed. Regardless of the severe alterations in thylakoid organization, photosynthetic oxygen evolution was virtually not compromised in *dgd1* mutant leaves.

The primary reactions of photosynthesis in higher plants are associated with thylakoids, the inner membrane system of chloroplasts. Thylakoid membranes are unique in their lipid composition. Most abundant are the neutral galactolipids MGDG and DGDG, with a relative amount for both galactolipids of 60 to 80 mol% of the total thylakoid lipid fraction, followed by the anionic lipids phosphatidylglycerol and sulfoquinovosyl diacylglycerol (Douce and Joyard, 1990). Individual thylakoid lipids are thought to play an important role for the ordered assembly and structural maintenance of the photosynthetic apparatus. However, hypotheses on thylakoid lipid function are primarily based on the analysis of their physicochemical properties in defined mixtures with water (for review, see Webb and Green, 1991), their specific association with isolated pigment-protein complexes (e.g. Gounaris et al., 1983b; Murata et al., 1990; Trémolierès et al., 1994), and in vitro reconstitution experiments (Paulsen et al., 1990; Nußberger et al., 1993). The harsh detergent treatments involved in the in vitro procedures raised the question of whether respective results represent the situation in vivo. A possible solution to this problem is provided by a genetic approach that has been successfully applied in the case of the sulfolipid sulfoquinovosyl diacylglycerol.

The analysis of sulfolipid-deficient mutants of photosynthetic bacteria (Benning et al., 1993; Güler et al., 1996) and the unicellular alga *Chlamydomonas reinhardtii* (Sato et al., 1995) has proven very valuable for the evaluation of the role of sulfolipid in photosynthesis. In higher plants fatty acid desaturase mutants of *Arabidopsis thaliana* were employed to study the role of specific fatty acid substituents of thylakoid lipids with regard to the function of the light-harvesting antenna or chilling sensitivity (McCourt et al., 1985; Wu and Browse, 1995). Likewise, working with transgenic tobacco plants Murata and co-workers concluded that the amount of desaturated phosphatidylglycerol is an important determinant for the stabilization of the photosynthetic apparatus against low-temperature photoinhibition (Murata et al., 1992).

Unlike the desaturase mutants, the primary genetic defect in the recently isolated *dgd1* mutant of *A. thaliana* affects not just a subset of molecular species, but an entire thylakoid lipid class, because it causes a reduction to 10% of the wild-type level in the relative amount of DGDG (Dörmann et al., 1995). The *dgd1* mutant shows a pale-green leaf color, reduced growth, and alterations in photosynthesis-related parameters, such as chloroplast ultrastructure and Chl *a*/*b* ratio. Therefore the *dgd1* mutant provides, for the first time, a tool to study the possible function of DGDG in vivo. Because the genetic background of the *dgd1* mutant has been carefully cleared of secondary

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Abbreviations: Chl, chlorophyll; CP43, CP47, Chl *a*-binding protein complexes with a molecular mass of 43 and 47 kD, respectively; ΔF, fluorescence yield changes; DGDG, digalactosyl diacylglycerol; F_o, F_m, F_v, dark-level, variable, and maximum Chl fluorescence, respectively; F_v/F_m, ratio of variable to maximum Chl fluorescence; LHCII, peripheral light-harvesting Chl *a*/*b*-binding protein complexes associated with PSII; MGDG, monogalactosyl diacylglycerol; σ, photoreduction efficiency of the primary plastoquinone acceptor Q_A.

mutations, we assume that a causal relationship exists between the observed mutant traits and the primary defect, the DGDG deficiency (Dörmann et al., 1995). The present study focuses on alterations in the composition and stoichiometry of the pigment-protein complexes of the thylakoid membrane.

Higher plants possess intricate light-harvesting antennae for both photosystems. Three functional layers can be distinguished (Jansson, 1994). The innermost (core) antenna consists of Chl *a*-binding proteins closely connected to the reaction centers. By far the most Chl is bound to peripheral LHCII, which is known to be organized in trimeric units and which presumably consists of a mixture of two proteins, Lhcb1 and Lhcb2 (Jansson, 1994; Kühlbrandt et al., 1994). The minor polypeptides Lhca1 to Lhca4 of PSI and Lhcb3 to Lhcb6 of PSII are probably monomeric, constitute the inner light-harvesting antennae, and serve as linkers, transferring excitation energy from LHCII to the respective core antennae (e.g. Bassi et al., 1993; Härtel and Lokstein, 1995).

A characteristic set of pigments is bound to each apoprotein (Thayer and Björkmann, 1992; Bassi et al., 1993; Ruban et al., 1994; Lee and Thornber, 1995) and it appears that lipids are specifically associated as well. For example, the predominant lipid in PSII reaction center preparations is MGDG (Murata et al., 1990), with up to 30 mol% of DGDG found in some preparations (Trémolierès et al., 1994). The minor light-harvesting complexes of PSII are approximately 10-fold enriched in lipids, particularly in highly unsaturated galactolipids, as compared with the other complexes (Trémolierès et al., 1994). All four thylakoid lipids are present in the peripheral LHCII but, in particular, two seem to play a specific role. Phosphatidylglycerol stabilizes the trimeric form of LHCII in vitro and the presence of DGDG is required for the formation of two- or three-dimensional crystals of LHCII trimers (Nußberger et al., 1993; Flachmann and Kühlbrandt, 1996).

In the past the analysis of numerous mutants deficient in either the biosynthesis of apoproteins or pigments has provided novel insights into the highly regulated, but still poorly understood processes of assembly and maintenance of the pigment-protein complexes in thylakoid membranes of higher plants (for a recent review, see Paulsen, 1995; see also Flachmann and Kühlbrandt, 1996; Härtel et al., 1997). Here we extend this genetic approach to elucidate the possible function of the galactolipid DGDG in thylakoid membranes. By comparing the galactolipid-deficient *dgd1* mutant and the wild type of *A. thaliana*, we observed pronounced alterations in the mutant photosynthetic apparatus at the level of reaction centers and the light-harvesting antennae.

MATERIALS AND METHODS

Sterilized seeds of *Arabidopsis thaliana* ecotype Columbia (Col-2, wild type) and a four-times-backcrossed *dgd1* mutant (Dörmann et al., 1995) were germinated on 0.7% (w/v) agar-solidified Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 1% (w/v) Suc. The seedlings were kept on agar for 10 d prior to the transfer to pots containing a soil mixture as described previously (Dörmann et al., 1995). Regardless of the growth medium, the plants were cultivated under a 16-h light/8-h dark regime at a PPFD of 100 μmol m^{-2} s^{-1} and 18°C per day and 15°C per night cycle. If not otherwise stated, fully expanded rosette leaves of 3- to 4-week-old plants were used for the experiments just prior to the bolting of the primary inflorescence stalk. The leaf number at this stage was 11.6 (± 0.6) for the wild type and 8.4 (± 0.9) for the *dgd1* mutant. Usually, bolting in the mutant lagged behind the wild type by 2 to 3 d.

Pigment and Protein Assays

Leaves were rapidly frozen in liquid nitrogen for subsequent pigment and protein determination. Pigments were extracted and analyzed by HPLC, as previously described (Thayer and Björkman, 1992) with some modifications (Härtel et al., 1996). Total leaf protein was extracted according to Kruse et al. (1995) and immunoblotting was performed as described by Härtel et al. (1996), except that total leaf protein extracts were used. Values represent the average of the whole-leaf tissue of all fully expanded rosette leaves of a single plant.

Preparation of Thylakoids and Quantification of Electron Transport Components

Freshly harvested rosette leaves were ground using a blender in isolation buffer containing 330 mM sorbitol, 5 mM $MgCl_2$, 50 mM Tricine/NaOH (pH 7.8), and 0.1% (w/v) BSA. Following filtration through cheesecloth, the suspension was centrifuged at 5000*g* for 5 min. The pellet was resuspended in hypotonic buffer containing 10 mM Tricine/NaOH (pH 7.8) and centrifuged at 500*g* for 1 min to remove remaining cell debris. The supernatant was centrifuged at 5000*g* for 5 min, and the pellet consisting mainly of thylakoid membranes was resuspended in the isolation buffer and kept on ice until use. For low-temperature fluorescence measurements $MgCl_2$ was omitted in the resuspension buffer.

The assays of PSII and PSI reaction centers were as previously described (Härtel et al., 1997). The number of Q_B-binding sites was determined by linear regression through a double-reciprocal plot of the concentrations of free and bound [^{14}C]atrazine. Atrazine binds specifically to the D1 protein of the PSII reaction center. The amount of P700 was determined from the absorbance difference observed at 702 nm between ferricyanide-oxidized and ascorbate-reduced thylakoids.

Chl Fluorescence Measurements

Low-temperature (77 K) Chl fluorescence emission spectra of thylakoid suspensions were recorded using a spectrophotometer (F-4500, Hitachi, Tokyo, Japan). The suspensions were adjusted to 2.5 μg Chl mL^{-1}. Excitation and emission slit widths were 5 and 2.5 nm, respectively. Fluorescence (77 K) was measured in the presence and absence of 10 mM $MgCl_2$.

Table I. *Pigment contents in mature leaves*

Pigments were extracted with acetone and separated by HPLC analysis as described in "Materials and Methods." Values represent the means (± SD) of 25 measurements using leaves of 24- to 27-d-old plants.

Pigment	Wild Type	*dgd1* Mutant
	μmol m⁻² leaf area	
Chl *a*	193 ± 26	176 ± 28
Chl *b*	62 ± 11	69 ± 10
Chl (*a* + *b*)	256 ± 37	245 ± 36
Chlide (*a* + *b*)[a]	n.d.[b]	6.9 ± 3.8
Neoxanthin	11.5 ± 2.5	18.4 ± 4.2
Lutein	28.9 ± 3.6	27.3 ± 3.7
β-Carotene	25.2 ± 4.3	22.7 ± 3.6
V + A + Z[c]	8.6 ± 1.4	14.5 ± 3.0
Chl *a*/*b*	3.12 ± 0.04	2.54 ± 0.08

[a] Chlide (*a* + *b*) values were calculated using the conversion factors for Chl *a* and Chl *b*, respectively. [b] n.d., Not detectable. [c] V, Violaxanthin; A, antheraxanthin; Z, zeaxanthin.

In vivo Chl fluorescence was monitored at room temperature with a pulse amplitude modulation fluorometer as described by Lokstein et al. (1994). The maximum photochemical efficiency of PSII was assessed by the ratio of F_v/F_m. The parameter σ as a measure for the relative effective absorption cross-section of PSII was derived from light dosage-response curves of the fluorescence yield increase induced by a single-turnover flash (Falkowski et al., 1986). Flashes with a half-peak width of 12 μs were generated by a Xenon lamp (XST 103, Walz, Effeltrich, Germany) equipped with a blue-green filter (BG 18, Schott, Mainz, Germany). ΔF were recorded 120 μs after the flash. Sequences of 30 flashes spaced 10 s apart were applied and averaged. The plot of $\Delta F/\Delta F_{max}$ versus I/I_{max} can be described by an exponential saturation function, $\Delta F/\Delta F_{max} = 1 - e^{\sigma I/Imax}$. ΔF corresponds to the increase in fluorescence due to a flash of intensity I. ΔF_{max} is ΔF induced by the maximum light intensity I_{max}. Background far-red light with a photon flux density of 15 μmol m^{-2} s^{-1} was applied to avoid a limitation of electron transport by PSI. For all room temperature fluorescence measurements plants were dark-adapted for at least 1 h prior to the measurements.

O_2 Evolution and Electron Transport Activity Measurements

In vivo O_2-evolution rates under saturating CO_2 were determined using a leaf disc electrode (Bachofer, Reutlingen, Germany). Uncoupled whole-chain (PSII plus PSI) electron transport in thylakoids (equivalent to 10 μg Chl) was determined polarographically as previously described (Härtel et al., 1997). PAR was provided by a cold light source (KL 1500, Schott) and measured as incident photon flux density with a quantum sensor (LI-189A, Li-Cor, Lincoln, NE).

RESULTS

Changes in Absolute Amounts and Stoichiometry of Pigments

The pigment composition of leaf extracts was compared on a leaf area basis for the wild type and the *dgd1* mutant (Table I). The total amount of Chl *a* and *b* was similar in the *dgd1* mutant and the wild type. However, if calculated on a leaf fresh weight basis the amount of Chl (*a*+*b*) was reduced to approximately 75% in the *dgd1* mutant (data not shown). Independent of the respective reference system is the Chl *a*/*b* ratio, which was lowered from 3.12 in the wild type to 2.54 in the *dgd1* mutant. Moreover, the absolute amounts of neoxanthin and the sum of the xanthophyll-cycle pigments violaxanthin, antheraxanthin, and zeaxanthin were 1.7-fold increased in the *dgd1* mutant, if compared on a leaf area basis (Table I) or even on a total Chl basis (data not shown). Additionally, small amounts of Chlide *a* and *b* were detected in the *dgd1* mutant.

Altered Photosystem Stoichiometry

The PSI and PSII contents of thylakoids were estimated from measurements of the differences in absorption at 702 nm of reduced P700 compared with oxidized P700 and the capacity of the membranes to bind atrazine, respectively (Table II). The *dgd1* mutant thylakoid preparations showed an increased Chl to PSII ratio and a reduced Chl to PSI ratio in comparison with the wild type, indicating an increase in PSII and a reduction in PSI antenna Chl in the mutant. Taking into account the amount of Chl per leaf area (Table I), we calculated the number of reaction centers on a leaf area basis. The number of PSII reaction centers in the *dgd1* mutant was only 70% of the wild-type level, whereas the number of PSI reaction centers was 1.2 times higher in the mutant. Assuming that the number of Q_B-binding sites and P700 molecules is proportional to the number of PSII and PSI units, respectively, the PSII to PSI ratio is reduced to approximately 60% in the *dgd1* mutant (Table II).

Table II. *Numbers of PSI and PSII reaction centers*

The number of PSII centers was calculated by the binding capacity of thylakoid membranes for radiolabeled atrazine. The number of PSI centers was estimated from the absorption difference at 702 nm of reduced minus oxidized P700. The data represent the means (± SD) of four different preparations.

Parameter	Wild Type	*dgd1* Mutant
Chl per PSII (mol Chl mol^{-1} atrazine bound)	482 ± 46	662 ± 79
PSII per leaf area (μmol atrazine bound m^{-2})	0.531 ± 0.051	0.371 ± 0.044
Chl per PSI (mol Chl mol^{-1} P700)	535 ± 42	442 ± 23
PSI per leaf area (μmol P700 m^{-2})	0.478 ± 0.038	0.554 ± 0.029
PSII to PSI ratio	1.11	0.67

Chl Fluorescence Characteristics

As shown above, Chl *a/b* ratios were reduced in the mutant. It is well established that Chl *b* is exclusively associated with the light-harvesting complexes, in particular the peripheral LHCII, for which a characteristic Chl *a/b* ratio between 1.2 and 1.4 has been obtained (Bassi et al., 1993; Kühlbrandt et al., 1994; Paulsen, 1995). To examine whether the molecular environment or arrangement of the pigment-binding complexes and thus excitation energy distribution is affected in the *dgd1* mutant, fluorescence emission spectra of thylakoid preparations were recorded at 77 K. Figure 1 shows fluorescence emission spectra for thylakoid preparations of the wild type (Fig. 1A) and the *dgd1* mutant (Fig. 1B) upon excitation in the Soret band of Chl *b* at 472 nm in the absence (dashed line) and presence (continuous line) of Mg^{2+}. Three main maxima are present. The maximum at 685 nm represents primarily overlapping emissions of LHCII and the PSII core antenna complex CP43 (Nakatani et al., 1984) with maxima at 681 and 685 nm, respectively. The shoulder at 698 nm can be attributed to the PSII core antenna complex CP47 (Vermaas et al., 1986) and the maximum at 730 nm to the light-harvesting complex of PSI (Murata and Satoh, 1986). The mutant spectrum (Fig. 1B, dashed line) is characterized by a slight blue shift of the PSI emission maximum from 730 toward 727 nm and an increase in the magnitude of the fluorescence emission at 685 and 698 nm, respectively. This effect could be caused by a less efficient excitation energy transfer from LHCII to the core complexes, or taken as an indication for structural changes (enlargement) of the PSII antenna. The unchanged PSII fluorescence maxima positions suggest that the molecular environment of the PSII core antenna complexes is not affected by the DGDG deficiency in the mutant.

In an attempt to further address the nature of fluorescence changes, the effect of $MgCl_2$ on fluorescence emission spectra was investigated. The addition of Mg^{2+} cations is thought to induce the stacking of granal membranes (Murata and Satoh, 1986; Staehelin and van der Staay, 1996). For the wild type and the mutant the ratio of F_{685}/F_{730} increased after addition of $MgCl_2$ (continuous line); for the wild type the ratio increased from 0.35 to 0.48 and for the *dgd1* mutant from 0.61 to 1.20. The changes are due to an increase in PSII and a concomitant decrease in PSI fluorescence emission. The apparent blue shift in the PSI emission maximum at 727 toward 718 nm in the *dgd1* mutant is due to the strong suppression of the PSI emission at 727 nm. The more pronounced Mg^{2+} effect on mutant thylakoids presumably reflects a more extensive cation-dependent reorganization of pigment-protein complexes that causes an even stronger increase in energy distribution toward PSII.

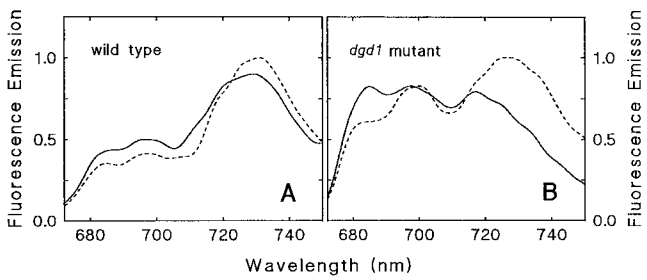

Figure 1. Low-temperature (77 K) Chl fluorescence emission spectra of thylakoid suspensions of *A. thaliana* wild type (A) and the *dgd1* mutant (B) in the absence (dashed line) or presence (continuous line) of $MgCl_2$ (10 mM). Suspensions were adjusted to a Chl concentration of 2.5 μg mL^{-1}. The spectra were adjusted to yield a similar magnitude of the red-most PSI emission maximum in the absence of $MgCl_2$. Excitation was at 472 nm (Chl *b* excitation).

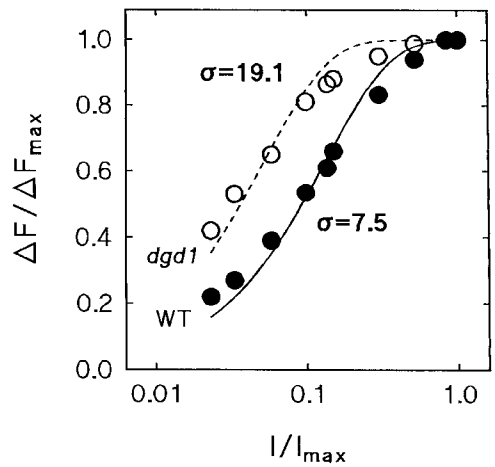

Figure 2. Flash energy-response curves of the fluorescence yield increase in leaves of *A. thaliana* wild type (WT) and the *dgd1* mutant (*dgd1*). Changes in fluorescence yield are expressed as $\Delta F/\Delta F_{max}$, where ΔF is the fluorescence yield induced with a single-turnover flash of an intensity I, and ΔF_{max} is the maximum fluorescence yield produced by the nonattenuated flash of I_{max}. An exponential saturation function ($\Delta F/\Delta F_{max} = 1\text{-}e^{\sigma I/I max}$) can be fitted to the data with r^2 values of 0.99 and 0.98 for the wild type and the mutant, respectively.

A direct measure for the efficiency of excitation energy transfer from LHCII to the PSII reaction centers in vivo is provided by the parameter σ, as deduced from the light-saturation curves for the flash-induced fluorescence yield (Fig. 2). A cumulative single-hit Poisson distribution function can be reasonably well fitted to the experimental data ($r^2 \geq 0.98$), and the value for σ was more than doubled for the *dgd1* mutant. This result provides a strong indication for an increase in the effective absorption cross-section of PSII and implies that excitation energy transfer from LHCII to the PSII reaction center is highly efficient in the *dgd1* mutant.

Stoichiometry of Pigment-Binding Proteins

To investigate possible differences in the amount of pigment-binding proteins of the PSII core complex and of the peripheral LHCII apoproteins, we quantified individual proteins by immunoblotting. Antibodies raised against the respective proteins were employed to probe the corresponding proteins of *A. thaliana* in total leaf extracts. Because turnover rates of D1 proteins are known to be particularly fast and sensitive to any kind of photoinhibitory stress, the 9-kD subunit of Cyt-b_{559} was used as a measure

for PSII abundance. Cyt-b_{559} is an integral part of the PSII reaction center and it is known to be of paramount importance for the assembly and stabilization of the PSII complex (Whitmarsh and Pakrasi, 1996). For the sake of a direct comparison the amount of sample loaded onto the gels was adjusted to yield comparable amounts of Cyt-b_{559}, as deduced from the staining intensity of a dilution series. As shown in Figure 3, the stoichiometry of the proteins D1, D2, CP43, and CP47 was, except for an apparent slight decrease in the D2 content in the *dgd1* mutant, not appreciably altered both relative to Cyt-b_{559} and to each other. This result demonstrates the tight coupling in the assembly of the proteins of the PSII core complex, even under the condition of severe reduction of DGDG in the *dgd1* mutant. In contrast, a clear increase in the amount of peripheral LHCII proteins relative to that of the reaction center/core complex of PSII was detectable in the mutant extract (Fig. 3).

Accumulation of Pigments and Pigment-Binding Proteins during Leaf Development

To rule out the possibility that the differences between the wild type and the mutant were only due to a different stage of leaf development, the amount of individual apoproteins and pigments was monitored during an extented time course (Fig. 4; Table III). A set of monoclonal antibodies has been recently developed to distinguish individual light-harvesting complex subunits of barley (Król et al., 1995) and was employed in this study to estimate the amount of the minor light-harvesting proteins Lhcb4 and Lhcb5 of PSII and the proteins Lhca1 and Lhca2 of PSI in *A. thaliana* leaf extracts. Polyclonal antibodies were used for the quantification of D1 and peripheral LHCII (Lhcb1 and Lhcb2) proteins. All antibodies showed a specific cross-reaction with individual pigment-binding proteins, as indicated by the expected molecular masses of the proteins in the *A. thaliana* extracts. Extracts of the wild type and of the mutant were adjusted to yield a comparable protein concentration. Apart from a few minor immune-reacting bands of high molecular mass obtained with some antibodies, no further cross-reactivity to other proteins was observed.

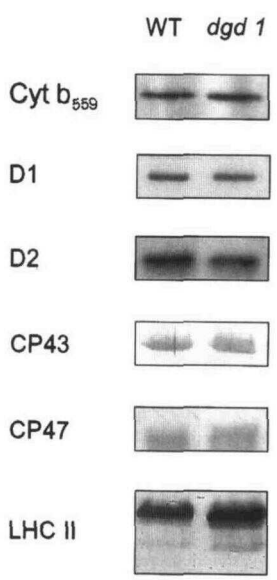

Figure 3. Quantification of pigment-binding proteins of PSII in mature leaves of *A. thaliana* wild type (WT) and the *dgd1* mutant. Samples were probed with antibodies directed against the reaction center apoproteins D1 and D2, the core antenna proteins CP43 and CP47, and the peripheral LHCII apoproteins (Lhcb1/Lhcb2). All samples were adjusted to yield a comparable immunostaining intensity of the Cyt-b_{559} band.

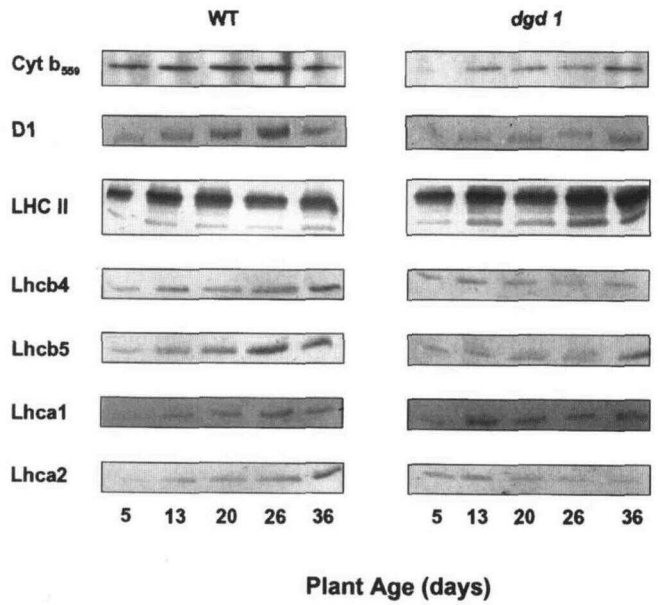

Figure 4. Quantification of individual light-harvesting apoproteins in developing leaves of *A. thaliana* wild type (WT) and the *dgd1* mutant. Total protein was extracted from leaves of 5-, 13-, 20-, 26-, and 36-d-old plants and analyzed by western blotting. Equal amounts of leaf protein were loaded. Samples were probed with polyclonal antibodies raised against the peripheral LHCII and with monoclonal antibodies against the Lhcb4 and Lhcb5 proteins of PSII and the Lhca1 and Lhca2 proteins of PSI.

The most intriguing observation shown in Figure 4 was the increase in the ratio of LHCII to the reaction center proteins Cyt-b_{559} and D1 throughout development in the mutant. The amount of proteins Lhcb4 and Lhcb5, which are part of the inner PSII light-harvesting antenna, increased continuously in wild-type leaf extracts and reached a maximum at 26 d after sowing (Fig. 4). In contrast to the wild type, a slow decline in the amount of Lhcb4 and Lhcb5 during leaf development was observed for the mutant, despite a continuous increase in the amount of peripheral LHCII proteins (Fig. 4). A similar trend was observed for the PSI antenna proteins Lhca1 and Lhca2, although the extent of the decrease of Lhca1 was only marginal.

Considering the differences in the accumulation of the apoproteins during leaf development, we expected the respective pigments to show a corresponding accumulation pattern, because Chl *b*, lutein, neoxanthin, and the xanthophyll-cycle pigments violaxanthin, antheraxanthin, and zeaxanthin are known to be preferentially associated with the light-harvesting complexes. The pigment compo-

Table III. *Pigment contents and maximum photochemical efficiency of PSII in leaves of different developmental stages*

Pigment contents were determined by HPLC analysis and normalized to the Chl (*a* + *b*) content. F_v/F_m ratios were determined with leaves after 60 min of predarkening immediately prior to pigment extraction. All values represent the means (± SD) of six measurements using leaves of independent plants.

Plant Age	Wild Type					*dgd1* Mutant				
	Neoxanthin	Lutein	V + A + Z[a]	Chl *a/b*	F_v/F_m	Neoxanthin	Lutein	V + A + Z	Chl *a/b*	F_v/F_m
d	*mmol mol*$^{-1}$ *Chl (a+b)*			*ratio*		*mmol mol*$^{-1}$ *Chl (a+b)*			*ratio*	
5	42.7 ± 1.8	131.6 ± 7.2	47.4 ± 4.8	3.09 ± 0.04	0.61 ± 0.05	43.1 ± 4.3	127.4 ± 3.8	51.1 ± 5.8	2.67 ± 0.05	0.52 ± 0.04
13	42.5 ± 5.8	118.2 ± 1.7	42.0 ± 4.2	3.13 ± 0.06	0.67 ± 0.01	48.9 ± 5.5	109.3 ± 7.9	55.0 ± 13.1	2.65 ± 0.05	0.57 ± 0.03
20	45.1 ± 6.2	114.4 ± 2.3	38.5 ± 2.8	3.11 ± 0.07	0.75 ± 0.01	54.2 ± 5.0	112.5 ± 5.6	60.2 ± 7.7	2.72 ± 0.04	0.64 ± 0.03
26	41.8 ± 3.1	115.1 ± 4.4	42.1 ± 4.3	3.12 ± 0.04	0.80 ± 0.01	68.8 ± 6.6	121.5 ± 4.3	63.1 ± 8.3	2.54 ± 0.08	0.70 ± 0.02
36	47.4 ± 2.5	122.7 ± 12.0	38.2 ± 4.6	2.85 ± 0.12	0.78 ± 0.01	70.8 ± 8.7	149.5 ± 10.7	65.3 ± 6.7	2.27 ± 0.17	0.72 ± 0.02

[a] V, Violaxanthin; A, antheraxanthin; Z, zeaxanthin.

sition of leaves of a different age normalized to total Chl is shown in Table III. The difference in the Chl *a/b* ratios between the wild type and the *dgd1* mutant was already fully established in 5-d-old plants and remained largely constant throughout d 26. In contrast to the wild type, the amount of neoxanthin increased continuously during development of the *dgd1* mutant, whereas lutein was present in stoichiometric amounts to the Chls in both lines throughout the testing period. There is a steady increase of the pool size of xanthophyll-cycle pigments in mutant leaves from 108% in 5-d-old leaves to 171% in 36-d-old leaves of the respective wild-type level.

As a simple measure of PSII function, the maximum efficiency of PSII was assayed by the Chl fluorescence ratio F_v/F_m after 1 h of dark adaptation in parallel to pigment determination. F_v/F_m ratios have been shown to be linearly correlated with the quantum yield of light-limited O_2 evolution (Demmig and Björkmann, 1987) and the number of functional PSII reaction centers (Russell et al., 1995; Park et al., 1996). The fact that F_v/F_m amounted to 85 to 90% of the respective wild-type level in the mutant at all stages of development (Table III) points to a relatively high photochemical competence of PSII even in the mutant.

Photosynthetic Performance

To evaluate the consequences of the observed structural alterations in the photosynthetic apparatus of the *dgd1* mutant, photosynthetic performance was determined for intact leaves. Light-response curves of O_2 evolution are shown in Figure 5. Regardless of the structural alterations, there were virtually no differences in maximum photosynthetic O_2-evolution rates on a leaf area basis. Because of the similar Chl content per unit area (Table I), O_2-evolution rates were also indistinguishable when expressed on a unit Chl basis. However, it should be noted that in an attempt to measure whole-chain electron-transport activities with isolated thylakoids, only 20 to 30% of the wild-type activity was recovered with mutant thylakoids (data not shown). These differences between the in vivo and in vitro measurements are probably due to a reduced stability of the isolated mutant thylakoids, prompting us to employ the least-invasive approach in this study.

DISCUSSION

The galactolipid DGDG accounts for approximately 20% of the thylakoid acyl lipids. Assuming that the pigment-protein complexes of the thylakoids are associated with specific membrane lipids, it seems plausible that a 90% reduction in the amount of this particular lipid in the *dgd1* mutant has an impact on the assembly or the function of the photosynthetic apparatus. In the past, the functional analysis of thylakoid lipids has often been accompanied by drastic experimental measures, such as lipase or detergent treatment of thylakoids, to manipulate the lipid composition of isolated thylakoid membranes, followed by reconstitution experiments of solubilized pigment-protein complexes. Whereas this approach has shed some light on the effects of thylakoid lipids in vitro, the significance of the obtained data with regard to the function of thylakoid lipids in vivo remains unclear. The availability of the *dgd1* mutant provides an opportunity to study the function of one of the major thylakoid lipid classes in vivo. A prerequisite for the validity of the employed approach is our assumption that the observed effects are solely caused by DGDG deficiency and not by secondary mutations. With regard to the *dgd1* mutant the likelihood of secondary

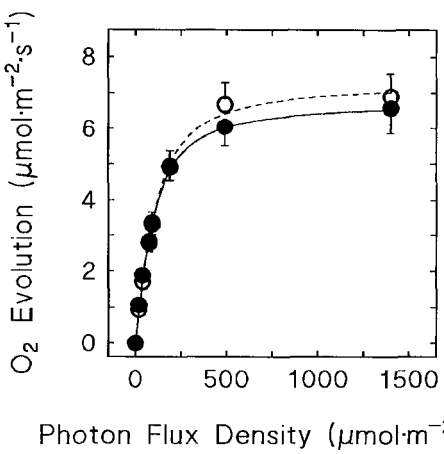

Figure 5. Light-response curves of CO_2-saturated O_2 evolution in leaves of *A. thaliana* wild type (●) and the *dgd1* mutant (○) on a leaf area basis. Values represent the means of at least eight measurements. SDs are as indicated.

mutations has been minimized by multiple backcrossing of the mutant to the wild type and by careful analysis of the genetic background of the five-times-backcrossed mutant during our efforts toward the map-based cloning of the *DGD1* locus. At this time many recombinants were analyzed, some closer than 50 kb to the *DGD1* locus on both sides and showing the same complex phenotype without exception (P. Dörmann and C. Benning, unpublished results).

A combination of biochemical and biophysical techniques was applied to investigate the effect of the DGDG deficiency in the *dgd1* mutant, with particular focus on the organization of individual pigment-protein complexes. Quantification by immunoblotting showed that apoproteins of the PSII reaction center/core complex and the inner LHCs of PSI and PSII were less abundant in the *dgd1* mutant, whereas the peripheral LHCII apoproteins accumulated to higher amounts (Figs. 3 and 4). Apparently, the abundance of specific pigment-protein complexes, particularly those closer to the core of PSII, appears to be limited by the reduced amount of DGDG present in the membrane, suggesting that DGDG is required for the stabilization of these complexes. Accordingly, the increase in the amount of peripheral LHCII proteins in parallel with the decrease in the amount of the inner complexes during leaf development in the *dgd1* mutant (Fig. 4) could mean that lower proportions of DGDG are necessary for assembling (stabilizing) of the trimeric LHCII. Unfortunately, no quantitative data on the association of lipids with different pigment-protein complexes are available for *A. thaliana*. However, in maize leaves the inner PSII antenna complexes seem to contain a greater relative proportion of lipids than the peripheral LHCII (Trémolières et al., 1994). Assuming that this observation is also valid for *A. thaliana*, one would expect a particularly high impact of DGDG deficiency on the inner light-harvesting antenna of PSII in the *dgd1* mutant. This seems to be the case.

Corroborating evidence for an increase in the size of the peripheral LHCII relative to the PSII core complex in the *dgd1* mutant is provided by the pigment analyses. Two observations were most intriguing: the decreased Chl *a/b* ratios in the *dgd1* mutant throughout development and the accumulation of xanthophylls (in particular neoxanthin and xanthophyll-cycle pigments) that occurred in parallel with an accumulation of peripheral LHCII apoproteins. The increased relative amounts of neoxanthin and xanthophyll-cycle pigments along with the decreased Chl *a/b* ratio in leaves of the *dgd1* mutant could reflect either an increase in the PSII to PSI ratio or an increase in the number of LHCs of PSII relative to PSII core complexes. Because we actually observed a decrease in the PSII to PSI ratio in the *dgd1* mutant (Table II), the changes in the pigment composition point toward the second possibility. It is well documented that nearly all (>90%) of the leaf neoxanthin is associated with PSII, with the bulk being bound to the peripheral LHCII proteins (Thayer and Björkman, 1992; Bassi et al., 1993; Ruban et al., 1994). Therefore, the increased abundance of neoxanthin along with the increased amount of Chl *b* in the *dgd1* mutant may be due to an increased size of the peripheral LHCII. For xanthophyll-cycle pigments it seems more likely that the increased amounts are due to an increased excitation energy pressure on PSII, as indicated by the decreased actual quantum efficiency of PSII (Dörmann et al., 1995). However, currently we cannot exclude the possibility that a proportion of xanthophylls is present as free (not bound to antenna proteins) pigment in the mutant.

The quantification of apoproteins and pigments does not necessarily provide a clue to the number of completely assembled complexes efficiently transferring energy to the reaction centers. However, in vivo Chl fluorescence measurements suggest an increased effective absorption cross section of PSII in mature leaves of the *dgd1* mutant (Fig. 2). These data represent an independent line of evidence for an increased PSII antenna size (i.e. an increased ratio of LHCII to PSII core) in the *dgd1* mutant in support of the conclusions drawn from the pigment and apoprotein analyses. In view of the notion that several reaction centers of PSII may share a common pool of antenna pigment-protein complexes (Joliot et al., 1973; Melis and Anderson, 1983), the possibility remains that a reduction in the number of photochemically competent reaction centers in the *dgd1* mutant may increase the effective antenna size for the remaining competent ones. However, a comparison of the photosynthetic O_2-evolution rates (Fig. 5) with the number of PSII reaction centers (Table II) determined on a leaf area basis suggests a high functional competence of the assembled PSII centers in the *dgd1* mutant in vivo.

A quantitative analysis of the immunoblots reveals a 60 to 70% reduction of the D1 content on a protein basis in the mutant (Fig. 4), but a 30% reduction of Q_B-binding sites on a leaf area and, hence, Chl basis (because Chl content was not different) when compared with the wild type (Table II). A similar phenomenon was observed by Russell et al. (1995) investigating *A. thaliana* leaves under light stress and was explained by a preferred proteolysis of D1 at the donor side of the PSII reaction center, as compared with the acceptor side harboring the Q_B-binding site. However, in our case, part of the apparent differences can be simply explained by the fact that protein/Chl ratios are about 1.5 times higher in mutant leaves at all stages of development. On the other hand, an almost linear correlation between the functional PSII centers and F_v/F_m in the dark-adapted state has been shown (Russell et al., 1995; Park et al., 1996). According to this relationship, the reduction in F_v/F_m by 15% in the *dgd1* mutant would imply that about 25 to 35% of all PSII centers may be nonfunctional. This value would be in agreement with the decrease in the amount of Q_B-binding sites in the mutant. However, it has to be emphasized that an estimation in this way does not consider possible alterations in the F_v/F_m ratios due to the structural changes in antenna composition and in the stoichiometry between PSII and PSI units, which would particularly affect the F_o level and thereby also F_v. For example, an increase in the PSII antenna size would be expected to increase the F_o level and thereby decrease F_v and hence F_v/F_m in the mutant. On the other hand, the F_o level is known to be also influenced by PSI fluorescence emission. The relative (to PSII) contribution of PSI fluorescence emission is lower in the mutant (Fig. 1), which, in turn, should result in a decrease in the F_o level.

The possibility that the decrease in F_v/F_m could simply arise from a higher excitation energy distribution to PSI due to a decreased grana stacking in the mutant is unlikely because both the number and size of grana and the extent of stacking was increased in the *dgd1* mutant, as observed by electron microscopy (Dörmann et al., 1995). Furthermore, the Mg^{2+}-dependent fluorescence changes are thought to be a consequence of stacking and unstacking of granal membranes (Murata and Satoh, 1986). Accordingly, thylakoid stacking is stimulated by cations, a process that was frequently found to depend on the size of LHCII (e.g. Armond et al., 1976; Mullet and Arntzen, 1980). The mutant showed an increase in the 77 K fluorescence emission ratio of PSII to PSI, and this increase was more pronounced after addition of Mg^{2+} (Fig. 1). This observation is consistent with a less efficient excitation energy transfer from PSII to PSI due to a higher extent of cation-induced changes in the spatial separation of the various pigment-protein complexes in the *dgd1* mutant.

The nonbilayer-forming properties of MGDG are thought to be important for grana formation or for the packing of hydrophobic proteins, such as the LHCs (Roughan and Boardman, 1972; Gounaris et al., 1983a; Murphy and Woodrow, 1983; Webb and Green, 1991). In this respect the higher MGDG to DGDG ratio of the *dgd1* mutant should be of interest (Dörmann et al., 1995). Our results imply that the ability for membrane appression is virtually not restricted despite the DGDG deficiency. This result supports the view that DGDG is of minor importance and that MGDG is essential with regard to grana stacking.

It has been observed that removal of DGDG inhibits O_2-evolution activity of PSII-enriched samples in vitro, an effect that could be reversed by subsequent resupplementation of the lipid, suggesting an essential role for the water-splitting process (Gounaris et al., 1983b). This finding seems to conflict with the similar O_2-evolution rates on a leaf area basis in wild-type and DGDG-deficient mutant leaves (compare Fig. 5 and Table II). On the other hand, we could determine only a very low O_2-evolution activity in isolated *dgd1*-mutant thylakoids. Taken together, these observations can be reconciled by assuming a stabilizing role of DGDG for PSII activity in vitro rather than a specific function in the overall O_2-evolving process in the intact system. However, based on these measurements alone one cannot exclude the possibility that the water-splitting system, in particular the donor side of PSII, is affected in vivo. The similar maximum O_2-evolution rates in leaves also suggest that other factors must account for the reduced growth of the mutant (Dörmann et al., 1995). The most obvious would be that the limited availability of DGDG may prevent the assembly of the functional thylakoid membrane at a proper rate.

Changes in the stoichiometry of the two photosystems in plants have been shown to depend on the intensity and quality of light (Chow et al., 1990; Kim et al., 1993). Furthermore, it is well established that Chl *b* deficiency in higher plants results in a reduction of the light-harvesting antenna size of PSII and changes in PS stoichiometry (Ghirardi et al., 1986; Kim et al., 1993). To our knowledge, effects of lipid deficiency on PS adjustment have not been previously reported. Here we show that plants deficient in a major lipid class, DGDG, are characterized by a substantial decrease in the PSII to PSI ratio (Table II), that is brought about by a decrease in the amount of PSII and a concomitant increase in the amount of PSI. As shown by Chow et al. (1990), adjustment of PS stoichiometry improves the quantum efficiency of photosynthesis. In the case of the *dgd1* mutant the relatively (to the PSII core complexes) higher LHCII content presumably favors preferential excitation of PSII, which may trigger an increased PSI content to overcome the imbalance in electron transport. Hence, the lowered PSII to PSI ratio in the mutant seems to also represent an adaptive response of the photosynthetic apparatus to the altered light absorption/utilization by the photosystems.

In conclusion, we show here that the strong reduction in a complete thylakoid lipid class is accompanied by dramatic changes in the composition of the photosynthetic apparatus. A surprising outcome of this study is the increase in the size of the light-harvesting antenna of PSII in the *dgd1* mutant. This conclusion is based on several independent observations: (a) alterations in the abundance and stoichiometry of pigment-binding proteins, (b) alterations in pigment stoichiometry, (c) higher numbers of Chl molecules per Q_B-binding site, (d) changes in the 77 K fluorescence emission spectra, and (e) increases in the effective absorption cross-section of PSII in the *dgd1* mutant. Regardless of these marked structural alterations, a large proportion of PSII reaction centers remains functional, as indicated by photosynthetic O_2-evolution rates and the F_v/F_m ratios. Thus, our results suggest that DGDG plays an important role in vivo in the structural organization of the photosynthetic apparatus. However, further analysis will be required to corroborate our present findings once the *DGD1* locus has been isolated by map-based cloning, because the availability of the gene will allow us to construct DGDG-deficient antisense lines that should show a range of phenotypes resembling that of the *dgd1* mutant.

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