Modification of the Water Oxidizing Complex in Leaves of the *dgd1* Mutant of *Arabidopsis thaliana* Deficient in the Galactolipid Digalactosyldiacylglycerol[†]

F. Reifarth,[‡] G. Christen,[‡] A. G. Seeliger,[‡] P. Dörmann,[§] C. Benning,[§] and G. Renger*,[‡]

Max-Volmer-Institut für Biophysikalische Chemie und Biochemie, Technische Universität Berlin, Strasse des 17. Juni 135, D-10623 Berlin, Germany, and Institut für Genbiologische Forschung Berlin GmbH, Ihnestrasse 63, 14195 Berlin, Germany

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ABSTRACT: The primary biochemical defect in the genetically well characterized *dgd1* mutant of *Arabidopsis thaliana* causes a 90% reduction in the relative amount of the galactolipid digalactosyldiacylglycerol (DGDG). To study the effect of this DGDG deficiency on photosystem II (PS II), time-resolved transients of laser-flash-induced changes of the relative fluorescence quantum yield $F_{var,rel}(t)$ were measured in whole leaves from wild-type and the *dgd1* mutant. The results obtained reveal (i) in untreated leaves the decay kinetics of $F_{var,rel}(t)$ reflecting $Q_A^{\bullet-}$ reoxidation by endogenous plastoquinone are very similar in wild-type and the *dgd1* mutant at room temperature, (ii) the Arrhenius plot of the temperature dependence of electron transfer from $Q_A^{\bullet-}$ to Q_B exhibits a break point at about 19 °C in wild-type and about 12 °C in the *dgd1* mutant, (iii) in leaves treated with DCMU the slow reoxidation of $Q_A^{\bullet-}$ by the PS II donor side is blocked to a much higher extent in the *dgd1* mutant (about 50%) compared to wild-type (about 10%), and iv) the normalized amplitude of $F_{var,rel}(t = 1\ \mu s)$ reflecting the percentage of fast $P680^{\bullet+}$ reduction by Y_Z exhibits a characteristic period four oscillation in wild-type while this feature is strongly damped in the *dgd1* mutant. Presumably, the severe DGDG deficiency is causing the thermal down shift of a lipid phase transition that affects the $Q_A^{\bullet-}$ reoxidation by Q_B. Most strikingly, the properties of the WOC are modified as a result of reduced DGDG content. Thus, the lipid DGDG appears to be of structural relevance for the WOC.

The essential steps of solar energy exploitation take place in pigment protein complexes that are anisotropically incorporated into lipid bilayer membranes with a comparatively high protein content. This anisotropy gives rise to vectorial electron transport processes that lead to an electric energetization of the membrane. The coupling of redox processes with protolytic reactions generates an electrochemical potential difference that can be used for ATP synthesis [for a latest review, see Gräber (1997) and references therein]. In this way, the membrane acts as a functional element of energy transduction with the lipid phase providing a low permeability barrier to dissipative proton transport. This property is clearly related to the lipid bilayer structure and therefore a bulk phase phenomenon. In addition, lipids can exert specific interactions with protein complexes as is known for cardiolipin in cytochrome oxidase [for a review, see Babcock and Wikström (1992) and references therein] or for the role of phosphatidylglycerol (PG)[1] and digalactosyldiacylglycerol (DGDG) in the structural organization of light harvesting II (LHC II) complexes [Krupa et al., 1992; Nussberger et al., 1993; for a review, see Paulsen (1995)]. The lipid composition of the chlorophyll-a containing thylakoid membranes in all oxygenic photosynthetic organisms is unique by the dominance of uncharged galactolipids and the presence of the charged lipid sulfoquinovosyldiacylglycerol (SQDG) [for reviews, see Murphy (1986), Douce and Joyard (1990), and Webb and Green (1991)]. It is therefore attractive to speculate on the possibility of a special role of one of these lipids for PS II where the essential steps of water oxidation to molecular oxygen take place [for a latest review, see Renger (1997) and references therein].

Two approaches have proven most successful to elucidate the role of specific thylakoid lipids with regard to the function of different protein complexes involved in the light reactions of photosynthesis: (i) extraction of protein complexes from the membrane and reconstitution in a specific lipid environment and (ii) genetically induced changes in lipid composition followed by careful analysis of the respective mutant. The advantage of the mutant approach would be that effects can be studied *in vivo* without disturbing the system by harsh treatments. Applying the *in vitro* approach, it has been shown that, in preparations of PSII membrane fragments, oxygen evolution rates could be stimulated by the addition of DGDG or phosphatidylcholine while SQDG exerts an inhibitory effect (Gounaris et al., 1983). This result suggested that, contrary to SQDG, DGDG may be important for the functional integrity of PSII. Applying the genetic approach by construction of a SQDG-deficient null-mutant of a cyanobacterium, Güler et al. (1996) could demonstrate that the complete absence of SQDG had only small effects on PS II activity and the water oxidizing complex. Mutants of the weed *Arabidopsis thaliana* as well as transgenic plants

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* Corresponding author.
[‡] Technische Universität Berlin.
[§] Institut für Genbiologishe Forschung Berlin GmbH.

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[1] Abbreviations: Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS II, photosystem II; P680, photoactive Chl of the reaction center of PS II; Q_A and Q_B, primary and secondary plastoquinones of PS II; WOC, water-oxidizing complex; Y_Z, tyrosine 161 of the PSII D1 polypeptide; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; SQDG, sulfoquinovosyldiacylglycerol.

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were successfully used to study the effects of an altered fatty acid composition of thylakoid lipids on photosynthesis and chilling sensitivity (Gibson et al., 1994; Murata et al., 1992). Likewise, the recently isolated DGDG-deficient *dgd1* mutant of *A. thaliana* (Dörmann et al., 1995) may provide a valuable tool to study the function of DGDG in thylakoid membranes of a higher plant. This mutant contains only 10% of DGDG amounts found in the wild-type. Most notable are the stunted growth, the pale green leaf color, and changes in the thylakoid ultrastructure. Steady-state chlorophyll fluorescence measurements revealed a decreased quantum yield of photosynthesis (Dörmann et al., 1995). These preliminary results already suggest that DGDG may be important or even essential to support growth and optimal photosynthesis in *A. thaliana*.

In order to analyze effects owing to DGDG deficiency in whole leaves under *in vivo* conditions on the reaction pattern of PS II, noninvasive analytical tools were used in the present study. Fluorometric methods are most suitable to achieve this goal [for a review, see Govindjee (1995) and references therein]. The results obtained revealed that in the mutant the properties of the water-oxidizing complex (WOC) are significantly modified while the acceptor side exhibits only minor changes.

MATERIAL AND METHODS

Surface sterilized seeds of *A. thaliana* ecotype Columbia (Col-2, wild-type) and four times back-crossed *dgd1* mutant (Dörmann et al., 1995) were germinated on 0.7% (w/v) agar solidified MS medium (Murashige & Skoog, 1962) supplemented with 1% (w/v) sucrose. The seedlings were kept on agar for 10 days prior to the transfer to pots containing a soil mixture as previously described (Dörmann et al., 1995). Regardless of the growth medium, the plants were cultivated under a 16 h light/8 h dark regime at a photon flux density of 100 μmol m^{-2} s^{-1}. Only fully expanded rosette leaves of 3−4 week old plants were used. The plants were dark adapted for at least 1 h prior to the measurements.

Flash-induced changes of the fluorescence quantum yield were monitored with two types of home-built equipment that markedly differ with respect to their time resolution. Measurements on $P680^{\bullet+}$ reduction were performed with the equipment that permits a time resolution down to 500 ns [for details, see Reifarth et al. (1997)]. For determination of the kinetics of the electron transfer, reactions from Q_A^- to $Q_B(Q_B^-)$ and the recombination reactions of Q_A^- with the PS II donor side in the presence of DCMU equipment was used that is described by Gleiter et al. (1990). Deconvolution of multiphasic kinetics into monoexponential components was performed by using a special evolution strategy fit method according to Ostermeier (1992).

NH_2OH or DCMU treatment was performed by incubating the leaves for about 1 h in an aqueous solution of NH_2OH (5 mM) or DCMU (100 μM, 1% methanol). For control experiments, the leaves were incubated with the respective solvent only. Virtually no differences were observed between the solvent treated (control) and untreated leaves, indicating that the observed effects of NH_2OH or DCMU incubation were not induced by the solvent.

For measurements of the temperature dependence, the samples were placed on a sample holder with adjustable temperature. The sample holder temperature was controlled by a Peltier element with an accuracy of about ±1 °C. If not indicated otherwise, all other measurements were performed at room temperature.

RESULTS

The time dependent population of redox states $P680^{\bullet+}$ and $Q_A^{\bullet-}$ generated by flash excitation can be monitored via measurements of the relative fluoresence quantum yield, because $P680^{\bullet+}$and Q_A are potent quenchers of Chl-a fluorescence while P680 and $Q_A^{\bullet-}$ are nonquenching states (Govindjee 1995, and references therein). This method is used in the present study to analyze the kinetics of donor and acceptor side reactions in leaves of *Arabidopsis thaliana* from the wild-type and the *dgd1* mutant recently discovered by Dörmann et al. (1995).

$Q_A^{\bullet-}$ Reoxidation by Endogeneous Plastoquinone and/or Other Redox Components in Untreated Leaves. In order to investigate the $Q_A^{\bullet-}$ reoxidation, flash-induced changes of the fluorescence quantum yield in the 5 ms domain were measured for leaves from wild-type and the *dgd1* mutant [for further details, see Govindjee (1995) and Renger et al. (1995) and references therein]. A comparison of the curves shown in Figure 1 reveals two features: (a) the level of maximum fluorescence F_m induced by a saturating laser flash is lower in the mutant compared to the wild-type (the ratio F_m/F_o is about 85% of that of the wild-type), and (b) the decay kinetics and the level of F_t that persists above F_o even 10 s after the flash (dashed line) are not much affected in the mutant.

The first feature is in line with steady state fluorescence measurements; the second will be quantatively analyzed in terms of the $Q_A^{\bullet-}$ reoxidation kinetics. The relationship between the normalized population of state $Q_A^{\bullet-}$, $[Q_A^{\bullet-}(t)]$, and the relative transient fluorescence $F_{var,rel}(t) = (F(t) - F_o)/F_o$ is nonlinear owing to excitation energy transfer between photosynthetic units (Joliot & Joliot, 1964). Accordingly, the relation between both quantitites is given by

$$F_{var,rel}(t) = bQ_A^{\bullet-}(t) + (1-b)\frac{(1-p)Q_A^{\bullet-}(t)}{1-pQ_A^{\bullet-}(t)} \qquad (1)$$

where $(1 - b)$ is the fraction of PS II units which are connected via excitation energy transfer and p is the probability of this process as described previously (Dohnt & Renger, 1984). In spinach thylakoids, typical values are $(1 - b) = 0.7$ and $p = 0.5$. Corresponding data are lacking for leaves of *A. thaliana* but in a first approximation it seems reasonable to use the above mentioned values for data analysis. A satisfying fit of the $Q_A^{\bullet-}(t)$ decay can be achieved by a triexponential decay kinetics plus a time independent fraction:

$$Q_A^{\bullet-}(t) = Q_A^{\bullet-}(t=0)\sum_{i=1}^{3} a_i \exp(-t/\tau_i) + a_4 \qquad (2)$$

where a_4 represents the terminal concentration of $Q_A^{\bullet-}$ that is not reduced within the time range of the measurements (usually 10 s). The values of a_i and τ_i obtained by a numerical fit of the data are summarized in Table 1. An inspection of the values of a_i and τ_i reveals that (a) the overall decay is dominated by kinetics with lifetimes of 290 μs (wild-

Table 1: Kinetic Parameters of the Q_A^- Reoxidation (See Eq 2) after the First Flash in Untreated Leaves from Wild-Type and *dgd1* Mutant of *Arabidopsis thaliana*[a]

	wild-type	*dgd1* mutant
a_1 (τ_1/ms)	0.70 (0.29)	0.63 (0.32)
a_2 (τ_2/ms)	0.14 (2.50)	0.17 (2.00)
a_3 (τ_3/ms)	0.09 (770)	0.11 (580)
a_4 (τ_4 > 10 s)	0.07	0.09

[a] The experiments were performed at room temperature. For further experimental details see Materials and Methods.

type) and 320 μs (*dgd1*) and a normalized extent of 0.70 and 0.63, respectively. These lifetimes τ_1 are typical for electron transfer kinetics from $Q_A^{\bullet-}$ to Q_B bound to its binding niche (Bowes & Crofts, 1980; Weiss & Renger, 1984). Within the experimental error the values of a_1 and τ_1 are the same in wild-type and *dgd1* mutant leaves. (b) A fraction of about 15−20% of the PS II centers exhibit a $Q_A^{\bullet-}$ reoxidation that is slower by a factor of about 10 than that of direct electron transfer from $Q_A^{\bullet-}$ to Q_B. These kinetics are characteristic for PS II complexes where $Q_A^{\bullet-}$ reoxidation is limited by diffusion of endogenous PQ-molecules to an empty Q_B site (Crofts & Wraight, 1983). Likewise, the normalized extent of this reaction is very similar to that found in spinach thylakoids (Renger et al., 1995). Virtually no difference is observed between wild-type and *dgd1* mutant. (c) A small percentage of PS II complexes (order of 10% of the overall population) is characterized by much slower kinetics. It seems likely that in these PS II complexes the Q_B site is nonfunctional so that $Q_A^{\bullet-}$ becomes reoxidized by other endogenous redox groups, e.g., donor site components. The present data do not permit an unambiguous assignment. (d) In a fraction of less than 10% of the PS II complexes, $Q_A^{\bullet-}$ remains in its reduced form even at 10 s after the flash, owing to the redox equilibrium between $Q_A^{\bullet-}Q_B$ and $Q_AQ_B^{\bullet-}$. In the case of spinach thylakoids, values of about 20 are reported for the equilibrium constant of $Q_A^{\bullet-}Q_B \Leftrightarrow Q_AQ_B^{\bullet-}$, corresponding to $[Q_A^{\bullet-}]_t$ population of about 5% (Robinson & Crofts, 1983). On the basis of findings a−d it can be concluded that the acceptor side of PS II is at most only marginally affected in the *dgd1* mutant compared to the wild-type.

Temperature Dependences. In general, lipids are known to affect the temperature dependence of photosynthetic electron transport (Murata, 1989), chilling tolerance of plants (Wada et al., 1990), and thermal stability (Murata, 1989). It therefore appeared worth analyzing the pattern of acceptor side reactions as a function of temperature. Analogous traces as shown in Figure 1 were monitored within the temperature range 0−52 °C. An inspection of the data readily shows that the variable fluorescence $F_v = F_m - F_o$ starts to decline steeply at higher termperatures. In order to illustrate this effect, Figure 2 shows the normalized variable fluorescence $[F_m(\vartheta) - F_o(\vartheta)]/[F_m(\vartheta = 0\ °C) - F_o\ (\vartheta = 0\ °C)]$ as a function of temperature ϑ. Two features emerge from this figure: (i) in $0° < \vartheta < 30$ °C the normalized variable fluoresecence remains almost constant and above 30° C it starts to decline approaching a value close to zero at 52° C, and (ii) the *dgd1* mutant is more susceptible to high temperature stress than the wild-type.

For a more detailed kinetic analysis, the data were evaluated by using eqs 1 and 2 as outlined in the former

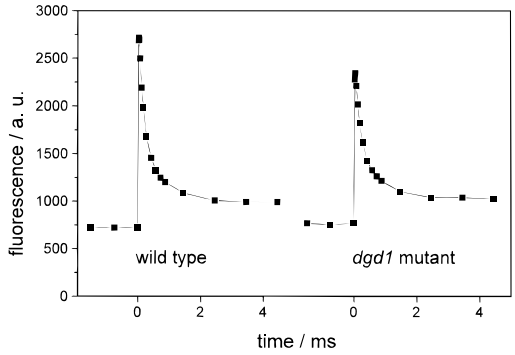

FIGURE 1: Laser-flash-induced change of the relative fluorescence quantum yield at room temperature as a function of time in dark-adapted leaves from wild-type and *dgd1* mutant of *Arabidopsis thaliana*.

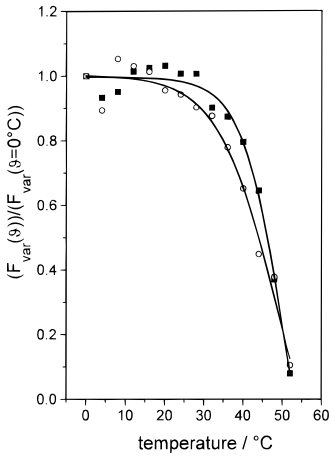

FIGURE 2: Temperature dependence of the variable fluorescence normalized to the value at $\vartheta = 0$ °C, $F_{var}(\vartheta)/F_{var}(\vartheta = 0\ °C)$, in dark-adapted leaves from wild-type and *dgd1* mutant of *Arabidopsis thaliana*.

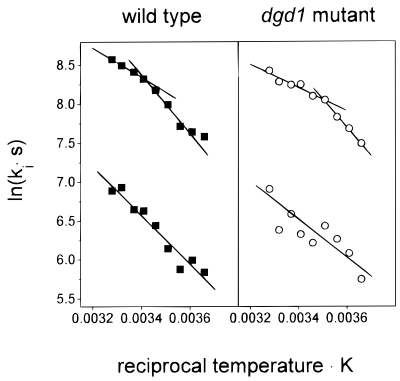

FIGURE 3: Semilogarithmic plot of the rate constants k_1 (top) and k_2 (bottom) of $Q_A^{\bullet-}$ reoxidation as a function of reciprocal temperature in dark-adapted leaves from wild-type (left side) and *dgd1* mutant (right side) of *Arabidopsis thaliana*. For details see text.

section for room temperature measurements. In terms of the $Q_A^{\bullet-}$ reoxidation by endogenous PQ, only the rate constants k_1 and k_2 are of interest. An Arrhenius plot of the data is shown in Figure 3. In order to avoid any interference with thermal degradation effects (see Figure 2), only results were used that were obtained at temperatures $\vartheta < 32°$. A closer inspection of Figure 3 reveals that the relation of k_1 as a function of the reciprocal temperature cannot be satisfactorily described by a straight line. A much better fit is achieved by the assumption that two regions exist, which are characterized by different activation energies E_A for $Q_A^{\bullet-}$ reoxi-

Table 2: Activation Energies of the Q_A^- Reoxidation Reaction after the First Flash in Untreated Leaves from Wild-Type and *dgd1* Mutant of *Arabidopsis thaliana*[a]

	wild-type	*dgd1* mutant
ϑ_c/°C	19	12
$E_A(k_1)$ $\vartheta > \vartheta_c$/kJ mol^{-1}	16 ± 1	13 ± 2
$E_A(k_1)$ $\vartheta < \vartheta_c$/kJ mol^{-1}	31 ± 5	30 ± 2
$E_A(k_2)$/kJ mol^{-1}	26 ± 3	20 ± 4

[a] The break point temperature of the Arrhenius plot of the Q_A^- reoxidation reaction is denoted by ϑ_c. For further experimental details see Materials and Methods

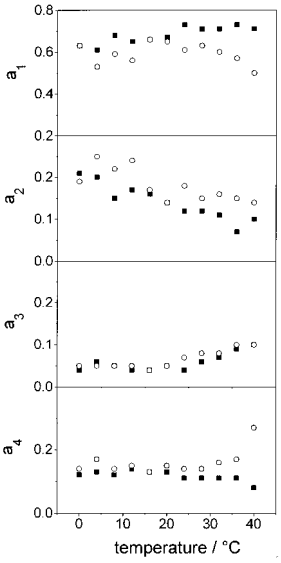

FIGURE 4: Temperature dependence of the normalized amplitudes a_i of different kinetic components of $Q_A^{\bullet-}$ reoxidation in dark-adapted leaves from wild-type (filled symbols) and *dgd1* mutant (open symbols) of *Arabidopsis thaliana*. For details see text.

dation by Q_B. The E_A values gathered from this type of analysis are summarized in Table 2. A striking feature emerging from a comparison of the data is the close similarity of the activation energies in both lines while the transition temperature seems to be significantly lower in the *dgd1* mutant.

A less clear picture arises for the temperature dependence of the rate constant k_2 that reflects the diffusion controlled $Q_A^{\bullet-}$ reoxidation by PQ in PS II with an empty Q_B site (*vide supra*). The data scattering is much larger and therefore it is difficult to arrive at clear conclusions. It seems that a straight line provides a rough description with activation energies of 26 ± 3 kJ/mol and 20 ± 4 kJ/mol for wild-type and *dgd1* mutant, respectively. These values are very similar. Taking into account the experimental error, it appears most likely that the mutation does not markedly affect the PQ diffusion to the Q_B site.

Further information on the reaction pattern of the acceptor side can be obtained from a brief inspection of the temperature dependence of the normalized amplitudes a_i (see eq 2). The data in Figure 4 show that, up to 20 °C, virtually no changes are observed within the error limit and no differences exist between wild-type and *dgd1* mutant. Between 20 and 30 °C, only minor changes arise. Above 30 °C a significant decrease of a_1 and a concomitant increase of a_4 are observed in the mutant with no corresponding changes in the wild-type. This feature is indicative of a higher susceptibility of $Q_A^{\bullet-}$ reoxidation by Q_B and possibly

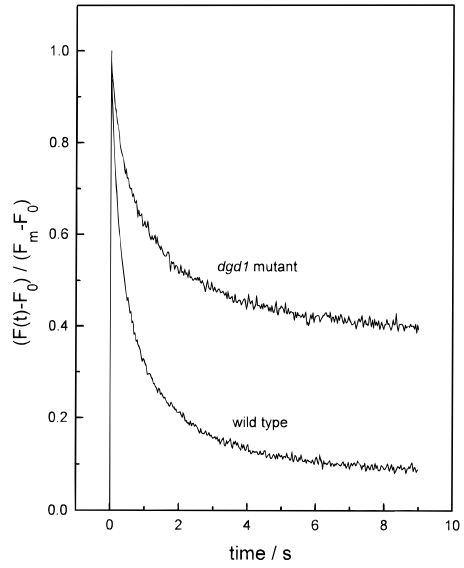

FIGURE 5: Laser-flash-induced changes of the normalized variable fluorescence quantum yield at room temperature as a function of time in dark-adapted and DCMU-treated leaves from wild-type and *dgd1* mutant of *Arabidopsis thaliana*. For experimental details see Materials and Methods.

a shift of the equilibrium constant toward lower values in the mutant. Accordingly, the thermal stability of the Q_B site seems to be decreased presumably due to reduced DGDG levels in the mutant.

Reoxidation of $Q_A^{\bullet-}$ in DCMU-Treated Leaves. The results reported so far describe the pattern of $Q_A^{\bullet-}$ reoxidation in leaves that were not affected by exogeneous inhibitors. A drastic change arises when leaves are treated with DCMU and related compounds of similar action because they bind at the Q_B site, thereby preventing reoxidation of $Q_A^{\bullet-}$ by endogenous PQ [for reviews, see Renger (1986) and Oettmeier (1992) and references therein]. In this case, $Q_A^{\bullet-}$ becomes reoxidized mainly by interaction with the donor side. Therefore, this type of approach provides additional information on possible effects due to changes of the DGDG content.

In the presence of DCMU, the reoxidation of $Q_A^{\bullet-}$ is highly retarded and, therefore, the flash-induced changes of the relative fluorescence quantum yield have to be monitored in a much wider time domain. Figure 5 shows typical traces measured in DCMU-treated leaves (time window extended by a factor of 2000 compared to Figure 1). For the sake of direct comparison of the kinetics in wild-type and mutant leaves, the normalized variable fluorescence is depicted. A first inspection readily shows that significant differences exist between both curves, in marked contrast to untreated leaves (see Figure 1). Data analysis revealed that again a satisfying description could be achieved by a triexponential decay plus a nonrelaxing fraction of $[Q_A^{\bullet-}(t)]$, i.e., by formally the same expression (eq 2) as for untreated leaves but with drastically altered lifetimes τ_i. The results of the numerical fit are summarised in Table 3. These data show that a small fraction of 5−10% of the total PS II population is characterized by decay kinetics with τ_1 of 70−80 ms. This time is typical for a recombination between $Q_A^{\bullet-}$ and Y_Z^{OX} in PS II complexes that are deprived of a functionally competent water oxidizing complex (Weiss & Renger, 1984). Therefore, the 70−80 ms is inferred to indicate that in leaves of

Table 3: Kinetic Parameters of the Q_A^- Reoxidation (See Text and Eq 2) after the First Flash in DCMU-Treated Leaves from Wild-Type and *dgd1* Mutant of *Arabidopsis thaliana*[a]

	wild-type	*dgd1* mutant
a_1 (τ_1/s)	0.09 (0.07)	0.06 (0.08)
a_2 (τ_2/s)	0.39 (0.47)	0.19 (0.66)
a_3 (τ_3/s)	0.39 (2.20)	0.25 (3.00)
a_4 (τ_4 > 10 s)	0.13	0.50

[a] The experiments were performed at room temperature. For further experimental details see Materials and Methods.

both wild-type and *dgd1* mutant, a minor fraction of PS II centers is lacking a fully intact WOC. The two slower decay kinetics exhibit lifetimes of 500−700 ms and 2−3 s, respectively. The latter values are virtually the same as those reported for the reoxidation of $Q_A^{\bullet -}$ by the WOC in redox state(s) $S_2(S_3)$ in spinach [for a review, see Debus (1992) and references therein]. Accordingly, the same reaction is assumed to be responsible for the 2−3 s kinetics in DCMU treated leaves of *A. thaliana* (wild-type and *dgd1* mutant). The situation is somewhat less clear for the PS II subpopulation with a 500−700 ms decay component. This reaction is also ascribed to $Q_A^{\bullet -}$ reoxidation by the donor side. Depending on the functional and structural integrity of the WOC, two possibilities have to be considered: (i) the WOC is nonfunctional as electron donor and simultaneously the rate of recombination between $Q_A^{\bullet -}$ and Y_Z^{OX} is retarded by a factor of 5−10 compared to centers with a 70−80 ms kinetics, or (ii) the WOC remains intact but its interaction with Q_A^- is kinetically faster than that of the subpopulation with a 2−3 s component. Although *a priori* neither of these alternatives can be excluded, the latter appears to be much more reasonable because the subpopulation of centers with these kinetics is larger by a factor of 2 in the wild-type than in the mutant. It can be expected that the wild-type is the more healthy line; and, therefore the 500−700 ms is ascribed to PS II complexes with a mostly intact WOC. This idea is supported by measurements of flash-induced fluorescence transients in untreated leaves (*vide infra*).

The most striking feature emerging from the data of Table 3 is the pronounced increase of the nonrelaxing part (a_4) that arises in the *dgd1* mutant at the expense of the 500−700 ms and 2−3 s components. If one takes into account that the acceptor side is only marginally affected in nontreated leaves (*vide supra*), this pronounced effect observed in DCMU-treated samples is indicative of major changes in the WOC or its environment in the *dgd1* mutant.

In order to analyze possible donor side effects in more detail, measurements of flash-induced changes of the relative fluorescence quantum yield were performed at higher time resolution.

Analysis of Redox Reactions at the Donor Side. In PS II complexes with a fully competent WOC, the reduction of $P680^{\bullet +}$ by Y_Z takes place via a multiphasic kinetics with half-lifetimes of 20−50 ns, 200−300 ns, 5 μs, and 35 μs (Renger et al., 1978; van Best & Mathis, 1978; Renger et al., 1983; Brettel und Witt, 1983; Eckert et al., 1984). The relative extent of these kinetics depends on the redox state S_i of the WOC giving rise to characteristic period four oscillation patterns (Gläser et al., 1976; Brettel et al., 1984; Eckert & Renger, 1988). As $P680^{\bullet +}$ is a strong fluorescence quencher (Butler, 1972), the reduction kinetics can be monitored via flash-induced changes of the relative fluorescence quantum yield provided that the time resolution of the equipment is sufficiently high. Some complications of data interpretation arising from the formation of carotenoid triplets that act as potent fluorescence quenchers can be solved by using appropriate excitation conditions [for details, see Reifarth et al. (1997)]. Figure 6 shows traces of the flash-induced fluorescence quantum yield at a time resolution of 1 μs. For the sake of direct comparability between wild-type and *dgd1* mutant, the normalized variable fluorescence is presented. The signals of both types of leaves exhibit an instantaneous rise owing to the unresolved fast components of $P680^{\bullet +}$ reduction in the ns domain, followed by slower, well-resolved kinetics. A comparison between the traces readily reveals that the *dgd1* mutant is characterized by a marked increase of the extent of microsecond kinetics compared with the wild-type. This finding is indicative of changes at the PS II donor side in the *dgd1* mutant that give rise to modifications of $P680^{\bullet +}$ reduction by Y_Z. In order to obtain further information, the characteristic period four oscillation of $P680^{\bullet +}$ reduction was analyzed. In this case, actinic flashes of saturating intensity have to be used for achieving most pronounced oscillation patterns. The normalized fluorescence yield at 1 μs after the actinic flash was used as indicator because under these conditions $P680^{\bullet +}$ is almost completely reduced by Y_Z via the nanosecond kinetics while only a very small fraction of this reaction can occur through 5 and 35 μs kinetics. Therefore, the parameter $F_{1\mu s}(n)$ is a suitable measure of the extent of nanosecond kinetics contributing to $P680^{\bullet +}$ after excitation with the nth flash of a train of actinic flashes. Furthermore, for the sake of direct comparability of the data measured in wild-type and *dgd1* mutant, the values of $F_{1\mu s}(n)$ were normalized to the steady state level that is approximately given by the average of the first eight flashes, i.e., the quantity $F_{1\mu s}(\text{mean}) = 1/8 \sum_{n=1}^{8} F_{1\mu s}(n)$. Figure 7 shows the values of $F_{1\mu s}(n)/F_{1\mu s}(\text{mean})$ as a function of flash number n. The wild-type leaves exhibit a pronounced period four oscillation that is typical for systems with an intact WOC [for further details, see Reifarth

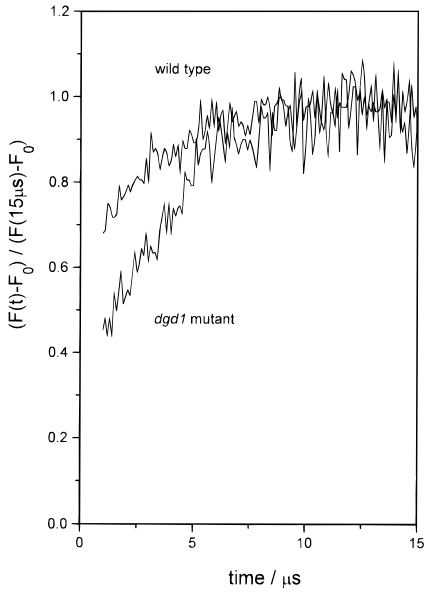

FIGURE 6: Laser-flash-induced changes of the normalized variable fluorescence quantum yield as a function of time in dark-adapted leaves from wild-type and a *dgd1* mutant of *Arabidopsis thaliana*. For experimental details see Materials and Methods.

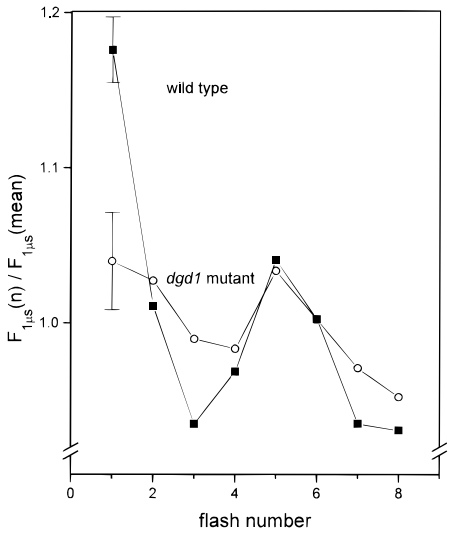

FIGURE 7: Fluorescence quantum yield at 1 μs after an actinic flash normalized to the mean values of eight flashes, $F_{1\mu s}(n)/F_{1\mu s}$(mear) as a function of flash *n* in dark-adapted leaves from wild-type (filled symbols) and *dgd1* mutant (open symbols) of *Arabidopsis thaliana*. Experimental conditions as in Figure 6. For further details see text.

et al. (1997)]. A drastically different pattern, however, is observed for the *dgd1* mutant. In this case, the oscillations are highly damped. This finding suggests that significant changes occur at the PS II donor side and strongly support the conclusions gathered from the measurements in DCMU-treated leaves.

Effects on the kinetics of $P680^{\bullet+}$ reduction can arise due to a modification of the coupling between P680 and Y_Z or indirectly via alteration or destruction of the WOC. In an attempt to address this problem, experiments were performed with leaves that were incubated with NH_2OH to disturb the WOC. Numerous studies have shown that, in PS II deprived of the WOC, the $P680^{\bullet+}$ reduction by Y_Z in the nanosecond domain is replaced by a 5−20 μs kinetics depending on the pH value (Conjeaud & Mathis, 1980; Renger et al., 1984). Measurements of flash-induced changes of the fluorescence quantum yield were recently shown to provide a suitable analytical tool for monitoring these reactions (Reifarth et al., 1997). The results obtained for NH_2OH treated leaves of *A. thaliana* are shown in Figure 8. A comparison with the traces of Figure 6 readily reveals that the contribution of the fast unresolved rise kinetics of $F(t) - F_o/F(15\ \mu s) - F_o$ disappear to a large extent and that only minor differences exist between wild-type and *dgd1* mutant. On the basis of these findings, it is inferred that the WOC in the mutant is somewhat more susceptible to degradation by NH_2OH while the kinetics of electron transfer from Y_Z to $P680^{\bullet+}$ remains almost the same as those of the wild-type.

DISCUSSION

In the present study, redox reactions of the donor and acceptor side of PS II were analyzed in leaves of *A. thaliana* by using noninvasive fluorometric methods. As a resumé of the obtained results, three major conclusions can be drawn from the effects induced by reduction of the digalactosyldiacylglycerol (DGDG) content in a mutant of *A. thaliana*.

(1) The kinetics of $Q_A^{\bullet-}$ reoxidation by endogenous plastoquinone and the equilibrium constant between the states $Q_A^- Q_B$ and $Q_A Q_B^{\bullet-}$ are very similar if not identical in wild-type and the *dgd1* mutant.

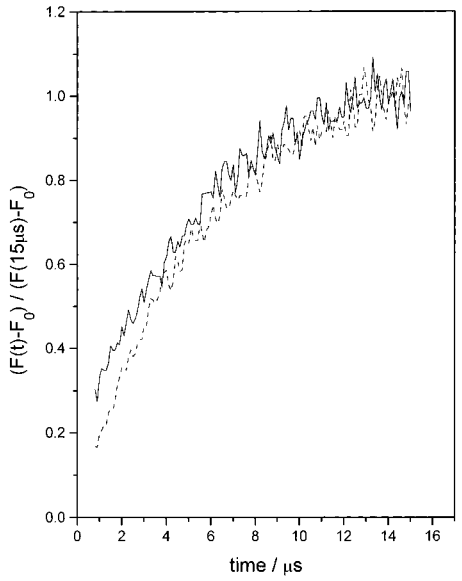

FIGURE 8: Laser-flash-induced changes of the normalized variable fluorescence quantum yield as a function of time in dark-adapted and NH_2OH-treated leaves from wild-type and *dgd1* mutant of *Arabidopsis thaliana*. Other experimental conditions as in Figure 6.

(2) The *dgd1* mutant exhibits a small but discernible enhancement of the susceptibility to high-temperature stress and a shift of the break point of the Arrhenius plot for electron transfer from $Q_A^{\bullet-}$ to Q_B toward lower temperatures.

(3) In contrast to the acceptor side, the donor side is markedly altered in the *dgd1* mutant compared to wild-type.

The finding that in the *dgd1* mutant the acceptor side is only marginally affected suggests that DGDG does not play a key role in establishing the structure of the Q_B site. Therefore, the drastic impairment of the function of the Q_B site in PS II core complexes isolated by the use of detergents (Gleiter et al., 1993; van Leeuwen et al., 1993) is most likely not due to a replacement of DGDG by detergent molecules but originates from another structural modification. Likewise, the enhanced susceptibility to higher temperatures is inferred not to be caused by a specific interaction of DGDG with the protein matrix of the Q_B site but reflects a general effect on the structure of the PS II complex in the *dgd1* mutant that leads to higher thermal instability.

A complementary support for the existence of this type of structural modification is the finding that the Arrhenius plot for $Q_A^{\bullet-}$ reoxidation by Q_B exhibits a clearly discernible break point at about 19 °C in wild-type and 12 °C in the *dgd1* mutant. In general, this break point phenomenon is likely to reflect a structural change that gives rise to a modified reaction coordinate. Analogous features have been previously observed for the permeability of the thylakoid membrane to ion transport (Kraayenhof et al., 1971; Gräber & Witt, 1975) and the decay kinetics of redox states S_2 and S_3 in the WOC catalyzed by lipophilic protonophores (Renger, 1975). These phenomena probably reflect structural changes in the lipid bulk phase of the thylakoid membrane. It is conceivable that changes of the DGDG content in the mutant affect these properties because DGDG is a major component of the lipid phase of the thylakoid membrane [for a review, see Webb and Green (1991) and references therein].

Apart from these interesting phenomena, the most striking feature emerging from a change of the DGDG content is

the marked effects on the PS II donor side. Taking into account the different facets of changes observed under various conditions (untreated, DCMU-, and NH_2OH-incubated leaves) it appears to be most plausible to localize the major effect to the WOC. As a consequence DGDG is inferred to modulate the functional pattern of the WOC either by direct interaction or via indirect structural effects. This conclusion is in line with previous findings showing that, in PS II membrane fragments with comparatively low oxygen evolution rates, the addition of DGDG or phosphatidylcholine causes a stimulatory effect while acidic lipids are of inhibitory action (Gounaris et al., 1983).

Two questions arise for the underlying mechanism(s) of the observed effects with regard to the DGDG deficiency: (i) are all PS II complexes affected in the same way or does a heterogenous population exist with WOCs that attain different stages of modification and (ii) what is the possible mode of changes in the WOC?

Although an unambiguous answer cannot be given for the first question, it seems more likely that DGDG depletion lead to heterogenous effects, because already wild-type plants contain PS II complexes with different properties [for a review on "static" and "dynamic" heterogeneties of PS II, see Lavergne and Briantais (1996) and references therein].

With regard to the second question, several possibilities exist for a modification of the WOC. The most drastic effect would be the occurrence of nonfunctional WOCs. In this case, Y_Z^{OX} could not be reduced by electrons from the WOC. As a consequence, a marked increase of the normalized extent of the 70−80 ms is expected to arise in DCMU-treated leaves of the *dgd1* mutant, in contrast to the experimental finding (see Table 3). Therefore, it is concluded that the significant changes in the *dgd1* mutant are not due to an increased population of PS II with a completely nonfunctional WOC. Less severe modifications can lead to a destabilization of oxidized manganese states in the WOC. Recent studies have shown that the WOC is destabilized in extensively dark-adapted mutants of cyanobacteria where either the whole extrinsic 33 kDa protein is deleted (Engels et al., 1994; Burnap et al., 1996) or the CP 47 is modified by deletion of 3−8 amino acid residues from the large lumen exposed loop (Gleiter et al., 1994, 1995). It appears very attractive to speculate that not only proteins but also lipids (DGDG) contribute to the stability of oxidizing redox equivalents stored in manganese centers of the WOC. Thus, depletion of DGDG in the mutant could lead to a population of more reduced states of the WOC in the dark and/or an accelerated decay of S_2 and S_3 between the flashes. In the first case, the state(s) $Q_A^{\bullet-}S_2(S_3)$ could not be formed after excitation with a laser flash and $Q_A^{\bullet-}$ not reoxidized via recombination with the WOC, i.e., $Q_A^{\bullet-}$ stays reduced in DCMU-treated leaves of the *dgd1* mutant as is shown in Figure 5 and Table 3. The same effect is expected in the second case when the dark decay of $S_2(S_3)$ by other endogenous or exogenous redox components efficiently competes with $Q_A^{\bullet-}$ reoxidation (Hanssum et al., 1985). Likewise, the oscillation pattern of the S_i states in dark adapted samples excited with a flash train disappears. This phenomenon is consistent with the results observed for $P680^{\bullet+}$ reduction in the *dgd1* mutant (Figure 6). Regardless of the detailed mechanism, the data of the present study provide first direct evidence for a significant modification of the WOC in the *dgd1* mutant. It could be a hint for a more specific role of DGDG for the stability of oxidizing redox equivalents stored in the WOC. This effect is certainly not related to a bulk phase of lipids because isolated PS II core complexes containing only about 10 lipid molecules including 2−3 DGDG (Murata et al., 1990) are fully competent in O_2 evolution and exhibit only very minor effects on the reaction coordinate of the individual redox steps (Karge et al., 1997). In this respect, it is interesting to note that isolated cytochrome oxidase which catalyzes the reverse reaction of water oxidation to O_2 requires cardiolipin for full activity [for a review, see Babcock and Wikström (1992)].

The data of the present study provide the first direct evidence for the idea that specific lipids may play an important role for the functional and structural integrity of the WOC. Neverthless, it should be pointed out that at this time the possibility cannot be completely ruled out that a secondary mutation may be involved which is independent of that causing the DGDG deficiency. However, with regard to the *dgd1* mutant, the likelyhood of secondary mutations has been minimized by multiple back-crossing of the mutant to the wild-type and by careful analysis of the genetic background of the five times back-crossed mutant during our efforts toward the map-based cloning of the *DGD1* locus. At this time, many recombinants were analyzed, some closer than 100 kilobasepairs to the *DGD1* locus on both sides that show the same complex phenotype without exception (Dörmann, P., and Benning, C., unpublished results). Further analysis will be required to corroborate our present findings once the *dgd1* lines have been isolated by map based cloning. The availability of the gene will allow to construct DGDG deficient antisense lines with an unambigous genetic background. In future studies on antisense lines, attempts will be made to unravel the possible mechanism underlying the observed effects of DGDG deficiency on the WOC.

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