The biosynthesis of thylakoid lipids in eukaryotic photosynthetic organisms often involves enzymes in the endoplasmic reticulum (ER) and the chloroplast envelopes. Two pathways of thylakoid lipid biosynthesis, the ER and the plastid pathways, are present in parallel in many species, including Arabidopsis, but in other plants, e.g., grasses, only the ER pathway is active. The unicellular alga Chlamydomonas reinhardtii diverges from plants like Arabidopsis in a different way because its membranes do not contain phosphatidylcholine, and most thylakoid lipids are derived from the plastid pathway. Here, we describe an acylated derivative of sulfolipid, 2'-O-acylsulfoquinovosyldiacylglycerol (ASQD), which is present in C. reinhardtii. Although the fatty acids of sulfoquinovosyldiacylglycerol (SQDG) were mostly saturated, ASQD molecular species carried predominantly unsaturated fatty acids. Moreover, directly attached to the head group of ASQD was preferentially an 18-carbon fatty acid with four double bonds. High-throughput robotic screening led to the isolation of a strain of Chlamydomonas reinhardtii, designated Δsqd1, which lacks ASQD as well as SQDG. In this mutant, the SQD1 ortholog was completely deleted and replaced by plasmid sequences. It is proposed that ASQD arises from the sugar nucleotide pathway of sulfolipid biosynthesis by acylation of the 2'-hydroxyl of the sulfoquinovosyl head group. At the physiological level, the mutant showed increased sensitivity to a diuron herbicide and reduced growth under phosphate limitation, suggesting a role for SQDG and/or ASQD in photosynthesis as conducted by C. reinhardtii, particularly under phosphate-limited conditions.

The thylakoid membrane of oxygenic photosynthetic organisms provides the lipid matrix in which the photosynthetic electron transport complexes are embedded. The lipid portion of the thylakoid membrane consists of four glycerolipids, namely monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), phosphatidylglycerol (PG), and the sulfolipid sulfoquinovosyldiacylglycerol (SQDG). The focus here is on SQDG and its derivative 2'-O-acylsulfoquinovosyldiacylglycerol (ASQD). The genes encoding enzymes for the biosynthesis of SQDG have recently been isolated in Arabidopsis (Essigmann et al., 1998; Yu et al., 2002). Furthermore, sulfolipid-deficient mutants in bacteria (Benning et al., 1993; Güler et al., 1995; Minoda et al., 2002) have provided important clues toward the function of SQDG in photosynthetic membranes. The growth of the sulfolipid-deficient mutants in the aforementioned bacteria and Arabidopsis is impaired after phosphate starvation, whereas the sulfolipid deficiency does not seem to have an adverse effect on growth under optimal conditions. In the respective wild types, the biosynthesis of SQDG is up-regulated under phosphorus limitation, apparently to compensate for a loss of the anionic phospholipid PG. This adaptation results in conservation of phosphate, while maintaining the anionic character of the thylakoid membrane. In the mutants, this compensatory mechanism is inactivated and PG levels are maintained under phosphate deficiency. Experiments with a C. reinhardtii mutant deficient in SQDG suggested that this lipid may be required for the proper function of photosystem II (PSII) under optimal growth conditions (Sato et al., 1995; Minoda et al., 2002). Sulfolipid might also be more critical for photosynthesis in the cyanobacteria Synechocystis sp. PCC6803 because previous attempts to disrupt a sulfolipid gene failed in this particular organism without exogenous supplementation of SQDG (Güler et al., 2000).

Sulfolipid biosynthesis in plants proceeds in two steps via the sugar nucleotide pathway (Pugh et al., 1995; Benning, 1998). The first reaction, the biosynthesis of UDP-sulfoquinovose from UDP-Glc and sul-
fite, is catalyzed by SQD1 (Sanda et al., 2001) and the second reaction, the transfer of sulfoquinovose from UDP-sulfoquinovose to diacylglycerol, is carried out by SQD2 (Yu et al., 2002). Here, we describe a mutant of *C. reinhardtii* in which the SQD1 ortholog has been deleted and replaced by plasmid sequences. To our surprise, not only was SQDG absent from this mutant, but also a second minor sulfolipid, which has not been previously described, was missing. The structure of this lipid was analyzed and its biosynthesis is discussed. Furthermore, the absence of two lipids in this mutant raises questions regarding the interpretation of physiological data obtained for sulfolipid mutants of *C. reinhardtii*.

### RESULTS

#### Accumulation of ASQD in *C. reinhardtii*

Thin-layer chromatograms of lipids from 35S-sulfate-labeled cells showed the expected lipid band for the plant sulfolipid SQDG, and, in addition, a band of approximately 5% intensity of SQDG (Fig. 1, ASQD). The chromatographic behavior of the compound running with higher mobility suggested that it has a decreased polarity. The ratio of ASQD to SQDG was comparable under normal and phosphate-depleted growth conditions with increased labeling in the two compounds after phosphate starvation (Fig. 1).

To determine the identity of the unknown sulfur-labeled compound, we purified approximately 100 mg from 20 liters of phosphate-starved *C. reinhardtii* cultures using a combination of column and thin-layer chromatographic techniques. We hypothesized that this compound may be a derivative of SQDG and we used a modification of a purification procedure developed for sulfolipid (O’Brien and Benson, 1964). Fatty acid methyl ester analysis was used to determine the fatty acid composition of ASQD, SQDG, and entire cell extracts as shown in Table I. Although SQDG carried mostly palmitate (16:0) and oleate (18:1), ASQD was strikingly different and contained more unsaturated fatty acids, including an 18:4 fatty acid as subsequently confirmed by mass spectrometry (MS). This fatty acid was originally identified in extracts of *C. reinhardtii* by Giroud et al. (1988) as 18:4\(^{\Delta 5,9,12}\). A corresponding 18:3\(^{\Delta 5,9,12}\) fatty acid distinguishable in our chromatograms from more typical \(\alpha\)-linolenic acid (18:3\(^{\Delta 9,12,15}\)) was present as well. The whole-cell extract composition was noticeably different from that observed for ASQD and SQDG (Table I). Negative-ion fast-atom bombardment (FAB)-MS (Fig. 2A) of the ASQD sample showed a major molecular ion species at approximately \(m/z\) 1074. Closer inspection of this region (Fig. 2, insert) revealed two major species at \(m/z\) 1073.5.

#### Table I. Comparison of the fatty acid composition of the two sulfolipids and cells

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>ASQD</th>
<th>SQDG</th>
<th>Whole Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>39.5 ± 7.0</td>
<td>72.5 ± 7.1</td>
<td>25.7 ± 8.4</td>
</tr>
<tr>
<td>16:1</td>
<td>nd</td>
<td>1.4 ± 0.2</td>
<td>7.9 ± 2.8</td>
</tr>
<tr>
<td>16:2</td>
<td>nd</td>
<td>nd</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>16:3</td>
<td>nd</td>
<td>nd</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>16:4</td>
<td>nd</td>
<td>nd</td>
<td>11.1 ± 0.5</td>
</tr>
<tr>
<td>18:0</td>
<td>4.5 ± 1.0</td>
<td>2.5 ± 1.6</td>
<td>3.7 ± 0.0</td>
</tr>
<tr>
<td>18:1</td>
<td>2.5 ± 1.6</td>
<td>20.5 ± 5.5</td>
<td>19.2 ± 0.9</td>
</tr>
<tr>
<td>18:2</td>
<td>6.6 ± 1.0</td>
<td>1.1 ± 0.1</td>
<td>7.4 ± 0.7</td>
</tr>
<tr>
<td>18:3(^{\Delta 5,9,12})</td>
<td>10.3 ± 1.6</td>
<td>0.9 ± 0.1</td>
<td>9.2 ± 3.6</td>
</tr>
<tr>
<td>18:3(^{\Delta 9,12,15})</td>
<td>19.4 ± 4.5</td>
<td>1.6 ± 0.5</td>
<td>11.8 ± 0.6</td>
</tr>
<tr>
<td>18:4</td>
<td>17.5 ± 2.5</td>
<td>nd</td>
<td>1.1 ± 0.5</td>
</tr>
</tbody>
</table>

* nd, Not detected. Two independent experiments were averaged (±SD).
and 1075.5 corresponding to triacylated SQDG molecular ions with total fatty acid compositions (carbons:double bonds) of 52:7 and 52:6, respectively. Based on analysis of fatty acid methyl esters (Table I), it was hypothesized that the 52:7 species represents a triacylated SQDG form composed of 18:3/16:0/18:4, and the 52:6 species most likely represents a mixture of 18:3/16:0/18:3 and 18:2/16:0/18:4 molecular species. The major peak of the FAB-MS at m/z 1073.5 was selected as the parent ion for FAB-CAD-MS/MS (Fig. 2B). Diagnostic secondary ions consistent with a triacylated SQDG molecule were present throughout the spectrum. Only two in support of the structure for ASQD are discussed here. The secondary ion at m/z 209 was tentatively interpreted as a sulfoquinovose head group fragment (Fig. 2, B and C, compound 2), and a prominent fragment at m/z 543 and its neighbor at m/z 541 (not resolved in Fig. 2B) corresponded to a monoacyl SQDG derivative after the loss of both diacylglycerol acyl groups from a triacylated SQDG. The fatty acyl groups in these ions are 18:4 or 18:3, respectively, proposed to be esterified to one of the sulfoquinovose hydroxyl groups (Fig. 2C, compound 3).

To further corroborate the structure and to determine the position of the third acylation on the sulfoquinovosyl head group, 1H-NMR spectra were recorded for ASQD and SQDG (Fig. 3). The 1H spectrum of SQDG given in Figure 3B is essentially identical to that described by Cedergren and Hollingsworth (1994). For ASQD (Fig. 3C), assignment of glycerol and carbohydrate ring proton resonances was aided by a 1H-1H homonuclear decoupling experiment (data not shown). This experiment revealed that the 2-sulfoquinovose proton is shifted downfield by approximately 1.2 ppm relative to SQDG, and that the 1- and 3-protons are shifted downfield by approximately 0.17 ppm, consistent with an acyl function esterified to the 2-hydroxyl. The region from 5.2 to 5.4 ppm provides information on the acyl substituents, as this region contains the vinyl proton signals of the fatty acyl groups in addition to the glyceryl-2-proton signal. Integration of this region (using the anomeric proton as reference) showed approximately three protons for SQDG, indicating that the average SQDG molecule contains just one double bond (two vinyl protons in addition to the glyceryl-2-proton). Conversely, ASQD had a much stronger...
signal of approximately 14 protons (13 vinyl protons plus glycerol 2), in agreement with the molecular species observed by MS and fatty acid analysis.

Isolation of a Sulfolipid-Deficient Insertional Mutant of *C. reinhardtii*

To begin to understand the biosynthesis of this new lipid and to identify genes essential for its biosynthesis, a genetic approach was applied. For this purpose, we developed a robotic high-throughput screening procedure (see “Materials and Methods” for details) allowing the direct one-dimensional thin-layer chromatography (TLC) analysis of lipid extracts from a large number of mutagenized *C. reinhardtii* colonies. To be able to quickly identify a locus of interest, insertional mutagenesis by plasmid transformation was used. Among approximately 10,000 primary transformants screened, one putative sulfolipid-deficient strain was isolated along with a number of other putative mutants screened, one putative sulfolipid-deficient mutant strain was isolated along with a number of other putative mutant strains showing aberrant lipid profiles. To use the most stringent and sensitive test for the presence of sulfolipids in the putative SQDG-deficient mutant, we labeled the cells with $^{35}$S-sulfate. Cells were grown under replete and phosphate-limited conditions that typically induce the synthesis of sulfolipids in plants (Essigmann et al., 1998; Yu et al., 2002). As presented in Figure 1, *C. reinhardtii* dw15.1 showed increased labeling of SQDG and ASQD under phosphate-limited growth conditions, when approximately equal amounts of total lipids were loaded. However, the sulfolipid-deficient mutant strain was completely devoid of sulfolipid, and SQDG and ASQD could not be detected under either growth condition (Fig. 1).

A Putative SQD1 Ortholog of *C. reinhardtii* Is Deleted in the Sulfolipid-Deficient Strain

Plasmid rescue was used to identify genomic DNA flanking the mutagenic plasmid insertion. The resulting plasmid, pSqdl-R (Fig. 4, bottom), carrying a greater than 10 kb insert, was partially sequenced in a single run from the right end of the pUC119 vector. The resulting sequence could be placed on scaffold 22 of the recently released genomic sequence of *C. reinhardtii* (http://genome.jgi-psf.org/chlre1/chlre1.home.html). This sequence was approximately 30 kb downstream from a putative ortholog of the *AtSQD1* gene, which is known to be essential for sulfolipid biosynthesis in Arabidopsis (Essigmann et al., 1998). After restriction mapping of the plasmid insert and comparison with the predicted map of the genomic sequence of scaffold 22 (Fig. 4), the left end of the plasmid vector was placed approximately 10 kb downstream of the putative *CrSQD1* gene. To test genomic DNA flanking the inserted plasmid on the opposite side, a PCR marker-based strategy was used because these genomic sequences could not be recovered by plasmid rescue. Oligonucleotides were designed to amplify segments of DNA of defined length, which are denoted as markers A through E. Markers A through D (Fig. 4) were present in DNA isolated from dw15.1 but could not be detected in reactions using template DNA from the sulfolipid-deficient mutant (data not shown). However, Marker E (Fig. 4) was present in both strains. Based on this analysis, the sulfolipid-deficient strain carried a deletion of nearly 20 kb centered around the putative *CrSQD1* gene. Therefore, the mutant line was designated Δsqd1. The mutant is lacking the entire *CrSQD1* gene as suggested by the negative result for PCR markers A and B designed against the two ends of *CrSQD1* (Fig. 4). Beyond the 3’ end of this gene, no additional predicted open reading frames were affected by the deletion (Fig. 4). However, beyond the

![Figure 4](http://www.plant.org) Comparison of genomic sequences surrounding the *CrSQD1* locus in the wild type (WT), the Δsqd1 mutant, and the rescued plasmid (pSqdl-R). Five predicted genes/elements are shown as black arrows/box. They are annotated to encode: snRNP, small nuclear ribonuclear particle; DHPR, dihydropicolinate reductase; *CrSQD1*, *SQD1/SQDB* ortholog; T, repetitive element with similarity to transposases; and ABC, putative ABC lipid transporter. Plasmid sequences derived from pMN-24 are the pUC119 vector and the nitrate reductase marker gene (*NIT1*). Diagnostic PCR products used to delineate the extent of the deletion in Δsqd1 are indicated by capital letters above the wild-type genomic sequence. The arrow in pSqdl-R depicts the left end sequence used to place the rescued genomic DNA onto the genome. Restriction enzymes indicated by two vertical bars are: E, EcoRV; H, Hind III; N, NodI; S, SalI; Sp, SpeI; and X, Xbal.
5’ end of CrSQD1, a repetitive element (Fig. 4T), possibly the remnant of a transposon, was completely missing as well. This element is present more than 100 times in the available genomic sequence of C. reinhardtii and showed sequence similarity to transposases. More importantly, the 5’ end of a putative ABC transporter gene with similarities to phospholipid transporters (Fig. 4, A–C) was also deleted, as indicated by a negative result for marker D in the mutant. Because marker E, just outside the predicted ABC transporter open reading frame was present, it is concluded that the deletion ends within this putative gene. Taken together, more than one gene is affected in the Δsqd1 mutant, complicating the interpretation of the resulting biochemical and physiological phenotypes.

CrSQD1 Clusters with Bona Fide Plant SQD1 Proteins

As mentioned above, a putative ortholog of the plant SQD1 gene, tentatively designated CrSQD1, was found deleted in the sulfolipid-deficient Δsqd1 mutant. Figure 5 shows a lineup of representative SQD1/SQDB orthologs from plants, bacteria, and archaea, and an unrooted tree with sequence similarity clusters. The orthologs from R. spheroides (Benning and Somerville, 1992a), S. meliloti (Weissenmayer et al., 2000), Synechococcus PCC7942 (Güler et al., 1996), and the two plants Arabidopsis (Essigmann et al., 1998) and spinach (Shimojima and Benning, 2003) have been experimentally verified by mutant and complementation analysis or by biochemical analysis of the recombinant protein. As shown in Figure 5B, the known and presumed enzymes catalyzing the first reaction of sulfolipid biosynthesis are highly conserved, even across kingdoms. The CrSQD1 protein clusters with the two plant proteins on a major branch of the unrooted tree that also holds the cyanobacterial cluster (Fig. 5A).

The Δsqd1 Mutant Shows Reduced Growth under Phosphate Limitation and Increased Herbicide Sensitivity

Relative lipid compositions of the control strain dw15.1 and the Δsqd1 mutant derived from dw15.1 were compared for cells grown under phosphate-replete and phosphate-limited conditions (Table II). In the dw15.1 strain, the relative SQDG content drastically increased under phosphate-limited growth conditions, whereas the amount of PG decreased. In the Δsqd1 mutant, which completely lacks SQDG and ASQD, PG amounts were higher in cells grown in replete medium and did not decrease under phosphate limitation to the same extent as observed for strain dw15.1. Qualitatively similar observations have been made for SQDG-deficient mutants of Arabidopsis (Yu et al., 2002) and bacteria (Benning et al., 1993; Güler et al., 1996). Furthermore, the relative content of DGDG increased in both strains under phosphate-limitation as was observed for Arabidopsis (Härter et al., 2000). Thus, glycolipid/phospholipid substitution as an adaptive mechanism in response to phosphate deprivation is a common phenomenon in species of bacteria, seed plants, and unicellular green algae. Interestingly, the relative amount of the nonphosphorous extraplastidic betaine lipid (Table II, DGTS) did not change in either strain under the two growth conditions. The ASQD form of sulfolipid chromatographed with DGTS in this experiment and could not be separately analyzed. However, from our labeling experiments, we estimate that the relative amount of this lipid did not represent more than 1% (w/v) of the polar lipids analyzed here. Thus, most of the lipid in the DGTS/ASQD mixture was representative of the betaine lipid.

Growth under the two conditions was compared for dw15.1 and Δsqd1, as shown in Figure 6. The growth of both strains was essentially indistinguishable under phosphate-replete conditions. Growth of dw15.1 was delayed and growth of Δsqd1 ceased at much lower density under phosphate-limited conditions. Unfortunately, growth data on phosphate-replete and -limited medium are not available for another SQDG-deficient mutant of C. reinhardtii, hf-2 (Sato et al., 1995; Minoda et al., 2002). However, based on the analysis of the hf-2 mutant, it was proposed that sulfolipid deficiency in general causes an impairment of photosynthesis under standard growth conditions. As an indirect test for PSII structure and function, we compared the growth of control strain dw15.1 and the Δsqd1 mutant on 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a herbicide binding to the Qα acceptor site of PSII and thereby blocking PSII activity. As shown in Figure 7, the Δsqd1 mutant was more sensitive to DCMU than dw15.1, suggesting alterations in PSII, thus making it more accessible to DCMU or less stable under the experimental growth conditions. A similar result was observed for the hf-2 mutant (Minoda et al., 2002).

DISCUSSION

The Presence of ASQD in Lipid Extracts of C. reinhardtii Is Not an Extraction Artifact

Acylated glycolipids have been previously observed in plant extracts (Heinz, 1967), but it was concluded that these are formed during extraction of the plant tissues at low pH by an enzymatic transacylation reaction (Heinz et al., 1978). Several lines of evidence suggest that the ASQD observed in extracts of C. reinhardtii was not the result of the extraction conditions, but was present in the cell membranes. First, we tested several extraction conditions, leaving formic acid out of our extraction solvent to keep the pH high or extracting first with hot isopropanol, which should immediately destroy any enzymatic
activity, especially in a cell wall-deficient strain like dw15.1. Applying these conditions did not change the abundance of ASQD in the extracts (data not shown). Second, we did not observe acylated galactolipids in the extracts as previously described for plant extracts. Third, the diacylglycerol fatty acid composition of ASQD was highly specific and very different from SQDG, making it unlikely that ASQD originated from the general SQDG pool. Fourth, the third fatty acid attached directly to the head group of ASQD was also very specific, inconsistent with a random transacylation of SQDG acyl groups from other lipids. Taken together, these observations strongly suggest that ASQD is a minor lipid in cellular membranes of C. reinhardtii.

A Hypothesis for the Biosynthesis of ASQD in C. reinhardtii

Important clues for the biosynthesis of ASQD can be derived from its fatty acid composition, which differs from SQDG by its much higher ratio of unsaturated fatty acids (Table I). Analyzing gas chromatograms obtained for ASQD-derived fatty acid methyl esters, we could detect two unusual compounds with slightly smaller and slightly larger retention times than that for 18:3/9,12,15-linolenate methyl esters (data not shown). Two unusual fatty acids, 18:3/5,9,12 and 18:4/5,9,12,15, were previously reported for C. reinhardtii (Giroud et al., 1988), and the observed retention times of the unknown compounds would agree with retention times predicted for the methyl esters of these two unusual fatty acids. Although we did not obtain direct structural data on the position of double bonds in the acyl groups of ASQD, MS clearly suggested that the major molecular species has an 18:4 fatty acid attached directly to the head group. It seems likely that this is the 18:4/5,9,12,15 fatty acid, the only 18:4 fatty acid previously reported to be present in C. reinhardtii. Furthermore, it may be inferred that the 18:3 fatty acid attached to the head group of the second most abundant molecular species is the 18:4 precursor 18:3/5,9,12, also previously described. Our data do not allow us to conclude whether this fatty acid or 18:3/9,12,15-α-linolenic acid is present in the diacylglycerol moiety of ASQD. However, one might predict that linoleic and α-linolenic acids are esterified in the diacylglycerol moiety of ASQD, whereas the two unusual fatty acids

Figure 5. Comparison of SQD1/SQDB from bacteria, archaea, and plants. A, Unrooted tree produced from the alignments in B using ClustalW software. A, Clusters of closely related organisms are shaded. B, Identical amino acids are indicated by black boxes, similar ones with gray boxes. Amino acids marked with an asterisk participate in the reaction and are absolutely conserved. The following proteins were included in the analysis (GenBank accession nos. in parentheses): AtSQD1, Arabidopsis (T05311); SoSQD1, spinach (Spinacia oleracea; AA039667); TaSQDB, Thermoplasma acidophilum (NP 394533); SultokSQDB, Sulfolobus tokodaii (NP 343918); SulsolSQDB, Sulfolobus solfataricus (NP 343918); PCC6803SQDB, Synechocystis sp. PCC6803 (NP 440474); SmeSQDB, Sinorhizobium meliloti (NP 386851); RspSQDB, Rhodobacter sphaeroides (B45729); PCC7942SQDB, Synechococcus sp. PCC7942 (AAC43899); TelSQDB, Thermosynechococcus elongatus BP-1 (NP 681188); and PCC7120SQDB, Nostoc sp. PCC7120 (NP 485784). The C. reinhardtii ortholog, CrSQD1, is shown as predicted from the genome sequence (http://genome.jgi-psf.org/chlre1/chlre1.home.html; scaffold 22, gene 22.14).
are esterified to the 2'-hydroxyl of the sulfoquinovose head group.

A hypothesis for ASQD biosynthesis taking into account the different molecular species for ASQD and SQDG is shown in Figure 8. There are clearly two pools of SQDG, 16:0/16:0 and 18:1/16:0. ASQD biosynthesis seems to have access only to the latter pool, which can give rise to the 18:3-containing diacylglycerol moiety by lipid-linked desaturation. The most unsaturated 18:3/16:0 molecular species is also the most abundant in ASQD. Interestingly, the two unusual fatty acids 18:3\(^{5,9,12}\) and 18:4\(^{5,9,12,15}\) proposed to be esterified directly to the head group of ASQD are known as acyl groups primarily in extraplastidic lipids, such as phosphatidylethanolamine and the betaine lipid DGTS (Giroud et al., 1988) and it was proposed that these fatty acids are a product of the endoplasmic reticulum (ER) desaturation pathway. A second aspect of the hypothesis derives from the fact that the deletion of the \(CrSQD1\) gene in \(sqd1\) affects SQDG as well as ASQD biosynthesis. If we assume that \(CrSQD1\) catalyzes the conversion of UDP-Glc and sulfite to UDP-sulfoquinovose as described for its presumed spinach and Arabidopsis orthologs (Sanda et al., 2001; Shimojima and Benning, 2003), the lack of both lipids in the mutant suggests that ASQD is synthesized by acylation of UDP-sulfoquinovose or by acylation of SQDG. The biosynthesis of SQDG

### Table II. Lipid composition of \(dw15.1\) and \(\Delta sqd1\) mutant under normal and phosphate-deprived growth conditions

<table>
<thead>
<tr>
<th></th>
<th>1.0 mM Pi</th>
<th>0.02 mM Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(dw15.1)</td>
<td>(\Delta sqd1)</td>
</tr>
<tr>
<td>%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MGDG</td>
<td>38.2 ± 4.5</td>
<td>34.5 ± 3.1</td>
</tr>
<tr>
<td>Diacylglycerol-N-trimethylhomoserine (DGTS)/ASQD</td>
<td>25.8 ± 5.7</td>
<td>27.2 ± 4.2</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>6.1 ± 0.6</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>PG</td>
<td>7.7 ± 1.1</td>
<td>12.4 ± 0.6</td>
</tr>
<tr>
<td>DGDG</td>
<td>14.8 ± 2.3</td>
<td>16.3 ± 2.5</td>
</tr>
<tr>
<td>SQDG</td>
<td>5.0 ± 0.9</td>
<td>nd</td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td>2.3 ± 0.7</td>
<td>2.7 ± 0.5</td>
</tr>
</tbody>
</table>

* ASQD and DGTS were not separated in this experiment. Three independent experiments were averaged (±sd).

Figure 6. Growth of \(dw15.1\) (squares) and \(\Delta sqd1\) (circles) under phosphate-replete (closed symbols) and phosphate-limited (open symbols) conditions.

Figure 7. Growth of \(dw15.1\) and \(\Delta sqd1\) on agar-solidified medium containing different amounts of the herbicide DCMU at concentrations as indicated.
occurs in the plastid in all plants investigated and like its presumed orthologs, the CrSQD1 sequence contains a predicted N-terminal chloroplast transit peptide. Thus, SQDG biosynthesis in *C. reinhardtii* is likely to be a function of chloroplasts, as it is in seed plants. Because UDP-sulfoquinovose synthesis occurs in the plastid and the acyl groups found attached to the sulfoquinovose head group of SQDG appear to be of extraplastidic origin, it seems unlikely that UDP-sulfoquinovose is directly acylated, but that SQDG is the substrate for acylation. Analysis of SQDG molecular species indicates that there are two pools of SQDG, 16:0/16:0 and 18:1/16:0, of which only the second appears to be the substrate for an acyltransferase forming ASQD (Fig. 8). The acyltransferase involved in acylating the sulfoquinovose head group apparently has access to the unusual fatty acids 18:3<sup>5,9,12</sup> and 18:4<sup>5,9,12,15</sup> derived from the ER pathway. Thus, it seems possible that this enzyme is localized in the outer envelope of the plastid to gain access to SQDG made in the plastid and to fatty acids derived from the ER. Whether ASQD itself is present only in plastid envelopes or also in extraplastidic membranes remains to be investigated.

The Function of ASQD and SQDG in *C. reinhardtii*

To study the function of a particular membrane lipid, it would be ideal to have access to a genetic null mutant, which is well defined at the molecular level such that effects from secondary mutations can be ruled out. For a previously described SQDG-deficient mutant of *C. reinhardtii*, hf-2 (Sato et al., 1995; Minoda et al., 2002), derived from UV mutagenesis and identified based on its high chlorophyll fluorescence phenotype, the exact molecular defect is not known. The physiology of this mutant was investigated in great detail, and based on its photosynthesis related phenotype, it was concluded that SQDG plays a crucial role in photosynthesis of *C. reinhardtii* (Sato et al., 1995; Minoda et al., 2002). However, whether this mutant is impaired in growth has not been reported. Here, we describe a new SQDG-deficient mutant of *C. reinhardtii*, which provides a new challenge for the interpretation of the physiological data obtained for this and possibly other SQDG-deficient mutants. The Δsqd1 mutant completely lacks not only SQDG, but also ASQD. Therefore, any physiological phenotype observed for the mutant could be due to the lack of SQDG, ASQD, or both. To distinguish between the functions of ASQD and SQDG, one would have to isolate a mutant specifically affecting ASQD biosynthesis. It should be possible to isolate a strain carrying a mutation in the sulfoquinovose acyltransferase gene proposed to be involved in ASQD biosynthesis, which would specifically lack ASQD. On the other hand, because SQDG appears to be the precursor for ASQD, we would predict that it will not be possible to isolate a strain lacking just SQDG but still containing ASQD. It is not known at this time whether the independently isolated SQDG-deficient mutant hf-2 also lacks ASQD.

Figure 8. Hypothesis for ASQD biosynthesis in *C. reinhardtii*. The width of arrows indicates the extent of flux into the different molecular species. End products of the pathway are boxed. Different molecular species can be distinguished based on their acyl composition. Carbon number followed by double bond number are provided for the respective acyl groups. Two acyl groups separated by/indicate the two acyl groups at the *sn*-1 and *sn*-2 positions of the diacylglycerol moiety, respectively. The head group acyl groups are indicated by (2^′). These two fatty acids are also marked with an asterisk to indicated that they represent the two unusual acyl groups 18:3<sup>5,9,12</sup> and 18:4<sup>5,9,12,15</sup>. The block in the Δsqd1 mutant is indicated. DAG, Diacylglycerol; UDP-SQ, UDP-sulfoquinovose.
which does not show a growth impairment unless the respective mutant becomes phosphate deprived (Yu et al., 2002). When we grew the Δsqd1 mutant of *C. reinhardtii* under phosphate-depleted conditions (Fig. 6), we observed a reduction in growth and no decrease in the relative amount of the anionic lipid PG, indicating its inability to adjust the membrane lipid composition in response to phosphate starvation. However, Δsqd1 is clearly more sensitive to DCMU than dw15.1, as was observed for hf-2 (Minoda et al., 2002). Unfortunately, no DCMU sensitivity data are available for bacteria or Arabidopsis for comparison. However, it should be pointed out that slight changes in the photochemistry of PSII were observed for an SQDG-deficient mutant of *Synechococcus* sp. PCC7942 without an effect on growth rates under optimal conditions (Güler et al., 1996). Therefore, SQDG deficiency in general may have subtle effects on PSII, which leads to visible growth impairments only when additional stress factors such as phosphate limitation are present.

**The Identity of CrSQD1 and Evolution of Sulfolipid Biosynthesis**

Two arguments can be provided that CrSQD1 represents an ortholog of SQD1/SQDB found in plants and bacteria. First, its deletion causes the complete loss of sulfolipid in the Δsqd1 mutant in agreement with the inactivation of a gene essential for sulfolipid biosynthesis. Second, as the sequence lineup in Figure 5 shows, SQD1/SQDB proteins are highly similar and display the expected relational clusters when presented as an unrooted tree. The degree of experimentally confirmed orthology is high with five bona fide members in different clusters. The presumed ortholog of *C. reinhardtii*, CrSQD1, clusters closely with the experimentally verified Arabidopsis ortholog, for which crystal structure and reaction mechanism are known (Mulichak et al., 1999). All the unique active site residues Thr145, Tyr182, and His183 (numbering refers to the crystallized recombinant AtSQD1 protein) participating in the reaction mechanism are absolutely conserved in all orthologs (Fig. 5). Thus, the currently available evidence strongly suggests that CrSQD1 is the SQD1/SQDB ortholog of *C. reinhardtii*.

The close similarity of the SQD1/SQDB proteins suggests that the biosynthesis of UDP-sulfoquinovose evolved only once, presumably in a common ancestor of bacteria, archaea, and plant chloroplasts. Surprisingly, the SQDB protein from the cyanobacterium *Synechococcus* sp. PCC7942 is more closely related to orthologs from the α-proteobacteria group than those from the other cyanobacteria included in the analysis. Considering that *Synechococcus* sp. PCC7942 is a simpler cyanobacterium relative to the others, lacking tocopherols and higher unsaturated fatty acids, the clustering of its SQDB protein with those from α-proteobacteria is revealing. It also supports the predictive value of similarity tree building based on the SQD1/SQDB protein orthologs.

The presence of sulfolipid in archaea has not yet been described, despite the fact that members of the archaea appear to have orthologs equally distantly related to the SQDB proteins from bacteria and the SQD1 proteins from plants. However, there is a report of a sulfoquinovosylated protein in the archaeaon *Sulfolobus acidocaldarius* (Zähringer et al., 2000). Thus, archaea probably have the capability to synthesize UDP-sulfoquinovose from UDP-Glc and sulfitic catalyzed by their apparent SQDB orthologs, but they may lack the glycosyltransferase needed to actually synthesize SQDG. Instead, they have a transferase capable of adding sulfoquinovose as the terminal residue in an oligosaccharide chain.

Lipid metabolism in the unicellular alga *C. reinhardtii* has several unique features distinguishing it from other model systems such as Arabidopsis. Most prominently, it lacks phosphatidylcholine but contains instead the betaine lipid diacylglycerol-N-trimethylhomoserine. In addition to the previously described lipids, *C. reinhardtii* also synthesizes a 2'-O-acyl derivative of the plant sulfolipid. Based on the fatty acids present in ASQD, it was concluded that this lipid most likely arises from a distinct pool of SQDG by acylation of the sulfoquinovose head group with fatty acids of extraplastidic origin. It was proposed that the responsible enzyme may be located in the plastid outer envelope to explain its access to plastidic and extraplastidic substrates. Plasmid insertional mutagenesis was used in combination with a high-throughput (TLC) screen to isolate a mutant, Δsqd1, carrying a deletion of the CrSQD1 gene proposed to encode the UDP-sulfoquinovosyl synthase of *C. reinhardtii*. This mutant lacks both sulfolipids, ASQD and SQDG. Physiological analysis of the mutant led to the conclusion that the sulfolipid SQDG and/or its derivative ASQD are required for optimal growth and photosynthesis under phosphate-limited conditions. As previously concluded for photosynthetic bacteria and plants, anionic sulfolipid compensates for losses in PG in *C. reinhardtii* as part of the adaptive response to phosphate-limited growth conditions.

**MATERIALS AND METHODS**

**Growth and Labeling of *Chlamydomonas reinhardtii* Cell Cultures**

The cell wall-less strain dw15.1 (cw15, nit1, and mt +) was obtained from Arthur Grossman (The Carnegie Institution of Washington, Stanford, CA). This strain and its derivatives were grown in Tris-acetate-phosphate (TAP) medium (Harriss, 1989) modified to contain phosphate (Pi) at varying concentrations as indicated. For growth on plates, this medium was solidified with 1% (w/v) purified agar. Small liquid cultures were grown under constant illumination as 30- to 40-ml cultures in closed 50-ml conical centrifuge tubes, or larger cultures as 5-liter cultures in 6-liter flasks under cool-white fluorescent lights at 24°C under a 16-h day/8-h night regime.
Cultures were agitated by shaking twice daily, and cells were harvested by centrifugation in the late logarithmic phase of growth. Growth rates were determined by measuring the optical density at 720 nm of 30-mL cultures grown in TAP medium under Pi-replete (initial concentration of 1 mM Pi) or Pi-limited (initial concentration of 0.02 mM Pi) conditions. 35S- and 14C-labeling experiments were carried out by addition of 50 µCi [35S]Na2SO4 (carrier-free 61406 Ci mmol−1) or 25 µCi (47.5 mCi mmol−1) 1-14C acetate to a 30 mL culture. Cells were also grown photoautotrophically on high salt (HS) medium agar plates (Harris, 1989) at three times strength and with varying concentrations of DCMU, added as a concentrate in 50% (w/v) slurry of corn starch in TAP (Shimogawara et al., 1998). Sucrose was utilized as a carbon source for the cultures grown in TAP medium under Pi-replete (initial concentration of 1 mM Pi) or Pi-limited conditions. 35S- and 14C-labeled 30-L cultures were extracted immediately after harvesting with chloroform/methanol/88% formic acid (10/10/1, v/v) and the phases were separated by addition of 3 volumes of 0.2 M H3PO4 and KCl. Radioactivity in an aliquot of the chloroform phase was determined by scintillation counting and a fixed amount of radioactivity was used for analysis. For 14C-acetate labeling, two-dimensional TLC analysis (Benning et al., 1995) was followed by exposure to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA), and for 35S-labeling, a one-dimensional TLC system was used as described (Hartel et al., 2000) followed by quantification of the signals on the phosphorimager.

Quantitative Lipid Analysis

Lipids from cell pellets of 35S- or 14C-labeled 30-L cultures were extracted with chloroform/methanol/88% formic acid (10/10/1, v/v) and the phases were separated by addition of 3 volumes of 0.2 M H3PO4 and KCl. Radioactivity in an aliquot of the chloroform phase was determined by scintillation counting and a fixed amount of radioactivity was used for analysis. For 14C-acetate labeling, two-dimensional TLC analysis (Benning et al., 1995) was followed by exposure to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA), and for 35S-labeling, a one-dimensional TLC system was used as described (Hartel et al., 2000) followed by quantification of the signals on the phosphorimager.

Generation and Screening of Insertional Mutants

Strain dw15.1 was used for insertional mutagenesis with plasmid pMN24 containing the wild-type nitrate reductase (NIT1) gene (Fernandez et al., 1989). Transformation by the glass bead method was essentially as described by Kindle (1998), except that TAP medium containing nitrate as the sole N source (TAP-NO3) was used for selection of transformants, pMN24 was linearized with BamHI before transformation, and cells were plated in 0.5 mL of a 50% (w/v) slurry of corn starch in TAP (Shimogawara et al., 1998). Sucrose was omitted to reduce fungal contamination of the plates. Primary transformants were screened for lesions in lipid metabolism by a high-throughput robotic screening strategy. Briefly, individual transformed colonies were picked with sterile toothpicks, inoculated into 96-well plates containing 1.5 mL of TAP-NO3 medium, and grown to plateau stage. The cells in each 96-well plate were replicated onto two agar plates with a 48-prong replica plating tool, followed by centrifugation of the remainder to collect the cells. Cell pellets were extracted with 300 µL of chloroform/methanol/88% formic acid (10/10/1, v/v) and the phases were separated by addition of 150 mL of chloroform/methanol/88% formic acid (10/10/1, v/v) and 1 mL KCl and were separated into two phases by centrifugation. Anionic lipids were purified based on a procedure modified from O’Brien and Benson (1964). The lower organic phase was applied to a column (2 cm × 12 cm) of Florisil (J.T. Baker, Phillipsburg, NJ) equilibrated in CHCl3. Pigments and some MGDG were washed from the column with three column volumes of CHCl3, and the bound polar lipids were then eluted with two column volumes of CHCl3/CH3OH (2:1, v/v). Eluate from the Florisil column was applied directly to a column (3 cm × 9 cm) of diethylaminoethyl-cellulose (acetate form) pre-equilibrated in CHCl3/CH3OH (2:1, v/v). Uncharged polar lipids and residual pigments were washed from the column with two column volumes of the same solvent, and anionic lipids were eluted with chloroform/methanol/concentrated ammonia (16:8:1, v/v), resulting in a preparation enriched in anionic lipids, with traces of contaminating MGDG and DGDG. This eluent was phase partitioned by the addition of water, followed by centrifugation, and the organic phase was concentrated under a stream of N2 and was further purified by preparative TLC (Benning et al., 1995). Compounds of interest were identified by light staining with I2 vapor and were scraped from the plate into glass tubes, followed by elution of the silica twice with chloroform/methanol (1:3, v/v).

MS

FAB-mass spectra were acquired in the negative ion mode on a mass spectrometer (HX-110; JEOL, Peabody, MA) essentially as described (Gage and Lefebvre, 1993). Approximately 1 µg of sample dissolved in chloroform/methanol (1:1, v/v) was mixed with triethylamine as matrix on the FAB probe tip. Ions were produced by bombardment with a beam of Xenon atoms (6 keV) and were accelerated by a voltage of 10 kV. For CAD-tandem MS/MS, helium was used as the collision gas in a cell located in the first field-free zone, and a data system (DA-5000; JEOL) was used to generate linked scans at a constant B/E ratio.

Fatty Acid Analysis

Samples were prepared by filtering the TLC-purified material through glass wool, evaporating it to dryness under N2, and further drying it over desiccant under vacuum for at least 3 h. Samples were then dissolved in 0.6 mL of chloroform/methanol-d6/acidic acid-d4 (10/1/1, v/v) and dispensed into NMR tubes. Standard 1H-1H NMR spectra were acquired on a 500 MHz instrument (VXR-500; Varian, Palo Alto, CA) at 25°C, and 1H-1H homonuclear decoupled spectra were recorded at 600 MHz on an INOVA...
instrument (Varian). Acquisitions were made by using the signal of internal methanol as lock signal, and chemical shift values were referenced to the internal methanol resonance at 3.30 ppm.

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LITERATURE CITED


