MEMBRANE LIPID BIOSYNTHESIS IN *CHLAMYDOMONAS REINHARDTII*

By

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ABSTRACT

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Lipids play essential roles in all cellular organisms, most prominently as components of cellular membranes, which delineate the boundary between the cell and its environment, and between subcellular compartments. The synthesis of membrane lipids in the unicellular flagellated alga *Chlamydomonas reinhardtii* was studied using biochemical techniques, forward and reverse genetics, and genomics.

The major component of extraplastidic membranes in *Chlamydomonas* is the betaine lipid diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS). As a model system for the study of betaine lipid biosynthesis, the DGTS biosynthesis proteins BtaA and BtaB from *Rhodobacter sphaeroides* were studied at the biochemical level, and were shown to be responsible for all reactions of DGTS biosynthesis. Sequencing of the *Chlamydomonas* genome revealed a protein containing BtaA- and BtaB- like domains on the same polypeptide, leading to the hypothesis that this fusion protein, *Cr*BTA1, catalyzes all reactions of DGTS biosynthesis in this alga. This hypothesis was confirmed by characterization of the recombinant enzyme, and site directed mutagenesis was used to identify active site residues in the two domains. *In vivo* functional analysis of the corresponding gene was carried out by using RNA interference to block gene expression, which led to pleiotropic effects on growth rate and temperature sensitivity.

A forward genetic screen was carried out in *Chlamydomonas* by using plasmid tagging insertional mutagenesis. This led to the discovery of a mutant defective in synthesis of one of the thylakoid lipids, sulfoquinovosyldiacylglycerol. Taken together, these results answer some previously unresolved questions regarding membrane lipid biosynthesis in *Chlamydomonas*, and establish this organism as a useful complement to the more thoroughly characterized models of seed-plant lipid metabolism, such as Arabidopsis.

ACKNOWLEDGMENTS

My time at Michigan State has been the most exciting, stimulating, and interesting four years of my life, and that fact is due to a great number of wonderful people with whom I have interacted. First and foremost among them is my thesis advisor, Dr. Christoph Benning. I owe a great deal to him for giving me a project which was a little bit outside of the mainstream, allowing me a lot of freedom to roam with it, and encouraging me to take my ideas and make something of them. I'm also indebted to him for not charging me for all the broken glassware, dropped jugs of toluene, and similar blunders I make on a regular basis.

Without exception, all of my co-workers have been wonderful, and coming to work everyday was always exciting. The Benning lab has been my home away from home, and my lab mates have been like a second family, so leaving is bittersweet.

The work on *Chlamydomonas* mutant screening was carried out by a motley crew consisting of Todd Lydic, Mike Ruckle, and (sometimes) the author, with guidance from Dr. C. Benning and Dr. Barbara Sears, MSU Department of Plant Biology. I will always fondly recall the many hours spent standing idly by while we watched the Tecan robot tirelessly spotting TLC plates. We all also owe a debt of gratitude to the custodial staff for not emptying the trash on one particular Friday, thus allowing us to recover the replica plate containing the mutant described in Chapter 5 on the following Monday.

I have had the good fortune to be involved with Drs. Changcheng Xu and Koichiro Awai in their studies on lipid trafficking mutants of Arabidopsis (Appendix B), and I look forward to reading about the continuation of this exciting work after I leave the

lab. Another project that was especially fun and interesting was the annotation of EST sequences of the red alga *Galdieria sulphuraria* (Appendix A), conducted in collaboration with Dr. Andreas Weber and his laboratory. Still another side project with another experimental organism was conducted in collaboration with Dr. Helmut Bertrand, MSU Dept. of Microbiology and Molecular Genetics, who I worked with in showing that *Neurospora crassa* synthesizes betaine lipid under phosphate stress.

Chapters 3, 4 and 5 make use of data generated by the *Chlamydomonas* Genome Project, and the work described in those chapters would have been made much more time consuming and tedious (if not impossible) if it were not for this data being publicly available. I was lucky enough to be involved on Christoph's behalf in the annotation phase of this project (described in Chapter 4), part of which was done at the "JGI Chlamy Jamboree" held in December 2003 at the DOE- Joint Genome Institute in Walnut Creek, CA.

My guidance committee, Drs. Gregg Howe, Bob Hausinger, Doug Gage, and John Ohlrogge, have been very helpful and supportive over the years, and have given me good feedback and advice from which I have benefited greatly. I also was given a good start at MSU in the Plant Research Lab through rotations with Drs. Gregg Howe and Lee McIntosh.

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Last and most importantly, I want to thank my wife Stephanie, who makes me a much better person than I would otherwise be, and without whom (according to my mother, at least) I would not have made it in Michigan. I also want to thank my parents, who recognized and encouraged my love of science from an early age, and have encouraged me at all stages of my academic career.

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Chapter 1

Lipid biosynthesis in plants and algae

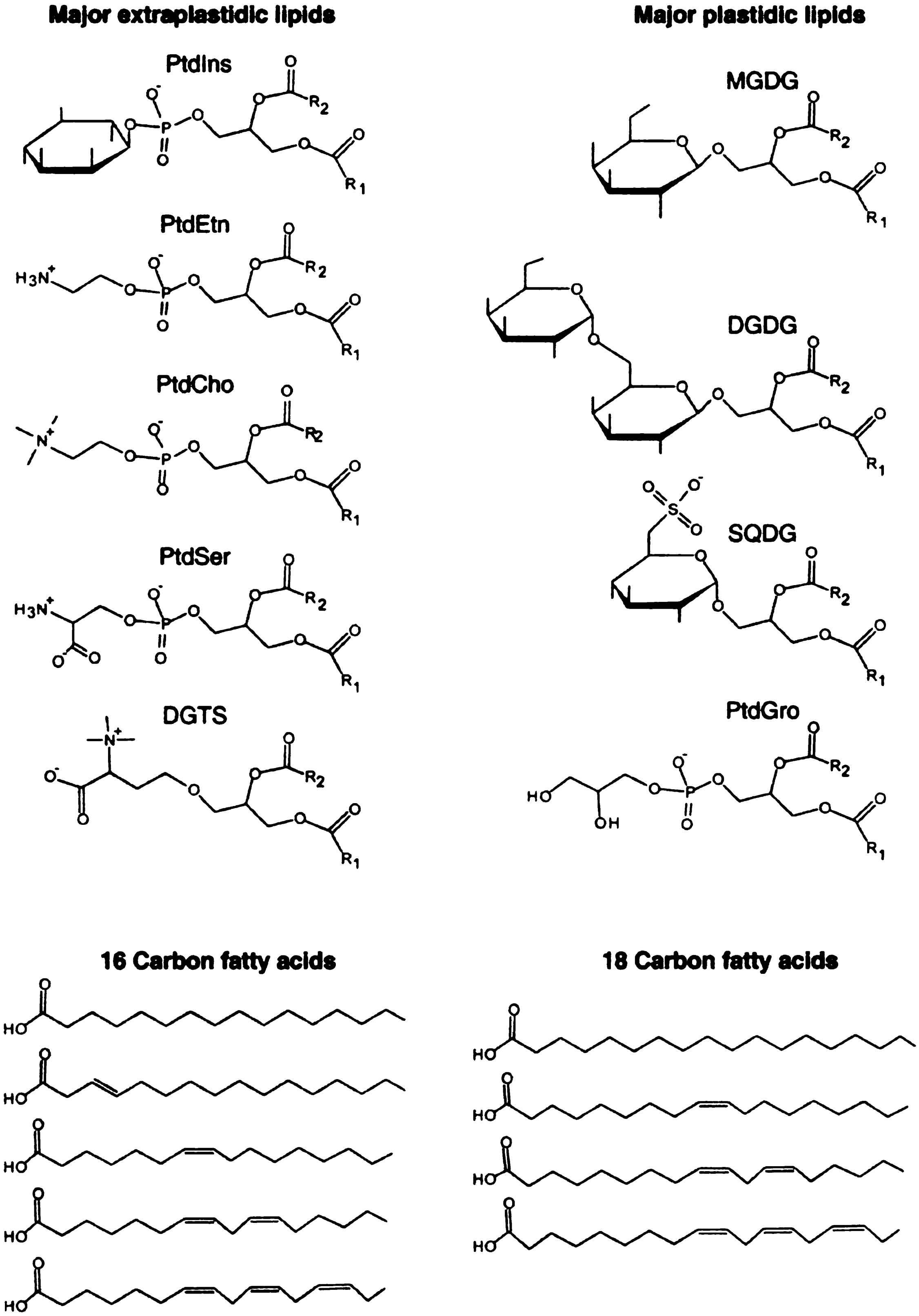
Introduction.

The molecular biology and biochemistry of membrane lipid biosynthesis in plants has been extensively studied over the past several decades, leading to detailed hypotheses regarding fatty acid synthesis, glycerolipid assembly, fatty acid desaturation, and trafficking of lipids between membranes. (Somerville et al., 2000). This chapter will summarize the salient points of these critical cellular processes with a focus on recent advances in the isolation of genes and proteins responsible for the biochemical activities, and will also attempt to show which topics are in need of clarification and further study. Additionally, the lipid metabolism of higher plants (typified by *Arabidopsis thaliana*) and green algae (e.g. *Chlamydomonas reinhardtii*) will be compared and contrasted, and I will introduce the questions that have been addressed in the following chapters.

Composition of plant membranes.

The polar lipid and fatty acid composition of plant cells is complex, and is influenced substantially by the presence of the plastid. This organelle was derived from an endosymbiotic relationship between a cyanobacterium and the eukaryotic progenitor of plant cells. As a consequence, most plants cells contain polar lipid species that are characteristic of eukaryotes, such as phosphatidylcholine and phosphatidylethanolamine, and also lipids characteristic of cyanobacteria, such as mono- and digalactosyldiacylglycerol (MGDG and DGDG), and the sulfolipid sulfoquinovosyldiacylglycerol (SQDG). The chemical structures of these polar lipids, as well as other species commonly found in plants and algae, are presented in Figure 1.1.

Figure 1.1: **Structures of polar lipids and fatty acids in plants and algae.** MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol, PtdGro, phosphatidylglycerol; PtdCho, phosphatidylcholine; DGTS, diacylglyceryl-*N*,*N*,*N*,-trimethylhomoserine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol. Fatty acids, from top down, (carbons:double bonds$^{\Delta\text{positions}}$) 16:0 (palmitate), 16:1$^{\Delta 3\textit{trans}}$, 16:1$^{\Delta 7}$ (palmitoleate), 16: 2$^{\Delta 7,10}$, 16:3$^{\Delta 7,10,13}$, 18:0 (stearate), 18:1$^{\Delta 9}$ (oleate), 18:2$^{\Delta 9,12}$ (linoleate), 18:3$^{\Delta 9,12,15}$ (linolenate).


Major extraplastidic lipids
PtdIns
PtdEtn
PtdCho
PtdSer
DGTS
Major plastidic lipids
MGDG
DGDG
SQDG
PtdGro
R1
R2
H3N+
HO
OH
16 Carbon fatty acids
18 Carbon fatty acids

Among those molecules not present in seed-plants are the betaine lipids, such as diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS), and diacylglyceryl-*N*,*N*,*N*-trimethylhydroxymethy-β-alanine (DGTA). These lipids have been hypothesized to take on the functions of PtdCho in species that synthesize them (Sato, 1992), and the biosynthetic pathways involved in their synthesis will be discussed in detail.

The fatty acid complement of plant cells is also complex (Figure 1.1). The bulk of fatty acids present in plant cell membranes consist of saturated, monounsaturated, and polyunsaturated 16 and 18 carbon species, the unsaturated forms being derived from the saturated chains by the action of soluble and membrane-bound fatty acid desaturases. While the bulk of membrane fatty acids are made up of relatively simple linear chains, other species, e.g. cyclopropene, hydroxyl, and epoxide containing derivatives are found to occur predominantly in the seed oil of some species.

Fatty acid biosynthesis in the plastid.

Figure 1.2 gives a schematic representation of fatty acid biosynthesis and membrane lipid assembly in the plastid. The plastid is known to be the major site of fatty acid synthesis in plant cells (Ohlrogge and Browse, 1995) and most of the genes encoding enzymes involved in plastidic fatty acid synthesis have been isolated and studied. Fatty acid synthesis in the plastid occurs via a system very similar to that of bacteria. As the committed step in the pathway, acetyl-CoA is carboxylated to form malonyl-CoA by the action of acetyl-CoA carboxylase. The predominant plastidic isoform of ACCase is a multimeric bacterial-type enzyme consisting of four proteins, including biotin carboxylase, biotin carboxyl carrier protein, α-carboxyltransferase, and β-

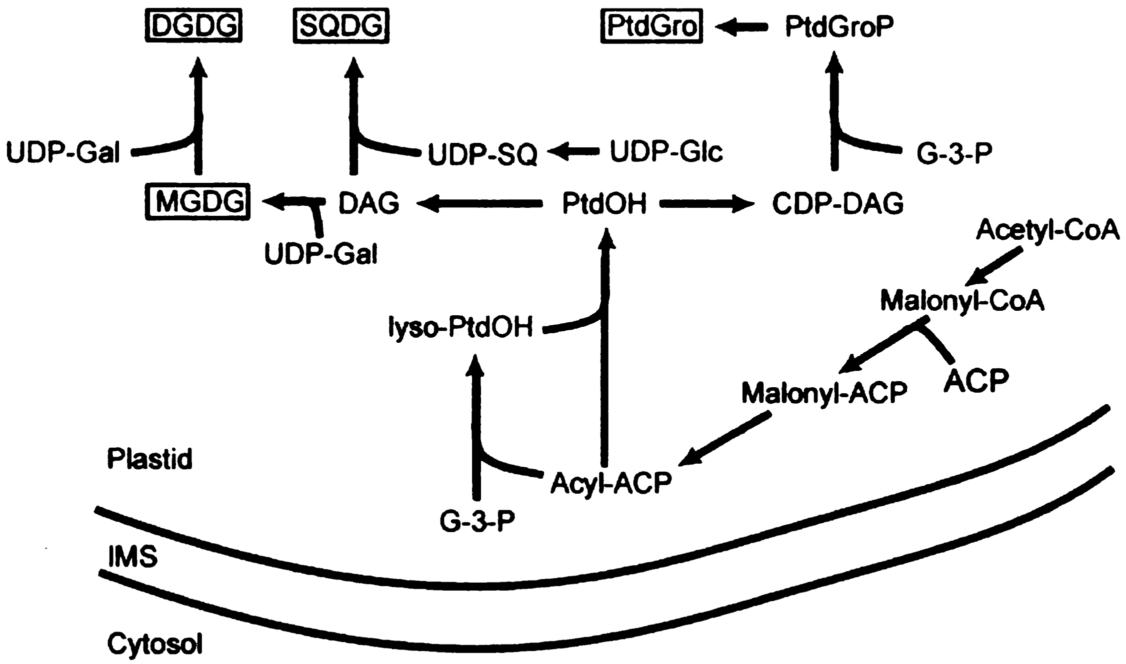


Figure 1.2: **Schematic for plastid membrane lipid synthesis.** Fatty acids are produced in the plastid and incorporated into the 4 plastid lipids as outlined in this scheme. PtdGroP, phosphatidylglycerolphosphate; UDP-Gal, uridine-5'-diphosphate-galactose; DAG, diacylglycerol; UDP-Glc, uridine-5'-diphosphate-glucose; UDP-SQ, uridine-5'-diphosphate-sulfoquinovose; CDP-DAG, cytidine-5'-diphosphate-diglyceride; G-3-P, glycerol-3-phosphate; PtdOH, phosphatidic acid; ACP, acyl-carrier protein; IMS, intermembrane space.

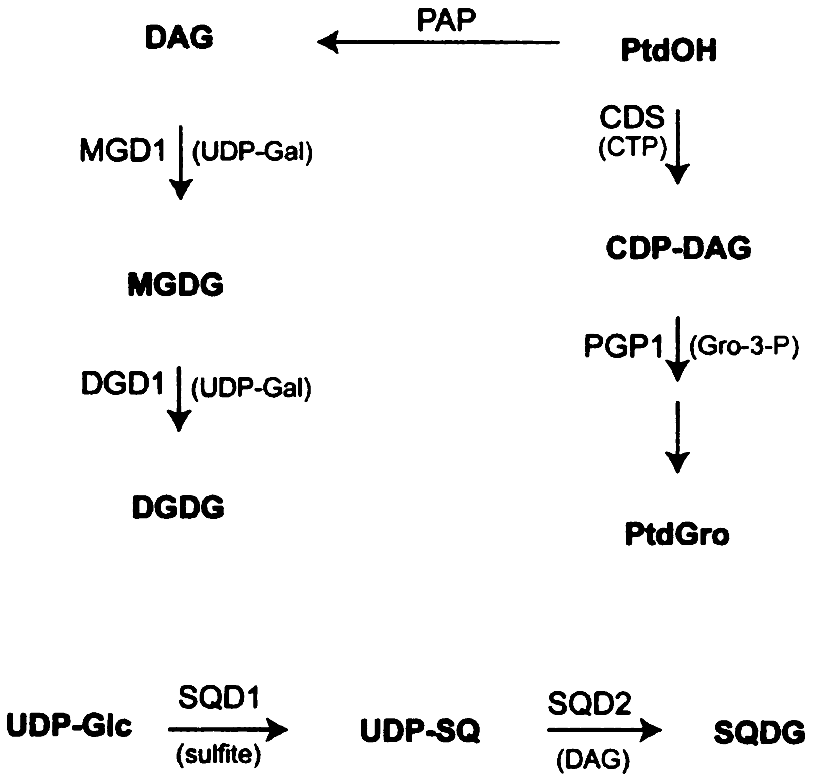


Figure 1.3: **Synthesis of the 4 major plastid lipid classes.** Major biosynthetic pathways for the four major plastid lipids, as described in the text. MGD1, MGDG synthase; DGD1, DGDG synthase; PGP1, phosphatidylglycerophosphate synthase; SQD1, UDP-SQ synthase, SQD2, sulfolipid synthase.

carboxyltransferase subunits. ACCase is regarded as a control point in initiation of fatty acid biosynthesis, and is under multiple regulatory controls (Somerville et al., 2000).

Malonate can be transferred from CoA to a small phosphopantetheine- containing polypeptide called acyl carrier protein (ACP). This reaction is catalyzed by malonyl-CoA: ACP transacylase, and the protein from *Chlamydomonas* has recently been identified in a proteomic screen for thioredoxin interacting proteins (Lemaire et al., 2004). This may indicate that its enzymatic activity is under the control of the plastidic thioredoxin system, which would probably inhibit its activity through oxidation of a disulfide bridge in the dark, when energy charge is low. Conversely, it would be activated in the light during optimal growth conditions by reduction of this putative disulfide bridge, and this cycle would represent another layer of regulation in fatty acid biosynthesis.

After synthesis of malonyl-ACP, fatty acid synthesis in plastids continues by the action of a type II (bacterial) multimeric fatty acid synthase, located in the chloroplast. Using acyl-ACP and malonyl-ACP as substrates, the first component of this complex is 3-ketoacyl-ACP synthase, which catalyses a Claisen condensation of the malonyl and acyl substituents, releasing carbon dioxide as a byproduct, and forming a carbon-carbon bond to increase the chain length by two carbons. The next steps involve reduction of the 3-keto group, elimination of water from the resulting 3-hydroxy acyl species, and finally reduction of the enoyl-ACP species by the activities of 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydratase, and enoyl-ACP reductase (Somerville et al., 2000). This sequence of reactions is repeated, resulting in the elongation of the growing fatty acid chain by two carbons each cycle until the chain reaches 16 or 18 carbons. At this point, the 18 carbon species, stearoyl-ACP, can be acted upon by a soluble fatty acid desaturase, the strearoyl-ACP Δ9 desaturase (Shanklin and Somerville, 1991) giving oleoyl-ACP as a product.

The long chain fatty acyl end products (predominantly palmitoyl-ACP and oleoyl ACP) are then partitioned into one of two major pathways of polar lipid biosynthesis. The acyl groups either become part of the plastidic membrane lipids by the action of the plastidic glycerol-3-phosphate: acyl-ACP acyltransferase (GPAT, (Murata and Tasaka, 1997)) and lyso-phosphatidate acyltransferase (LPAAT, (Kim and Huang, 2004; Yu et al., 2004)), or are directed to the cytosol by the action of fatty acyl-ACP thioesterases. In the former pathway, fatty acids are retained in the plastid and used for plastidic membrane lipid synthesis. In the latter pathway, free fatty acids are released by the action of a thioesterase, and these are channeled across the inner and outer envelopes of the chloroplast and incorporated into acyl-CoA by the action of a long chain acyl-CoA synthetase (Koo et al., 2004).

Incorporation of FA into the plastidic pathway of membrane lipid synthesis.

As described above, the acyl-ACPs produced from plastidic fatty acid synthase may become substrates for the plastidic GPAT and LPAAT enzymes. GPAT has been isolated from a number of species (Murata and Tasaka, 1997) and shown to prefer oleoyl-ACP as a substrate. Mutations in the Arabidopsis plastidic GPAT (encoded by the *ACT1*(*ATS1*) gene) give rise to plants that are unable to synthesise diacylglycerol moieties in the plastid, thus forcing an increase in flux through the so called "eukaryotic" pathway of thylakoid lipid synthesis (Kunst et al., 1988), which will be discussed below.

The second step in this sequence is the LPAAT (ACT2), and this enzyme shows higher affinity for palmitoyl-ACP (Bourgis et al., 1999; Kim and Huang, 2004). Interestingly, GPAT (*act1*) mutants are viable and grow essentially like wild type, presumably due to the increase in flux through the eukaryotic pathway, whereas null alleles of the LPAAT (*ats2*) gene cause an embryo lethal phenotype (Kim and Huang, 2004; Yu et al., 2004). The combined substrate specificities of the two plastidic acyltransferases result in a diglyceride *sn*1/*sn*2 molecular species preference of 18:1/16:0, and this is the predominant phosphatidic acid (PtdOH) species initially produced by the plastidic or "prokaryotic" pathway. PtdOH produced by this sequence can then be converted to CDP-diglyceride and used directly for phosphatidylglycerol (PtdGro) biosynthesis, or can be dephosphorylated by the action of phosphatidic acid phosphatase, forming DAG as a substrate for galactolipid and sulfolipid biosynthesis.

Galactolipid synthesis.

The most abundant membrane lipid in the biosphere is thought to be MGDG (Figures 1.1 and 1.3) (Dörmann and Benning, 2002), which makes up about 50% of the lipid component of the thylakoid membrane in most oxygenic photosynthetic organisms. The biochemical activity responsible for synthesis of MGDG was characterized as a component of the chloroplast inner envelope membrane, and a thorough biochemical characterization was carried out on partially purified fractions from spinach (Marechal et al., 1994). Cloning of the gene encoding MGDG synthase was accomplished by purifying the enzyme from expanding cucumber leaves, partial sequencing of the protein, and using this information to amplify a fragment of the gene by degenerate PCR (Shimojima et al., 1997). Sequence analysis revealed that MGDG synthase is similar to the bacterial UDP-*N*-acetylglucosaminyl transferase MurG, and that, by analogy with the MurG mechanism, MGD1 condenses UDP-galactose (produced by the action of UDP-glucose epimerase, (Dörmann and Benning, 1996)) and diacylglycerol with inversion of anomeric configuration (e.g. producing a β-galactoside linkage). Subsequent studies in Arabidopsis identified two additional MGDG synthase isoforms that are regulated strongly by phosphorus deprivation (Awai et al., 2001), and these will be discussed later in the section on phosphate regulation of lipid biosynthesis.

The next most abundant lipid in the plastid is derived from MGDG by addition of a second galactose moiety. DGDG (Figures 1.1 and 1.3) is a bilayer-forming lipid. That is, when suspended in water, it tends to form a lamellar phase due to the packing together of roughly cylindrical adjacent monomers. In contrast, MGDG is a non-bilayer forming lipid, and its roughly conical shape allows pure preparations to attain inverted micellar phases in aqueous solution (Sprague and Staehelin, 1983). The bilayer:nonbilayer ratio of biological membranes is typically under tight control (Vikstrom et al., 1999), therefore it follows that the enzyme which forms DGDG from MGDG is a major control point in determining this ratio in the thylakoid membranes, therefore determining a basic biophysical property of the system.

The older literature on DGDG synthesis is mainly focused on the formation of DGDG by a galactolipid:galactolipid galactosyltransferase (GGGT) mechanism, by which the galactose moiety of one MGDG is transferred to the 6' hydroxyl of a second MGDG molecule, with inversion of anomeric configuration (e.g. (Heemskerk et al., 1988; van Besow and Wintermans, 1978)). This "classical" mechanism is at odds with recent findings based on the cloning and characterization of DGDG synthase isoforms from Arabidopsis.

A brute force biochemical screen involving TLC of lipids extracted from mutagenized plants was used to isolate a line, designated *dgd1*, with a 90% reduction in the amount of DGDG present in membranes (Dörmann et al., 1995). Positional cloning was used to isolate the *DGD1* gene, and the activity of its product was confirmed in *E. coli* by co-expression of the *MGD1* gene from cucumber, which led to synthesis of DGDG in *E. coli.* (Dörmann et al., 1999). Although originally thought to *be* the GGGT, DGD1 is characterized by a glycosyltransferase motif in its C-terminal domain, implying that it uses UDP-galactose as a substrate in a retaining glycosyltransferase reaction. The role of the N-terminal domain of DGD1 is unknown, but may have functions in lipid trafficking (Dörmann et al., 1999).

Since the mutation in *dgd1* is a null allele, the presence of DGDG at 10% of the wild-type level was interpreted to mean that there is a second, DGD1 independent pathway of DGDG biosynthesis. Further studies identified this pathway as inducible by phosphorus starvation (Härtel et al., 2000), and the enzyme responsible has recently been identified as DGD2 (Kelly and Dormann, 2002). This aspect of galactolipid synthesis will be discussed further below.

Sulfolipid synthesis and function.

The plant sulfolipid, SQDG (Figure 1.1), was described by Benson and co-workers nearly 50 years ago (Benson et al., 1959). As a component of the thylakoid membrane, it is one of the most abundant sulfonate compounds in the biosphere, and much effort has been dedicated to elucidating the molecular biology and biochemistry of its biosynthetic pathway (Benning, 1998). Beginning with studies on *Rhodobacter sphaeroides* and cyanobacteria, (Benning and Somerville, 1992a; Benning and Somerville, 1992b; Güler et al., 1996; Güler et al., 2000), and later Arabidopsis (Essigmann et al., 1998; Sanda et al., 2001; Yu et al., 2002) and spinach (Shimojima and Benning, 2003), it became apparent that a sugar-nucleotide pathway is operative in which UDP-glucose and sulfite act to form UDP sulfoquinovose (Pugh et al., 1995; Sanda et al., 2001), followed by transfer of the sulfoquinovose moiety to diacylglycerol (Güler et al., 2000; Yu et al., 2002). This pathway is diagrammed in Figure 1.3.

The function of SQDG in photosynthetic reactions is still a contentious topic. It is clear that sulfolipid deficient mutants of *Rhodobacter*, *Synechococcus*, and Arabidopsis are photosynthetically competent, and present very little difference in photosynthetic parameters relative to the respective wild-types, (Benning et al., 1993; Güler et al., 1996; Yu et al., 2002). However, a mutant of *Synechocystis* PCC 6803 in which the *sqdB* gene is insertionally inactivated behaves as a sulfolipid auxotroph, indicating that SQDG is an essential factor for growth of this species (Aoki et al., 2004). This leads to the conclusion that there is no universal requirement for SQDG in photosynthesis, but that it is conditionally important in a species-specific manner. This is also true of photosynthetic eukaryotes, such as *Chlamydomonas*, as discussed below.

The *hf2* mutant of *Chlamydomonas* was isolated in a screen for strains showing high chlorophyll fluorescence, and was later shown to be deficient in the biosynthesis of SQDG (Sato et al., 1995b). Further characterization of this mutant has shown that it has defects in the heat stability of PSII (Sato et al., 1995a; Sato et al., 2003a), and that it is more sensitive to the diuron herbicide DCMU, a competitive inhibitor of the Q_B site of PSII. Taken together, these data led to the conclusion that SQDG is involved in maintaining the stability of PSII.

In addition to PSII, a recent X-ray structural study of the cytochrome b_6f complex of *Chlamydomonas* reveals sulfolipid in a tight association with the cytochrome *f* subunit (Stroebel et al., 2003). In this structure, the sulfonate group forms a salt bridge with a lysine residue that was shown to be critical for the co-translational insertion of this polypeptide into the thylakoid membrane (Choquet et al., 2003). Therefore, in *Chlamydomonas*, sulfolipid has been implicated in the function of two of the core components of the photosynthetic electron transport chain.

Another function of SQDG is in maintenance of the anionic character of the thylakoid during phosphate deprivation (Benning, 1998). SQDG and the other anionic lipid of the thylakoid, phosphatidylglycerol, are regulated in a reciprocal relationship such that the net negative charge on the membrane is kept constant (Yu and Benning, 2003). This compensatory mechanism will be explored below.

Chapter 5 gives the results of a forward genetic screen for mutants of *Chlamydomonas* with defects in lipid metabolism. Although a major focus of the work presented here is on the elucidation of DGTS biosynthesis in *Chlamydomonas* (see below), the most tractable line in which there were obvious differences in polar lipid content was an SQDG deficient mutant. This line also lacked a previously unknown 2'-*O*-acylated derivative of SQDG. Chapter 5 presents the structural elucidation of this novel species, the characterization of the mutation in the SQDG deficient line as a deletion of *SQD1*, and the functional consequences of SQDG deficiency on the growth of *Chlamydomonas*.

Phosphatidylglycerol synthesis and function.

As mentioned above, PtdGro is one of the two anionic phospholipids in the plastid, and along with SQDG, it acts to maintain the negative charge density and proper function of this membrane. Two PGP synthases (Figure 1.3) have been cloned from Arabidopsis and biochemically characterized (Muller and Frentzen, 2001), and recent years have seen several studies that show a direct link between PtdGro deficiency and abnormal plastid development and photosystem function (Xu et al., 2002; Gombos and Murata, 1998; Hagio et al., 2002; Babiychuk et al., 2003). These studies have been based on a mutant of Arabidoposis, *pgp1-1*, which has an 80% reduction in the PGP synthase activity (Figure 1.3), and on T-DNA insertional mutants, which represent null alleles. Studies on the *pgp1-1* mutant showed that the pigment composition was altered, and that the plants were generally smaller and less photosynthetically compentent than wild type (Xu et al., 2002). Studies on T-DNA alleles of the *PGP1* gene have shown that its function is critical to photosynthetic growth and plastid development, as the null mutant of this gene requires sucrose for growth, and has severe defects in the organization of the photosynthetic membranes (Babiychuk et al., 2003; Hagio et al., 2002). Additionally, PGP1 has been shown to be targeted to both plastids and mitochondria (Babiychuk et al., 2003). While PGP1 is essential for plastid function, the mitochondrial role appears to be redundant, as the mitochondria in the *jovtenky* (*pgp1*) mutant are capable of forming PtdGro and cardiolipin, and there are no great dysfunctions in the mitochondrial electron transport chain.

PtdGro is unique among chloroplast lipids in that it contains a $16{:}1^{\Delta3trans}$ acyl species (Figure 1.1). This particular PtdGro molecular species has been implicated in maintaining the stability of the PSII complex of *Chlamydomonas*, and the photosynthetic defects of the mutant lacking PtdGro:$16{:}1^{\Delta3trans}$ can be partially rescued by culturing this strain in the presence of liposomes containing this lipid (Dubertret et al., 1994). Analysis of these mutants, *mf1* and *mf2*, also implies a role for PtdGro in the co-translational insertion of PSII core polypeptides into the photosynthetic membrane (Pineau et al., 2004), however the pleiotropic nature of these mutations calls into question whether a PtdGro biosynthesis gene or fatty acid desaturase is involved, or whether the lipid phenotypes are an indirect result of some other lesion in a gene required for optimal photosynthesis.

As alluded to above, PtdGro and SQDG are linked by their anionic character, and they exhibit an inverse relationship in terms of quantity. For example, in the *sqd2* mutant of Arabidopsis, which lacks SQDG, PtdGro content is maintained at a level so as to replace the lost sulfolipid (Yu et al., 2002). Likewise, under phosphate deprived conditions, PtdGro levels drop, and SQDG biosynthesis is up-regulated to compensate (Essigmann et al., 1998; Yu et al., 2002). It follows that a double mutant with lesions affecting both pathways would lack this compensatory ability, and would be compromised in growth. This was shown to be the case in a study (Yu and Benning, 2003) in which a double mutant of *sqd2* and *pgp1* was constructed by repeated back-crossing (to normalize differences between the Ws and Col-0 ecotypes) to create nearisogenic lines segregating the wild-type, single, and double mutants. The double mutant plants were severely compromised in autotrophic growth, and were hypersensitive to the PSII inhibitor DCMU. Additionally, the single mutants of *pgp1* and *sqd2* showed increased sensitivity to DCMU, implying that, while SQDG is not required for photosynthetic growth and shows no growth phenotype under laboratory conditions, there may be subtle changes in the organization of PSII that render it more sensitive to this herbicide.

Fatty acid export from the plastid.

The section on fatty acid biosynthesis above gave a brief overview of the pathway, but another critical step in plant lipid metabolism is the export of fatty acids and their incorporation into extraplastidic membrane lipids such as PtdCho, PtdEtn, and in certain non-seed plants, the betaine lipids, such as DGTS. Hypotheses regarding the steps in acyl-chain export from the plastid have been tested using in vivo ^{18}O labeling (Pollard and Ohlrogge, 1999). This study showed that fatty acids produced in the chloroplast are incorporated into plastidic lipids by direct acyl-transfer from acyl-ACP, but that those in the extraplastidic membranes show a 50% reduction in ^{18}O content. This result is interpreted as being consistent with the model that the free fatty acids are released via hydrolysis in the plastid, and transported out to the site of "eukaryotic" lipid synthesis. At least two thioesterases in Arabidopsis, FatA and FatB, mediate the hydrolysis of predominantly 18:1 and 16:0 ACPs, respectively (Salas and Ohlrogge, 2002). To further elucidate the mechanism of fatty acid export from the plastid, (Koo et al., 2004) used a

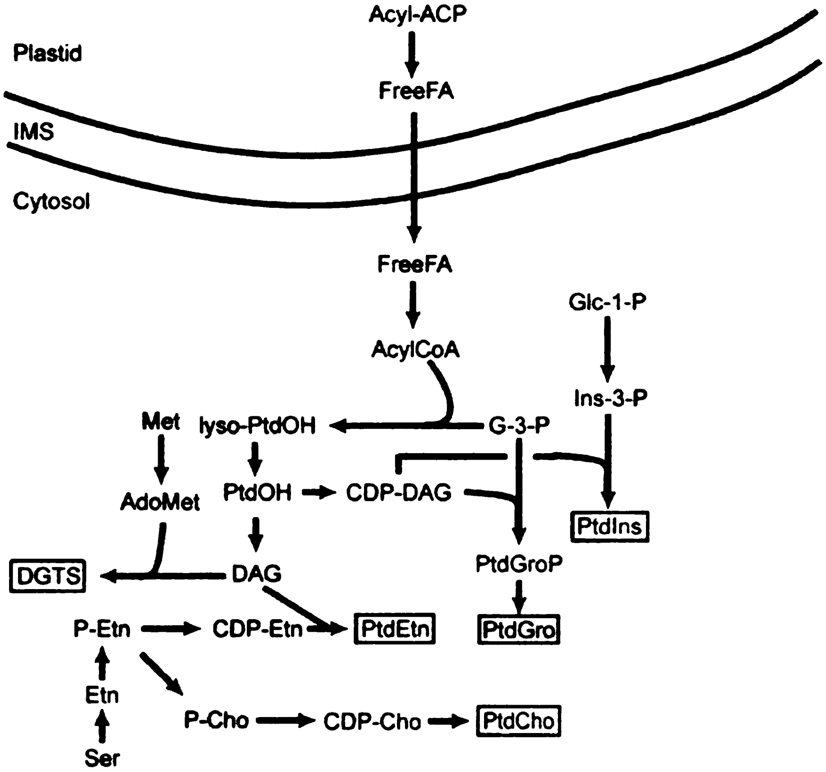


Figure 1.4: **Scheme for fatty acid export and incorporation into extraplastidic lipids.** IMS, intermembrane space; ACP, Acyl carrier protein; FA, fatty acid; PtdOH, phosphatidic acid; CDP-DAG, cytidine 5'-diphosphate-diacylglycerol; Met, methionine; AdoMet, S-adenosylmethionine; DGTS, diacylglyceryl-N,N,N-trimethylhomoserine; DAG, diacylglycerol; G-3-P, glycerol-3-phosphate; PtdGro, phosphatidylglycerol; PtdGroP, phosphatidylglycerophosphate; Glc-1-P, glucose-1-phosphate; Ins-3-P, *myo*-inositol-3-phosphate; PtdIns, phosphatidylinositol; Ser, serine; Etn, ethanolamine; P-Etn, phosphoethanolamine; CDP-Etn, cytidine 5'-diphosphate-ethanolamine; CDP-Cho, cytidine 5'-diphosphate-choline; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.

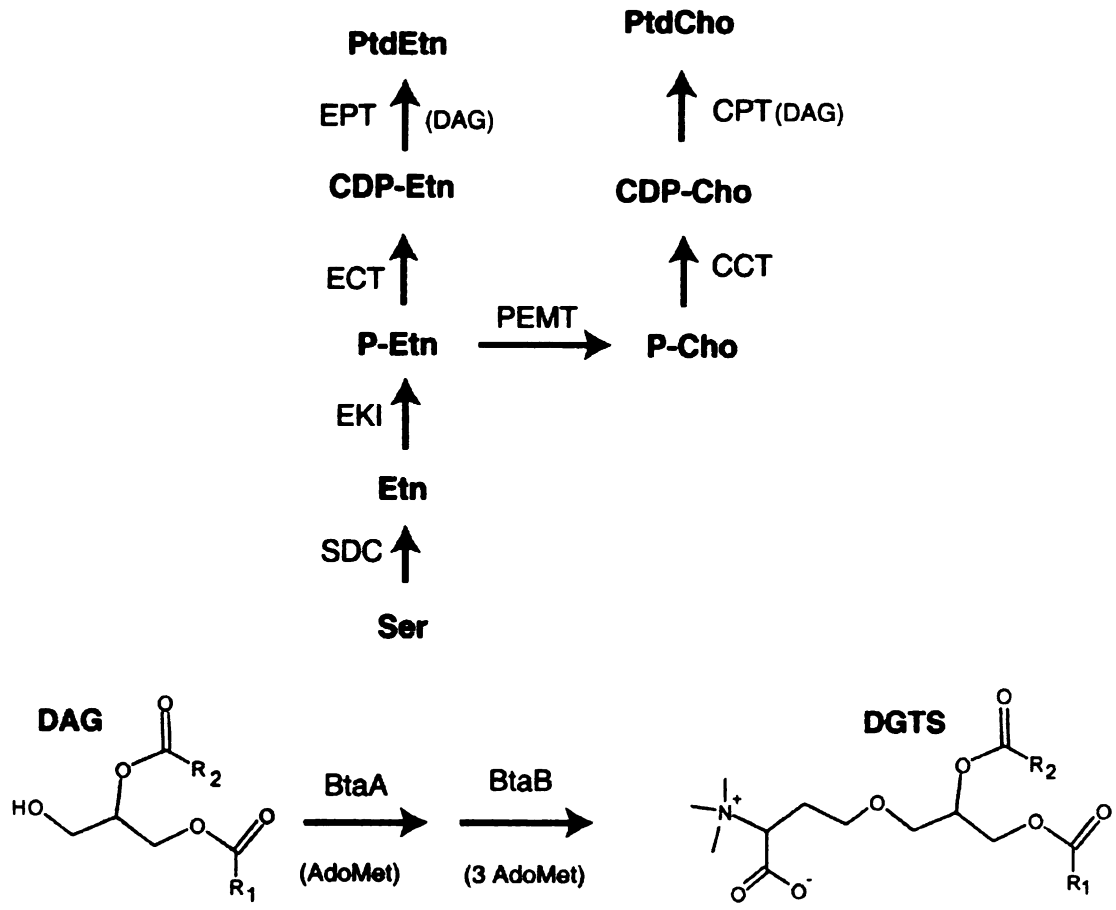


Figure 1.5: **Biosynthesis of PtdCho, PtdEtn and DGTS in the extraplastidic membranes.** Chemical species are identified in bold, and correspond to the abbreviations in Figure 1.4. SDC, serine decarboxylase; EKI, ethanolamine kinase; ECT, ethanolamine phosphate cytidylyltransferase; EPT, CDP-Etn:DAG ethanolaminephophotransferase; PEMT, phosphoethanolamine methyltransferase; CCT, phosphocholine cytidylyltransferase; CPT, CDP-Cho:DAG cholinephosphotransferase; BtaA, AdoMet:DAG 3-amino-3-carboxypropyltransferase (diacylglycerylhomoserine synthase); BtaB, diacylglycerylhomoserine *N*-methyltransferase.

labeling approach to determine whether fatty acids simply diffuse out of the plastid, or whether there is a specific transport mechanism. The results indicate that the free pool of fatty acid destined for export has a very short half life, on the order of ◁ second, and that there is a specific channeling of this pool to an acyl-CoA synthetase (ACS). This final step in export, activation of the free fatty acid to an acyl-CoA, has been studied by analysis of the Arabidopsis ACS gene family (Schnurr et al., 2002). This study showed that the LACS9 isoform is likely to be the major activity in developing seeds and expanding leaves, where the fatty acid synthesis rate is high. The protein is localized to the chloroplast envelopes, consistent with its proposed function, however a T-DNA insertional mutant showed essentially no effect on the composition of leaf lipids. Given that there are multiple members of this ACS family, there is probably considerable functional redundancy. Central metabolic pathways such as fatty acid synthesis and export from the plastid are likely to be conserved among Arabidopsis and *Chlamydomonas*, as evidenced by, for example, the similarity between Arabidopsis and *Chlamydomonas* ω-6 fatty acid desaturases (Sato et al., 1997).

Phosphatidylcholine and Phosphatidylethanolamine synthesis.

The phospholipids PtdCho and PtdEtn are the most abundant lipids in yeast and mammalian cells, and much is understood about their biosynthesis and function. In *Saccharomyces cerevisiae*, there are two pathways of biosynthesis for PtdCho, referred to as the methylation pathway, and the CDP-choline or Kennedy pathway. In the methylation pathway, phosphatidylserine is produced in the ER by the action of PtdSer synthase (Kohlwein et al., 1988) and then transported to the sites of the two PtdSer decarboxylases (PSD) for conversion into PtdEtn. The two PSD activities are encoded by the *PSD1* gene, encoding the mitochondrial isoform, and the *PSD2* gene, which specifies the golgi/vacuolar activity (Trotter et al., 1993; Trotter et al., 1995). The PtdEtn resulting from PSD action is then transferred back to the ER for PtdCho production by *N*-methylation via the PtdEtn methyltransferases Pem1p/Cho2p (catalyzing the first methylation) and Pem2p/Opi3p (catalyzing the final two methylations) (Howe and McMaster, 2001).

The Kennedy or CDP-Cho pathway is operative when yeast are fed Cho, but under "normal" circumstances, the flux into this pathway is only a minor component of PtdCho biosynthesis. This pathway involves phosphorylation of Cho, forming phosphocholine (P-Cho), activation of P-Cho with CTP to form CDP-Cho, and finally condensation of CDP-Cho with DAG to form PtdCho (Howe and McMaster, 2001). The relative flux into the two pathways is largely dependant on the supply of choline, however other factors such as temperature (Howe et al., 2002) and inositol supply (Loewen et al., 2004) also play roles in the regulation of these pathways.

In plants, PtdCho is important as an intermediate in the eukaryotic pathway of thylakoid lipid synthesis (Figure 1.6), and also in triacylglycerol biosynthesis in seeds (Roughan and Slack, 1982). As in yeast, multiple pathways of PtdCho synthesis are operative in plant cells, however the CDP-Cho pathway seems to be the major route to synthesis of PtdCho, with methylation of PtdEtn being almost unknown in plants (Somerville et al., 2000). Plants, however, have the capability to produce Etn and Cho directly for use as precursors. Unique to plants is a serine decarboxylase, which allows the production of Etn from Ser (Rontein et al., 2001). The Etn may then be phosphorylated by an ethanolamine kinase, followed by N-trimethylation of the phospho-Etn to form P-Cho (the pathways outlined in Figure 1.5). The gene encoding the trimethylation activity in this pathway was isolated from spinach and Arabidopsis by cDNA complementation of *Schizosaccharomyces pombe cho2* and *Saccharomyces cerevisiae opi3* mutants, respectively (Bolognese and McGraw, 2000; Nuccio et al., 2000).

The control of PtdCho has been studied in mammalian cells, and it is widely regarded that PtdCho synthesis is regulated at the level of formation of CDP-Cho in most tissues. The major exception to this is in hepatic tissues, where formation of PtdCho by the PtdEtn methylation is the major route of synthesis (Vance et al., 1997). The major isoform of CDP-Cho synthetase is the CT-α isoform, and its transcription is regulated coordinately with the cell cycle (Golfman et al., 2001). The CT-α protein is also regulated in a multivalent manner by phosphorylation (Arnold et al., 1997) and reversible membrane binding. This last point has been the subject of some very elegant biochemistry by Cornell and co-workers. It has been shown that membrane binding activates CT-α (Cornell, 1991), and that the membrane binding is stimulated by non-bilayer lipids in the membrane (Davies et al., 2001). This leads to the conclusion that CT-α is itself a sensor of membrane bilayer/non-bilayer lipid ratios, in much the same way that other biochemical pathways are allosterically regulated by their end products. While the information on control of yeast and mammalian PtdCho synthesis is far from complete, it is still much more than is known of the analogous regulatory processes in plants.

Betaine lipid synthesis.

Another group of polar glycerolipids, the betaine lipids, consists of diacylglycerol bound in an ether linkage to a quaternary amine alcohol. These lipids are widely distributed among marine and freshwater algae, mosses, ferns, and other non-vascular plants, as well as among many fungal species (Eichenberger, 1982; Sato and Furuya, 1984; Vaskovsky et al., 1998). The structure of one member of this class, DGTS, is given in Figure 1.1.

The quaternary amine containing, zwitterionic betaine lipids are similar in overall structure to PtdCho, and it has been postulated that betaine lipids such as DGTS can functionally replace PtdCho in membranes (Sato, 1992). Evidence for this hypothesis is given by the fact that organisms that contain betaine lipids, such as *Chlamydomonas*, often lack PtdCho. Further correlative evidence comes from studies on *Rhodobacter sphaeroides* which, when deprived of phosphate, decreases the amount of PtdCho and other phospholipids in its membranes and induces *de novo* synthesis of DGTS and other nonphosphorous lipids (Benning et al., 1995).

This last point is the key observation that spurred work on bacterial DGTS biosynthesis. The initial discovery of phosphate-deprivation induced production of DGTS in *Rhodobacter* was made in the context of studies to determine the function of SQDG (Benning et al., 1993). In general, the complement of polar lipids in *Rhodobacter* was observed to drastically change under phosphate limited growth conditions, and several unidentified non-phosphorous lipids, one of which was later shown to be DGTS (Benning et al., 1995), were produced concomitantly with a decrease in the amount of phospholipids. A similar scenario has been shown to occur in several marine pseudomonad species (Minnikin et al., 1972; Minnikin et al., 1974).

Studies following up on the discovery of DGTS in *Rhodobacter* focused on defining the pathway by which this lipid is synthesized (Hofmann and Eichenberger, 1996), identifying the genes encoding enzymes of DGTS biosynthesis (Klug and Benning, 2001) and defining the regulatory elements that control the induction of DGTS accumulation in *Sinorhizobium meliloti* (Geiger et al., 1999). The DGTS biosynthetic pathway has also been studied in the eukaryotic algae *Ochromonas danica* (Vogel and Eichenberger, 1992) and *Chlamydomonas reinhardtii* (Moore et al., 2001; Sato, 1988), and was proposed to follow the scheme outlined in Figure 1.5. DGTS biosynthesis in *Rhodobacter* was also postulated to follow this pathway based on labeling studies outlined in Hofmann and Eichenberger (1996).

Geiger et al. (1999) showed that phosphate stress-induces DGTS production in *Rhizobium meliloti,* and that this induction is dependent on *phoB* which, in *E. coli*, encodes the response regulator of the two-component system controlling expression of genes involved in phosphate acquisition and utilization under limiting conditions (Wanner, 1993). This result showed that DGTS accumulation is a function of the pho regulon, but the regulated genes encoding the proposed functions were not known at that time. Studies described by Klug and Benning (2001) allowed the tentative placement of two enzymes into the biosynthetic scheme, as outlined in Figure 1.5.

Briefly, screening of a mutagenized population of *Rhodobacter* grown under phosphate limitation yielded several strains lacking the ability to accumulate DGTS, and complementation cloning of one of the mutants revealed an operon consisting of two open reading frames, designated *btaA* and *btaB*. The product of *btaA* was proposed to be the S-adenosylmethionine (AdoMet) dependent 3-amino-3-carboxypropyl transferase (see Figure 1.5), on the basis that over-expression of *btaA* alone leads to accumulation of the precursor diacylglycerylhomoserine (DGHS), while disruption of the gene in the RKL3 mutant abolishes DGTS (and DGHS) production. This function represents an unusual reaction of AdoMet, which is primarily a donor of methyl groups, however similar reactions have been noted in the biosynthesis of the antibiotic nocardicin A (Reeve et al., 1998), and the plant siderophore nicotianamine (Ling et al., 1999). The *btaB* gene product is homologous to AdoMet-dependent methyltransferases, and insertional disruption of the open reading frame leads to accumulation of DGHS under phosphate limitation, hence the function of this protein is likely the catalysis of one or more of the three methyl group transfers outlined in Figure 1.5. It is worth noting that the analogous methyltransferase involved in PtdCho biosynthesis in *Rhodobacter*, encoded by the *pmtA* gene (Arondel et al., 1993), is responsible for all three methylation events leading to the formation of PtdCho from phosphatidylethanolamine (PtdEtn), so it is feasible that the BtaB protein acts in a similar manner.

As mentioned above, the unicellular chlorophyte alga *Chlamydomonas reinhardtii* has previously been shown to contain DGTS (Eichenberger and Boschetti, 1977) and to lack PtdCho (Giroud et al., 1988). Aside from the replacement of PtdCho by DGTS, *C. reinhardtii* has a complement of polar lipids quite similar to higher plants, and the positional fatty acid analysis of individual lipid classes indicates that the eukaryotic pathway of glycerolipid synthesis is involved only in assembly of extraplastidic membranes, i.e. there is no transfer of diacylglycerol species from the endoplasmic reticulum to the chloroplast (Giroud et al., 1988). This lack of a eukaryotic complement of thylakoid molecular species contrasts with the situation in grasses and dicots such as spinach and Arabidopsis, and correlates well with a lack of PtdCho, as this lipid is thought to be important in lipid trafficking from the endoplasmic reticulum to the plastid ((Somerville et al., 2000); see Fig 1.6 below). Studies using [^{14}C]-methionine feeding (Sato, 1988) and *in vitro* analysis of crude microsomal preparations (Moore et al., 2001) indicated that the pathway for biosynthesis of DGTS is consistent with that given in Figure 1.5. At the time of those studies, no genes or proteins specifying DGTS biosynthesis had been isolated from any eukaryote, however a detailed analysis of the fatty acid distribution in different lipids of *C. reinhardtii* presented strong evidence for an extraplastidic origin for this lipid (Giroud et al., 1988), consistent with exclusion of the biosynthetic activities from chloroplast membrane fractions (Moore et al., 2001).

The core focus of the work presented in the following chapters was to biochemically characterize the BtaA and BtaB enzymes from *Rhodobacter*, and to identify the cognate eukaryotic activities. Chapters 2 and 3 present the biochemical data addressing these points, and to this end the work was successful. Chapter 2 details the functional analysis of BtaA and BtaB, details the development of an assay system for BtaA, and proposes a model for the active site based on similarity to methyltransferases. Chapter 3 follows with the discovery that the DGTS biosynthetic machinery in *Chlamydomonas* (BTA1) is a multidomain fusion protein of BtaA- and BtaB- like modules. Site-directed mutagenesis is also used to identify residues that are involved in substrate binding by the individual domains, by analogy with the models proposed for bacterial BtaA and BtaB.

Chapter 4 follows the biochemical data with an *in vivo* functional analysis, using RNAi to reduce the accumulation of *BTA1* transcript and examine the effects of down-regulating the machinery required for the bulk of extraplastidic membrane synthesis.

Cellular organization of lipid biosynthesis: The eukaryotic pathway of thylakoid lipid synthesis.

Studies of lipid metabolism in plants in late 1970s and early 1980s revealed that there are both eukaryotic and prokaryotic pathways of lipid biosynthesis involved in the production of thylakoid lipid species, as reviewed by Roughan and Slack (1982) and presented schematically in Figure 1.6. This scheme was proposed to involve the export of fatty acid from plastids and their incorporation into PtdCho in the endomembrane system, followed by the import of the diacylglycerol moiety of PtdCho back to the plastid, and its incorporation into plastidic glycerolipid species. Further studies in Arabidopsis by Browse, Somerville, and co-workers showed that the flux into the prokaryotic and eukaryotic pathways of thylakoid biogenesis is approximately equal in this species (Browse et al., 1986). As discussed above, corroborating evidence for these pathways was obtained from studies on the *act1* mutant (Kunst et al., 1988), which is deficient in the acyl-ACP dependent GPAT activity, the first step in the prokaryotic pathway. The actual identity of the species being transported back to the plastid is still controversial, but strong evidence points to the role of PtdCho as the donor of this species.

The situation in *Chlamydomonas* is not as convoluted, in that this organism contains DGTS in place of PtdCho, as described above, and this is in agreement with the

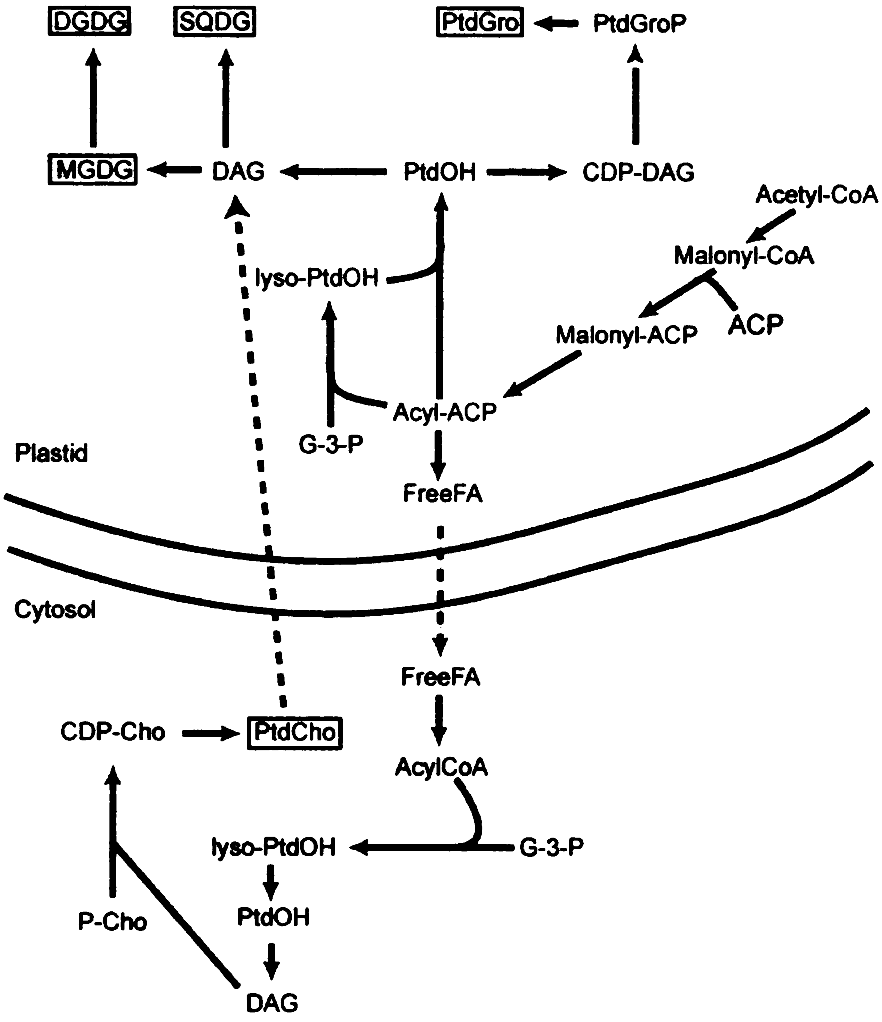


Figure 1.6: **Schematic representation of lipid transport pathways present in plants.** Abbreviations for the metabolites are the same as Figures 1.3 and 1.5. Export of fatty acid from the plastid is hypothesized to take place by direct channeling of free fatty acid hydrolyzed from acyl-ACP to an acyl-CoA synthetase in the outer envelope membrane. Import of the DAG moiety of PtdCho back into the chloroplast (the "eukaryotic pathway" of thylakoid lipid synthesis) is also indicated, and takes place by an unknown mechanism.

fact that there are no eukaryotic galactolipid species in *Chlamydomonas* (Giroud et al., 1988). This may not be true of all green algae, however, as recent evidence suggests that the chlorophyte alga *Chlorella kessleri* possesses a eukaryotic pathway for thylakoid lipid synthesis (Sato et al., 2003b). This study was performed by the classical method of ^{14}C acetate pulse-chase labeling, followed by a time course of measurement of radioactivity in each lipid class. Early in the chase, label was mostly detected in labeled MGDG and DGDG species with a 16 carbon FA at the *sn*-2 position, which is typical of prokaryotic lipids, and in the extraplastidic lipid PtdCho. Later in the chase, there was a reduction of label in PtdCho, concomitant with an increase in MGDG and DGDG species with 18 carbon FA at the *sn*-2 position of glycerol. This is interpreted as the PtdCho DAG moiety being transferred back to the chloroplast via the eukaryotic pathway.

This finding of a eukaryotic pathway in a Chlorophyte alga such as *C. kessleri* raises questions about the evolutionary origin of this pathway. Apparently, either this pathway was operative very early in the evolution of *Viridiplantae*, before the split between Chlorophytes (such as *Chlorella*) and Streptophytes (such as Arabidopsis), or this mechanism of lipid synthesis is the result of convergent evolution after this division. Until there is a mechanistic molecular description of the eukaryotic pathway in both phyla, this question will remain unresolved.

The enigma of the transport of eukaryotic fatty acid species to the plastid is currently the subject of intense study, and the report of a mutant with a blockage in this process has been reported (Xu et al., 2003). Data in Appendix B show that an atypical set of galactolipids containing all-β-linked oligosaccharide chains accumulate in this mutant, and a hypothesis for their biosynthesis is given.

Phosphate regulation of lipid metabolism.

As touched on above, the availability of phosphorus can have a profound impact on the lipid composition of plant cell membranes. This is apparent in Arabidopsis, in which phosphate limitation induces the synthesis of an extraplastidic DGDG species (Härtel et al., 2000), and recent studies on plasma membranes from phosphate starved oat (Andersson et al., 2003), and in maple suspension culture cells (Jouhet et al., 2003), show that a major proportion of PtdCho is replaced by DGDG during phosphate starvation. Since DGDG is a bilayer-forming lipid with roughly similar biophysical properties to PtdCho, these studies imply that, under phosphate deprivation, PtdCho is replaced by DGDG so that phosphate can be partitioned into more critical metabolites, such as nucleic acids. This also implies a new or adaptable set of lipid trafficking mechanisms which would be required to transport DGDG to the plasma membrane and ER (Dörmann and Benning, 2002).

As discussed briefly above, this lipid-substitution phenomenon is also observed to occur in the plastid, e.g. substitution of PtdGro with SQDG under phosphate limitation. These lipid-substitution phenomena are inducible by phosphate deprivation, as are other cellular responses to phosphate starvation such as the development of high affinity transport systems, secretion of phosphatases to hydrolyze and release phosphate from organic phosphate esters (Grossman, 2000), and increases in RNAse activity due to induction of *RNS1* to increase turnover of cellular RNAs (Bariola et al., 1994). A Myb domain transcription factor that potentially mediates the activation of a subset of the plant PHO-regulon, and which is conserved between *Chlamydomonas* and Arabidopsis, has been identified genetically in the respective organisms. The *Chlamdomonas psr1* mutants were isolated based on their inability to induce phosphatase secretion and resist killing by $^{32}PO_4$ (Shimogawara et al., 1999), and the Arabidopsis mutant *phr1* was isolated based on its inability to induce GUS expression from a phosphate-stress inducible promoter (Rubio et al., 2001). The regulatory mechanisms involved in the induction of the Arabidopsis SQD1/2, MGD2/3, and DGD2 genes are unresolved, and may or may not be under the control of the PSR1/PHR1 system. This point could be resolved easily by analysis of lipid content and gene expression in these mutants grown in phosphate limiting conditions.

***Chlamydomonas* and Arabidopsis as complementary models of plant lipid metabolism.**

The outline of plant lipid metabolism presented here has shown that there is fertile ground for comparative biochemical genomics of Arabidopsis and *Chlamydomonas*. These two organisms are representative of the two most deeply branching phylogenetic clades in green plants, namely the *Streptophyceae* (Arabidopsis) and *Chlorophyceae* (*Chlamydomonas*). Given that the genome sequences are available for both models (Grossman et al., 2003; The Arabidopsis Genome Initiative, 2001), it could prove useful to systematically compare the biosynthetic pathways involved in lipid metabolism in these two organisms to find universal concepts applicable to all plants. It seems that several are already evident, namely that the core pathways of fatty acid biosynthesis and plastid membrane lipid assembly are the same, and that fatty acids are exported from the plastid for the synthesis of extraplastidic lipids. These points are addressed in Chapter 4, which, in addition to the functional analysis of BTA1, presents a set of genes that have been annotated as having potential activities in lipid biosynthesis. This allows the formation of hypotheses regarding lipid biosynthesis pathways, and reveals that some of the components that are central to plastid fatty acid synthesis may also be targeted to the mitochondrion.

This approach of using bioinformatics to identify similarities and differences between *Chlamydomonas* and Arabidopsis shows several areas where apparently conserved processes could perhaps be studied in *Chlamydomonas* more easily than Arabidopsis. Fatty acid synthesis and export would be one example. It is known that *Chlamydomonas* can take up and incorporate palmitate and oleate very rapidly into both plastidic and cytoplasmic lipids (Giroud et al., 1988), and it is reasonable to think that fatty acid auxotrophs could be isolated in this organism, as they have been for *E. coli* and yeast. One would expect to find strains that are blocked at any of the various steps of fatty acid biosynthesis outlined above, and also mutants defective in fatty acid export from the plastid. Among these hypothetical mutants, those defective in fatty acid synthesis could be identified by their inability to incorporate labeled acetate into fatty acid, and fatty acid export mutants would be identified as those that incorporate this label into plastidic lipids such as MGDG, but not extraplastidic lipids such as DGTS. The fact that *Chlamydomonas* has fewer complicating factors than Arabidopsis (such as tissue specific gene expression as in the LACS9 example (Schnurr et al., 2002) above), and that a gene-tagging approach has been used to isolate lipid biosynthesis mutants (Chapter 5) makes this sort of approach all the more attractive.

The differences in lipid metabolism between the two model organisms are also informative. For example, the ER membranes of Arabidopsis and *Chlamydomonas* should share many analogous functions, however PtdCho dominates the lipid composition of the ER in Arabidopsis, while that of *Chlamydomonas* is composed primarily of DGTS. The regulation of membrane composition in any organism, especially in plants, is a topic that has proved fairly intractable, however, it may be the case that biosynthesis of DGTS in *Chlamydomonas* and PtdCho in Arabidopsis are under the control of a conserved membrane sensing mechanism. In other organisms, this sort of membrane sensing takes the form of a membrane-bound transcription factor being released and activated upon a change in membrane composition, as in the SREBP system of metazoans (Brown and Goldstein, 1999; Dobrosotskaya et al., 2002), and the Opi1p protein in yeast (Loewen et al., 2004). Systems such as these are completely unknown in plants, but it seems highly probable that an analogous system would be in play in the plant kingdom. The identification of such a regulatory mechanism would certainly benefit from a more thorough understanding of lipid biosynthesis and turnover in multiple plant model systems.

Development of Chlamydomonas as a model system for lipid metabolism. Taken together, the results in Chapters 3, 4 and 5 give the starting point for development of *Chlamydomonas* as a model for plant lipid metabolism. Gene tagging will be useful in forward genetic screens for lipid mutants or auxotrophs, as shown in Chapter 5, and RNAi techniques are useful for reverse genetic experiments targeted to specific genes, as outlined in Chapter 4. This last method may be especially helpful for determining the functions of potentially essential genes, as is probably the case for *BTA1* in Chapter 4. These technologies, combined with biochemical approaches such as those in Chapter 3, as well as the use of genomic resources, as demonstrated in Chapter 4 (and Appendix A, in the case of *Galdieria sulphuraria*), will make *Chlamydomonas* a useful complement to seed-plant models of lipid metabolism.

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Chapter 2

Functional characterization of the bacterial betaine lipid biosynthetic enzymes BtaA and BtaB

Abstract

Betaine lipids are non-phosphorous glycerolipid analogs of phosphatidylcholine. The biosynthesis of the betaine lipid diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS) has previously been studied in phosphate-starved cells of the purple bacterium *Rhodobacter sphaeroides*, and a genetic approach identified two proteins that are necessary for this process. Here we show that all reactions of DGTS biosynthesis in *R. sphaeroides* are attributable to *Rs*BtaA and *Rs*BtaB, as co-expression of the respective genes leads to DGTS formation in *E. coli*, which normally lacks this lipid. The recombinant *Rs*BtaA protein was membrane-associated and showed S-adenosylmethionine:diacylglycerol 3-amino-3-carboxypropyl transferase activity. *Rs*BtaA directed the transfer of label from 1-[^{14}C]-S-adenosylmethionine (AdoMet) into the DGTS precursor diacylglycerylhomoserine (DGHS) allowing an analysis of the biochemical and kinetic properties of this enzyme. Comparative analysis of *Rs*BtaA and its bacterial homologs revealed a motif with similarity to the AdoMet binding pocket of methyltransferases, and allowed the prediction of residues involved in substrate binding.

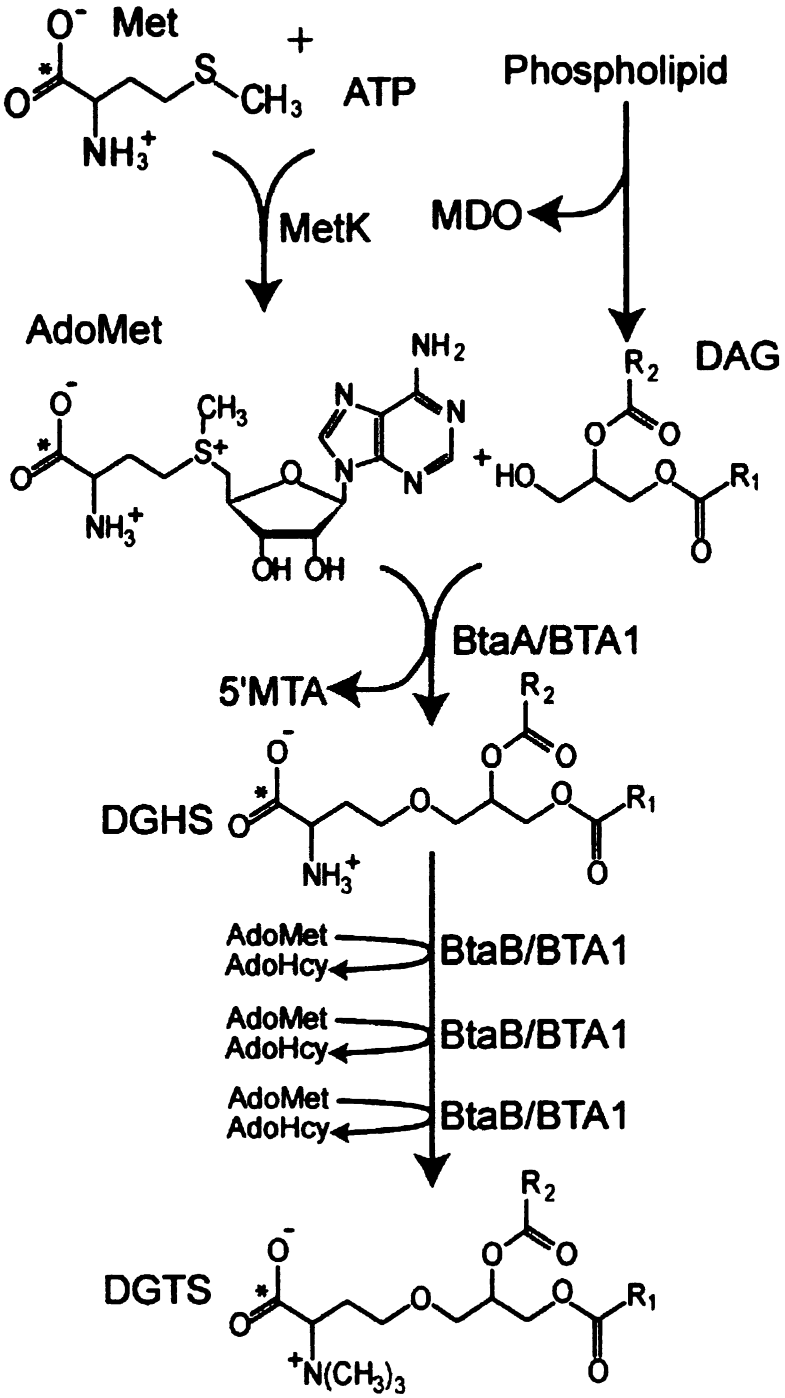
Introduction

Many organisms, such as bacteria, plants, and fungi, rely on mineral nutrients taken directly from the soil or aquatic environment. As such, these organisms tend to have exquisitely specialized mechanisms to cope with limitation of a given essential nutrient. For example, most organisms have well defined responses to phosphate limitation, including the induction of extracellular phosphatases and high affinity transport systems (Grossman, 2000), the reorganization of fundamental biochemical pathways, e.g. glycolysis (Plaxton, 1996), and even the replacement of cellular membrane phospholipids with non-phosphorous lipids. This last point has been well documented in the plants Arabidopsis (Essigmann et al., 1998; Härtel et al., 2000) and oat (Andersson et al., 2003), and most pronouncedly in the α-proteobacteria *Rhodobacter sphaeroides* and *Sinorhizobium meliloti*, which under phosphate stress become depleted of membrane phospholipids and induce the synthesis of non-phosphorus lipids, such as glycolipid species, the betaine lipid diacylglyceryl-*N,N,N*-trimethylhomoserine (DGTS), and ornithine containing lipids. (Benning et al., 1995; Geiger et al., 1999).

DGTS, which was first discovered in the unicellular alga *Ochromonas danica* (Brown and Elovson, 1974) and thereafter in *Chlamydomonas reinhardtii* (Janero and Barrnett, 1982), and other lower plants and fungi (Furlong et al., 1986; Kunzler and Eichenberger, 1997; Rozentsvet et al., 2000), has been proposed to take the place of phosphatidylcholine (PtdCho) in membranes of these organisms. Studies of DGTS biosynthesis using radioisotope feeding experiments identified methionine as a source of both the C_4 homoserine moiety and methyl groups of DGTS (Sato, 1988)(see structure in Figure 2.1), leading to the conclusion that S-adenosylmethionine (AdoMet) could be the active donor of the four carbon unit of methionine to form the precursor diacylglycerylhomoserine (DGHS), followed by lipid-linked methylation of DGHS by an AdoMet dependent methyltransferase (Hofmann and Eichenberger, 1996)(Figure 2.1). A closely related lipid, diacylglyceryl-*N*,*N*,*N*-trimethylhydroxymethyl-β-alanine (DGTA), is derived from DGTS as judged by pulse-chase radiolabelling studies (Vogel and Eichenberger, 1992).

Recently, Klug and Benning (2001) used a genetic approach in *R. sphaeroides* to gain access to genes that are required for the biosynthesis of DGTS. This work resulted in the identification of an operon containing two open-reading frames, designated *btaA* and *btaB*, which are necessary for DGTS accumulation during phosphate deprivation. The product of *btaA* was proposed to function as an AdoMet-dependent 3-amino-3-carboxypropyl-transferase, producing the intermediate DGHS. *Rs*BtaB showed a high degree of similarity to methyltransferases, and was proposed to be a trifunctional AdoMet-dependent *N*-methyltransferase, adding 3 methyl units to the amino function of DGHS to form DGTS (see Figure 2.1). In fact, *Rs*BtaB shows a high degree of sequence similarity to *Rs*PmtA, which produces phosphatidylcholine by *N*-trimethylation of phosphatidylethanolamine (Arondel et al., 1993), indicating that *Rs*BtaB and *Rs*PmtA may share analogous functions in separate pathways. In order to understand DGTS biosynthesis in greater detail, we initiated a biochemical characterization of the BtaA and BtaB proteins from *R. sphaeroides*, focusing in particular on *Rs*BtaA due to the unusual nature of the proposed reaction.

Figure 2.1. **DGTS biosynthetic pathway and involvement of *Rs*BtaA/B-*Cr*BTA1.** BtaA is proposed to catalyze the transfer of the 3-amino-3-carboxypropyl group of AdoMet to the 3-hydroxyl of DAG to form the intermediate DGHS. DGHS is then sequentially *N*-methylated by the BtaB methyltransferase to form DGTS. In *C. reinhardtii*, these functions would be carried out by the BtaA- and BtaB- like domains of the multifunctional *Cr*BTA1 protein. AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; DAG, diacylglycerol; DGHS, diacylglycerylhomoserine; DGTS, diacylglyceryl-(*N*,*N*,*N*)-trimethylhomoserine; MDO, membrane derived oligosaccharides; MetK, AdoMet synthetase; 5'MTA, 5'-methylthioadenosine.


Met
+
ATP
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MDO
AdoMet
DAG
+
BtaA/BTA1
5'MTA
DGHS
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AdoHcy
BtaB/BTA1
AdoMet
AdoHcy
BtaB/BTA1
AdoMet
AdoHcy
BtaB/BTA1
DGTS

Materials and Methods

Materials. Phospholipase C from *Bacillus cereus* was from Sigma (St. Louis, MO), mouse monoclonal anti-His tag antibody was from Qiagen (Valencia, CA). Restriction enzymes, T4 DNA ligase, calf-intestinal phosphatase, and Klenow fragment were from New England Biolabs (Beverly, MA), *Taq* DNA polymerase was from Roche (Indianapolis, IN), and *Pfu* DNA polymerase was from Stratagene (La Jolla, CA). All other chemicals and solvents were of reagent grade and were from Sigma, EM Science (Gibbstown, NJ) or J.T. Baker (Phillipsburg, NJ).

Expression of *RsbtaA* and *RsbtaB*. A summary of all strains and plasmids used in this study is presented in Table 1. The coding regions of *RsbtaA* and *RsbtaB* were PCR-amplified from plasmid pRKL323 (Klug and Benning, 2001) for expression in pQE-31 (Qiagen) or pACYC-31 (Dörmann et al., 1999) respectively, with the following primers (restriction sites underlined): *btaA* forward (*Sph*I), 5'-ACATGCATGCAGTGACGCAGTTCGCCCTC-3'; *btaA* reverse(*Kpn*I), 5'-CGGGGTACCAGGACGATCCGCTCGAACCG-3'; *btaB* forward (*Bam*HI), 5'-GCGGATCCGATGACCGACGCCACCCAT-3'; *btaB* reverse (*Hin*DIII), 5'-GCAAGCTTCTCTCACCGCGTGAGCGTG-3'. PCR was carried out with *Taq* DNA polymerase (Roche) according to the manufacturer's specifications, except that 10% DMSO (v/v) was added to each reaction to overcome difficulties in PCR due to the high G+C content of *R. sphaeroides* DNA. PCR products were first cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and sequenced at the MSU Genomics Technology Support Facility, followed by subcloning into the expression vectors using restriction sites as outlined in the PCR primers (see Table I). The resulting constructs were thus designated pBtaA (*btaA* sequence in pQE-31), pBtaB (*btaB* in pACYC-31), and pBtaA-LC (*btaA* in pACYC-31). PCR2.1 and pQE-31 derivatives were propagated in LB medium containing ampicillin at 100 μg/ml, and pACYC-31 derivatives in LB with chloramphenicol at 25 μg/ml. Strains containing compatible combinations of plasmids as indicated in individual experiments were constructed by co-transformation of heat-shock competent cells of TOP10F' (Invitrogen) with 100 ng of both plasmids followed by selection on LB containing both ampicillin and chloramphenicol.

***In vivo* production of DGHS and DGTS in *E. coli* and *R. sphaeroides*.** *E. coli* TOP10 F' cells harboring compatible combinations of plasmids as described in individual experiments were grown as 2 ml overnight cultures, and 0.1 ml of these cultures were used to inoculate 10 ml LB containing appropriate antibiotics. After growing to OD ~0.6, the cultures were harvested by centrifugation, and dispersed into 10 ml M9 minimal medium (Sambrook et al., 1989) containing 0.2 mM isoprpopyl-β-D-thiogalactoside (IPTG) and 0.5 μCi 1-[^{14}C]-methionine (American Radiolabeled Chemicals, St. Louis, MO). Cultures were incubated for 3 h and harvested by centrifugation, followed by extraction with 2 ml chloroform:methanol (1:1, v/v) and phase separation by addition of 0.5 ml 1M KCl, 0.2 M H_3PO_4. *R. sphaeroides* wild-type 2.4.1 and its mutant *btaB-dis* (Kan^r) (Klug and Benning, 2001) were grown to mid-log phase in 100 ml phosphate-replete or phosphate-limited Sistroms medium (Sistrom, 1960; Sistrom, 1962) as described (Benning et al., 1995; Klug and Benning, 2001). A 10 ml aliquot of each

Table 2.1. Strains and plasmids used in this work.

Strain or plasmid	*Description*	*Source*
R. sphaeroides		
2.4.1	Wild type	(Benning and Somerville, 1992)
btaB-dis	2.4.1 with Kan^R cassette disrupting *btaB*	(Klug and Benning, 2001)
E. coli		
TOP10F'	Cloning and expression strain, Φ80*lac*ZΔM15, F' {*lac*I^q Tn10(Tet^R)}	Invitrogen
Plasmids		
pCR2.1	PCR product cloning vector (Amp^R)	Invitrogen
pBluescript SK+	General cloning vector (Amp^R)	Stratagene
pRKL323	*R. sphaeroides btaAB* operon in pBluescript SK+	(Klug and Benning, 2001)
pACYC-31	Low copy *E. coli* expression vector (Cam^R)	(Dörmann et al., 1999)
pQE-31	High copy *E. coli* expression vector (Amp^R)	Qiagen
pBtaA	*Sph*I-*Kpn*I fragment of *btaA* amplified from pRKL323 in pQE-31	This work
pBtaB	*Bam*HI-*Hin*DIII fragment of *btaB* amplified from pRKL323 in pACYC-31	This work

culture was then harvested by centrifugation and suspended in 10 ml fresh Sistrom's medium containing 0.5 μCi 1-[^{14}C]-Met, followed by 3 h incubation at 28° C and harvesting and extraction as described for the *E. coli* cultures above. A portion of the organic phase from each extraction was spotted onto activated (120° C, 2 h), ammonium sulfate impregnated TLC plates (Silica-60, Baker) and resolved in acetone/toluene/water (91:30:7, v/v) followed by autoradiography (method A) or onto untreated plates and developed in the solvent system chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v) (method B).

Subcellular localization of BtaA protein. A 2 ml overnight culture of cells containing pBtaA was used to inoculate 100 ml of LB-ampicillin, and then incubated at 37° C to an OD_{600} of 0.7-0.9. IPTG was added at 50 μM to induce low level expression of *Rs*BtaA and to avoid the formation of inclusion bodies, and the culture was shifted to 28° C and incubated a further 4 hours. Cells were harvested by centrifugation and suspended in 4 ml lysis buffer (20 mM KP_i, pH 7.2, 500 mM NaCl, 15% (v/v) glycerol), frozen in liquid nitrogen and thawed slowly, and then lysed by sonication for 30 sec 3 times with a microprobe tip. The crude lysate was centrifuged at 2,000 g for 10 min to remove unbroken cells and cellular debris, and the supernatant was subjected to centrifugation at 20,000 g for 20 min. The soluble fraction was transferred to a new tube and the glassy pellet of membranes was gently washed with lysis buffer, followed by addition of 400 μl of lysis buffer and resuspension of the membrane fraction by sonication in a water bath sonicator. The resuspended membranes were then subjected to another round of centrifugation, washing and resuspension, resulting in a washed membrane fraction, and

20 μg of protein of each fraction (cell lysate, soluble, and membranes) were subjected to SDS-PAGE on a 10% gel, followed by either Coomassie staining or western blotting (Sambrook et al., 1989) with anti-His$_6$ antibody (Qiagen) at a dilution of 1:3000.

***In vitro* activity assays of *Rs*BtaA.** *E. coli* TOP10 F' harboring pBtaA was grown in 250 ml LB ampicillin at 37° C to an OD of 0.7, and induced with 0.25 mM IPTG followed by an additional 4 h of growth at 28° C. Cells were harvested by centrifugation and the cell pellet was suspended in 10 ml of cold buffer (50 mM HEPES, 1 mM DTT, 1 mM EDTA, pH 7.3). The resuspended cells were sonicated 3-4 times, 30 seconds each with a microprobe tip, and the lysate was centrifuged at 2000 g for 10 min to remove unbroken cells and cellular debris. 1 ml aliquots of the cell-free extract were then frozen in liquid N_2 and stored at –80° C prior to use. Activity under these storage conditions did not decrease appreciably for at least 1 month.

Assays for the pH versus activity profile were conducted in 100 μl final volume by combining 48.75 μl of cell free extract with 48.75 μl of 100 mM HEPES, TrisCl, or MES, 1 mM DTT, 1 mM EDTA, at varying initial pH to give a final pH in the range of 5.5-8.6 when mixed with the cell free extract (initial pH 7.3). Reactions were initiated by addition of 25,000 dpm 1-[^{14}C]-AdoMet (American Radiolabeled Chemicals, 2.5 μl, final concentration of AdoMet of 2.1 μM), incubated for 30 min at 28° C, and terminated by addition of 400 μl of chloroform/methanol (1:1, v/v) and100 μl 0.9% (w/v) NaCl to separate aqueous and organic phases. The organic phase was transferred to a new tube, dried under a stream of nitrogen, and dissolved in 50 μl chloroform/methanol (1:1, v/v). This lipid extract was then spotted onto silica-60 TLC plates (Baker) and developed with the solvent chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v), followed by quantification of the signal for DGHS (and methylated products in the case of *Cr*BTA1) on a phosphorimager screen (Molecular Dynamics) with the ImageQuant software package. Determination of K_M was carried out at pH 7.6 for *Rs*BtaA and 7.2 for *Cr*BTA1, with varying concentrations of AdoMet, and the value of K_M was determined by direct fitting of a hyperbola to the experimental data in the Origin software package (OriginLab, Northampton, MA). Assays for the effect of proposed inhibitors were performed with 4 μM AdoMet and 100 μM inhibitor added from a 1 mM stock solution in water, and the same volume of water was added to the uninhibited control reaction.

Effect of DAG production by phospholipase C on *Rs*BtaA activity. Phospholipase C (PLC) from *Bacillus cereus* was obtained as a 3.2 M $(NH_4)_2SO_4$ suspension (Sigma) and was desalted by several dilution-concentration cycles with assay buffer using an Centricon-5 microconcentrator (Amicon), and was diluted to a final concentration of ~1 unit/μl. A portion of this PLC preparation was heated at 95° C for 5 min and served as a heat inactivated control, i.e. the only difference between the experimental and control assays was whether or not the PLC had been heat inactivated, thus allowing direct comparison of the two treatments without consideration of residual salt or pH changes. Assays were conducted in triplicate by pre-incubation of the reaction mix (85 μl cell lysate, 10 μl PLC) for 10 min at 28°C followed by addition of 50,000 dpm of 1-[^{14}C]-AdoMet (final concentration of 4.2 μM), incubation for 30 min, and extraction and TLC as described above.

Results

***Rs*BtaA and *Rs*BtaB are sufficient to reconstitute DGTS biosynthesis in *E. coli*.** In Figure 2.2, the reconstitution of DGTS biosynthesis in *E. coli* is shown, in agreement with the proposed functions of the two putative enzymes. As positive controls for accumulation of various lipids, the *R. sphaeroides* wild-type 2.4.1 and *btaB*-dis strains (Klug and Benning, 2001) were grown in phosphate-replete or phosphate-limited Sistrom's medium to induce synthesis of DGHS in the *btaB*-dis strain, or DGTS in the wild-type. Compatible combinations of expression plasmids were used to introduce *Rs*BtaA and *Rs*BtaB singly or together into *E. coli*, followed by analysis of polar lipid content.

To demonstrate the function of *Rs*BtaA, we expected that *E. coli* expressing *Rs*BtaA could use endogenous DAG and AdoMet to accumulate DGHS, the non-methylated precursor of DGTS (Figure 2.1). In Figure 2.2*A* we show that this is indeed the case, as a primary-amine containing lipid which co-migrates with authentic DGHS was present upon expression of *btaA* in *E. coli*. This result firmly establishes the role of *Rs*BtaA as the first step of DGTS biosynthesis in the pathway outlined in Figure 2.1, and excludes the possibility that *Rs*BtaA acts as a component of a heteromeric complex with a protein specific to *R. sphaeroides*, or requires a substrate or co-factor present in *R. sphaeroides* but not in *E. coli*.

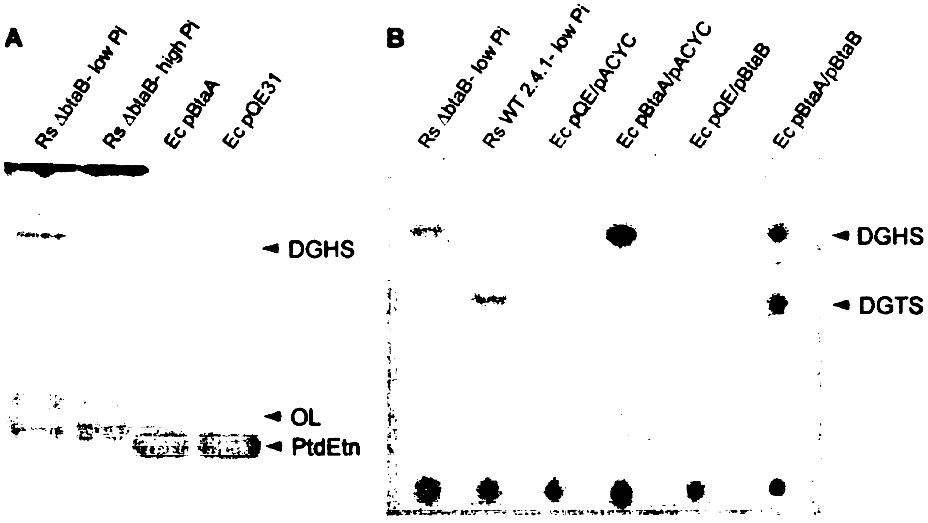


Figure 2.2. **Reconstitution of DGTS biosynthesis in *E. coli.*** *A*, *E. coli* expressing *btaA* accumulate the DGTS precursor DGHS. Lipids were extracted and resolved as described in the Experimental Procedures method A, and TLC plates were sprayed with ninhydrin reagent to reveal primary amine-containing lipids. The *btaA* expression strain (lane 3) shows a ninhydrin positive band co-migrating with authentic DGHS from a phosphate stressed *R. sphaeroides btaB* KO strain (lane 1). Phosphate replete *R. sphaeroides btaB* KO and empty pQE-31 serve as negative controls (lanes 2 and 4, respectively). *B*, BtaB produced by expression of *btaA* on a compatible plasmid reconstitutes DGTS biosynthesis. 1-[^{14}C]-methionine labels DGHS in the *btaA* expression strain, and co-production of the BtaB protein converts a portion of this precursor to DGTS in cells harboring both pBtaA and pBtaB.

To show the function of the putative trifunctional methyltransferase *Rs*BtaB, a compatible expression plasmid containing the *RsbtaB* gene was introduced into *E. coli* along with the pBtaA expression plasmid. Our expectation was that a portion of the DGHS produced by *Rs*BtaA would be converted into *N*-methylated products as shown in Figure 2.1. Figure 2.2*B* shows the results of a metabolic labeling strategy aimed at testing the function of *Rs*BtaB. *R. sphaeroides* and *E. coli* strains were incubated with 1-[^{14}C]-methionine, which is taken up and converted into AdoMet by the action of MetK (AdoMet synthetase, see Figure 2.1 (Cohen and Saint-Girons, 1987)), and then used by *Rs*BtaA for DGHS synthesis. The fate of the radiolabel was determined by analysis of the resulting lipids with TLC and autoradiography. The pBtaA/pACYC lane shows accumulation of label in DGHS, and the pBtaA/pBtaB strain shows that a portion of DGHS is converted to DGTS by the action of *Rs*BtaB, as judged by co-migration of the new species with authentic DGTS. A representative result using the solvent system described in Experimental Procedures is given in Figure 2.2*B*, however this species co-migrated in several other solvent systems with authentic DGTS (data not shown). In addition, a less abundant species migrating just below the DGHS spot presumably represents a partially methylated intermediate in this particular solvent system (Figure 2.2*B*). These results suggest that *Rs*BtaB acts as the sole methyltransferase in the pathway.

RsBtaA is associated with membranes. Analysis of the localization of *Rs*BtaA within the *E. coli* cell is given in Figure 2.3. Prediction based on hydropathy analysis (Figure 2.3*A*), (Kyte and Doolittle, 1982) and with the TMHMM algorithm (not shown)

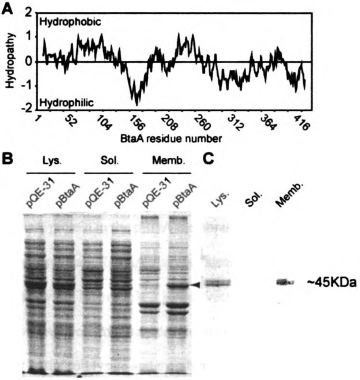


Figure 2.3. **The *Rs*BtaA protein is associated with cellular membranes.** *A*, Hydropathy analysis of *Rs*BtaA. *B* and *C*, *E. coli* cells harboring pBtaA or empty pQE-31 vector were induced, harvested, and separated into a soluble and membrane fraction as described in the text. Twenty μg of protein of each fraction were electrophoresed on a 10% SDS-PAGE gel and (*B*) stained with Coomassie Blue or (*C*) transferred to a PVDF membrane and probed with anti-His tag antibody. The position of His$_6$-BtaA protein (~45 KDa) is indicated.

(Krogh et al., 2001) suggests that *Rs*BtaA is devoid of membrane spanning helices and is very hydrophilic throughout. Based upon this analysis, *Rs*BtaA was predicted to be a soluble protein. Figure 2.3*B* shows the results of the fractionation of a *RsbtaA*-expressing *E. coli* culture by differential centrifugation with the parental pQE-31 vector serving as control. Comparison of the cell lysate (Figure 2.3*B*, left two lanes) and soluble (Figure 2.3*B*, middle two lanes) fractions shows no discernable difference between the two protein profiles, but the membrane fraction (right two lanes) shows enrichment of a ~45 kDa putative *Rs*BtaA protein in the pBtaA membrane fraction (Figure 2.3*B*, arrowhead). The His_6-tagged recombinant *Rs*BtaA was also identified by immunoblotting with anti-His_6 antibody as shown in Figure 2.3*C*. Empty vector controls were not present on the immunoblot shown, but were routinely devoid of cross-reacting bands. Apparently, *Rs*BtaA is present in the cell lysate and membrane fractions, but absent from the soluble fraction, suggesting that it is a membrane-associated protein. The presence of multiple basic residues at the termini of some of the BtaA homologs suggests that the protein is bound to the membrane though electrostatic interactions, similar to some peripheral membrane proteins, e.g. (Wang et al., 2000). This hypothesis is presented schematically in Figure 2.7*A*.

RsBtaA has AdoMet:diacylglycerol 3-amino-3-carboxypropyltransferase activity. *In vitro* assays of *Rs*BtaA enzyme activity were conducted using 1-[^{14}C]-AdoMet to follow product formation, and results are presented in Figures 2.4 and 2.5. Initially we attempted to solubilize and purify *Rs*BtaA utilizing the engineered His_6-tag, but our

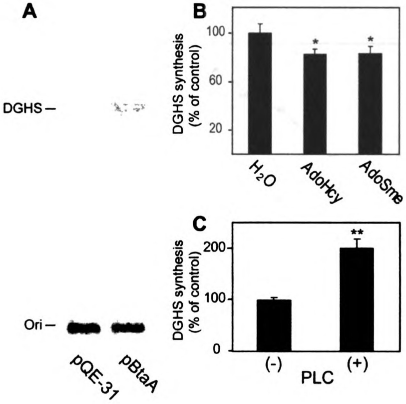


Figure 2.4. **In vitro assay of BtaA activity.** *E. coli* cells expressing *Rs*BtaA were broken by sonication and assayed for the ability to incorporate label from 1-[^{14}C]-AdoMet into DGHS as described in Experimental Procedures. *A*, Lysates from cells harboring pQE-31 or pBtaA plasmids were incubated with labeled AdoMet and products analyzed by TLC. *B*, Incubation of assay mixtures with potential inhibitors of the reactions. *C*, Generation of DAG *in situ* by pre-incubation of assay mixtures with phospholipase C. Statistical significance was assessed with a 2-tailed students-*t* test, and P< 0.05 is indicated by "*", while P<0.01 is indicated by "**". AdoHcy, S-adenosylhomocysteine; AdoSme, 5'-methylthioadenosine; DGHS, diacylglycerylhomoserine; PLC, Phospholipase C.

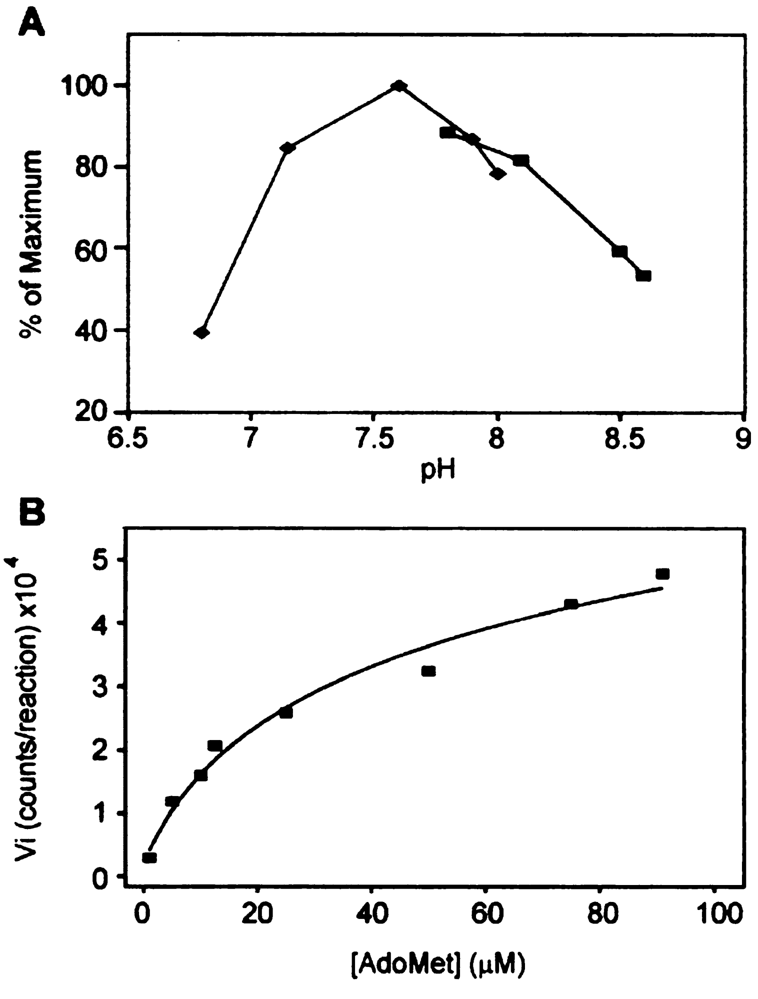


Figure 2.5. **pH dependence of BtaA and affinity for AdoMet.** *A*, Assays were carried out in HEPES or HEPES/Tris buffer at the indicated pH values as described in the text. Reaction mixtures were incubated at 28° C for 1 h, followed by extraction, TLC (method B), and quantification by exposure to a phosphorimager screen *B*, Assays were carried out at pH 7.6 as in the determination of pH optimum, and the concentration of AdoMet was varied.

attempts were unsuccessful. While we were able to purify small amounts of apparently soluble *Rs*BtaA protein using various detergents in the lysis buffer, we were unable to demonstrate activity in a reconstituted liposome system. To circumvent this problem, we developed a system to minimize the steps between expression and enzyme assay to demonstrate the proposed reaction catalyzed by *Rs*BtaA (Figure 2.1). *E. coli* membranes contain small amounts of DAG derived from the synthesis of membrane-derived oligosaccharides (Kennedy, 1987). Thus as the source of enzyme and DAG, a cell-free lysate from pBtaA expressing *E. coli* was used. As demonstrated in Figure 2.4*A*, label from AdoMet was efficiently incorporated into DGHS by transfer of the 3-amino-3-carboxylpropyl moiety to DAG. To probe the effect of potential inhibitors of the reaction (Figure 2.4*B*), S-adenosylhomocysteine (AdoHcy), 5'-methylthioadenosine (5'MTA), and methionine (Met) were tested for their ability to reduce incorporation of label into DGHS. AdoHcy, a product and potent inhibitor of methyltransferases (Fauman et al., 1999), and 5'-methylthioadenosine (the presumed product of *Rs*BtaA) were slightly inhibitory, but Met had no significant effect on the reaction.

In order to determine that DAG is the lipid substrate of BtaA, the cell lysate was incubated with active or heat inactivated phospholipase C (PLC), thus generating DAG *in situ*. Results of this experiment (Figure 2.4*C*) demonstrate that *Rs*BtaA activity is stimulated at least two-fold in the assay mixtures that were pretreated with 10 units of PLC, which corroborates the role of DAG as the lipid substrate.

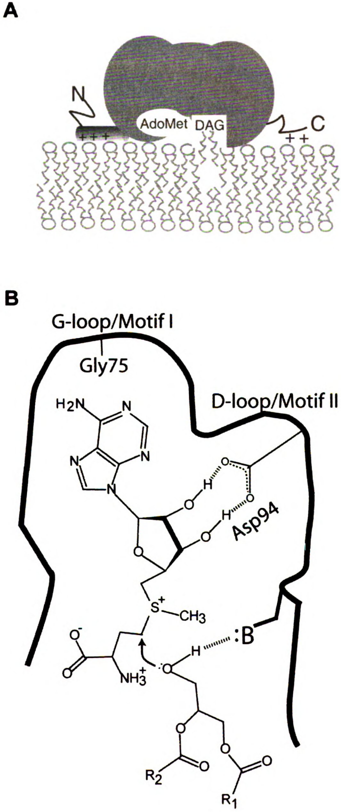
A number of divalent metals including Mg^{++}, Ca^{++}, and Mn^{++} were tested at 1 mM for their ability to stimulate the reaction, but no stimulatory effect was observed, with the possible exception of slight increases in activity by calcium ions (data not shown). This stimulation was variable between different preparations of enzyme, indicating that if Ca^{++} does have a stimulatory effect, it might be indirect, e.g. Ca^{++} could act as a counter ion to membrane phospholipids and thus stabilize the membrane upon which *Rs*BtaA is acting. The metal chelators EDTA and EGTA were tested and were found to have no effect on enzyme activity, corroborating the idea that *Rs*BtaA is probably not a metalloenzyme. *Rs*BtaA was found to have a slightly basic pH optimum of ~7.6 (Figure 2.5*A*), and the K_M toward AdoMet was 27 μM (Figure 2.5*B*).

BtaA is related to methyltransferases. As shown in the multiple sequence alignment of Figure 2.6*A*, BtaA from *R. sphaeroides* has orthologs in a number of other bacteria, predominantly its relatives in the Gram-negative family of α-proteobacteria. The orthologs from *S. meliloti* and *A. tumefaciens* were cloned into pQE-31 essentially as described above for *btaA* from *R. sphaeroides*, and recombinant *E. coli* carrying these constructs also accumulated DGHS (data not shown), indicating that the bacterial *btaA* genes are truly homologous in both sequence and function. All of these BtaA orthologs are encoded in a putative operon of the same structure as that of *R. sphaeroides* (predicted to be transcribed as a bicistronic *btaAB* message (Klug and Benning, 2001)) except for the BtaA-like protein of *Xylella fastidiosa*, which is not accompanied by a *btaB* gene in the predicted operon. Given the fact that BtaB is similar to other small-molecule methyltransferases, is was impossible to unequivocally identify the BtaB ortholog elsewhere in this genome. Using PSI-BLAST (Altschul et al., 1997) to search for other

Figure 2.6. **Multiple sequence alignment of bacterial BtaA orthologs and methyltransferases.** BLAST searching using *R. sphaeroides* BtaA (*Rsp*, AAK53560) as query was used to identify orthologous proteins in finished genomes of *Agrobacterium tumefaciens* (*Atu*, NP_355081), *Sinorhizobium meliloti* (*Sme*, NP_386300), *Mesorhizobium loti* (*Mlo*, NP_103130), *Rhodopseudomonas palustris* (*Rpa*, NP_946080). PSI-BLAST revealed that a domain in the N-terminal half of BtaA is similar to the AdoMet binding motifs of some bacterial methyltransferases. Regions corresponding to methyltransferase motifs I and II are indicated with (****), and GenBank protein accession numbers are used as identifiers for the methyltransferases in the pileup.

```
                           Motif I         Motif II
                            ****            *****
AtuBtaA     58 AMELGEGHRIVTIGSGGCNMLAYLSRNPASIDVVDLNPHHIAL
SmeBtaA     59 AMQIRPGHRIVTIGSGGCNMLTYLSAEPARIDVVDLNPHHIAL
MloBtaA     58 AMQLGQGHRIVTIASGGCNILAYLTRSPARIDAVDLNAAHIAL
RspBtaA     60 ALAIRPGDRLVAIASGGCNVLSYLTQGPGSILAVDLSPAHVAL
RpaBtaA     80 ALQLPIGSTIVTISSGGCNALSYLTAKPAQVFAVDLNEAHLAL
ZP_00081747    YLRSRPGGRLLEIGCGSGTMLSYLGSLGWRTEGIDVDPSAVAN
NP_616375      AAELSPQDTILEIGAGIGNLTERLARRAKKVIAVELDPALVSV
NP_637624      ARVALPGARVLDVGCGGGLLSEAMARLGAQVTAIDLAPELVKV
ZP_00010354    AGRLSPSDRVLEIGCGTGGTAIRLAPGVAQWTATDLSPEMVRI
ZP_00098885    SEVLRADMRVLEVATGTGLMALGIAKFVRQVEATDFSPKMIET
NP_442452      NLAISPGQKVLDLCCGGGQATVYLAQSGATVVGLDASPKALGR
consensus      a--l--g-riv-ig-G---m---l----a-i-avdl-p--i-l
```

Figure 2.7. **Schematic representation of BtaA protein and active site.** *A*, BtaA is proposed to be a peripheral membrane protein interacting with the negatively charged membrane through basic regions present at the N- and C- termini (Figure 2.6A). *B*, Schematic model for the active site of BtaA, with AdoMet binding through motifs I and II as has been shown for methyltransferases. The involvement of a hypothetical general base (:B) to position and activate the 3-hydroxyl of DAG is also proposed.


A
N
AdoMet
DAG
C
+ + +
+ +
B
G-loop/Motif I
Gly75
D-loop/Motif II
Asp94
H2N
N
S+
CH3
:B
H
O
NH3+
R2
R1

protein families that might be related to BtaA, we discovered a number of related, but as yet uncharacterized bacterial methyltransferase-like proteins. A sequence alignment of the pertinent regions, as well as an analysis of putative motifs involved in AdoMet binding (Martin and McMillan, 2002) is shown in Figure 2.6*B*, leading to a prediction of residues involved in AdoMet binding, as presented schematically in Figure 2.7*B*.

Discussion

This work was undertaken to determine the biochemical function of two proteins, BtaA and BtaB, which had both previously been shown by genetic means to be necessary for phosphate starvation-induced DGTS biosynthesis in *R. sphaeroides* (Klug and Benning, 2001). A key result presented here is the demonstration that, not only are BtaA and BtaB necessary for DGTS biosynthesis in *R. sphaeroides*, but that they are also sufficient to provide all the activities in the biosynthetic pathway, as judged by their ability to convey DGTS production in *E. coli*, which normally lacks this lipid. Genetic analysis following the isolation of these genes left this point somewhat unresolved, in that disruption of the *RsbtaB* gene with a kanamycin resistance cassette resulted in the accumulation of both DGHS and a monomethylated form during phosphate starvation, leaving the possibility open that the *Rs*BtaB protein may only act as the methyltransferase for the second and/or third *N*-methylation(s) of the precursor (Klug and Benning, 2001). A more detailed analysis of *Rs*BtaB was prevented by the apparent toxicity of this protein in *E. coli* cells. While low expression sufficient for DGTS biosynthesis was achieved, it was not possible to purify active *Rs*BtaB in amounts permitting further characterization.

However, the *Rs*BtaA protein was conclusively shown to transfer the 3-amino-3-carboxypropyl moiety from AdoMet to DAG. This result is in agreement with the role that had been tentatively assigned based on mutagenesis and ectopic expression in the previous work, and rules out any other contingencies for the activity of *Rs*BtaA. Attempts to solubilize, purify, and reconstitute BtaA into liposomes of defined composition were unsuccessful. In fact, the BtaA activity is sensitive to low concentrations of detergent when, e.g. 0.5 % (w/v) octyl-glucoside or Tween-20 was added to an assay as described above. For this reason, we took the approach of keeping the recombinant enzyme in an as native a state as possible and minimizing the steps between expression and enzyme assay. *E. coli* membranes contain a small amount of DAG derived from the synthesis of membrane derived oligosaccharides (Kennedy, 1987), thus we used the endogenous *E. coli* DAG pool from membranes in the cell-free lysate as substrate to obviate the need for reconstitution into liposomes.

Previous work in a cell-free system on the cognate BtaA-type transferase activity in *C. reinhardtii* had given conflicting results as to the identity of the hydrophobic substrate, indicating that DAG might not be the direct substrate because the addition of exogenous DAG strongly inhibited the reaction (Moore et al., 2001). However, the point was raised that excess DAG might simply disrupt the membrane environment in which the enzyme(s) are working, and the decrease in activity might not be a result of enzyme inhibition, *per se*. The results we present for recombinant *Rs*BtaA seemingly provide a solution to this question, in that incubation of our enzyme/membrane system with PLC stimulated the rate of DGHS formation, and probably represents a milder way of generating DAG *in situ*, versus adding this lipid directly to the reaction.

The kinetic properties of *Rs*BtaA toward AdoMet and its analogs are similar to those of methyltransferases. The apparent K_M for AdoMet of 27 μM that was measured for *Rs*BtaA is in line with values obtained for methyltransferases, which range from ~5-50 μM (Edwards and Dixon, 1991; James et al., 1995; Preisig et al., 1989). *Rs*BtaA was weakly inhibited by *S*-adenosylhomocysteine (AdoHcy), which lacks the activated methyl unit of AdoMet and competitively inhibits methyltransferases (Fauman et al., 1999), and also by its presumed product, 5'-methylthioadenosine, but not by methionine itself. The pH profile showed an optimum at ~pH 7.6, which is similar to that measured for the activity from *C. reinhardtii* (Moore et al., 2001).

Having established that *Rs*BtaA is active in the proposed reaction, and given that the substrate DAG is a minor component of bacterial cell membranes (Kennedy, 1987) we wanted to determine if *Rs*BtaA associates with membranes. Hydropathy analysis using a standard Kyte-Doolittle algorithm (Kyte and Doolittle, 1982) (Figure 2.3*A*) or a more sensitive hidden-Markov model based approach (TMHMM, (Krogh et al., 2001)) to predict transmembrane helices revealed that *Rs*BtaA is actually quite hydrophilic and predicted to be a soluble protein. However fractionation by differential centrifugation (Figure 2.3*B* and *C*) revealed that it is probably a peripheral membrane protein, as it is not present in a soluble form in preparations that are active in the *Rs*BtaA assay, but is instead present solely in membrane containing fractions. Analysis of the multiple sequence alignment in Figure 2.7*A* showed that a segment of 12-15 basic and hydrophobic residues is present in the N-terminus of some of the homologs, as well as a polybasic stretch at the C-terminus. This finding led to the hypothesis that these regions might target and anchor *Rs*BtaA to the inner membrane by forming a basic amphipathic α-helix and providing favorable electrostatic interactions with the anionic membrane surface, as is the case for some other peripheral membrane proteins (Bernstein et al., 2000; Wang et al., 2000).

The *Rs*BtaA enzyme is a member of a small group of enzymes using the ubiquitous alkylating agent AdoMet as a donor of a 3-amino-3-carboxypropyl moiety rather than the methyl group. Other characterized enzymes that catalyze a similar reaction include one involved in antibiotic biosynthesis in *Nocardia uniformis* (Reeve et al., 1998), and spermidine synthase, which transfers the aminopropyl moiety of decarboxylated-AdoMet to putrescine in the polyamine biosynthesis pathway (Korolev et al., 2002). Sequence and structural similarity of these enzymes to AdoMet-dependent methyltransferases has been demonstrated (Korolev et al., 2002; Reeve et al., 1998) and it is clear from these results that these proteins share a methyltransferase fold (Fauman et al., 1999; Martin and McMillan, 2002) with a conserved AdoMet binding pocket. The identity of BtaA as a methyltransferase fold protein was not readily apparent on the basis of sequence analysis, but a detailed comparison of the bacterial BtaA orthologs allowed the identification of putative AdoMet binding motifs. The multiple sequence alignments in Figure 2.6*A* and *B* show that residues corresponding to G75 and D94 in the *R. sphaeroides* protein are completely conserved among the BtaA homologs, and correspond to the conserved residues in AdoMet binding motifs I and II, analogous to the "G-loop" and "D-loop" in other nucleotide binding motifs (Kagan and Clarke, 1994; Martin and McMillan, 2002). This, coupled with the kinetic similarities of BtaA and methyltransferases, provides strong evidence that BtaA also binds AdoMet in a methyltransferase fold. A schematic representation of the active site of BtaA is presented in Figure 2.7*B*, and a model for the predicted topology and binding sites of DAG and AdoMet are represented in Figure 2.7*A*. The involvement of a hypothetical general base in activating the nucleophilic hydroxyl of DAG is also presented in Figure 2.7*A*, however the presence and/or identity of this residue was not predictable from the sequence analysis given the very limited similarity of BtaA to methyltransferases outside of the putative AdoMet binding domain. Taken together, the results presented here represent an initial mechanistic analysis of the enzymes of DGTS biosynthesis, identifying the key step in the pathway, DGHS synthesis, as being catalyzed by a highly divergent methyltransferase-like enzyme.

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Chapter 3

Functions of the bacterial betaine lipid biosynthetic enzymes BtaA and BtaB are carried out by the multifunctional polypeptide BTA1 in *Chlamydomonas reinhardtii*.

Abstract

In bacteria, biosynthesis of the betaine lipid diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine (DGTS) has been shown to be the function of two proteins, BtaA and BtaB, which catalyze all steps of the biosynthetic pathway. Analysis of the genome sequences of the green alga *Chlamydomonas reinhardtii* led to the discovery of a gene encoding a protein with bacterial BtaA- and BtaB-like domains, suggesting that in this organism, DGTS biosynthesis is the function of a single gene product. To test this hypothesis, the coding region of the *CrBTA1* gene was expressed in *E. coli*, which led to DGTS accumulation. Site-directed mutagenesis of the predicted active sites of the BtaA- and BtaB-like domains of *Cr*BTA1 demonstrates the function of the individual domains, and also demonstrates the importance of residues that had been predicted to be involved in AdoMet binding. It is thus concluded that betaine lipid enzymes are highly divergent members of the AdoMet-dependent methyltransferase family.

Introduction

The betaine lipid diacylglyceryl-*N,N,N*-trimethylhomoserine (DGTS) is a major component of the extraplastidic membranes of *Chlamydomonas reinhardtii* (Giroud et al., 1988). Due to its structural similarity to the phospholipid phosphatidylcholine (PtdCho), and the lack of PtdCho in *Chlamydomonas*, DGTS is presumed to take on the functions of PtdCho as the bilayer-forming component of the extraplastidic membranes (Moore et al., 2001). Previous work has shown that DGTS biosynthesis in bacteria is the function of two gene products, BtaA and BtaB, which are required for phosphate stress induced DGTS production in *Rhodobacter sphaeroides* (Klug and Benning, 2001). This work reports the identification and functional characterization of a protein from *Chlamydomonas*, BTA1, containing BtaA and BtaB- like domains in a single polypeptide. This leads to the conclusion that, in *Chlamydomonas*, a single protein is responsible for the bulk of extraplastidic membrane biogenesis.

Materials and Methods

Materials. Restriction enzymes, T4 DNA ligase, calf-intestinal phosphatase, and Klenow fragment were from New England Biolabs (Beverly, MA), *Taq* DNA polymerase was from Roche (Indianapolis, IN), and *Pfu* DNA polymerase was from Stratagene (La Jolla, CA). All other chemicals and solvents were of reagent grade and were from Sigma, EM Science (Gibbstown, NJ) or J.T. Baker (Phillipsburg, NJ).

Cloning and expression of *RsbtaA*, *CrBTA1*, and site directed mutants in *E. coli*. A summary of all strains and plasmids used in this study is presented in Table 3.1. The coding region of *RsbtaA* was PCR-amplified from plasmid pRKL323 (Klug and Benning, 2001) for expression in pACYC-31 (Dörmann et al., 1999) with the following primers (restriction sites underlined): *btaA* forward (*SphI*), 5'-ACATGCATGCAGTGACGCAGTTCGCCCTC-3'; *btaA* reverse(*KpnI*), 5'-CGGGGTACCAGGACGATCCGCTCGAACCG-3'; PCR was carried out with *Taq* DNA polymerase (Roche) according to the manufacturer's specifications, except that 10% DMSO (v/v) was added to each reaction to overcome difficulties in PCR due to the high G+C content of *R. sphaeroides* DNA. The PCR product was first cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA) and sequenced at the MSU Genomics Technology Support Facility, followed by subcloning into pACYC-31 using restriction sites as outlined in the PCR primers (see Table 3.1). The resulting construct was designated pBtaA-LC (*btaA* in pACYC-31). PCR2.1 and pQE-31 derivatives were propagated in LB medium containing ampicillin at 100 µg/ml, and pACYC-31 derivatives in LB with chloramphenicol at 25 µg/ml. Strains containing compatible combinations of plasmids as indicated in individual experiments were constructed by co-transformation of heat-shock competent cells of TOP10F' (Invitrogen) with 100 ng of both plasmids followed by selection on LB containing both ampicillin and chloramphenicol.

C. reinhardtii strain CC125 (wild-type mt+, *Chlamydomonas* culture center, Duke University) was grown to mid-log phase in 50 ml TAP medium (Harris, 1989) and harvested by centrifugation. RNA was purified with Trizol reagent (Invitrogen) according to the manufacturer's instructions. 1.0 µg of total RNA was reverse-transcribed with SuperScript II Rnase H$^-$ reverse transcriptase (Invitrogen), and the coding region of *CrBTA1* was amplified with *Pfu* DNA polymerase (Stratagene) from an aliquot of cDNA with the primers *CrBTA1* forward: 5’-CAGGATCCA**ATG**GGGTCGGGTCGT-3’; and *CrBTA1* reverse: 5’-CAGGTACCGCCGCCAGCTGCTTA-3’. The native start codon of *CrBTA1* in the forward primer is in bold, and *Bam*HI and *Kpn*I sites are underlined in the forward and reverse primers, respectively, and were used to clone the product in-frame with the N-terminal His$_6$-tag of pQE-31, giving rise to pBTA1.

Site directed mutagenesis of pBTA1 was carried out with the Quikchange II XL kit (Stratagene) according to the manufacturer’s instructions. Mutagenic primers were as follows: for the V322A, D323A mutant version of pBta1 (pBTA1µA), primers were µA-fw: 5’-GCCAGGTGGTGTCGGCGGCATGCAACCCCGCGCAG-3’, and its reverse complement, µA-rev. For the V103A, D104A mutant (pBTA1µB), primers were µB-fw: 5’-CAAGTCCATCTACGTGGCCGCCCTGTGCCACTCGCTG-3’, and its reverse complement, µB-rev. Primers were designed such that, in addition to the missense mutations for changing codons, a diagnostic restriction site was created or eliminated, e.g. “µB” destroys a *Sal*I site and “µA” introduces an *Sph*I site, in order to easily identify strains carrying the mutated plasmids.

In vivo production of DGHS and DGTS in *E. coli*. *E. coli* TOP10 F’ cells harboring compatible combinations of plasmids as described in individual experiments were grown as 2 ml overnight cultures, and 0.1 ml of these cultures were used to inoculate 10 ml LB containing appropriate antibiotics. After growing to OD ~0.6, the cultures were harvested by

Table 3.1. Strains and plasmids used in this work.

Strain or plasmid	*Description*	*Source*
C. reinhardtii		
CC125	Wild type	*Chlamydomonas* Genetics Center, Duke University
E. coli		
TOP10F’	Cloning and expression strain, Φ80*lac*ZΔM15, F’ {*lac*I^q Tn10(Tet^R)}	Invitrogen
Plasmids		
pBtaA-LC	*Sph*I-*Kpn*I fragment of *btaA* amplified from pRKL323 in pACYC-31	This work
pBTA1	*Bam*HI-*Kpn*I fragment of *CrBTA1* coding sequence in pQE-31	This work
PBTA1-μA	V322A, D323A derivative of pBTA1	This work
PBTA1-μB	V103A, D104A derivative of pBTA1	This work

centrifugation, and dispersed into 10 ml M9 minimal medium (Sambrook et al., 1989) containing 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) and 0.5 μCi 1-[^{14}C]-methionine (American Radiolabeled Chemicals, St. Louis, MO). Cultures were incubated for 3 h and harvested by centrifugation, followed by extraction with 2 ml chloroform:methanol (1:1, v/v) and phase separation by addition of 0.5 ml 1M KCl, 0.2 M H_3PO_4. A portion of the organic phase from each extraction was spotted onto activated (120° C, 2 h) TLC plates (Silica-60, Baker) and resolved in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v) followed by autoradiography.

In vitro activity assays of *Cr*BTA1. *E. coli* TOP10 F' harboring pBtaA was grown in 250 ml LB ampicillin at 37° C to an OD of 0.7, and induced with 0.25 mM IPTG followed by an additional 4 h of growth at 28° C. Cells were harvested by centrifugation and the cell pellet was suspended in 10 ml of cold buffer (50 mM HEPES, 1 mM DTT, 1 mM EDTA, pH 7.3). The resuspended cells were sonicated 3-4 times, 30 seconds each with a microprobe tip, and the lysate was centrifuged at 2000 g for 10 min to remove unbroken cells and cellular debris. 1 ml aliquots of the cell-free extract were then frozen in liquid N_2 and stored at –80° C prior to use. Activity under these storage conditions did not decrease appreciably for at least 1 month.

Assays for the pH versus activity profile were conducted in 100 μl final volume by combining 48.75 μl of cell free extract with 48.75 μl of 100 mM HEPES, TrisCl, or MES, 1 mM DTT, 1 mM EDTA, at varying initial pH to give a final pH in the range of 5.5-8.6 when mixed with the cell free extract (initial pH 7.3). Reactions were initiated by addition of 25,000 dpm 1-[^{14}C]-AdoMet (American Radiolabeled Chemicals, 2.5 μl, final concentration of AdoMet of 2.1 μM), incubated for 30 min at 28° C, and terminated by addition of 400 μl of chloroform/methanol (1:1, v/v) and 100 μl 0.9% (w/v) NaCl to separate aqueous and organic phases. The organic phase was transferred to a new tube, dried under a stream of nitrogen, and dissolved in 50 μl chloroform/methanol (1:1, v/v). This lipid extract was then spotted onto silica-60 TLC plates (Baker) and developed with the solvent chloroform/acetone/ methanol/acetic acid/water (10:4:2:2:1, v/v), followed by quantification of the signal for DGHS and methylated products on a phosphorimager screen (Molecular Dynamics) with the ImageQuant software package. Determination of K_M was carried out at pH 7.2 with varying concentrations of AdoMet, and determined by direct fitting of a hyperbola to the experimental data in the Origin software package (OriginLab, Northampton, MA). Assays for the effect of proposed inhibitors were performed with 4 μM AdoMet and 100 μM inhibitor added from a 1 mM stock solution in water, and the same volume of water was added to the uninhibited control reaction.

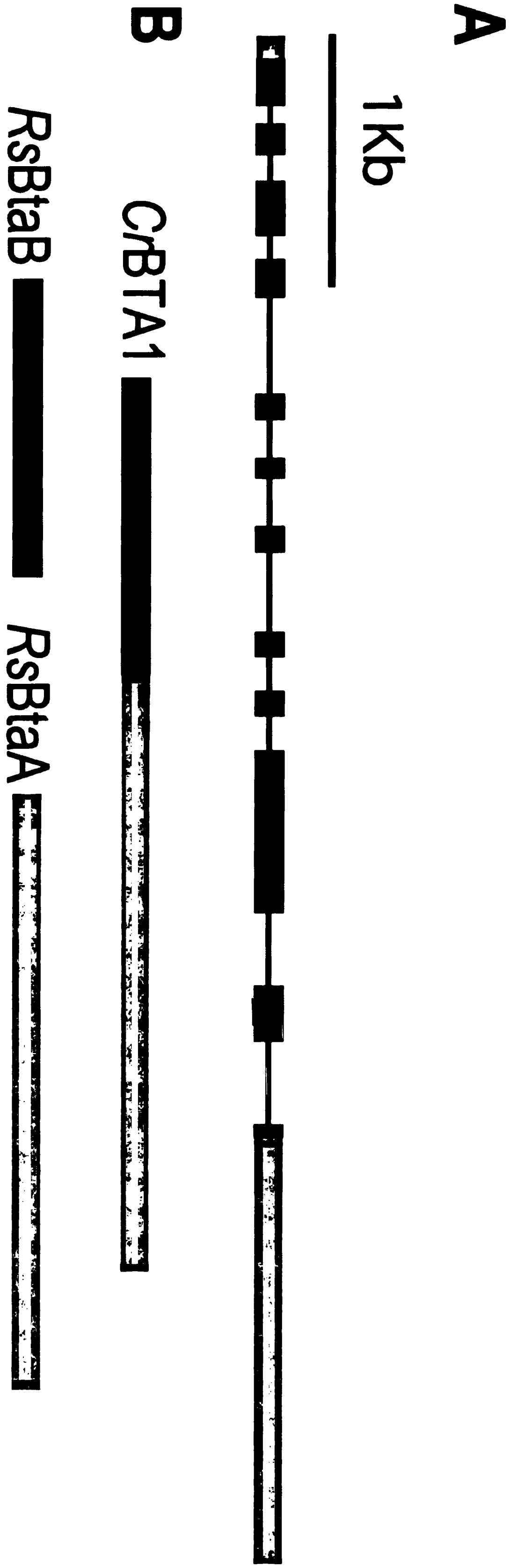
Mass spectrometry of DGTS. DGTS was isolated from lipid extracts of *Cr*BTA1 expressing *E. coli* by preparative TLC on ammonium-sulfate impregnated silica-60 TLC plates in the solvent chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v). The lipid spot was scraped from the plate and eluted in chloroform/methanol (1:1, v/v) and concentrated under a stream of nitrogen. Fast-atom bombardment-mass spectrometry was as previously described (Benning et al., 1995) except that nitrobenzyl alcohol was used as matrix.

Results

CrBTA1 is a multifunctional protein constituting the entire DGTS biosynthesis machinery in *C. reinhardtii*. The unicellular green alga *C. reinhardtii* is unusual among eukaryotes in that its membranes are devoid of phosphatidylcholine, and contain DGTS in its place. Given that a draft sequence of the *C. reinhardtii* genome is available (genome.jgi-psf.org/chlamy, (Grossman et al., 2003)), we took a genomic approach to elucidating the enzymes of DGTS biosynthesis in this organism. Figure 3.1*A* shows the genomic structure of *CrBTA1*, (genome locus ID 15059) encoding a protein consisting of an N-terminal domain with similarity to *Rs*BtaB, and a C-terminal portion with similarity to *Rs*BtaA (Figure 3.1*B* and *C*). The genomic locus is approximately 6 kb in length and the transcript is interrupted 11 times by introns (Fig, 3.1*A*). The predicted mRNA also contains a relatively long 3' untranslated region of ~1.6 kb, which is common to many *C. reinhardtii* transcripts.

The residues identified for *Rs*BtaA as being potentially important in AdoMet binding are conserved in *Cr*BTA1, and two residues in motif II (Kagan and Clarke, 1994) of the BtaA-like domain (V322, D323) are highlighted in Figure 3.1*C*. They were chosen for the mutagenesis experiments described below due to the fact that a V322A, D323A mutant would likely be incapable of binding AdoMet. This mutation would render the protein inactive in the DGHS synthesis reaction, but since the native conformation of the protein would probably be retained, the BtaB-like portion should still be active in the methylation steps. Additionally, the analogous positions in motif II (Kagan and Clarke, 1994) of the BtaB-like portion of the protein (V103, D104) were also targeted for alanine mutagenesis (Fig 3.1*C*). In this case, it was presumed that the

Figure 3.1. **Identification of *Cr*BTA1 as a fusion of BtaA- and BtaB-like domains.** *A*, BLAST searching of the draft genome sequence of *Chlamydomonas reinhardtii* (genome.jgi-psf.org/chlamy) revealed a ~6 kb locus encoding a protein with BtaA- and BtaB-like domains, referred to as BTA1. *B*, Alignment of the BTA1 sequence with BtaB and BtaA.


A
1Kb
B
CrBTA1
RsBtaB
RsBtaA

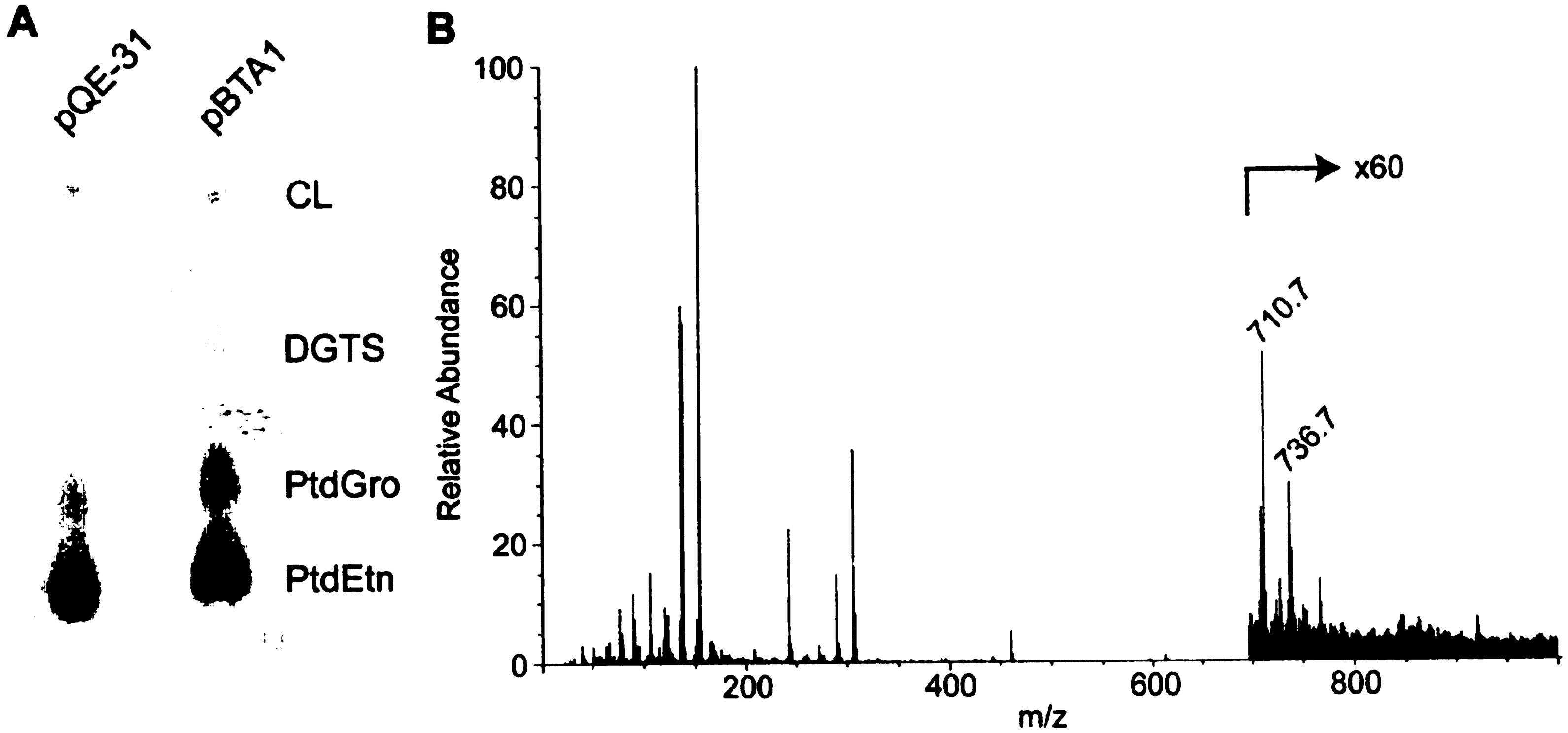
DGHS synthesis activity would be intact, but that the methyltransferase portion would be unable to bind AdoMet and therefore inactive, as a result of the alanine substitutions.

Cloning of the *Cr*BTA1 coding sequence (AY656806) and expression in *E. coli* led to accumulation of relatively large amounts of DGTS, allowing us to verify the structure by mass spectrometry. Fig 3.2A shows a TLC of lipids from *E. coli* expressing *CrBTA1*. It is clear that there is a new species accumulating in the expression strain, and this lipid is subsequently identified as DGTS by positive ion FAB-MS (Fig, 3.2B), and consists of molecular species that are typical of *E. coli* membrane lipids, e.g. the species at m/z 710.7 is DGTS carrying fatty acids totaling (carbons:double bonds) 32:1, and that of m/z 736.7 is a 34:2 species of DGTS. This result verifies the hypothesis that *Cr*BTA1 is capable of carrying out all reactions of DGTS biosynthesis in *C. reinhardtii*, given the fact that it has the same function as co-expression of *Rs*BtaA and *Rs*BtaB in *E. coli*.

Site directed mutagenesis of the AdoMetbinding domains of BTA1.

Site-directed mutagenesis was used to change residues that are predicted to be critical for AdoMet binding in the active sites of the BtaA-like and BtaB-like domains (Figure 3.3). The *Cr*BTA1μA protein is inactive in the synthesis of DGHS, as shown in Figure 3.3, lane 2, with background levels of label in the DGTS or DGHS regions of the chromatogram. BTA1μB, however, produces DGHS (Figure 3.3, lane 3), as confirmed by co-chromatography with the product of a strain carrying *Rs*BtaA on a low copy plasmid, which causes accumulation of DGHS. Lane 5 (Figure 3.3) shows DGTS accumulating in the pBTA1 containing *E. coli* strain, and Figure 3.3, lane 6 shows that the BtaB-like methyltransferase domain in pBTA1μA is still capable of methylating

Figure 3.2: ***Cr*****BTA1 catalyzes the full DGTS biosynthetic pathway.** *A*. *E. coli* cells harboring pBTA1 were grown and expressed as described in Experimental Procedures, and lipids were separated by TLC (method B) and visualized with iodine vapor. A culture containing the empty pQE-31 vector served as a negative control. PtdGro, phosphatidylglycerol; PtdEtn, phosphatidylethanolamine; CL, cardiolipin; DGTS, diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine. *B*. Fast atom bombardment mass spectrometry (FAB-MS) in the positive ion mode was used to confirm the identity of the putative DGTS spot in the TLC. Peaks at m/z 710.7 and 736.7 represent the most abundant molecular species, with acyl chain lengths and total double bonds (carbons: double bonds) of 32:1 and 34:2.


A
pQE-31
pBTA1
CL
DGTS
PtdGro
PtdEtn
B
Relative Abundance
100
80
60
40
20
0
200
400
600
800
m/z
x60
710.7
736.7

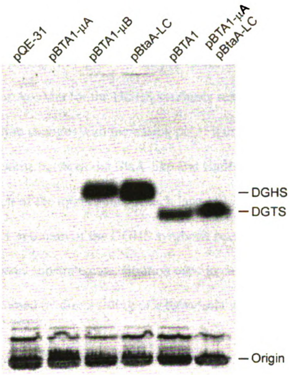


Figure 3.3: **Analysis of BtaA- and BtaB-like domains of BTA1 by site-directed mutagenesis.** *E. coli* harboring combinations of plasmids as described in the text were labeled with 1-[^{14}C]-Met and the lipids separated by TLC (Experimental Procedures, method B), followed by exposure to a phosphorimager. DGHS, diacylglyceryl-homoserine; DGTS, diacylglyceryl-*N*,*N*,*N*-trimethylhomoserine.

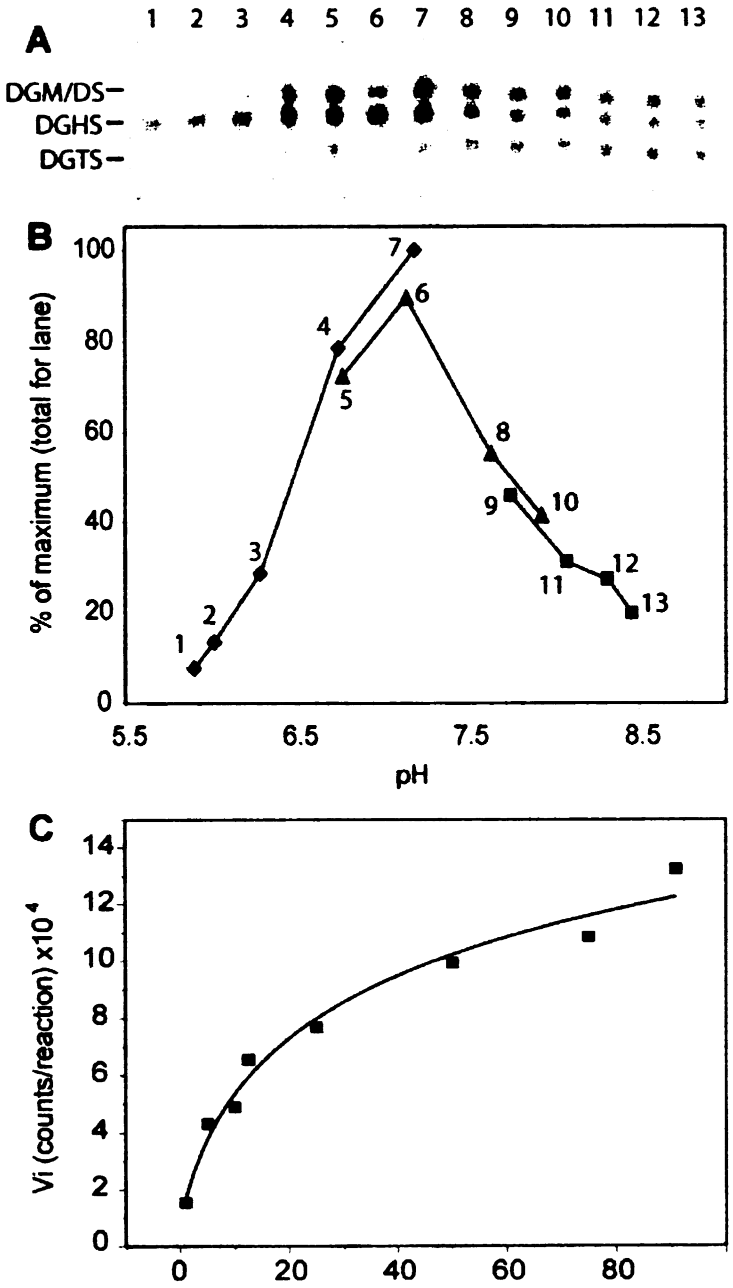
DGHS produced by *Rs*BtaA. These results establish the function of the two domains of *Cr*BTA1 as being comparable to those of the individual bacterial orthologs.

Kinetic analysis of CrBTA1. In Figure 3.4 the pH versus activity profile and determination of K_M for AdoMet for the DGHS synthesis reaction of *Cr*BTA1 are shown. The extent of methylation changes with increasing pH (Figure 3.4*A*), which indicates a difference in the pH optima between the BtaA-like and BtaB-like domains. Figure 3.4*B* shows the quantification of the total counts in each lane of Figure 3.4*A*, plotted versus the pH, which gives the pH optimum of the DGHS synthesis reaction as ~7.2. Figure 3.4*C* shows a plot of rate versus substrate concentration used to calculate the apparent K_M (16 μM), which was determined by direct fitting of a hyperbola to the experimental data.

Discussion

This work was undertaken to determine whether DGTS biosynthesis in eukaryotes is carried out in a similar manner as that of the BtaA/BtaB system of bacteria. Searching of the whole-genome shotgun assembly of *C. reinhardtii* (www.genome.jgi-psf.org/chlamy) with the *Rs*BtaA sequence revealed a predicted protein (locus ID 15059), which we have named *Cr*BTA1, showing 27% identity and 40% similarity to *Rs*BtaA over about 400 amino acids. In fact, not only was *Cr*BTA1 similar to *Rs*BtaA, but it also contained an N-terminal domain with similarity to *Rs*BtaB, leading to the hypothesis that in *C. reinhardtii*, DGTS biosynthesis is the function of a single protein with multiple activities. To test this hypothesis, we expressed the coding region of the *CrBTA1* cDNA (AY656806) in *E. coli*. This resulted in DGTS biosynthesis from expression of the single

Figure 3.4: **Biochemical properties of *Cr*BTA1.** *A*. TLC of products of *Cr*BTA1 at varying pH. Cell lysates were prepared and adjusted to the proper pH as described for *Rs*BtaA, followed by incubation with 1-[^{14}C]-AdoMet (4.2 μM) for 1 hour. Products were extracted and separated by TLC (Experimental Procedures, method B) followed by exposure to a phosphorimager (Molecular Dynamics) screen. *B*, The radioactivity in each lane of the image in (*A*) was quantified in the ImageQuant software package (Molecular Dynamics) and used determine the pH optimum for the DGHS synthase activity. *C*, Determination of apparent K_M (AdoMet) was carried out at pH 7.2 as in the determination of optimum pH, and the concentration of AdoMet]was varied. The apparent K_M was determined by direct fitting of a hyperbola to the experimental data in the Origin software package.


A
1 2 3 4 5 6 7 8 9 10 11 12 13
DGM/DS
DGHS
DGTS
B
% of maximum (total for lane)
0
20
40
60
80
100
5.5
6.5
7.5
8.5
pH
1
2
3
4
5
6
7
8
9
10
11
12
13
C
Vi (counts/reaction) x10^4
0
2
4
6
8
10
12
14
0
20
40
60
80

protein, thus confirming that *Cr*BTA1 contains all activities of the pathway (Figure 3.2). Expression of the BtaA-like portion of *Cr*BTA1 by itself was expected to result in DGHS accumulation, as was shown for *Rs*BtaA, however repeated attempts did not result in DGHS synthesis activity (data not shown). As an alternative way of showing the function of the two domains of *Cr*BTA1 and adding evidence to predictions of the BtaA active site residues, we altered residues in the predicted AdoMet binding site of the BtaA- and BtaB-like domains. The results of this experiment are informative on several levels. First, the fact that the *Cr*BTA1 V322A, D323A mutant (*Cr*BTA1µA) is inactive in synthesis of DGHS and DGTS lends credence to the model that BtaA binds AdoMet in a methyltransferase fold. Given the structure of the cognate binding site of methyltransferases, V322 is expected to make favorable hydrophobic interactions with the adenine moiety of AdoMet, and D323 is proposed to form hydrogen bonds to the 2' and 3' hydroxyls of the ribose portion. Mutation of these residues to alanine renders the DGHS synthase function of *Cr*BTA1 inactive, or at least compromises it to below the limits of detection. The DGHS tri-methylase function is, however, unaffected given that the *Cr*BTA1-µA protein is still capable of methylating DGHS produced by simultaneous expression of *Rs*BtaA (Figure 3.3, lane 6). This provides an additional piece of information regarding the sequence of reactions, showing that the *Cr*BTA1-B-domain is able to take DGHS from the membrane as a substrate, rather than it being transferred directly from the active site of the A-domain as would be the case for substrate channeling. This is in agreement with ***in vitro*** assays conducted on isolated *C. reinhardtii* membranes, as well as results presented in Figure 3.4, showing an accumulation of a transient pool of DGHS.

*Cr*BTA1 activity was measured in a cell-free system as for *Rs*BtaA, and similar enzymatic parameters were observed. The analysis is somewhat complicated by the fact that the methyltransferase is present on the same polypeptide, and thus multiple products are formed. However, measurement of all products on the same TLC plate (Figure 3.4*A*) made this a trivial point, in that the sum of DGHS and its methylated products gives the total flux through the BtaA-like domain, and this quantity was used in the analyses of Figure 3.4*B* and *C*. In fact, Figure 4*A* is informative as to the activity of the BtaB-like (*N*-methyltransferase) portion, showing that the formation of the mono- and dimethylated DGHS intermediates is preferred as the pH increases, and the final methylation is favored at a higher pH still (~pH 8). The pH dependence of these subsequent methylation reactions may reflect a change in pK_a values of the amine species with higher degrees of methylation.

The apparent K_M for the recombinant *Cr*BTA1 activity is presumably directly comparable with that of the activity that was measured in *C. reinhardtii* membranes (Moore et al., 2001), however the value obtained for the recombinant enzyme (~16 μM) was about 5 fold lower than that of the native activity, indicating that the membrane composition and environment of the enzyme may affect the affinity for AdoMet of the DGHS synthase module. This is not surprising, considering that a thorough kinetic description would require both AdoMet and DAG concentrations to be varied, i.e. at higher DAG concentrations (as might occur in *E. coli* membranes) the apparent K_M of the *Cr*BTA1-A domain for AdoMet may be lower. This question remains unresolved given the inability to reconstitute the proteins into the defined lipid environment of a synthetic liposome.

Taken together, the results presented here show that the enzymes and mechanisms of DGTS biosynthesis are conserved between eukaryotes and prokaryotes, with the exciting result that the eukaryotic activities are fused into a single polypeptide. This latter phenomenon is common among metabolic enzymes such as urease, which consists of 3 separate structural genes in *Klebsiella aerogenes* that are fused together on a single polypeptide in legumes (Goldraij et al., 2003), and acetyl-CoA carboxylase, which is present as a multimeric complex in bacteria, but consists of a single multifunctional polypeptide in eukaryotes (Nikolau et al., 2003). Apparently, the same regulatory advantages that are in play in those systems are important in DGTS biosynthesis as well, i.e. that having one gene to encode the machinery for the entire pathway presumably alleviates the coordination required for proper expression of multiple individual genes.

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Chapter 4

Molecular biology of lipid metabolism in *Chlamydomonas reinhardtii.* Analysis of the draft genome for pathways of lipid biosynthesis, and prospects for functional characterization of metabolic pathways by RNA interference. [1]

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[1] Parts of this work involving transformation of *Chlamydomonas* were performed in collaboration with Dr. Barbara Sears, Department of Plant Biology, MSU. The annotation of genes involved in lipid metabolism was carried out as part of the *Chlamydomonas* genome annotation project, under the direction of Drs. Arthur Grossmann (Carnegie Institution of Washington, Stanford University) and Dan Rokhsar (DOE-Joint Genome Institute).

Abstract

Lipid metabolism in seed-plants has been intensively studied at the biochemical, genetic, and genomic levels, and knowledge regarding the identity of genes encoding components of the major fatty acid and membrane lipid biosynthetic pathways is nearing completion. We now present a study of fatty acid and glycerolipid biosynthesis in an algal model, based on EST and genomic sequencing of *Chlamydomonas reinhardtii*. Genes encoding proteins involved in membrane biogenesis were analyzed on the basis of similarity to known proteins, and were organized so as to reconstruct the major pathways of glycerolipid synthesis in *Chlamydomonas*. This analysis accounts for the majority of genes involved in anabolic reactions of membrane lipid biosynthesis, and draws comparisons and contrasts between these pathways in *Chlamydomonas* and higher plants. As a case study in the functional analysis of the annotated genes, we used RNA interference to silence the *BTA1* gene, encoding the machinery for synthesis of the betaine lipid diacylglyceryl-*N,N,N*-trimethylhomoserine (DGTS), the major component of extraplastidic membranes. Silencing of this gene resulted in an attenuation of growth rate and increased sensitivity to heat stress, but showed little effect on the lipid composition of the cells. This may indicate a strong tendency toward membrane homeostasis, and that the reduced capacity for membrane biogenesis is compensated by a reduction in growth rate.

Introduction

Knowledge of genes and proteins involved in lipid metabolism in plants has increased at a phenomenal rate over the past decade. A large number of genes encoding enzymes of glycerolipid biosynthesis and fatty acid desaturation have been identified by genetic and biochemical means, e.g. references (Shimojima et al., 1997; Xu et al., 2002), and even lipid trafficking between the endoplasmic reticulum and plastid is beginning to be unraveled by forward genetic approaches (Xu et al., 2003). In addition, the availability of the Arabidopsis genome sequence (The Arabidopsis Genome Initiative, 2001) has facilitated the annotation of many uncharacterized gene products which are likely to have activity in lipid biosynthesis, trafficking, and catabolism (Beisson et al., 2003). Although Arabidopsis is undoubtedly the most widely used higher plant model system currently under investigation, the eukaryotic green alga *Chlamydomonas reinhardtii* is a well established model for many processes such as photosynthesis (Niyogi, 1999), phototaxis and flagellar function (Silflow and Lefebvre, 2001), post-transcriptional gene silencing (Wu-Scharf et al., 2000), and nutrient acquisition (Davies et al., 1996). The recent large-scale EST projects and sequencing of the *C. reinhardtii* genome (Shrager et al., 2003; Grossman et al., 2003), as well as the development of insertional mutagenesis (Tam and Lefebvre, 1993), RNA interference (RNAi) methods (Sineshchekov et al., 2002; Fuhrmann et al., 2001), and a molecular map (Kathir et al., 2003) make *Chlamydomonas* an attractive model in which to study gene function by both forward and reverse genetics in a unicellular photosynthetic eukaryote. Additionally, *Chlamydomonas* is ideal as a model organism in which to study the biosynthesis of non-phosphorous lipids such as the plastidic galacto- and sulfolipids, and the extraplastidic betaine lipid diacylglyceryl-

N,N,N-trimethylhomoserine (DGTS) (Giroud and Eichenberger, 1989; Moore et al., 2001), as well as phospholipids such as phosphatidylethanolamine (PtdEtn)(Yang et al., 2004). We have begun to use *Chlamydomonas* as a model for plant lipid metabolism, first using a high-throughput robotic screening methodology to find mutants with defects in lipid metabolism (Riekhof et al., 2003; Chapter 5), and using genomic resources to identify and characterize the pathway of betaine lipid biosynthesis (Chapter 3). We now present a study of genes involved in acyl-lipid metabolism, as gleaned from annotation of the v2.0 draft genome sequence available at http://genome.jgi-psf.org/chlamy/. Enzymes of fatty acid biosynthesis, glycerolipid synthesis, and fatty acid desaturation were identified and organized so as to reconstruct the lipid biosynthesis pathways and posit their subcellular localization. As a case study in the functional analysis of these annotated genes, we used RNAi to suppress the expression of the *BTA1* gene, encoding the protein responsible for synthesis of the major extraplastidic glycerolipid DGTS (Chapter 3). Suppression of *BTA1* led to pleiotropic effects on growth, while showing little effect on lipid composition. We discuss these results in the context of the coordinated regulation of membrane biogenesis and cellular growth.

Materials and Methods

Annotation of *C. reinhardtii* genes involved in lipid metabolism. Version 2.0 of the *C. reinhardtii* whole genome shotgun sequence assembly is available at http://genome.jgi-psf.org/chlamy. Genes encoding enzymes and other components of lipid metabolism (e.g. acyl-carrier proteins) were manually annotated based on keyword searches executed against the automatically annotated gene loci (Grossman et al., 2003), followed by verification of similarity by BLASTP searches (Altschul et al., 1997) and CLUSTALW multiple sequence alignments (Thompson et al., 1994) with verified protein sequences. In addition, direct BLASTP and TBLASTN searches of the v2.0 assembly were carried out with known protein sequences from plants, animals, fungi, and bacterial sources to add additional evidence and to identify genes that were incorrectly annotated by the automated system.

For prediction of subcellular localization, the programs TargetP and ChloroP were used (Emanuelsson et al., 1999; Emanuelsson et al., 2000). In cases where the N-terminus was not available due to sequence gaps and/or ambiguity in prediction of the first exon, tentative localization was predicted on the basis of their clustering with known isoforms from other species.

Construction of RNAi lines for suppression of *CrBTA1*. As the basis for the target of the anti-*BTA1* dsRNA construct, we chose a long EST from the middle section of the gene (GenBank accession BU651274). Briefly, nucleotides 1-467 were fused in the sense orientation to the RBCS2 5' promoter element with overlap-extension (OE) PCR, and this construct was ligated to the anti-sense oriented bases 1-697, forming a 467 bp dsRNA stem and a 230 bp loop (Figure 4.3).

Pfu Turbo DNA polymerase (Stratagene) was used according to the manufacturers protocol in all PCR reactions described below, except that 10% (v/v) DMSO was added to all reactions to remedy problems associated with amplification of the highly G+C rich sequences of *C reinhardtii*. The RBCS2 5' element was amplified from plasmid pSP124S (Lumbreras et al., 1998) with the primers RBCS fw-

CGAGCTCAAATGCCAGAAGGAGCG (*Sac*I underlined), and RBCS rev-CCTGGGTGTTCTGCTCCTCACCTGGCCATTTTAAG. For RT-PCR of *BTA1* sequences, RNA was isolated from mid-log phase *C. reinhardtii* strain CC125 with Trizol reagent (Invitrogen) according to the manufacturer's instructions, and 1.0 μg total RNA was used for first-strand cDNA synthesis with SuperScript II RNase H$^-$ reverse transcriptase (primed with oligo-dT) according to the manufacturer's protocol. 2 μl aliquots of the RT reaction were used as template in 50 μl PCR reactions. The sense-oriented portion of *CrBTA1* was amplified with the primers "*BTA1* sense fw"-CTTAAAATGGCCAGGTGAGGAGCAGAACACCCAGG; and "*BTA1* sense rev"-CGGATCCGCGCTTGGACCAGAAGT (*Bam*HI underlined). For overlap-extension PCR, the products of "RBCS fw/rev" and "*BTA1* sense fw/rev" were gel purified, and 10 ng of each product were mixed for use as the template in amplification of the fusion product with "RBCS fw" and "BTA1 sense rev" primers. Amplification of the antisense portion was with primers "*BTA1* anti fw"- CGGATCCCACCAGGCTCACGAACTT (*Bam*HI underlined), and "*BTA1* anti rev"- CGAATTCGGAGCAGAACACCCAGG (*Eco*RI underlined). All products were gel purified and cloned into the *Eco*RV site of pBluescript SK+and sequenced at the MSU genomics technology support facility. For RNAi plasmid construction, the RBCS::sense fusion and antisense segment were excised and cloned into pSP124S by a three piece ligation as indicated by the *Sac*I, *Bam*HI, and *Eco*RI sites in Figure 4.3, to give plasmid p*BTA1*-RNAi.

Transformation with RNAi construct and growth of cells.

The pSP124S plasmid and p*BTA1*-RNAi construct described above were used to transform strain dw15.1 (mt+, nit1-305, cw15) by the glass bead method (Kindle, 1998), except that TAP medium was used for cell growth, and transformation mixtures were plated in a slurry of cornstarch, as described (Shimogawara et al., 1998), onto TAP +1% yeast extract (TAPY) containing 10 μg/ml Zeocin (Invitrogen). Approximately 50 transformant colonies of each construct were propagated, and initial tests for growth phenotype were carried out in 1.5 ml TAP cultures in 24 well microtiter dishes.

For all physiological and growth experiments, cultures were grown in TAP medium with shaking (200 rpm) under constant cool white fluorescent light, at 24° C. Analysis of growth rates was carried out by inoculation of cells into 50 ml TAP medium to an initial density of OD_{750} =0.05, and growth was measured by taking the OD_{750} at regular intervals.

Temperature sensitivity was assessed by streaking a loop of freshly grown cells as patches onto TAPY plates, followed by incubation of the plates in the dark at 24, 37, or 42° C for 16 or 40 h. Following the challenge at high temperature, the plates were incubated in the light at 24° C for 3 days to assess survival.

RNA blot analysis

For the analysis of transcript levels in the RNAi and wild type strains, 50 ml cultures were inoculated and grown for two days, as in the growth rate analysis. Cultures were harvested by centrifugation, and total RNA was extracted from cell pellets with Trizol reagent (Invitrogen) according to the manufacturer's instructions. 5 μg of RNA was separated on a formaldehyde-agarose gel followed by blotting onto HyBond

(DuPont) membranes, and probing with the full length *CrBTA1* open reading frame (GenBank accession AY656806) (Sambrook et al., 1989).

Analysis of lipids

For the quantitative analysis of polar lipids, 50 ml cultures of the indicated strains were grown to late log phase in TAP medium, as for the growth rate analysis. Cells were collected by centrifugation and lipids were extracted from the cell pellets by vigorous vortexing in 4 ml of chloroform: methanol (1:1, v/v), followed by phase separation with 1 ml of 1 M KCl, 0.2 M H_3PO_4. For separation of polar lipid classes, a 2-dimensional thin-layer chromatography (silica 60 plates, J.T. Baker) system was employed consisting of chloroform: methanol: water (65/25/4, v/v) in the first dimension and chloroform: acetone: methanol: glacial acetic acid: water (10/4/2/2/0.75, v/v) in the second dimension. Polar lipid species were detected by brief exposure to iodine vapor, and the spots containing each lipid were scraped into a tube with a razor blade, followed by addition of 5.0 μg myristic acid (C14:0) as an internal standard. This preparation was then transmethylated and analyzed by GC as described (Rossak et al., 1997). As previously reported (Giroud et al., 1988), myristic acid is present in the total fatty acid pool (data not shown), however this fatty acid was absent from the polar lipid classes that we measured, and therefore 14:0 was suitable as an internal standard for quantification.

Results

Annotation of lipid biosynthesis genes in the *Chlamydomonas* genome.

We analyzed lipid biosynthetic pathways in *Chlamydomonas* by searching the whole genome shotgun sequence for proteins with significant similarity to known enzymes from plants and other organisms. Figures 4.1 and 4.2 and the accompanying Table 1 present a scheme for membrane lipid biosynthesis, starting with fatty acid synthesis in

the plastid (Figure 4.1, steps 1-4 and 16), plastidic glycerolipid biosynthesis (Figure 4.1, steps 5-15), extraplastidic phospholipid and betaine lipid biosynthesis (Figure 4.1, steps 16-31), and fatty acid desaturation, (Figure 4.2, steps 32-40).

Lipid metabolism in plastids.

Genes encoding enzymes of plastidic lipid metabolism are readily identifiable in the genome, and appear to be most similar to those of higher plants. For example, it is clear that all components of the multimeric bacterial-type acetyl-CoA carboxylase and fatty acid synthase (FAS) complexes are accounted for, similar to those that have been described for higher plant plastids. Interestingly, many of the enzymes central to fatty acid biosynthesis, e.g. the 3-hydroxyacyl-ACP-dehydratase and enoyl-CoA-reductase components of the type II FAS, are apparently present in a single copy in the genome, and have high and roughly equal probabilities of being targeted to both mitochondria and chloroplasts as judged by TargetP analysis (Emanuelsson et al., 2000).

The pathway of sulfoquinovosyldiacylglycerol (SQDG) biosynthesis has been thoroughly characterized in Arabidopsis and shown to be the function of two gene

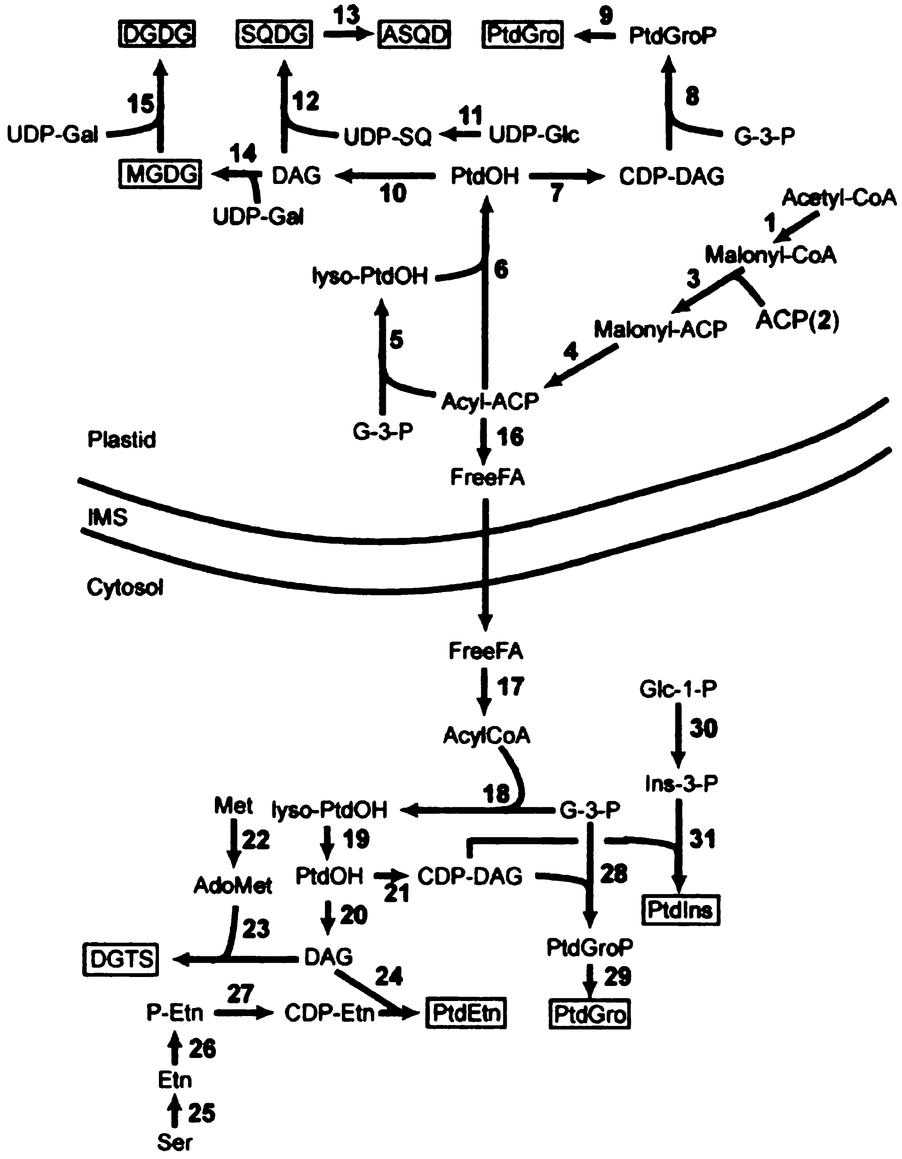

Figure 4.1: Pathways of lipid biosynthesis which are known or hypothesized to occur in *Chlamydomonas*, and their subcellular localization. Numbers in bold correspond to the activities summarized in TABLE 1, which also correlates gene models with their respective putative activities.

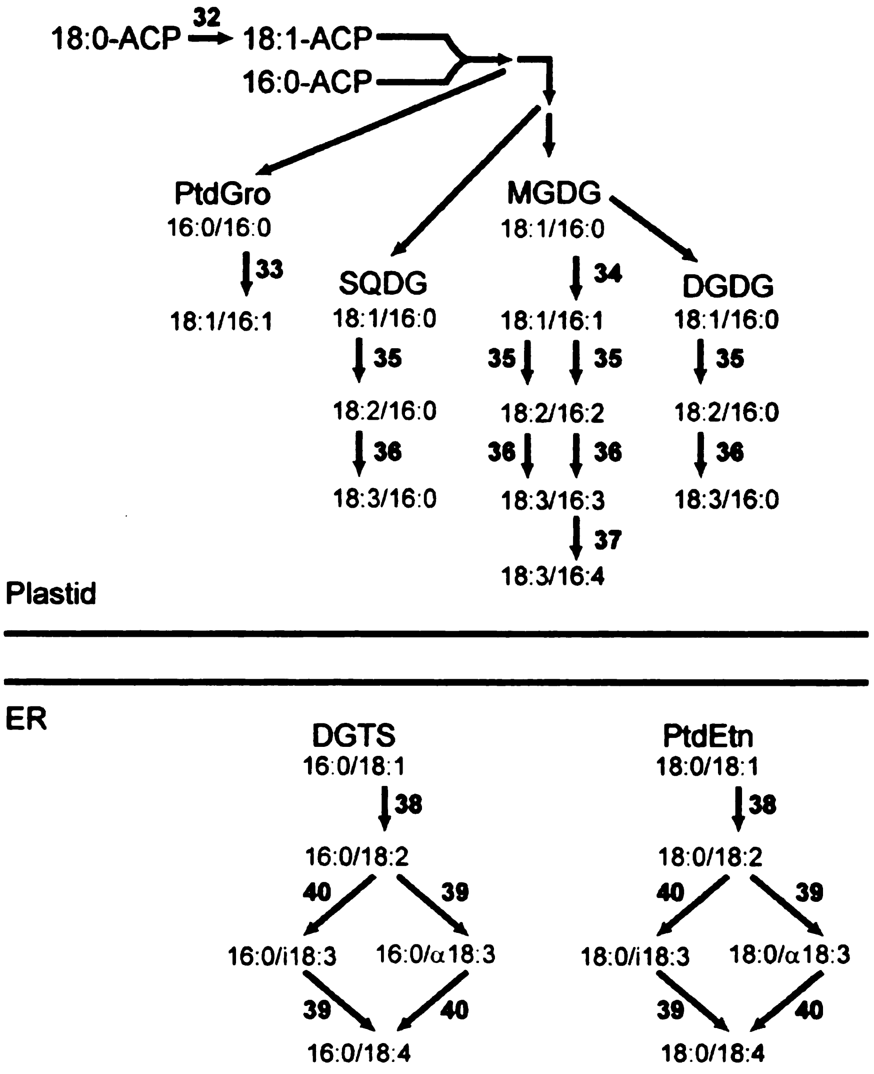

Figure 4.2: Pathways of acyl chain desaturation, with numbers in bold serving to cross reference the specific reaction with the gene model(s) in Table 1.

Table 4.1: Identification of the reactions diagrammed in Figures 4.1 and 4.2, and assignment of candidate genes on the basis of identity with known proteins and subcellular prediction based on analysis of the N-terminus. Candidates for dual targeting to chloroplast and mitochondria are highlighted.

PLASTID AND MITOCHONDRIAL PATHWAYS

Reaction	Description	Gene model	# ESTs
1	**Multimeric Acetyl-CoA carboxylase components:**		
1a	Alpha-carboxyltransferase	C_160059	23
1b	Beta-carboxyltransferase	C_1150054	39
1c	Biotin carboxylase	C_2010018	18
1d	Biotin carboxyl carrier protein	C_590042 C_250035	16 27
2	Acyl carrier protein	C_650040 C_210028	38 51
3	Malonyl-CoA: ACP transacylase	C_240122 C_7350001	4 16
4	**Type II Fatty acid synthase components:**		
4a	3-ketoacyl-ACP synthase	C_140066 C_720014 C_1320034	17 7 2
4b	3-ketoacyl-ACP reductase	C_460059	19
4c	3-hydroxyacyl-ACP dehydratase	C_120121	7
4d	enoyl-ACP-reductase	C_190023	64
5	Glycerol-3-P: Acyl-ACP acyltransferase	C_1210022	8
6	lyso-phosphatidate: Acyl-ACP acyltransferase	no candidate	
7	CDP-DAG synthetase	C_30067	12
8	phosphatidylglycerophosphate synthase	C_140036 C_2150006	21 4
9	phosphatidylglycerophosphate phosphatase	no candidate	
10	phosphatidate phosphatase	no candidate	
11	UDP-sulfoquinovose synthase	C_60107	46
12	sulfolipid synthase	C_250126 C_650023	5 0
13	sulfolipid 2'-O-acyltransferase	no candidate	
14	monogalactosyldiacylglycerol synthase	C_21260001	3
15	digalactosyldiacylglycerol synthase	C_230128	1
16	Acyl-ACP thiolase	C_200208	7

CYTOSOLIC PATHWAYS

Reaction	Description	Gene model	# ESTs
17	long chain acyl-CoA synthetase	C_1580041 C_7940001	11 0
18	Glycerol-3-P: Acyl-CoA acyltransferase	no candidate	
19	lyso-phosphatidate: Acyl-CoA acyltransferase	no candidate	
20	phosphatidate phosphatase	C_1170013 C_300097	1 4
21	CDP-DAG synthetase	C_630023	4
22	AdoMet synthetase	C_750005	82
23	Betaine lipid synthase	C_570065	42
24	CDP-Etn: DAG Etn phosphotransferase	C_960032	2
25	Serine decarboxylase	C_250130	43
26	Ethanolamine kinase	C_290062	0
27	CTP: phosphoethanolamine cytidylyltransferase	C_960052	15
28	Phsphatidylglycerophosphate synthase	C_1350041	0

29	phosphatidylglycerophosphate phosphatase	no candidate	
30	Inositol-3-phosphate synthase	C_860083	31
31	CDP-DAG: Inositol phosphotransferase	C_260084	3
FATTY ACID DESATURASES			
Plastidic Isoforms (as judged by ChloroP analysis)			
32	Stearoyl-ACP-Δ9-desaturase	C_170008	10
33	PtdGro palmitate-Δ3t-desaturase	no candidate	
34	MGDG palmitate-Δ7-desaturase	C_330009	70
35	ω-6 desaturase	C_790066	264
		C_290028	0
36	ω-3 desaturase	C_250125	39
37	MGDG 16 carbon Δ4-desaturase	no candidate	
Cytosolic isoforms			
38	Oleate desaturase	C_590003	20
		C_4630001	0
39	Linoleate desaturase	C_250037	4
40	Linoleate/linolenate-Δ5-desaturase	C_1000063	3
		C_1000033	4

products, namely SQD1 and SQD2 (Essigmann et al., 1998; Yu et al., 2002). SQD1 has been characterized in *Chlamydomonas* by both insertional inactivation (Riekhof et al., 2003) and complementation of a sulfolipid deficient *sqdB* cyanobacterial mutant (Sato et al., 2003), and the *SQD1* gene is highly expressed as judged by EST abundance. This apparent disproportionately high mRNA abundance might be due to the relatively large amount of SQDG in *Chlamydomonas* as compared to Arabidopsis, coupled with the fact that K_{cat} for the reaction catalyzed by SQD1 is very low (Sanda et al., 2001), which might necessitate increased abundance of the enzyme.

Genes encoding enzymes of galactolipid biosynthesis are also present in the genome, with MGD1 (monogalactosyldiacylglycerol synthase) and DGD1 (digalactosyldiacylglycerol synthase) genes present in the genome as single copies, as opposed to three MGDG and two DGDG synthase isoforms in Arabidopsis (Awai et al., 2001; Kelly and Dormann, 2002).

Several functions in plastidic lipid biosynthesis are without candidate genes including the phosphatidylglycerophosphate phosphatase (Figure 4.1, step 9), which is unknown in plants (Beisson et al., 2003), and a putative activity utilizing SQDG and an unidentified acyl donor to form 2'-*O*-acyl-SQDG (ASQD, Figure 4.1, step 13). This species has been shown to be a minor component of *Chlamydomonas* lipids, however it is apparently absent from higher plants (Riekhof et al., 2003; Chapter 5). While no candidate can be predicted, several serine carboxypeptidase-like enzymes responsible for catalyzing similar transacylation reactions in phenylpropanoid metabolism have been identified (Lehfeldt et al., 2000; Shirley et al., 2001), and it is conceivable that a similar mechanism is operative in ASQD biosynthesis. Additionally, phosphatidate phosphatase

(Figure 4.1, step 10) has at least two recognizable homologs in the genome, however neither of them are predicted to contain a plastid transit peptide.

Lipid metabolism in the endomembrane system.

Cytosolic membrane lipid metabolism in *Chlamydomonas* differs from that of higher plants by both the presence and absence of certain central pathways and branches of pathways. The most notable absence is phosphoethanolamine methyltransferase, which provides the pathway for methylation of phosphoethanolamine to form phosphocholine. This activity is responsible for the generation of phosphocholine for conversion into CDP-choline and then PtdCho, and the enzymes from spinach and Arabidopsis have been functionally identified by their ability to rescue a *cho2* mutant of *Schizosaccharomyces pombe* or an *opi3* mutant of *Saccharomyces cerevisiae*, respectively (Nuccio et al., 2000; Bolognese and McGraw, 2000). The apparent lack of a pathway for PtdCho precursor biosynthesis in *Chlamydomonas*, as well as a lack of the PtdEtn methylation pathway, correlates with a lack of this lipid from cellular membranes (Giroud et al., 1988).

PtdSer is a major precursor of PtdEtn in yeast, bacteria, and plants through the action of PtdSer decarboxylase (Voelker, 1997), however *Chlamydomonas* has been reported to lack PtdSer as a component of its membranes (Giroud et al., 1988). This fact is evident in the genome as an apparent lack of both phosphatidylserine synthase and phospholipid base exchange enzymes. This suggests that the biosynthesis of PtdEtn is the function of a single pathway, consisting of serine decarboxylase (Rontein et al., 2001) to generate Etn (Figure 4.1, step 25) followed by phosphorylation of

Etn (Figure 4.1, step 26), activation with CTP to form CDP-Etn (Figure 4.1, step 27), and transfer of the phosphoethanolamine moiety to DAG, forming PtdEtn (Figure 4.1, step 24). The *Chlamydomonas* CTP: phosphoethanolamine cytidylyl transferase enzyme has recently been characterized through expression in *E. coli* and biochemical characterization of the recombinant protein (Yang et al., 2004).

In the absence of PtdCho, the betaine lipid DGTS has been proposed as the bilayer forming and major structural component of extraplastidic membranes (Giroud et al., 1988; Sato, 1992). DGTS biosynthesis is catalyzed by a multifunctional enzyme utilizing DAG and 4 equivalents of AdoMet to form this lipid (Chapter 3) and this gene is annotated as *BTA1* (Figure 4.1, step 23).

Certain pathways are assumed to be present in multiple compartments. For example, PtdGro biosynthesis is proposed to be present in the plastid, mitochondria, and endoplasmic reticulum, and 3 isoforms of the phosphatidylglycerophosphate synthase are present in the genome. Two of these (Figure 4.1, step 8) are expressed as judged by EST analysis and both contain a predicted plastid or mitochondrial targeting sequence. The other is predicted to be cytosolic, but is not represented in the EST database. This may correlate with the fact that the mass of PtdGro present in the cytosol is predicted to be much less than that in the plastid (Giroud et al., 1988), and thus the gene encoding the cytosolic activity might not be as highly expressed as those directing PtdGro synthesis in the organelles.

Fatty acid desaturases.

In addition to the synthesis of the glycerolipids themselves, fatty acid desaturases (FAD) were identified and annotated in this survey. *Chlamydomonas* contains several fatty acids not found in Arabidopsis, namely $16{:}4^{\Delta 4,7,10,13}$, which is found predominantly in plastidic MGDG, as well as $18{:}3^{\Delta 5,9,12}$ and $18{:}4^{\Delta 5,9,12,15}$, the bulk of which are present as components of DGTS (Giroud et al., 1988; Giroud and Eichenberger, 1989). The FAD complement of Arabidopsis has been well characterized at the genetic and biochemical levels (Ohlrogge and Browse, 1995), and homologs are present in the *Chlamydomonas* genome, as outlined in Figure 4.2. Of note are the putative extraplastidic ω6 and ω3 desaturases (Figure 4.2, steps 38-39), as well as two additional, apparently extraplastidic FAD-like enzymes that are most similar to the plastidic palmitate specific Δ7 desaturase (FAD5).

Likewise, there are predicted plastidic homologs of the Arabidopsis FAD5, FAD6 and FAD7/8 enzymes ((Ohlrogge and Browse, 1995); Figure 4.2, steps 34-36), and the plastidic ω-6 desaturase of *Chlamydomonas* (Figure 4.2, step 35) has been previously identified in a mutant screen for high-chlorophyll fluorescence mutants, and was subsequently cloned and characterized (Sato et al., 1995; Sato et al., 1997). We were unable to assign a candidate for the Δ4 desaturase involved in generation of $16{:}4^{\Delta 4,7,10,13}$ (Figure 4.2, step 37), although the gene encoding this protein would likely be expressed at a high level, given the EST abundance for the other enzymes in this desaturation pathway.

Silencing of the *BTA1* gene is detrimental to growth.

To begin the functional analysis of genes annotated as having putative functions in acyl-lipid biosynthesis, we chose to target *BTA1* for silencing with a dsRNA hairpin

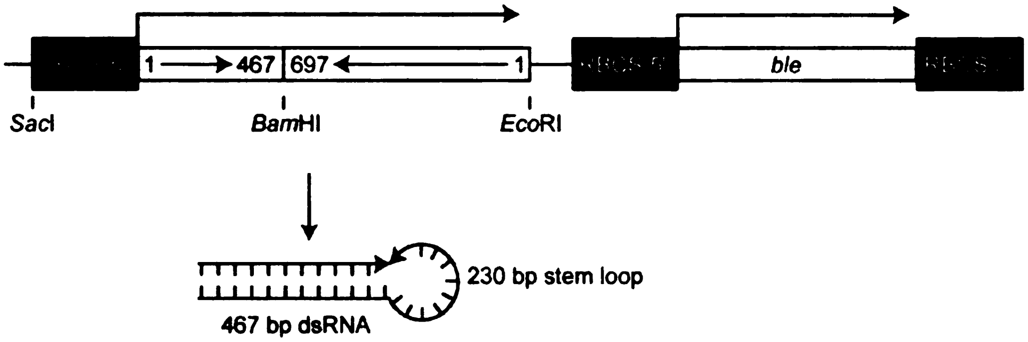

Figure 4.3: Schematic of RNAi construct. A long EST from the middle of the BTA1 gene was used as the basis for design of a dsRNA hairpin, as in described Materials and Methods. The parental vector was pSP124S, and the vector and RNAi constructs were transformed into Chlamydomonas dw15.1, and transformants selected on Zeocin (Invitrogen).

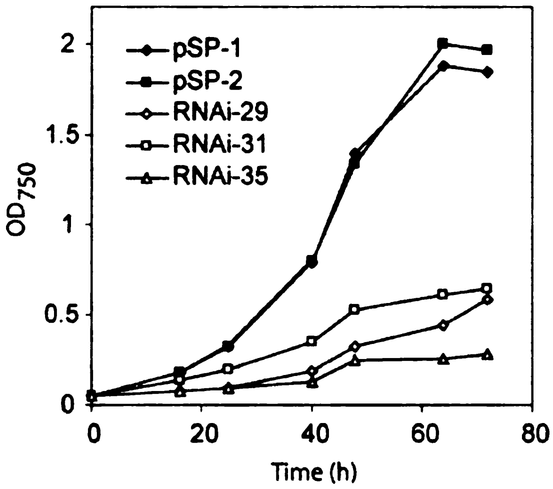

Figure 4.4: Growth curves of RNAi lines and vector controls. Cultures were inoculated at an initial OD_{750} of 0.05, and growth was monitored by measuring the OD_{750} at regular intervals.

construct (Figure 4.3). Transformation of this plasmid and the pSP124S vector into *Chlamydomonas* strain dw15.1 resulted in many Zeocin resistant colonies, and approximately 50 transformants of both the vector and RNAi constructs were subcultured and analyzed for defects in growth and lipid metabolism. Several lines of the RNAi transformants, designated i29, i31, and i35, showed alterations in growth rate and temperature sensitivity, growing at between 20 to 40 % of the rate of the control lines containing only vector (Figure 4.4). The growth of these lines on agar plates containing 1 % yeast extract was also slower than the vector controls, but the growth phenotype was slightly more pronounced in liquid culture. The growth rate defect also correlated with more pronounced temperature sensitivity. Lines i29, i31, and i35 were unable to survive incubation for 16 h at 42° C, while lines pSP1, pSP2 (vector controls), and i15 (containing the RNAi-construct but showing no growth phenotype) survived this treatment. i35 was unable to survive 40 h at 37° C, whereas the other strains tolerated this treatment, indicating that this line was both the most severely inhibited in growth rate and tolerance to high temperature.

Northern analysis of steady-state mRNA levels was conducted on the slow-growing RNAi lines and the vector controls. Cells were incubated for 48 h in 50 ml TAP cultures as for the growth rate analysis, to ensure that each strain was growing at its maximal rate. As shown in Figure 4.5, the growth phenotype correlates with a reduction of mRNA levels. The silencing of this gene was not complete, but one would not expect to recover strongly suppressed lines of what is likely to be an essential gene.

Figure 4.6 gives the polar lipid composition of the vector and RNAi lines. To our surprise, the relative amounts of the different polar lipid classes were essentially

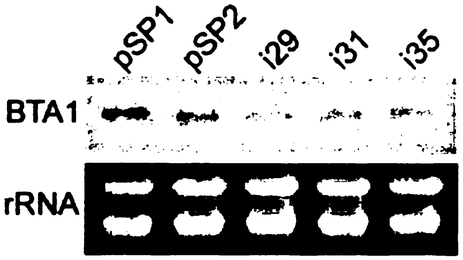

Figure 4.5: *BTA1* gene expression in RNAi lines. Cultures of pSP124S transformed controls and RNAi transformants were cultivated as in the growth curve experiment, and the cells were harvested after 48 h, followed by RNA isolation and northern blotting with 10 μg of RNA per lane. The respective lines are indicated above each lane, and the ethidium bromide stained gel serves as an indicator of equal loading as judged by rRNA intensity.

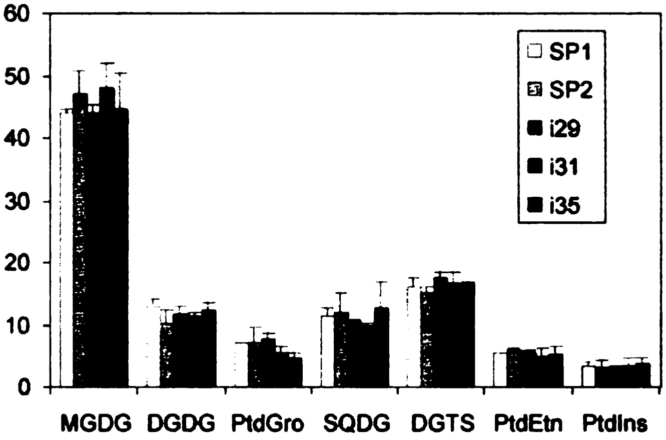

Figure. 4.6: Lipid composition of RNAi lines. Cultures were inoculated and grown as for the growth rate analysis, and were harvested after 72 h of incubation. Lipids were extracted from the cell pellets and resolved by TLC, followed by quantitative analysis by GC. Values are expressed as mole percentages (mol/100mol), and the quantities given are averages of two independent determinations, with error bars representing the range of the 2 values.

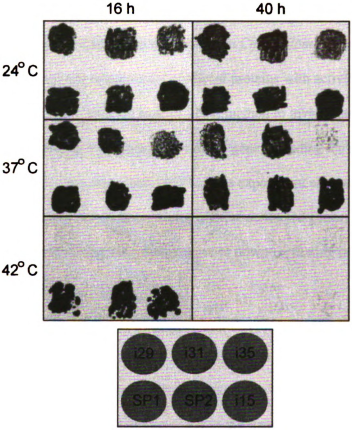

Figure 4.7: Temperature sensitivity of RNAi lines. The respective lines were freshly streaked onto TAPY plates and kept in the dark for 16 or 40 h at the indicated temperature. Following this treatment, plates were kept in the light at 24° C for several days before photographing the plates.

unchanged in the RNAi suppressors of *BTA1*. In addition, the total fatty acid profile of whole cells was largely unchanged in the 5 independent lines analyzed (data not shown).

Discussion

This work was initiated to explore lipid metabolism in *Chlamydomonas* by examination of the whole genome shotgun sequence for predicted proteins with activity in acyl-lipid metabolism. In addition, we used the hairpin RNAi method to investigate the functional consequences of suppressing expression of the *BTA1* gene, encoding the multifunctional enzyme for synthesis of the betaine lipid DGTS. This experiment also serves as a case study in the reverse-genetic functional analysis of these annotated genes, and shows the potential for rapidly determining the consequences of down-regulating other genes annotated in this study.

During the course of the annotation and analysis phase, several themes became apparent. First, the core pathways of fatty acid biosynthesis and glycerolipid assembly in *Chlamydomonas* appear to be somewhat simpler than those of Arabidopsis, both in the presence and/or absence of certain pathways, and in the size of the gene families that represent the various activities. A case in point is that of galactolipid synthesis. In the *Chlamydomonas* genome, only one MGDG-synthase (*MGD1*) and one DGDG-synthase (*DGD1*) is present, as opposed to three MGD isoforms (Awai et al., 2001) and two DGD isoforms (Kelly and Dormann, 2002) in the Arabidopsis genome. This may be thought of as a direct result of (or accompaniment to) the lack of PtdCho and its presumed replacement by the non-phosphorous analog DGTS in *Chlamydomonas*. The logic behind this statement is as follows. In Arabidopsis, phosphate deprivation has been shown to upregulate the expression of MGD2, MGD3, and DGD2, concomitant with a loss of PtdCho and accumulation of DGDG in extraplastidic membranes (Dörmann and Benning, 2002). This scenario has also been observed in phosphate starved Oat, where DGDG, presumably produced via the phosphate-starvation inducible biosynthetic pathway, replaces a large proportion of PtdCho in the plasma membrane (Andersson et al., 2003). This phenomenon is presumed to be an adaptation to phosphate limitation, in that a nonphosphorous lipid is substituting for a phospholipid, thus allowing reallocation of the limiting nutrient into more critical components, such as nucleic acids. Given that *Chlamydomonas* has no need to replace PtdCho with DGDG due to the presence of DGTS, the phosphate-inducible pathway of DGDG biosynthesis may have been rendered superfluous and lost over the course of evolution (assuming it was present in the common ancestor of angiosperms and *Chlamydomonas*), or may simply have evolved only in higher plants.

Another theme is apparent in the biosynthesis of fatty acids, in that some of the core components of this process appear to be targeted to both the plastid and mitochondrion. Several lines of evidence support this conjecture. First, it is known from studies on isolated organelles that fatty acid synthesis occurs in both plastids and mitochondria, the plastid being responsible for supplying the bulk of fatty acid biosynthesis to supply precursors for membrane lipid synthesis, and the mitochondrial pathway being a function of lipoic acid biosynthesis, a critical prosthetic group for certain mitochondrial enzymes. Several of the activities that are central to fatty acid synthesis, e.g components of the multimeric acetyl-CoA carboxylase (ACCase) and the type II fatty acid synthase, as well as acyl carrier proteins, are present in only one copy (two in the case of ACP) in the *Chlamydomonas* genome. This leads to the conclusion that, since the activities are present in multiple compartments, these proteins may be dually targeted both to mitochondria and to plastids, a phenomenon with a growing number of precedents, especially with proteins involved in core metabolic processes such as nucleotide biosynthesis and DNA replication, e.g. (Chabregas et al., 2001; Wall et al., 2004). Indeed, the *Chlamydomonas* proteins in question have a relatively high probability of dual targeting to mitochondria and plastids, as judged by TargetP analysis. The recent finding of a potentially dual targeted multifunctional ACCase in grasses (Focke et al., 2003) lends credence to this hypothesis. Additionally, Beisson et al. (2003) were unable to predict candidates for the mitochondrial 3-ketoacyl-ACP-reductase and 3-hydroxyacyl-CoA-dehydratase activities of Arabidopsis. Given that these activities have been demonstrated implicitly by the fact that mitochondria can synthesize fatty acids, it is reasonable to posit that dual targeting of these proteins could be operative in Arabidopsis as well.

Fatty acid desaturation has garnered much attention due to the potential for industrial uses of novel plant fatty acids, and the analysis of *Chlamydomonas* FAD enzymes presented here is based largely on work from Arabidopsis (reviewed by Ohlrogge and Browse, (1995)). Clear homologs of the Arabidopsis FADs are present in our survey. In addition, two putative cytosolic FADs are present which are somewhat divergent from those that have clear similarity to characterized isoforms in other organisms. *Chlamydomonas* synthesizes at least 2 extraplastidic fatty acids not found in Arabidopsis, and these proteins might be involved in the synthesis of these atypical acyl species (Figure 4.2, step 39). One approach to verifying hypotheses regarding the function of these putative FADs would be to use RNAi to silence the genes or, alternatively, to express the genes in Arabidopsis. For example, expression of the putative *Chlamydomonas* ER desaturases (Figure 4.2, step 40) in Arabidopsis has the potential to both show the function of these genes, and to demonstrate the consequences of increasing the desaturation level of Arabidopsis ER lipids.

Given the similarities and differences between *Chlamydomonas* and Arabidopsis, we used RNAi to investigate the consequences of down-regulating *BTA1*, encoding the protein responsible for synthesis of the PtdCho analog DGTS, as described above. We presumed that, given the role of PtdCho in animal and fungal cells, silencing of the *BTA1* gene would be detrimental to the growth of the *Chlamydomonas* cells, and that this would correlate with a decrease in DGTS content. In fact, most of the transformants showed no phenotype and probably do not express the dsRNA hairpin. However, several strains (approximately 10% of those propagated) showed a reduction in growth rate on both agar plates and in liquid culture (Figure 4.4). Northern analysis (Figure 4.5) reveals a significant decrease in the steady state transcript levels of the *BTA1* gene in the RNAi suppressor lines that show a growth phenotype. This rate of recovery of suppressors in an RNAi experiment is in line with other values reported in the literature (Soupene et al., 2004). The growth defects of the lines chosen for analysis probably represent comparatively mild phenotypes, as there were also many very small transformant colonies that we were unable to propagate or that died after several rounds of re-streaking, and which may have represented more severe alleles. Nonetheless, we investigated the growth properties and lipid profiles of three of the viable, yet poorly growing lines.

PtdCho biosynthesis defects are often accompanied by temperature sensitive growth and developmental phenotypes (Howe et al., 2002; Mou et al., 2002), and Figure 4.7 shows that this is also the case for suppressed expression of DGTS biosynthesis in the *BTA1*-RNAi lines. The degree of temperature sensitivity correlated with the severity of the growth rate defect, with lines i29, i31, and i35 being unable to survive 16 h at 42° C, and line i35 showing the most stringent temperature sensitivity, losing viability after 40 h at 37° C. Many studies have shown that a shift in temperature often induces changes in the composition of membranes and in the activities of lipid biosynthetic enzymes, e.g in *S. cerevisiae*, a shift in temperature from 24° to 37° was shown to induce an increased capacity for PtdCho biosynthesis via the CDP-Cho pathway (Dowd et al., 2001; Howe et al., 2002). In our experiment, by analogy with the situation in yeast, the temperature sensitive phenotype may result from an inability of the cells to respond to the heat stress by altering membrane lipid composition in a timely manner. While this seems a plausible explanation, further studies are needed to define the changes in lipid metabolism upon a shift in temperature in wild-type *Chlamydomonas* cells.

While there were definite consequences to growth when the *BTA1* gene is silenced, there were no profound effects on polar lipid composition under the conditions studied. Figure 4.6 shows the results of membrane lipid quantification by TLC followed by GC of fatty acid methyl esters, indicating that there are no statistically significant increases or decreases in the relative amounts of the polar lipid classes. This result was somewhat unexpected, but may be explained by a strong tendency toward membrane homeostasis. It appears that a reduced capacity for membrane biogenesis, as is presumably the case in the *BTA1*-RNAi suppressors, can be partially compensated by attenuation of growth rate. This phenomenon is similar to that observed in yeast with compromised PtdCho biosynthesis, in which growth cessation was hypothesized to be a mechanism to avoid cell death (Howe et al., 2002). In the yeast system, however, a profound reduction in PtdCho mass was observed after inactivation of both of its biosynthetic pathways. With proper experimental conditions, this might occur with DGTS in *Chlamydomonas*, however at the present time we have no way to abruptly shut off DGTS biosynthesis in rapidly growing cells. The RNAi lines under study are stably transformed and, as discussed above, probably represent weak alleles with only partial reduction in transcript levels (Figure 4.5).

The results of this study give a nearly comprehensive set of genes likely to be involved in membrane lipid biosynthesis in *Chlamydomonas*. However, the assembly of a biological membrane is more that the sum of its biosynthetic reactions, and there are undoubtedly layers of complexity which this study fails to reach. One area in which this gene set is lacking is lipid transport, which is likely to be critical in maintaining the proper lipid composition of a given membrane. For example, the case of a lipid being synthesized in one membrane and transported to another must be accounted for when devising a mechanistic description of membrane biogenesis. Likewise, simply knowing the genes involved in PtdEtn and DGTS biosynthesis (or any other set of lipids in a given membrane) says nothing of how the final stoichiometric ratio between the two is regulated so as to form a functional membrane with the correct biophysical properties. Given the lack of redundancy in the pathways described above, the absence of complicating factors such as tissue specificity, and the ease of functional analysis by gene silencing as described for *BTA1*, *Chlamydomonas* is poised to be a useful model for studying how plant cells sense and control the flux of precursors into different polar lipid biosynthesis pathways in order to produce a functional membrane.

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Chapter 5

The sulfolipids 2'-*O*-acyl-sulfoquinovosyldiacylglycerol and sulfoquinovosyldiacylglycerol are absent from a *Chlamydomonas reinhardtii* mutant deleted in *SQD1*[2]

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[2] This work has been published: Riekhof,W.R., Ruckle,M.E., Lydic,T.A., Sears,B.B., and Benning,C. (2003). The sulfolipids 2'-O-acyl-sulfoquinovosyldiacylglycerol and sulfoquinovosyldiacylglycerol are absent from a *Chlamydomonas reinhardtii* mutant deleted in SQD1. Plant Physiol *133*, 864-874.

Abstract

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*-acyl-sulfoquinovosyldiacylglycerol (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 plasmid disruption mutant of *C. reinhardtii*, designated Δ*sqd1*, 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.

Introduction

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 mono- and digalactosyldiacylglycerol (MGDG and DGDG), phosphatidylglycerol (PG), and the sulfolipid sulfoquinovosyldiacylglycerol (SQDG). The focus here is on SQDG and its derivative 2'-*O*-acyl-sulfoquinovosyldiacylglycerol (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., 1996; Weissenmayer et al., 2000), Arabidopsis (Yu et al., 2002), and *Chlamydomonas reinhardtii* (Minoda et al., 2002; Sato et al., 1995) 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 (Minoda et al., 2002; Sato et al., 1995). 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 (Benning, 1998; Pugh et al., 1995). The first reaction, the biosynthesis of UDP-sulfoquinovose from UDP-Glc and sulfite, 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*.

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 (Harris, 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. ^{35}S- and ^{14}C-labeling experiments were carried out by the addition of 50 μCi [^{35}S] Na_2 SO_4 (carrier-free 61606 Ci/mmol) or 25 μCi (47.5 mCi/mmol) 1-[^{14}C]acetate to a 30 mL culture. Cells were also grown photoautotrophically on high salt (HS) medium agar plates (Harris, 1989) at 1/3 strength and with varying concentrations of DCMU, added as a concentrated stock in dimethyl sulfoxide.

Quantitative Lipid Analysis

Lipids from cell pellets of ^{35}S- or ^{14}C-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 H_3PO_4 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 ^{14}C-acetate labeling, two-dimensional TLC analysis (Benning et al., 1995) was followed by exposure to a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA), and for ^{35}S-labeling, a one-dimensional TLC system was used as described (Härtel 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 (Kindle, 1998), except that TAP medium containing nitrate as the sole N source (TAP-NO_3) was used for selection of prototrophs, pMN24 was linearized with *Bam*HI 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). Suc 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-NO_3 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 with 150 μL of 1 M KCl and 0.2 M H_3PO_4, followed by centrifugation. Aliquots of the organic phase from each well were then spotted in rows of 24 along each side of an activated, ammonium sulfate-impregnated TLC plate (Benning and Somerville, 1992b) using a modified pipetting robot (Genesis RSP 100, Tecan US, Research Trangle Park, NC) as previously described (Xu et al., 2003). TLC plates were allowed to dry completely after spotting, cut in one-half, and developed in a solvent system containing chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, v/v). Lipids were visualized with α-napthol/sulfuric acid reagent to identify the glycolipids (Benning et al., 1995) and prolonged heating at 120°C to char the other polar lipids.

Identification and Molecular Characterization of the Site of Transgene Insertion

Plasmid rescue was accomplished essentially as described (Tam and Lefebvre, 1993). Briefly, genomic DNA was purified from the mutant by the method of Shimogawara et al. (Shimogawara et al., 1998). Subsequently, 20 μg of genomic DNA was digested with *Sal*I and was purified by ethanol precipitation. This DNA was resuspended in 900 μL of ligation buffer and was ligated for 16 h at 20°C with 10 units of T4 DNA ligase (Invitrogen, Carlsbad, CA). The ligated DNA was precipitated with ethanol and was suspended in water. A 50-μg aliquot was used to transform electrocompetent GeneHogs (Invitrogen) *Escherichia coli* cells according to the manufacturer's instructions. Transformants were selected on Luria-Bertani broth-ampicillin (100 μg/ml) plates and miniprep DNA was prepared and sequenced at the Michigan State University Genomics Technology Support Facility using the primer 5'-ATGACCATGATTACGCCAAG-3' designed to the pUC-119 multiple cloning site. The resulting sequence was used to query the *C. reinhardtii* draft genome sequence (http://genome.jgi-psf.org/chlre1/chlre1.home.html) to identify the site of pMN24 insertion.

Restriction mapping of the rescued plasmid was carried out with *Sal*I, *Hin*D III, *Eco*RV, *Not*I, *Xba*I, and *Spe*I. Because the rescued plasmid could only provide information to the left of the insertion point, several PCR markers, designated A through E, were designed to amplify regions of the chromosome to the right of the insertion site to determine the extent of the deletion caused by the pMN24 insertion. Primer pair sequences were:

marker A, 5'-GACAGCGCTACCGGTGTA-3', 5'-TGTTCCTCCTCGCACGTA-3'; marker B, 5'-AACGACATGTCGGGATCA-3', 5'-ACGTGCATTTCCTGCAAT-3'; marker C, 5'-CTCATCAAGCTGCTCCGT-3', 5'-TTCAGCAGGAGGAGCAC-3'; marker D, 5'-GTGCCTTGAATGCAAGCT-3', 5'-GCAAACTCCTTGAGCTCG-3'; and marker E, 5'-GTTAGCTGCTGCACATGC-3', 5'-GCATAACACCGCAGACAA-3'.

Large-Scale Extraction and Isolation of Compounds

Phosphate-limited cells from 20 liters of late log phase culture (TAP, 0.03 mM Pi, four cultures, 5 liters each) were collected by centrifugation and were extracted with 150 mL of chloroform/methanol/88% formic acid (10:10:1, v/v) followed by the addition of 37.5 mL of 0.2 M H_3PO_4 and 1 M KCl and were separated into two phases by centrifugation. Anionic lipids were purified based on a procedure modified from O'Brien and Benson (O'Brien and Benson, 1964). The lower organic phase was applied to a column (2 cm x 12 cm) of Florisil (J.T. Baker, Phillipsburg, NJ) equilibrated in $CHCl_3$. Pigments and some MGDG were washed from the column with three column volumes of $CHCl_3$, and the bound polar lipids were then eluted with two column volumes of $CHCl_3/CH_3OH$ (2:1, v/v). Eluate from the Florisil column was applied directly to a column (3 cm x 9 cm) of diethylaminoethyl-cellulose (acetate form) pre-equilibrated in $CHCl_3/CH_3OH$ (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 N_2 and was further purified by preparative TLC (Benning et al., 1995). Compounds of interest were identified by light staining with I_2 vapor and were scraped from the plate into glass tubes, followed by elution of the silica twice with chloroform/methanol (1:1, v/v).

Mass Spectrometry

Fast atom bombardment-mass spectra were acquired in the negative ion mode on a mass spectrometer (HX-110; JEOL, Peabody, MA) essentially as described (Gage et al., 1992). Approximately 1 μg of sample dissolved in chloroform/methanol (1:1, v/v) was mixed with triethanolamine 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 collisionally activated dissociation-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 dried and suspended in 1 mL of methanolic HCl, incubated for 1 h at 80°C, and the methyl esters were partitioned into hexane by the addition of 1 mL of hexane and 1 mL of 0.9% (w/v) NaCl. Gas-liquid chromatography was as previously described (Rossak et al., 1997).

NMR Spectroscopy

Samples were prepared by filtering the TLC-purified material through glass wool, evaporating it to dryness under N_2, and further drying it over desiccant under vacuum for at least 3 h. Samples were then dissolved in 0.6 mL of chloroform-d/methanol-d_4/acetic acid-d_4 (10:10:1, v/v) and dispensed into NMR tubes. Standard ^{1}H-NMR spectra were acquired on a 500 MHz instrument (VXR-500; Varian, Palo Alto, CA) at 25 °C, and ^{1}H-^{1}H 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.

Results

Accumulation of ASQD in *C. reinhardtii*

Thin-layer chromatograms of lipids from ^{35}S-sulfate-labeled cells showed the expected lipid band for the plant sulfolipid SQDG, and, in addition, a band of approximately 5 % intensity of SQDG (Figure 5.1, 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 (Figure 5.1).

To determine the identity of the unknown sulfur-labeled compound, we purified approximately 100 μg from 20 liters of phosphate-starved *C. reinhardtii* cultures using a combination of column and thinlayer 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

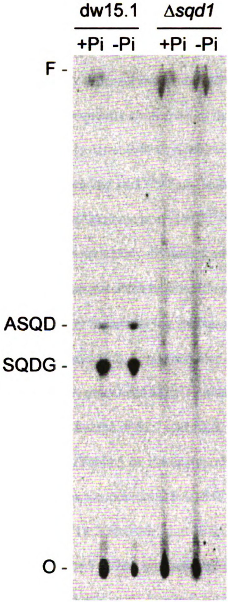


Figure 5.1. Separation of lipid extracts from ^{35}S-sulfate-labeled cells of *C. reinhardtii* wild-type (dw15.1) and mutant (*Δsqd1*) cells. Cells were either grown in phosphate-replete medium (+Pi) or phosphate-limited medium (-Pi). Lipids were visualized by autoradiography. ASQD, 2'-*O*-acyl-sulfoquinovosyldiacylglycerol; F, solvent front; O, origin; SQDG, sulfoquinovosyldiacylglycerol.

analysis was used to determine the fatty acid composition of ASQD, SQDG, and entire cell extracts as shown in Table 5.1. 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. (Giroud et al., 1988) as $18{:}4^{5,9,12,15}$. A corresponding $18{:}3^{5,9,12}$ fatty acid distinguishable in our chromatograms from more typical α-linolenic acid ($18{:}3^{9,12,15}$) was present as well. The whole-cell extract composition was noticeably different from that observed for ASQD and SQDG (Table 5.1). Negative-ion fast-atom bombardment (FAB)-MS (Figure 5.2A) of the ASQD sample showed a major molecular ion species at approximately *m/z* 1074. Closer inspection of this region (Figure 5.2, insert) revealed two major species at *m/z* 1073.5 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 5.1), 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 (Figure 5.2B). 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 (Figure 5.2, B and C, compound 2), and a prominent fragment at *m/z* 543 and its neighbor at *m/z* 541 (not resolved in Figure 5.2B) 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 (Figure 5.2C, compound 3).

To further corroborate the structure and to determine the position of the third acylation on the sulfoquinovosyl head group, ^{1}H-NMR spectra were recorded for ASQD and SQDG (Figure 5.3). The ^{1}H spectrum of SQDG given in Figure 5.3B is essentially identical to that described by Cedergren and Hollingsworth (1994). For ASQD (Figure 5.3C), assignment of glycerol and carbohydrate ring proton resonances was aided by a ^{1}H-^{1}H homonuclear decoupling experiment (data not shown). This experiment revealed that the 2-sulfoquinovose proton is shifted down-field by approximately 1.2 ppm relative to SQDG, and that the 1- and 3-protons are shifted down-field 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 glycerol-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.

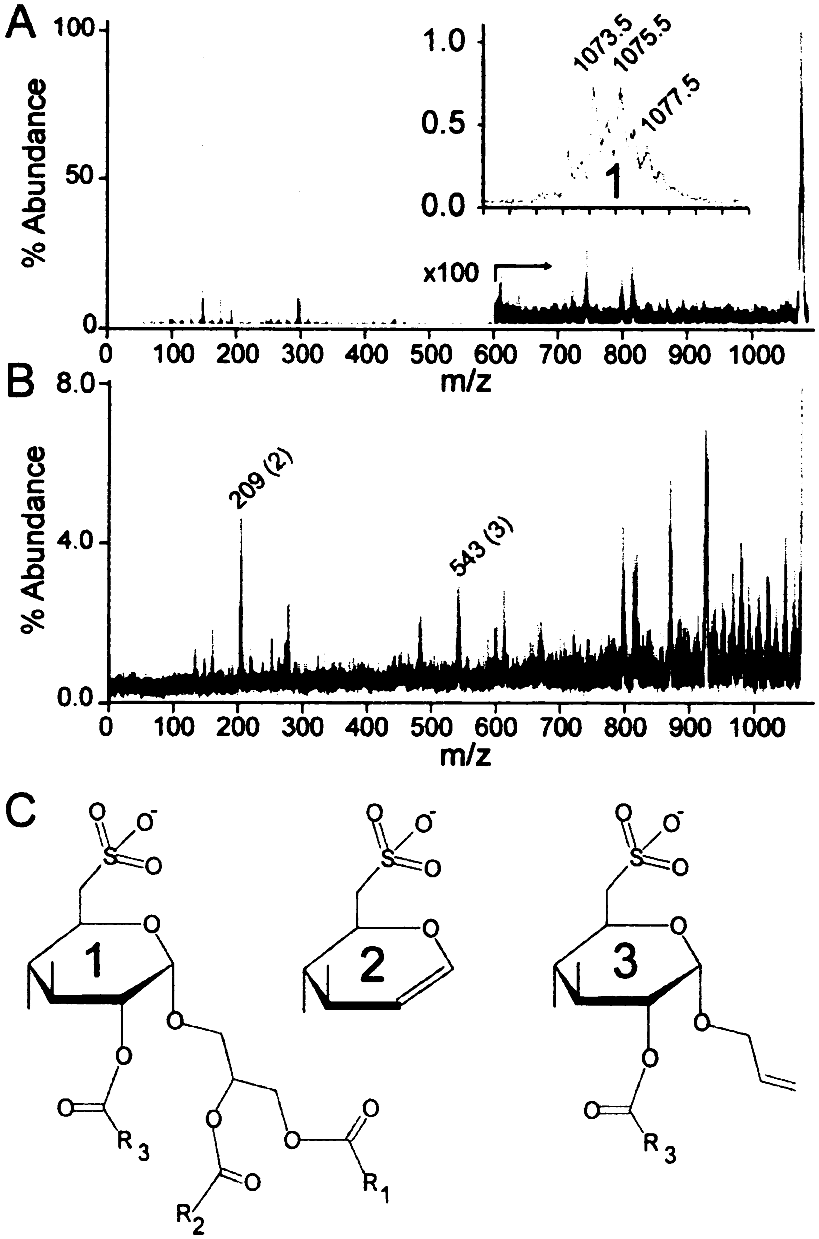


Figure 5.2. Mass spectrometric analysis of ASQD. (A) Total ion negative FAB-MS with insert showing an expansion of the molecular ion region. (B) FAB-CAD-MS/MS of the molecular ion at m/z 1073.5. C. Structure prediction for the molecular ion and two diagnostic fragments. The numbers in the two spectra refer to the three different structures. In addition m/z values for the respective fragments are indicated.

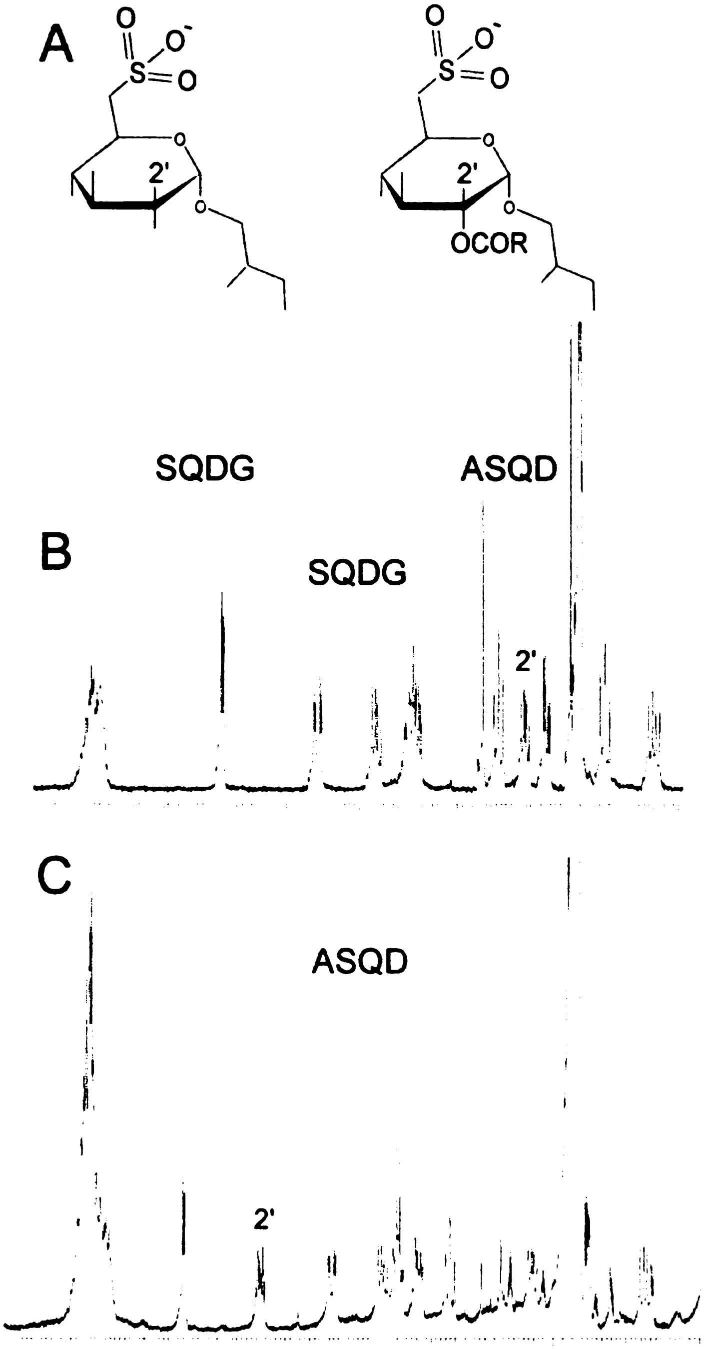


Figure 5.3. 500 MHz proton NMR spectra of SQDG and ASQD. (A) Structures for both lipids with proton designations as assigned to the signals in the spectrum for SQDG (B), and ASQD (C).

Table 5.1. Comparison of the fatty acid composition of the two sulfolipids and cells[a]

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

[a] nd, Not detected. Two independent experiments were averaged (±SD).

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

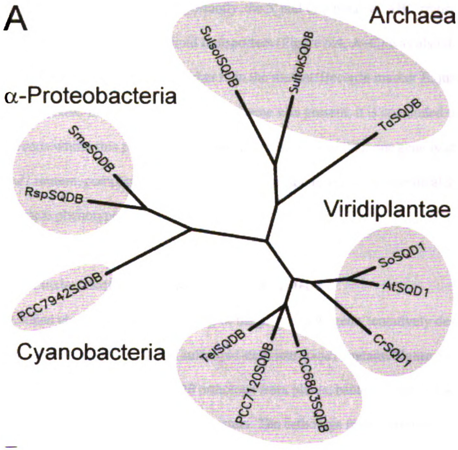
To begin to understand the biosynthesis of this new lipid and to identify genes essential for its biosynthesis, a genetic approach was applied. For this purpose, we developed a robotic high-throughput screening procedure (see "Materials and Methods" for details) allowing the direct one-dimensional thin-layer 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 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 5.1, *C. reinhardtii* dw15.1 showed increased labeling of SQDG and ASQD under phosphate-limited growth conditions, with ASQD present at approximately 5% intensity of SQDG under both 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 (Figure 5.1).

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, p*Sqd1*-R (Figure 5.4, 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 (Figure 5.4), 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 (Figure 5.4) 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 (Figure 5.4) 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 Δ*sqd1*. 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* (Figure 5.4). Beyond the 3' end of this gene, no additional predicted open reading frames were affected by the deletion (Figure 5.4). However, beyond the 5' end of *CrSQD1*, a repetitive element (Figure 5.4T), possibly the

Figure 5.4. Comparison of genomic sequences surrounding the *CrSQD1* locus in the wild type (WT), the *Δsqd1* mutant and the rescued plasmid (p*Sqd1*-R). Five predicted genes/elements are shown as black arrows/box. They are annotated to encode: *CrSQD1*, *SQD1/SQDB* ortholog; T, repetitive element with similarity to transposases; 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 p*Sqd1*-R depicts the left end sequence used to place the rescued genomic DNA onto the genome. Restricion enzymes indicated by two vertical bars are: E, *Eco* RV; H, *Hin*d III; N, *Not* I; S, *Sal* I; Sp, *Spe* I; X, *Xba* I.

Figure 5.5. Comparison of *SQD1/SQDB* from bacteria, archaea and plants. Unrooted tree produced from the alignments using ClustalW software. Clusters of closely related organisms are shaded. The following proteins were included in the analysis (GenBank accession number): AtSQD1, *Arabidopsis thaliana* (T05311); SoSQD1, *Spinacea oleracea* (AA039667); TaSQDB, *Thermoplasma acidophilicum* (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); PCC7120SQDB, *Nostoc* sp. PCC7120 (NP_485784). The *Chlamydomonas reinhardtii* ortholog, CrSQD1, was used as predicted from the genome sequence (http://genome.jgi-psf.org/chlre1/chlre1.home.html; scaffold_22, genie 22.14).


A
Archaea
SulsolSQDB
SultokSQDB
TaSQDB
α-Proteobacteria
SmeSQDB
RspSQDB
Viridiplantae
SoSQD1
AtSQD1
CrSQD1
PCC7942SQDB
Cyanobacteria
TelSQDB
PCC7120SQDB
PCC6803SQDB

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 (Figure 5.4, 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 Δ*sqd1* 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 Δ*sqd1* 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. sphaeroides* (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 (Figure 5.5A).

The Δ*sqd1* Mutant Shows Reduced Growth under Phosphate Limitation and Increased Herbicide Sensitivity

Relative lipid compositions of the control strain dw15.1 and the Δ*sqd1* mutant derived from dw15.1 were compared for cells grown under phosphate-replete and phosphate-limited conditions (Table 5.2). In the dw15.1 strain, the relative SQDG content drastically increased under phosphate-limited growth conditions, whereas the amount of PG decreased. In the *sqd1* 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 (Härtel 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 alga. Interestingly, the relative amount of the nonphosphorous extraplastidic betaine lipid (Table 5.2, DGTS) did not change in either strain under the two growth conditions. The ASQD form of sulfolipid co-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.

Table 5.2. Lipid composition of dw15.1 and Δsqd1 mutant under normal and phosphate-deprived growth conditions[a]

	1.0 mM Pi		0.02 mM Pi	
	dw15.1	*Δsqd*1	dw15.1	Δ*sqd*1
		%		
MGDG	38.2 ±4.5	34.5 ±3.1	35.7 ±10.3	32.5 ±7.9
Diacylglycerol-*N*-trimethylhomoserine (DGTS)/ASQD	25.8 ±5.7	27.2 ±4.2	26.9 ±8.4	24.3 ±3.2
Phosphatidylethanolamine	6.1 ±0.6	7.0 ±0.2	4.1 ±1.9	5.0 ±1.9
PG	7.7 ±1.1	12.4 ±0.6	3.6 ±1.8	10.6 ±1.5
DGDG	14.8 ±2.3	16.3 ±2.5	18.6 ±3.4	23.2 ±7.1
SQDG	5.0 ±0.9	nd	12.6 ±4.3	nd
Phosphatidylinositol	2.3 ±0.7	2.7 ±0.5	01.8 ±0.9	4.4 ±2.1

[a] ASQD and DGTS were not separated in this experiment. Three independent experiments were averaged (±SD).

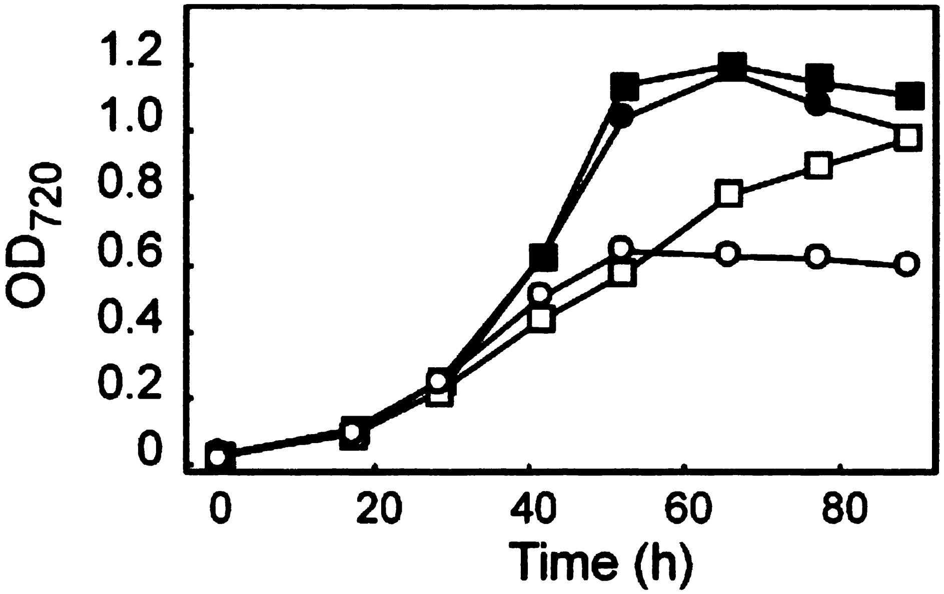


Figure 5.6. Growth of dw15.1 (squares) and *Δsqd1* (circles) under phosphate-replete (closed symbols) and phosphate-limited (open symbols) conditions.

Figure 5.7. Growth of dw15.1 and *Δsqd1* on agar-solidified medium containing different amounts of the herbicide DCMU at concentrations as indicated.

Growth under the two conditions was compared for dw15.1 and Δ*sqd1*, as shown in Figure 5.6. The growth of both strains was essentially indistinguishable under phosphate-replete conditions. Growth of dw15.1 was delayed and growth of *sqd1* ceased at much lower density under phosphate-limited conditions. Unfortunately, growth data on phosphatereplete and -limited medium are not available for another SQDG-deficient mutant of *C. reinhardtii, hf-2* (Minoda et al., 2002; Sato et al., 1995). 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 Δ*sqd1* mutant on 3(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), a herbicide binding to the Q_B acceptor site of PSII and thereby blocking PSII activity. As shown in Figure 5.7, the Δ*sqd1* 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).

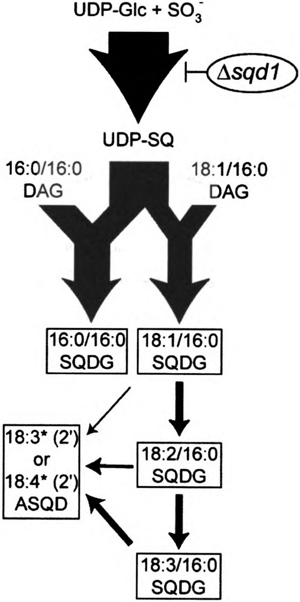
The Presence of ASQD in Lipid Extracts of *C. reinhardtii* is Not an Extraction Artifact

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 5.1). Analyzing gas chromatograms obtained for ASQD-derived fatty acid methylesters, we could detect two unusual compounds with slightly smaller and slightly larger retention times than that for $18{:}3^{9,12,15}$ -linolenate methylesters (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 methylesters 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

Figure 5.8. 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 unsual acyl groups $18{:}3^{\Delta 5,9,12}$and $18{:}4^{\Delta 5,9,12,15}$. The block in the *Δsqd1* mutant is indicated. ASQD, 2'-*O*-acyl-sulfoquinovosyldiacylglycerol; DAG, diacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; UDP-Glc, UDP-glucose; UDP-SQ, UDP-sulfoquinovose.


UDP-Glc + SO3-
Δsqd1
UDP-SQ
16:0/16:0 DAG
18:1/16:0 DAG
16:0/16:0 SQDG
18:1/16:0 SQDG
18:3* (2') or 18:4* (2') ASQD
18:2/16:0 SQDG
18:3/16:0 SQDG

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 $18{:}3^{9,12,15}$ -linolenic acid is present in the diacylglycerol moiety of ASQD. However, one might predict that linoleic and -linolenic acids are esterified in the diacylglycerol moiety of ASQD, whereas the two unusual fatty acids 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 5.8. 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 *Δsqd1* 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 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 ASQD 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 (Figure 5.8). The acyltransferase involved in acylating the sulfoquinovose head group apparently has access to the unusual fatty acids $18{:}3^{5,9,12}$ and $18{:}4^{5,9,12,15}$ 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* (Minoda et al., 2002; Sato et al., 1995), 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 phenotypes, it was concluded that SQDG plays a crucial role in photosynthesis of *C. reinhardtii* (Minoda et al., 2002; Sato et al., 1995). 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 Δ*sqd1* 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.

The *sqd1* mutant, although well defined at the molecular level, carries an extensive deletion affecting not only the *CrSQD1* gene, but also one neighboring structural gene (Figure 5.4). The specific effects of inactivating this second gene are not known, but as Figure 5.6 shows, growth under typical laboratory conditions in replete medium is not affected by the deletion of either gene. In general, the analysis of the Δ*sqd1* mutant presented here concurs with previous findings in other organisms such as Arabidopsis, which does not show a growth impairment unless the respective mutant becomes phosphate deprived (Yu et al., 2002). When we grew the Δ*sqd1* mutant of *C. reinhardtii* under phosphate-depleted conditions (Figure 5.6), 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, Δ*sqd1* 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 Δ*sqd1* mutant in agreement with the inactivation of a gene essential for sulfolipid biosynthesis. Second, as the sequence lineup in Figure 5.5 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 *At*SQD1 protein) participating in the reaction mechanism are absolutely conserved in all orthologs (Figure 5.5). 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. PCC7492 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 sulfite 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, Δ*sqd1*, carrying a deletion of the *CrSQD1* gene proposed to encode the UDP-sulfoquinovose 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.

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Appendix A

Analysis of lipid metabolism in a primitive red alga: EST analysis of the thermoacidophilic red alga *Galdieria sulphuraria* reveals conservation of central pathways and a complete pathway for lipid A biosynthesis [3]

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[3] Parts of this work have been published in: Weber, A.P.M., Oesterhelt, C., Gross, W., Bräutigam, A., Imboden, L.A., Krassovskaya, A., Linka, N., Truchina, J., Schneidereit, J., Voll, H., Voll, L.M., Zimmermann, M., Jamai, A., Riekhof, W.R., Yu, B., Garavito, R.M., and Benning, C. 2003. EST-Analysis of the Thermo-acidophilic red microalga *Galdieria sulphuraria* reveals potential for lipid A biosynthesis and unveils the pathway of carbon export from rhodoplasts. Plant Mol. Biol. *In press.*

Introduction

Lipids play crucial roles in all cellular organisms, principally as components of membranes that delineate the boundaries of the cell and its compartments, and also as storage compounds, components of cuticular waxes, and as precursors to signaling molecules in many organisms, among other functions. Lipid biosynthesis has been studied extensively in higher plants and algae, leading to detailed models for biosynthesis of fatty acids and their incorporation into glycolipids, phospholipids, and other fatty acid derived compounds (Ohlrogge and Browse, 1995; Somerville et al., 2000). While ubiquitous and critically important to life, lipid metabolism has traditionally been difficult to characterize due to the inherent insolubility of many of the enzymes and substrates involved. The combination of genetics, genomics, and biochemistry has allowed the identification and characterization of most of the key enzymes in plant lipid metabolism (Beisson et al., 2003; Frentzen, 2004; Ohlrogge and Browse, 1995; Somerville et al., 2000), and the present appendix follows in this vein by using genomic data to analyze lipid metabolism in a primitive red alga, *Galdieria sulphuraria*.

Materials and Methods

EST sequencing was carried out on phototrophic and heterotrophic cDNA libraries of *Galdieria sulphuraria* as described (A.P.M. Weber *et al*., *in press*), and is available at http://www.genomics.msu.edu/galdieria/index.cgi. Manual annotation of the *G. sulphuraria* dataset was carried out by keyword searches of the automated annotations, and by BLAST (Altschul et al., 1997) searching with relevant sequences of plant, animal, fungal, or bacterial origin. Significance values used for determining homology were generally set at a BLAST expectation value of less than E^{-20}, and were confirmed by multiple sequence alignments, conservation of active site residues, etc. when possible.

Results and Discussion

Fatty acid biosynthesis. Based on the EST dataset, the fatty acid and lipid metabolism of *Galdieria* is likely similar to that of seed plants. Production of malonyl-CoA through the action of acetyl-coA carboxylase (ACCase) is regarded as a rate limiting and highly regulated step in fatty acid biosynthesis (Somerville et al., 2000). In plants two ACCase isozymes exist: a homomeric multi-domain protein in the cytosol, and a multisubunit complex of four proteins in the plastid. Single ESTs for two of the subunits of plastidial ACCase (biotin carboxylase, A4_40C10; and β-carboxyltransferase, HET_25A08) were present in our database. Fatty acid synthase (FAS) in plants is also a plastidic multi-protein complex, and ESTs for the 3-ketoacyl-ACP-reductase (gs01440) and enoyl-ACP-reductase (HET_03C09) were present, representing clones from both the heterotrophic and autotrophic libraries. Other FA biosynthetic activities such as FA desaturases, acyl-CoA synthetases, and thiolases were also distributed in both libraries. These analyses indicate that this red alga, like higher plants, most likely synthesizes fatty acids in the rhodoplast via bacterial/plastidic type multimeric ACCase and FAS complexes, incorporates some of these FA into plastidic membrane lipids, and exports a portion to the cytosol for incorporation into extraplastidic lipids, as discussed below.

Fatty acid catabolism. β-oxidation, typically a function of the peroxisome, was represented in the ESTs as at least two acyl-CoA oxidase and three acetyl-CoA thiolase isozymes, while enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase, which are often present as a multifunctional polypeptide, were not detected.

Membrane lipid biosynthesis. *G. sulphuraria* synthesizes phospholipids and glycolipids typical of higher plants (T. Horlacher and C. Benning, unpublished results) and pathways for their biosynthesis were readily identifiable from the EST data. Two pathways for phosphatidylethanolamine biosynthesis were identified, consisting of phosphatidylserine decarboxylase (HET_44E08) and the CDP-ethanolamine (Kennedy) pathway (ethanolamine kinase, HET10E09; ethanolamine phosphate cytidylyltransferase, A4_10G06). Phosphatidylcholine is also apparently synthesized by two pathways in which either phosphoethanolamine is methylated to phosphocholine (phosphoethanolamine methyltransferase, A4_09F12) and then activated to CDP-Cho and used to form PtdCho, or by the lipid-linked methylation of PtdEtn to form PtdCho (PEMT, A4_16B08.)

Phosphatidylglycerol is present in seed plants in the plastid, mitochondria, and ER membranes, and phosphatidylglycerophosphate synthase (GS06870, 2 ESTs) was represented, although its localization could not be predicted, as was cardiolipin synthase (HET_11H01), typically a mitochondrial enzyme. Phosphatidylinositol biosynthesis was represented by inositol-3-phosphate synthase (HET_12A09), and implicitly by a PI-4-kinase (HET_10C06) although the actual PtdIns synthase was not readily identifiable.

Little is known about the biosynthesis and function of sphingolipids in plants, however putative orthologs encoding serine palmitoyltransferase (HET_31C05) and sphingolipid-Δ^4 desaturase (GS00710, 2 ESTs) were present.

Plastidial lipids typically consist of four species: mono- and digalactosyldiacylglycerol (MGDG and DGDG), sulfoquinovosyldiacylglycerol (SQDG), and PtdGro. MGDG synthase (HET_20D10) and SQD1 (HET_20F10) were represented, as was the aforementioned PGP synthase, however cDNAs encoding other enzymes in these pathways were not found. Typically, the expression of lipid genes is low and EST coverage may not be sufficient for these to be included in the dataset.

Lipid A biosynthesis. Lipid A is the core membrane anchor of bacterial lipopolysaccharide, the major component of the outer leaflet of the bacterial outer membrane (Raetz and Whitfield, 2002). Surprisingly, genes encoding a partial lipid A biosynthesis pathway are present in the Arabidopsis genome, although several steps are missing and nether lipid A nor derivatives have ever been unequivocally identified in seed plants. Here, we provide for the first time evidence for a complete lipid A biosynthetic pathway in plants (Figure A2). In *Galdieria*, the first 2 steps in the pathway (LpxA, LpxC, Figure A.2) are likely to be encoded in the plastid genome by analogy with *Cyanidioschyzon merolae* and *Cyanidium caldarium* RK1 (Glockner et al., 2000; Ohta et al., 2003). Later steps in the pathway appear to be nuclear encoded, and corresponding ESTs are present in our collection (LpxD, HET_35C04; LpxB, cn459 and A4_T7comboD04; LpxK, A4_18A10; WaaA, A4_10A08; KDO synthetase, A4_17B04; Figure A2), leading to the hypothesis that Galdieria has the capacity to synthesize a molecule similar to Kdo_2-Lipid IV_A. The pathway shown in Figure A2 represents the

Figure A.1: **Subcellular organization of fatty acid biosynthesis and glycerolipid assembly in *Galdieria sulphuraria*.** EST sequences corresponding to the activities in this scheme were annotated, and those present in the database are indicated by an asterisk.

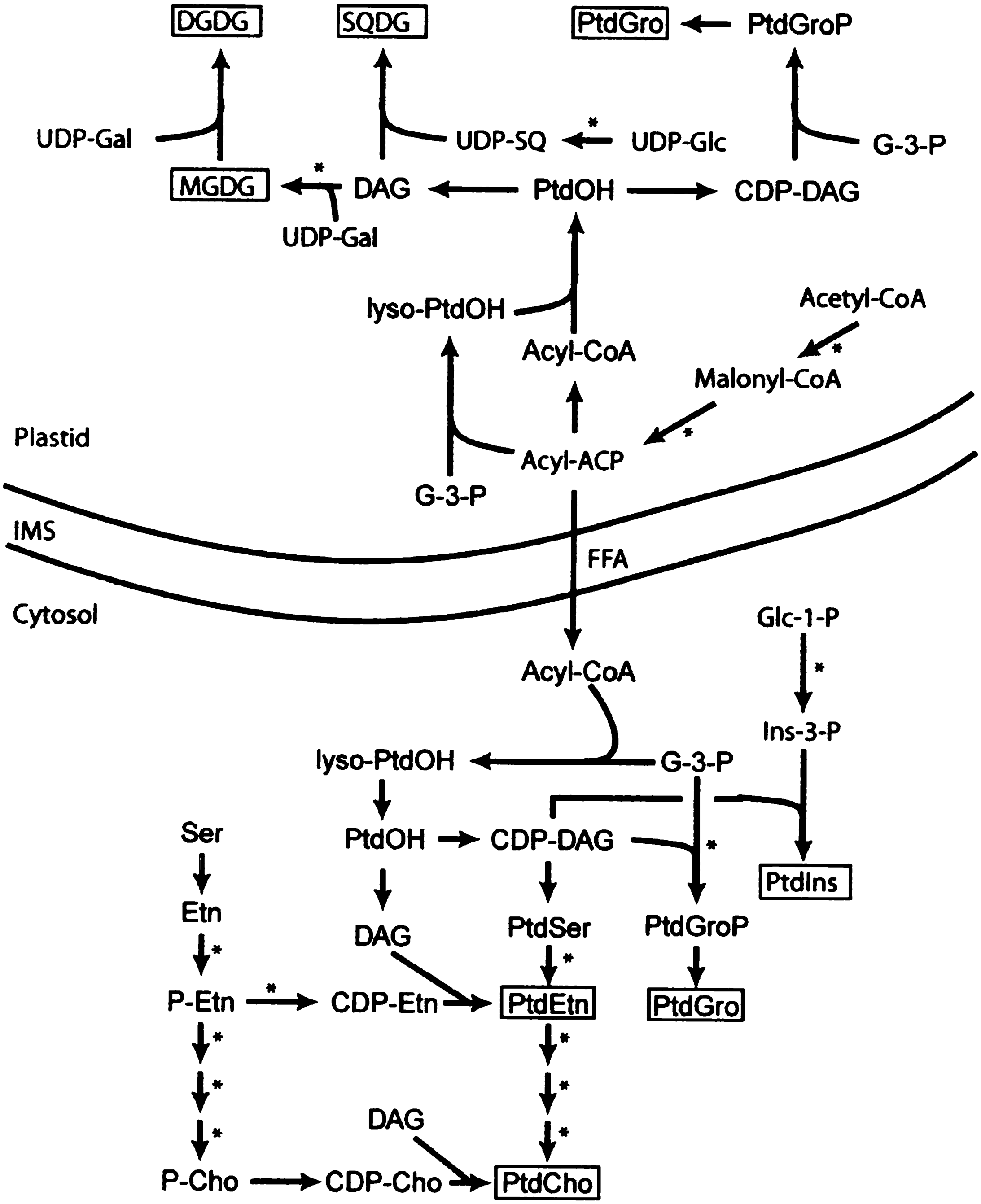
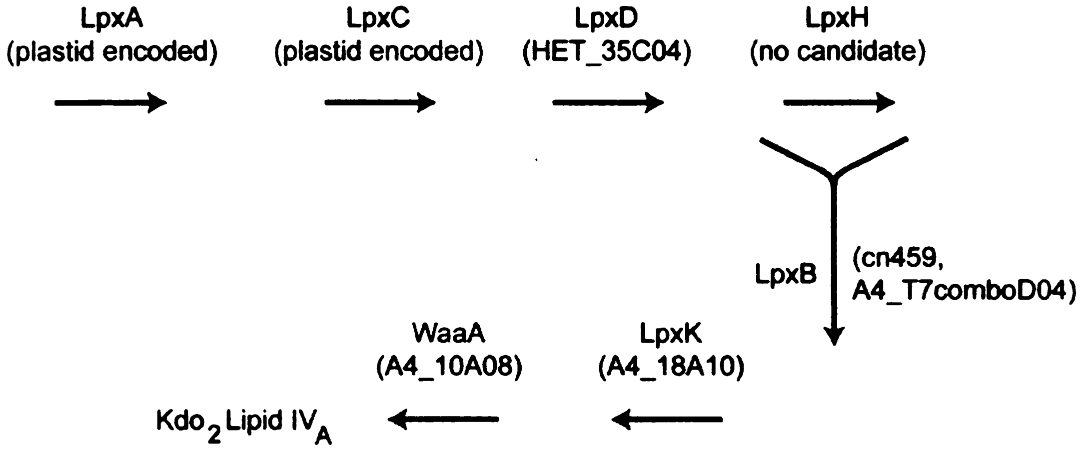

DGDG
SQDG
PtdGro
PtdGroP
UDP-Gal
UDP-SQ
*
UDP-Glc
G-3-P
MGDG
*
DAG
PtdOH
CDP-DAG
UDP-Gal
lyso-PtdOH
Acetyl-CoA
Acyl-CoA
*
Malonyl-CoA
Plastid
*
Acyl-ACP
G-3-P
IMS
FFA
Cytosol
Glc-1-P
*
Acyl-CoA
Ins-3-P
lyso-PtdOH
G-3-P
Ser
PtdOH
CDP-DAG
*
PtdIns
Etn
DAG
PtdSer
PtdGroP
*
*
P-Etn
*
CDP-Etn
PtdEtn
PtdGro
*
*
*
*
DAG
*
*
P-Cho
CDP-Cho
PtdCho

Figure A.2: **Lipid A biosynthetic pathway, and ESTs present in the database.** ESTs encoding enzymes with similarity to those involved in bacterial lipid A formation were annotated and indicated in the reaction scheme, as shown.


LpxA
(plastid encoded)
LpxC
(plastid encoded)
LpxD
(HET_35C04)
LpxH
(no candidate)
LpxB
(cn459,
A4_T7comboD04)
WaaA
(A4_10A08)
LpxK
(A4_18A10)
Kdo2 Lipid IVA

"typical" case as elucidated in *E. coli*, however other bacteria accumulate lipid A species with radically different structures, e.g. *Rhizobium etli* (Que et al., 2000a; Que et al., 2000b). Also, the lipid A structures of cyanobacteria, from which the plastid derives, are not well characterized, so while the core of the pathway appears to be present, the later steps leading to the final products remain to be elucidated.

If the proposed pathway is indeed operational, the function of lipid A-like molecules in plants is mysterious, and there are no published reports of lipid A or derivatives being detected in higher plants. It is tempting to posit that, given the extreme environment in which these primitive red algae live, lipid A is essential for stability of the plastid outer envelope, and therefore part of the pathway has been retained in the plastid genome. In fact, some of the ESTs annotated in the pathway show their greatest similarity to cyanobacterial sequences, further supporting the idea that in red algae and higher plants, the plastid may contain lipid A-like molecules as part of the plastid envelope membranes. Given the recent report of chloroplast transformation in *Porphyridium* (Lapidot et al., 2002), and nuclear transformation of *Cyanidioschizon merolae* (Minoda et al., 2004), it could be feasible to create a genetic null mutant at any point in the pathway to study the function of lipid A-like molecules in primitive red algae.

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Appendix B

Analysis of the structure of novel galactolipids accumulating in a lipid-trafficking mutant of Arabidopsis, and a hypothesis for their biosynthesis[4]

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[4] This work was done in collaboration with Dr. Changcheng Xu, and data from parts of this appendix have been published: Xu, C., Fan, J., Riekhof, W., Froehlich, J.E., and Benning, C. (2003). A permease-like protein involved in ER to thylakoid lipid transfer in Arabidopsis. EMBO J. *22*, 2370-2379

Introduction

In many plant species, biogenesis of the photosynthetic thylakoid membranes is reliant on pathways of glycerolipid biosynthesis present in the endoplasmic reticulum (ER) and plastid envelope membranes (Roughan and Slack, 1982). In the plastidic pathway (also referred to as the "prokaryotic pathway" due to similarities with the lipid metabolism of the cyanobacteria from which the plastid derives), fatty acids are synthesized in the plastid and used by plastidic acyltransferases to generate phosphatidic acid (PtdOH). PtdOH is then either used for phosphatidylglycerol (PtdGro) synthesis, or dephosphorylated to form diacylglycerol (DAG), which is then used for galactolipid and sulfolipid biosynthesis (Somerville et al., 2000).

In the ER localized "eukaryotic pathway," fatty acids are exported from the plastid and converted into acyl-CoA species for use by ER localized acyltransferases, giving rise to a cytosolic PtdOH pool. This pool of PtdOH becomes the precursor for phospholipid biosynthesis in the ER, giving rise to the typical membrane lipids phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn), among others. Based on pulse-chase labeling kinetics and analysis of molecular species, it has long been postulated that a portion of the cytosolic PtdCho pool is used as the donor of DAG moieties for galactolipid biosynthesis in the plastid (Browse et al., 1986), thus giving rise to "eukaryotic" thylakoid lipid species. While it is well established that the eukaryotic pathway of thylakoid lipid biosynthesis is operational in many plant species, a mechanistic description of the transfer of DAG moieties from cytosolic PtdCho into plastidic galactolipids is lacking.

During a screen for suppressors of the Arabidopsis *dgd1* mutant (Dörmann et al., 1995; Dörmann et al., 1999), approximately 25 mutant lines were discovered which accumulate novel tri- and tetra galactosyldiglycerides in the intact leaf, and one of these mutants was subsequently shown to have a defect in the eukaryotic pathway of thylakoid lipid synthesis (Xu et al., 2003). This work describes the structural elucidation of these oligo-galactolipids by nuclear magnetic resonance (NMR) as containing all-$\beta(1\rightarrow6)$ linked galactose residues, and a proposal for their biosynthesis via transgalactosylation by a β-galactosidase type mechanism is presented.

Materials and Methods

Isolation of lipids.

Lipids of the wild type and *tgd1-1* mutant were extracted and separated by preparative TLC on silica-60 plates in the solvent system chloroform/ methanol/ acetic acid/ water (85:15:10:5, v/v). Bands corresponding to the respective lipids were scraped from the plate and eluted in chloroform/methanol (1:1, v/v), followed by evaporation of the sample to dryness under a stream of nitrogen.

NMR.

Lipids purified as described above were further dried under vacuum over dessicant for several hours before dissolving in 0.7 ml of $CDCl_3/CD_3OD/CD_3OOD$ (10:10:1, by vol.). The addition of CD_3OOD shifts the water signal to 5.5–6 p.p.m. Data were collected on a Varian VXR-500 spectrometer at 500 MHz for protons and analyzed with the Varian VNMR software package. Chemical shifts were measured in relation to residual methanol as reference, defined as 3.30 p.p.m. For each sample, 128–512 FIDs were collected,

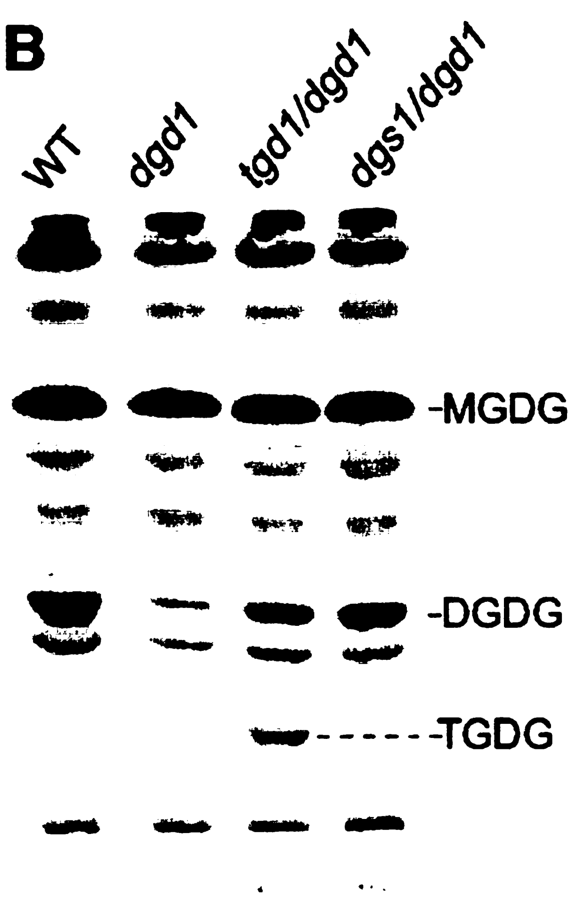

Figure B.1: **TLC of *tgd1* and wild type.** Lipids were extracted from the wild-type and mutants, as indicated above the image, and resolved by thin layer chromatography as outlined in the Materials and Methods section. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol.

depending on concentration. Suppression of the water signal was accomplished using a pre-saturating pulse in all mutant samples, which contained less lipid.

Results and Discussion

As shown in Figure 1, the *tgd1-1* mutant accumulates novel glycolipid species, which are absent in the wild-type. Gas-chromatography of alditol acetates derived from the hydrolyzed TGDG sample confirmed the identity of the monosaccharides as exclusively galactose, and fast-atom bombardment mass spectrometry (FAB-MS, data not shown) was consistent with a trigalactosyldiglyceride structure. NMR was used to determine the anomeric linkages of the trigalactosyl chain. Figure 2 shows the anomeric region of the 500 MHz NMR spectra of the galactolipids MGDG and DGDG isolated from the wild type and DGDG and TGTG from the *tgd1-1* mutant. MGDG has a β(1→3) linkage to diacylglycerol (Figure 1A), and DGDG has a Galα(1→6)-Galβ(1→3)-DAG (Figure 2B, αβDGDG) structure. Surprisingly, the structures of DGDG and TGDG isolated from the mutant did not contain these typical anomeric linkages, as shown in the spectra in Figures 2C and 2D. Whereas we expected the TGDG lipid to contain at least one α-linkage (that is, we assumed it would be derived by further galactosylation of αβDGDG already present in the membranes), we in fact observed three β-linkages. Given that the chemical shifts of all three β-protons were nearly identical, a linear all-β(1→6) chain is the most likely structure. Since the TGDG of the *tgd1-1* mutant is all β-linked, we assumed there to be a pool of ββDGDG giving rise to all-βTGDG. NMR of the DGDG isolated from the mutant (Figure 2B) shows clearly that it is a mixture of αβ- and ββDGDG, due to the

Figure B.2: **NMR of galactolipids isolated from wild-type and mutants.** Lipids were purified by TLC and NMR data was collected as described in Materials and Methods. A. MGDG (monoglacatosyldiacylglycerol) from the wild-type (Col-2) background. B. DGDG (digalactosyldiacylglycerol) from wild-type. C. DGDG from the *tgd1-1* mutant. D. TGDG (trigalactosyldiacylglycerol) from the *tgd1-1* mutant.

presence of a β-anomeric resonance at about the same position as one of the TGDG signals, and with about half the area as the α-anomeric resonance, indicating that the mixture is about 2/3 αβ- and 1/3 ββDGDG.

The phenomenology of TGDG synthesis deserves comment. It has been observed that Mg^{2+}ions stimulate the formation of TGDG in isolated chloroplasts, and NMR analysis of the TGDG accumulating in Mg^{2+}treated isolated spinach chloroplasts is of the all-β structure (data not shown), therefore both the TGDG being formed in the *tgd1-1* mutant and in isolated chloroplasts may derive from the same enzyme(s). It is also of note that the enzyme responsible for formation of TGDG resides on the outer chloroplast envelope, as judged by its susceptibility to thermolysin treatment (Xu et al., 2003), and also requires no addition of activated sugar donor (e.g. UDP-galactose). This leads to several requirements: the TGDG synthesis activity is attributable to a Mg^{2+}activated enzyme capable of removing the galactose from one galactolipid molecule and transferring it to a second, while retaining the β-configuration. This description is very similar to that of a β-galactosidase such as LacZ from *E. coli*, and a hypothetical scheme for the synthesis of all-β-oligogalactolipids by a galactosylhydrolase-like mechanism is given in Figure 4. Briefly, a catalytic aspartate residue in the active site cleaves the anomeric bond, resulting in a meta-stable α-linked galactosyl-enzyme intermediate (Juers et al., 2001). During the normal galactosidase cycle, a water molecule hydrolyzes the covalent intermediate, releasing galactose and free enzyme. If, however, the oxygen is part of an alcohol (e.g. the 6'-hydroxyl of an MGDG molecule) then the galactosylhydrolase functions as a galactosyltransferase. This activity for LacZ has been known for decades due to the formation of allolactose (Galβ(1→6)Glc), which proceeds

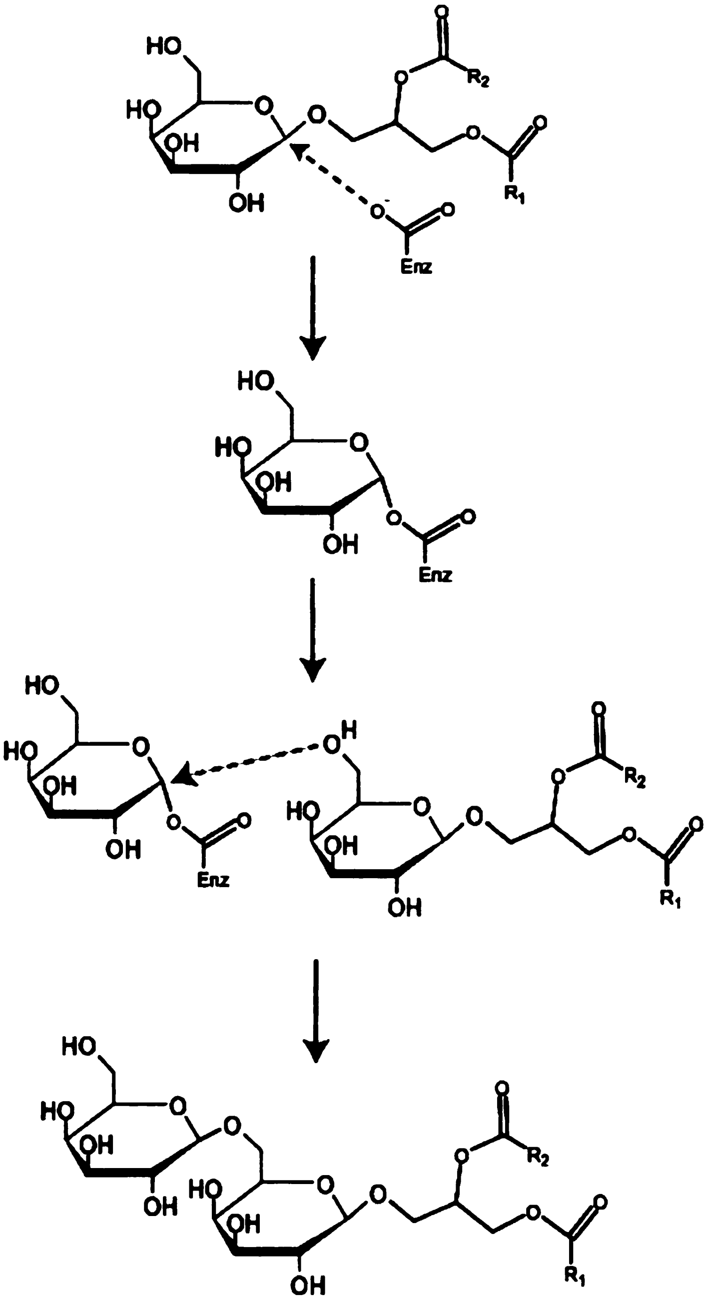

Figure B3: **Hypothesis for oligogalactolipid synthesis by a β-galactosidase mechanism.** As described in the text, (1) the catalytic aspartate in a β-galactosidase cleaves the galactose-glycerol linkage, forming (2) a covalent enzyme intermediate. This meta-stable intermediate is released by attack (3) of the 6' hydroxyl of a second MGDG molecule, leading to the accumulation of (4) ββ-DGDG. This cycle would create by breaking of the Galβ(1→4)Glc bond of lactose, forming the α-galactosyl-enzyme intermediate, followed by attack of the 6-hydroxyl of the same glucose on the galactosyl-intermediate. This reaction happens with the same glucose molecule when the lactose concentration is low, however at high concentrations of lactose, linear β(1→6) linked oligosaccharides are formed (Albayrak and Yang, 2002), i.e. the same structure as the headgroup portion of the oligogalactolipids described above. Therefore it seems feasible to conclude that the processive galactosyltransferase activity could be annotated as a glycosyl hydrolase family enzyme in the Arabidopsis genome database, perhaps even as a β-galactosidase.

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