Annotation of Genes Involved in Glycerolipid Biosynthesis in *Chlamydomonas reinhardtii*: Discovery of the Betaine Lipid Synthase BTA1<sub>Cr</sub>

Wayne R. Riekhof, Barbara B. Sears, and Christoph Benning

Department of Biochemistry and Molecular Biology, Department of Plant Biology, and Department of Energy-Plant Research Laboratory, Michigan State University, East Lansing, Michigan

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Lipid metabolism in flowering plants has been intensely studied, and knowledge regarding the identities of genes encoding components of the major fatty acid and membrane lipid biosynthetic pathways is very extensive. We now present an in silico analysis of fatty acid and glycerolipid metabolism in an algal model, enabled by the recent availability of expressed sequence tag and genomic sequences of *Chlamydomonas reinhardtii*. Genes encoding proteins involved in membrane biogenesis were predicted on the basis of similarity to proteins with confirmed functions and were organized so as to reconstruct the major pathways of glycerolipid synthesis in *Chlamydomonas*. This analysis accounts for the majority of genes predicted to encode enzymes involved in anabolic reactions of membrane lipid biosynthesis and compares and contrasts these pathways in *Chlamydomonas* and flowering plants. As an important result of the bioinformatics analysis, we identified and isolated the *C. reinhardtii* BTA1 (<em>BTA1<sub>Cr</sub></em>) gene and analyzed the bifunctional protein that it encodes; we predicted this protein to be sufficient for the synthesis of the betaine lipid diacylglycerol<sup>-</sup>N<sub>3</sub>N<sub>3</sub>-trimethylhomoserine (DGTS), a major membrane component in *Chlamydomonas*. Heterologous expression of <em>BTA1<sub>Cr</sub></em>, led to DGTS accumulation in <em>Escherichia coli</em>, which normally lacks this lipid, and allowed in vitro analysis of the enzymatic properties of BTA1<sub>Cr</sub>. In contrast, in the bacterium *Rhodobacter sphaeroides*, two separate proteins, Bta<sub>A</sub><sub>Rs</sub> and Bta<sub>B</sub><sub>Rs</sub> are required for the biosynthesis of DGTS. Site-directed mutagenesis of the active sites of the two domains of BTA1<sub>Cr</sub> allowed us to study their activities separately, demonstrating directly their functional homology to the bacterial orthologs Bta<sub>A</sub><sub>Rs</sub> and Bta<sub>B</sub><sub>Rs</sub>.

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Knowledge of genes and proteins involved in lipid metabolism in plants has greatly increased over the past decade. Many genes encoding enzymes of glycerolipid biosynthesis and fatty acid desaturation have been identified by genetic and biochemical means (10, 16, 33), and even lipid trafficking phenomena are beginning to be unraveled by genetic approaches (57). In addition, the availability of the *Arabidopsis* genome sequence (51) has facilitated the annotation of many uncharacterized gene products which are likely to have activities in lipid biosynthesis, trafficking, and catabolism (4). Although *Arabidopsis* is undoubtedly the most widely used plant system currently under investigation, the eukaryotic green alga *Chlamydomonas reinhardtii* is a well-established model for many processes, such as photosynthesis (31), phototaxis and flagellar function (47), posttranscriptional gene silencing (56), and nutrient acquisition (8). Recent large-scale expressed sequence tag (EST) projects and sequencing of the *C. reinhardtii* genome (21, 46) and the development of insertional mutagenesis (50), RNA interference methods (17, 48), and a molecular map (25) make *Chlamydomonas* an attractive genetic model. These attributes make *Chlamydomonas* also an ideal model organism with which to study the biosynthesis and physiological functions of nonphosphorous lipids, such as the plastidic galactolipids and sulfolipids and the betaine lipid diacylglycerol<sup>-</sup>N<sub>3</sub>N<sub>3</sub>-trimethylhomoserine (DGTS) (18, 29, 39), as well as phospholipids, such as phosphatidylethanolamine (PtdEtn) (58) and phosphatidylglycerol (PtdGro) (11, 34, 45).

We have begun to explore *Chlamydomonas* as a model for plant lipid metabolism by using a high-throughput robotic screening method to find mutants with defects in lipid metabolism (35). The initial goal of the study reported here was to identify and annotate genes involved in acyl-lipid metabolism in the *Chlamydomonas* version 2.0 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 lipid biosynthesis pathways and posit their subcellular localizations. As a specific example, this study allowed us to identify and characterize the enzyme responsible for the synthesis of the betaine lipid DGTS, which replaces the phospholipid phosphatidylcholine (PtdCho) in extraplastidic membranes of *Chlamydomonas*. Due to its structural similarity to PtdCho and the lack of PtdCho in *Chlamydomonas*, DGTS is presumed to take on the functions of PtdCho as the major component of the extraplastidic membranes of this organism (29, 40, 42).

Previous work elucidated the pathway for DGTS biosynthesis in *Rhodobacter sphaeroides* (23), and genetic analysis with this bacterium identified two gene products, Bta<sub>A</sub> and Bta<sub>B</sub>, which are required for phosphate stress-induced DGTS production in *R. sphaeroides* (27). The *btaA* gene was proposed to encode an <em>S</em>-adenosylmethionine (AdoMet):diacylglycerol (DAG) 3-amino-3-carboxypropyltransferase giving rise to the intermediate diacylglycerolhomoserine (DGHS), and the *btaB* gene was proposed to encode an AdoMet-dependent N-meth-
**TABLE 1. Strains and plasmids used in this work**

| Strain or plasmid | Description* | Source |
|-------------------|--------------|--------|
| **Strains**       |              |        |
| *C. reinhardtii*   |              |        |
| CC125             | Wild type; mt+ | Chlamydomonas Genetics Center, Duke University | Arthur Grossman |
| dw15.1            | nit1-305 cv15; mt+ |        |
| **E. coli TOP10F** | Cloning and expression strain: ψ800acZΔM15 F’ [lacI ΔTn10 (Tet‘)] | Invitrogen |
| **Plasmids**      |              |        |
| pBluescript SK(+)  | Cloning vector; Amp’ | Stratagene |
| pACYC-31          | Expression vector; Cam’ | Qiagen |
| pQE-31           | btaAB operon in pCHB500 | 27 |
| pRK1232          | Sphl-KpnI fragment of btaA amplified from pRK1232 in pACYC-31 | This work |
| pBTA1           | BamHI-KpnI fragment of the BTA1C, coding sequence in pQE-31 | This work |
| pBTA1-μA        | V340A-D341A derivative of pBTA1 | This work |
| pBTA1-μB        | V121A-D122A derivative of pBTA1 | This work |

* mt+, mating type +.

**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://www.jgi-psf.org/chlamy. Genes encoding enzymes and other components of lipid metabolism (e.g., acyl carrier proteins [ACP’s]) were manually annotated based on key word searches executed against the automatically annotated gene loci (21), followed by verification of similarity by BLASTP searches (1) and CLUSTALW multiple-sequence alignments (52) with verified protein sequences. In addition, direct BLASTP and TBLASTN searches of the version 2.0 assembly based on key word searches executed against the automatically annotated gene loci (21), followed by verification of similarity by BLASTP searches (1) and CLUSTAL-W multiple-sequence alignments (52) with verified protein sequences.

**Cloning and expression of BtaA, BTA1, and derivatives of BTA1 in Escherichia coli.** A summary of all strains and plasmids used in this study is shown in Table 1. The cloning region of btaA was PCR amplified from plasmid pRK1232 (27) for expression in pACYC-31. The coding region of btaA from plasmid pRK1232 (27) for expression in pACYC-31 (9) with the following primers (restriction sites are underlined): btaA forward (SpHl), 5’-ACATGACATCGAC GACGAGGTCGCTCCAACCG-3’; and btaA reverse (KpnI), 5’-GGGTCGGGTCGT-3’. PCR was carried out with Taq DNA poly- merase according to the manufacturer’s specifications, except that 10% (v/v) dimethyl sulfoxide was added to each reaction mixture to overcome difficulties in PCR due to the high G+C content of *R. sphaeroides* DNA. The PCR product was cloned into the EcoRI site of pBluescript SKI(+) into the Michigan State University (MSU) Genomics Technology Support Facility, and subcloned into pACYC-31 by using the restriction sites underlined in the PCR primer sequences shown above. The resulting construct was designated pBtaA-LC (btaA in pACYC-31).

**C. reinhardtii** strain CC125 was grown to mid-log phase in 50 ml of TAP medium (22) and harvested by centrifugation. RNA was purified with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. Total RNA (1.0 μg) was reverse transcribed with SuperScript II RNase H reverse transcriptase. The coding region of BTA1C, was amplified from an aliquot of cDNA with Pfu DNA polymerase and the following primers (the native start codon of BTA1C, in the forward primer is shown in bold type, and BamHI and KpnI sites are underlined in the forward and in the reverse primers, respectively): 5’-CAGGTACCTCAATTGGGGTTGGCTGCT-3’; and coding sequence in pOE-31, coding sequence in pQE-31.

**In vivo assays of DGHS and DGTS synthesis in E. coli.** Site-directed mutagenesis of pBTA1 was carried out with a Quikchange XL kit (Stratagene) according to the manufacturer’s instructions. For the V340A-D341A mutant version of pBTA1 (pBTA1-μA), the mutagenic primers were 5’-GCCAGGGTGTTGTCGGGCGCATGCACACCCGCGCA-3’ and its reverse complement μA-rev. The V121A-D122A mutant (pBTA1-μB), the mutagenic primers were 5’-CAAGTACCTCAGTGCCCGCTGTCAGTCTGCTTAA-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 Sall site and “μA” introduces an Sall site) in order to easily identify strains carrying mutated plasmids.

pBluescript and pOE-31 derivatives were propagated in Luria-Bertani (LB) medium containing ampicillin at 100 μg/ml and pACYC-31 derivatives were propagated in LB medium containing chloramphenicol at 25 μg/ml. Strains containing compatible combinations of plasmids were constructed by cotransformation of heat shock competent cells of TOP10F (Invitrogen) with 10 ng of each plasmid followed by selection on LB agar containing both ampicillin and chloramphenicol.

**In vivo assays of DGHS and DGTS synthesis in E. coli.** **E. coli** TOP10F cells harboring compatible combinations of plasmids were grown as 2-ml overnight cultures, and 0.1 ml samples of these cultures were used to inoculate 10 ml of LB medium containing appropriate antibiotics. After growth to an optical density at 600 nm of ~0.6, the cultures were harvested by centrifugation and dispersed into 10 ml of M9 minimal medium (37) containing 0.2 mM isopropyl-β-D-thiogalactoside (IPTG) and 0.5 μg of [1-14C)methionine (American Radiolabeled Chemicals, St. Louis, Mo.). Cultures were incubated for 3 h and harvested by centrifugation; these steps were followed by extraction with 2 ml of chloroform-methanol (1:1 [v/v]) and phase separation by the addition of 0.5 ml of M KCl-0.2 M H3PO4. A portion of the organic phase from each extraction was spotted on activated (120°C, 2 h) thin-layer chromatography (TLC) plates (Si 250; Baker), resolved with chloroform-
acetonemethanol-acetic acid-water (10:4:2:2:1 [vol/vol]), and subjected to autoradiography.

In vitro BTA1Cr activity assays. E. coli TOP10F cells harboring pBTA1 were grown in 250 ml of LB medium-ampicillin at 37°C to an optical density of 0.7 and induced with 0.25 mM IPTG; these steps were 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 dithiothreitol, 1 mM EDTA [pH 7.3]). The resuspended cells were sonicated three or four times for 30 s each time with a microprobe tip, and the lysates were centrifuged at 2,000 × g for 10 min to remove unbroken cells and cellular debris. Aliquots (1 ml) of the cell extracts were frozen in liquid N2 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-activity profile were conducted with a 100-μl final volume by combining 48.75 μl of cell extract with 48.75 μl of 100 mM HEPES, Tris-Cl, or morpholineethanesulfonic acid, 1 mM dithiothreitol, and 1 mM EDTA at various initial pHs to give a final pH in the range of 5.5 to 8.6 when mixed with the cell extract (initial pH, 7.3). Reactions were initiated by the addition of 25,000 dpm of [1-14C]AdoMet (American Radiolabeled Chemicals; 2.5 μCi; final concentration of AdoMet, 2.1 μM), incubated for 30 min at 28°C, and terminated by the addition of 400 μl of chloroform-methanol (1:1 [vol/vol]) and 100 μl of 0.9% (wt/vol) NaCl to separate the aqueous and organic phases. Under these conditions, product formation was linear over time up to 1 h at all concentrations of AdoMet tested. The organic phase was transferred to a new tube, dried under a stream of nitrogen, and dissolved in 50 μl of chloroform-methanol (1:1 [vol/vol]). This lipid extract was spotted on silica TLC plates (Baker) and developed with the solvent chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1 [vol/vol]), and subjected to autoradiography.

Structure confirmation for DGTS produced by BTA1Cr in E. coli. E. coli cells harboring pBTA1 were cultured as described above for the production of crude extracts for enzyme assays. DGTS was isolated from lipid extracts by preparative chromatography on a PhosphorImager screen (Molecular Dynamics, Piscataway, N.J.) with the ImageQuant software package. Determination of the Kₘ was carried out at pH 7.2 with various concentrations of AdoMet by direct fitting of a hyperbola to the experimental data in the Origin software package (OriginLab, Northampton, Mass.).

Structure confirmation for DGTS produced by BTA1Cr in E. coli. E. coli cells harboring pBTA1 were cultured as described above for the production of crude extracts for enzyme assays. DGTS was isolated from lipid extracts by preparative TLC on ammonium-sulfate-impregnated silica TLC plates with the solvent chloroform-acetone-methanol-acetic acid-water (10:4:2:2:1 [vol/vol]). The DGTS zone was scraped from the plates, eluted with chloroform-methanol (1:1 [vol/vol]), and concentrated under a stream of nitrogen. Fast-atom bombardment-mass spectrometry was done as previously described (5), except that ethanol was used as a matrix. 1H nuclear magnetic resonance (NMR) spectroscopy was conducted by dissolving approximately 200 μg of lipid in CDC13, and data were collected as previously described (5).

RESULTS

Annotation of genes involved in acyl-lipid biosynthesis in the Chlamydomonas genome. (i) Overall results. A metabolic reconstruction of glycerolipid metabolism in Chlamydomonas from genomic sequence data was performed in silico. For this purpose, we searched all available genomic sequences of Chlamydomonas, essentially covering the entire genome, for deduced protein sequences with significant similarity to lipid biosynthetic enzymes with experimentally verified activities from plants and other organisms. For building of the pathway models, we also considered analytical data published for the lipid compositions of various membranes in Chlamydomonas and plants in general. Figures 1 and 2, in conjunction with Table 2, show schemes for membrane lipid biosynthesis, starting with fatty acid synthesis in the plastid (Fig. 1, steps 1 to 4 and 16), plastidic glycerolipid biosynthesis (Fig. 1, steps 5 to 15), extraplastidic phospholipid and betaine lipid biosynthesis (Fig. 1, steps 16 to 31), and fatty acid desaturation (Fig. 2, steps 32 to 40). Genes are identified by their version 2.0 gene model transcript number, as well as by three-letter abbreviations based on the tentative assignment of function. These assignments were made in accordance with the recommendations of the Commission on Plant Gene Nomenclature and the Mendel database (28).

(ii) 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 flowering plants. For example, it is clear that all components of the multimeric bacterium-type acetyl coenzyme A (acetyl-CoA) carboxylase (Fig. 1 and Table 2, 1a to 1d) and fatty acid synthase complexes (Table 2, 4a to 4d) are accounted for and that these are most similar to those that have been described for flowering plant plastids. We identified single orthologs for many of the genes encoding enzymes central to fatty acid synthesis in the plastid.
biosynthesis, e.g., 3-hydroxyacyl-ACP-dehydratase and enoyl-CoA reductase (Table 2, 4c and 4d), components of the type II fatty acid synthase, and the predicted proteins have high and approximately equal probabilities of being targeted to both mitochondria and chloroplasts, as judged by TargetP analysis (12).

The pathway of sulfoquinovosyldiacylglycerol (SQDG) biosynthesis (Fig. 1, steps 11 and 12) has been thoroughly characterized in Arabidopsis and shown to be the function of the products of two genes, namely, SQD1 and SQD2 (14, 59). SQD1 has been characterized in Chlamydomonas by both insertional inactivation (35) and heterologous complementation of a sulfolipid-deficient sqdB cyanobacterial mutant (43), and the SQD1 gene, encoding UDP-sulfoquinovose synthase (Fig. 1, step 11), is highly expressed, as judged by EST abundance. This disproportionately high mRNA abundance might be due to the relatively large amount of SQDG in Chlamydomonas compared to that in Arabidopsis, coupled with the fact that the $k_{cat}$ for the reaction catalyzed by SQD1 is very low (38), a feature which might necessitate increased abundance of the enzyme. Genes encoding enzymes of galactolipid biosynthesis are also present in the genome, with MGD1 encoding monogalactosyldiacylglycerol synthase (Fig. 1, step 14) and DGD1 encoding digalactosyldiacylglycerol synthase (Fig. 1, step 15).

Several functions in plastidic lipid biosynthesis are still without candidate genes; these include the phosphatidylglycerol-phosphate phosphatase (Fig. 1, step 9), which is unknown in plants and algae (4), and a putative activity utilizing SQDG and an unidentified acyl donor to form 2'-O-acyl-SQDG (Fig. 1, step 13). 2'-O-Acyl-SQDG has been shown to be a minor component of Chlamydomonas lipids; however, it is apparently absent from flowering plants (35). It should also be noted that, unlike the endoplasmic reticulum (ER) isoforms, the plastidic isoform of phosphatidate phosphatase (Fig. 1, step 10), which is responsible for generating DAG for use in galactolipid and sulfolipid synthesis, is not known yet in flowering plants.

(iii) Extraplastidic lipid metabolism. Membrane lipid metabolism outside the plastid in Chlamydomonas differs from that in flowering plants by both the presence of unique pathways and the apparent absence of certain central pathways and branches of pathways. Most notable is the tentative absence of a gene for phosphoethanolamine methyltransferase, which provides the pathway for the 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 and an opi3 mutant of Saccharomyces cerevisiae, respectively (6, 32). The apparent lack of both a pathway for PtdCho precursor biosynthesis in Chlamydomonas and the PtdEtn methylation pathway, whereby PtdEtn is N trimethylated to form PtdCho, correlates well with the lack of PtdCho in cellular membranes (19).

Phosphatidylserine (PtdSer) is a major precursor of PtdEtn in yeasts, bacteria, and plants through the action of PtdSer decarboxylase (53); however, Chlamydomonas has been reported to lack PtdSer as a component of its membranes (19). This fact is corroborated by our survey of the genome, in that there is an apparent lack of genes encoding PtdSer synthase or phospholipid base exchange enzymes. These data lead us to propose that the biosynthesis of PtdEtn is the function of a single pathway, consisting of serine decarboxylase (36), to generate ethanolamine (Fig. 1, step 25); this step is followed by phosphorylation of ethanolamine (Fig. 1, step 26), activation with CTP to form CDP-ethanolamine (Fig. 1, step 27), and transfer of the phosphoethanolamine moiety to DAG to form PtdEtn (Fig. 1, step 24). The Chlamydomonas CTP:phosphoethanolamine cytidylyltransferase enzyme was recently characterized by expression in E. coli and biochemical characterization of the recombinant protein (58).

Certain pathways are assumed to be present in multiple compartments. For example, PtdGro biosynthesis is proposed to be present in the plastid, mitochondria, and ER, and genes
| Reaction or step | Description                      | Gene model(s) | Gene(s) | EST(s) |
|-----------------|----------------------------------|---------------|---------|--------|
| **Plastid and mitochondrial pathways** | Multimeric acetyl-CoA carboxylase components | C_160059 | ACX1 | 23 |
| 1a              | Alpha-carboxyltransferase        | C_1150054 | BCX1 | 39 |
| 1b              | Beta-carboxyltransferase         | C_2010018 | BCR1 | 18 |
| 1c              | Biotin carboxylase               | C_590042 | BXP1 | 16 |
| 1d              | Biotin carboxyl carrier protein  | C_250035 | BXP2 | 27 |
| 2               | Acyl carrier protein             | C_650040 | ACP1 | 38 |
| 3               | Malonyl-CoA:ACP transacylase     | C_240122 | MCT1 | 4 |
| 4               | Type II fatty acid synthase      | C_140066 | KAS1 | 17 |
| 4a              | 3-Ketoacyl-ACP synthase          | C_720014 | KAS2 | 7 |
| 4b              | 3-Ketoacyl-ACP reductase         | C_1320034 | KAS3 | 2 |
| 4c              | 3-Hydroxyacyl-ACP dehydratase    | C_650040 | KAR1 | 19 |
| 4d              | Enoyl-ACP reductase              | C_1210022 | ATSI | 8 |
| 5               | Glycerol-3-phosphate:acyl-ACP acyltransferase | C_240122 | MCT1 | 4 |
| 6               | Lyso-phosphatidate:acyl-ACP acyltransferase | C_120223 | ENR1 | 64 |
| 7               | CDP-DAG synthetase               | C_30067    | CDS1 | 12 |
| 8               | Phosphatidylglycerophosphate synthase | C_140066 | PGP1 | 21 |
| 9               | Phosphatidylglycerophosphate phosphatase | C_210023 | PGP2 | 4 |
| 10              | Phosphatidylglycerophosphate phosphatase | C_210023 | PGP2 | 4 |
| 11              | UDP-sulfoquinovose synthase      | C_60107    | SQD1 | 46 |
| 12              | Sulfolipid synthase              | C_250126 | SQD2a | 5 |
| 13              | Sulfolipid 2'':O-acetyltransferase | C_650023 | SQD2b | 0 |
| 14              | Monogalactosyldiacylglycerol synthase | C_210283 | DGD1 | 1 |
| 15              | Digalactosyldiacylglycerol synthase | C_210283 | DGD1 | 1 |
| 16              | Acyl-ACP thiolase                | C_210283 | FAT1 | 5 |
| 17              | Long-chain acyl-CoA synthetase   | C_1580041 | LCS1 | 11 |
| 18              | Glycerol-3-phosphate:acyl-CoA acyltransferase | C_7940001 | LCS2 | 0 |
| 19              | Lyso-phosphatidate:acyl-CoA acyltransferase | C_2010018 | No candidate |
| 20              | Phosphatidylphosphatase          | C_1170013 | PAP1 | 1 |
| 21              | CDP-DAG synthetase               | C_630223 | CDS2 | 4 |
| 22              | AdoMet synthetase                | C_750005 | SAS1 | 82 |
| 23              | Betaine lipid synthase           | C_570065 | BTA1 | 42 |
| 24              | CDP-ethanolamine:DAG-ethanolamine| C_900032 | EPT1 | 2 |
| 25              | Serine decarboxylase             | C_250130 | SDC1 | 43 |
| 26              | Ethanolamine kinase              | C_290062 | EKI1 | 0 |
| 27              | CTP:phosphoethanolamine cytidyltransferase | C_900032 | ECT1 | 15 |
| 28              | Phosphatidylglycerophosphate synthase | C_1350041 | PGP3 | 0 |
| 29              | Phosphatidylglycerophosphate phosphatase | C_260084 | No candidate |
| 30              | Inositol-3-phosphate synthase    | C_800063 | INO1 | 31 |
| 31              | CDP-DAG:inositol phosphotransferase | C_260084 | PIS1 | 3 |
| **Fatty acid desaturase plastidic isoforms** | Stearoyl-ACP-Δ9-desaturase | C_170008 | FAB2 | 10 |
| 32              | PufGro palmitate-Δ3t-desaturase   | C_350009 | FAD5 | 70 |
| 34              | MGDG palmitate-Δ7-desaturase     | C_790066 | FAD6b | 264 |
| 35              | α6-Desaturase                    | C_290028 | FAD6b | 0 |
| 36              | α3-desaturase                    | C_250125 | FAD7 | 39 |
| 37              | MGDG 16-carbon Δ4-desaturase      | C_260084 | No candidate |
| **Fatty acid desaturase cytosolic isoforms** | Oleate desaturase | C_590003 | FAD2a | 20 |
| 38              | Linoleate desaturase             | C_4630001 | FAD2a | 0 |
| 39              | Linoleate or linolenate-Δ5-desaturase | C_250037 | FAD3 | 4 |

*Proteins were identified on the basis of similarity to known proteins, and predicted subcellular targeting was based on an analysis of the N terminus and on similarity to isomers from other species for which the subcellular localization is known. Candidates for dual targeting to chloroplasts and mitochondria are in bold type; subunits are in italic type. 3t, 3-trans. Abbreviations are as in Fig. 1.

EST counts represent data available from the Joint Genome Institute genome browser in May and June 2004.
encoding three isoforms of phosphatidylglycerophosphate synthase are present in the genome. Two of these (Fig. 1, step 8) are expressed, as judged by EST analysis, and both proteins contain a predicted plastid or mitochondrial targeting sequence. The other isoform is predicted to be cytosolic but is not represented in the EST database. This finding may correlate with the fact that PtdGro is predicted to be present at a much lower abundance in the cytosol than in the plastid (19); thus, the gene encoding the cytosolic activity might not be as highly expressed as those directing PtdGro synthesis in the organelles. It is also noteworthy that we were unable to identify a gene for cardiolipin synthase, an enzyme typically present in the mitochondria of eukaryotes.

(iv) Fatty acid desaturases. In addition to the genes involved in the synthesis of the glycerolipids themselves, fatty acid desaturase genes were identified and annotated. The FAD gene complement of Arabidopsis has been well characterized at the genetic and biochemical levels (33), and orthologs are present in the Chlamydomonas genome, as outlined in Fig. 2. Of note are the putative extraplasmic ω6- and ω3-desaturase genes (Fig. 2, steps 38 and 39), as well as predicted plastidic orthologs of the Arabidopsis FAD5, FAD6, and FAD7 or FAD8 genes (33) (Fig. 2, steps 34 to 36). The plastidic ω6-desaturase gene of Chlamydomonas (Fig. 2, step 35) was identified previously in a mutant screen for high-chlorophyll fluorescence mutants and subsequently was cloned and characterized (41, 44). We were unable to assign candidate genes for the Δ4-desaturase involved in the generation of the unusual fatty acid 16:4Δ4,7,10,13 (carbon-double bonds, positions) in the plastid (Fig. 2, step 37) or the Δ5-desaturase involved in 18:3Δ5,9,12 and 18:4Δ5,9,12,15 synthesis in the ER (Fig. 2) (40).

BTA1_Cr is a bifunctional protein sufficient for DGTS biosynthesis. The pathway of betaine lipid biosynthesis in the bacterium R. sphaeroides and the proposed involvement of the enzymes BtaARs and BtaB Rs are shown in Fig. 3. In this bacterium, the betaine lipid DGTS is formed under phosphate-limited growth conditions (5). Chlamydomonas is unusual in that its membranes are devoid of PtdCho and constitutively contain DGTS in its place. Searching of the whole-genome shotgun sequence assembly with the bacterial BtaARs protein sequence (27) revealed a predicted protein (encoded by transcript C_570065) which we have named BTA1_Cr. It shows 27% identity and 40% similarity to BtaARs over about 400 amino acids. In addition, portions of BTA1_Cr were similar to the bacterial BtaB Rs protein. Indeed, examination of the genomic structure of BTA1_Cr (C_570065) revealed that the protein consists of an N-terminal domain with similarity to BtaB Rs and a C-terminal portion with similarity to BtaA Rs (Fig. 4A). The genomic locus is approximately 6 kb long, and the transcript is interrupted 11 times by introns (Fig. 4A). The predicted mRNA also contains a relatively long 3′ untranslated region of ~1.6 kb which is common to many Chlamydomonas transcripts.

Figure 4B shows a sequence alignment of BTA1_Cr and the BtaA Rs and BtaB Rs proteins. The residues identified for BtaA Rs as being potentially important in AdoMet binding are conserved in BTA1_Cr, and two residues in predicted AdoMet binding motif II (24) of the BtaA-like domain (V340 and D341) are indicated in Fig. 4B. To demonstrate functional homology between the two bacterial proteins and the two domains of BTA1_Cr, amino acids V340 and D341 were chosen for the mutagenesis experiments described below, because we expected that a V340A-D341A 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 would be expected to still be active in the methylation steps. Additionally, the analogous positions in AdoMet binding motif II (24) of the BtaB-like portion (V121 and D122) were targeted for alanine mutagenesis (Fig. 4B). In this situation, it was presumed that DGHS synthesis activity would be intact but that the methyltransferase portion would be unable to bind AdoMet and therefore would be inactive. Cloning of the BTA1_Cr cDNA (GenBank AY656806) and expression in E. coli led to the accumulation of relatively large amounts of DGTS, allowing structure elucidation. Figure 5A shows a TLC separation of lipids from E. coli expressing BTA1_Cr, and it is apparent that there is a new species accumulating in the expression strain. This lipid was subsequently identified as DGTS by positive-ion fast-atom bombardment-mass spectrometry (Fig. 5B) and NMR spectroscopy. It consists of molecular species that are typical of E. coli membrane lipids; e.g., the species at m/z 710.7 is consistent with DGTS carrying 32:1 (carbon-double bonds) fatty acids, and that at m/z 736.7 is a 34:2 species of DGTS. The 1H NMR spectrum (data not shown) exhibited resonances consistent with those previously reported (5), most notably, a strong nine-proton resonance at 3.22 ppm, which corresponds to the methyl protons of the DGTS quaternary ammonium group.
Kinetic analysis of BTA1_{Cr}. To characterize the activity of the BTA1_{Cr} protein, we expressed the gene in E. coli, broke the cells by sonication, and assayed the incorporation of [1-^{14}C]AdoMet into labeled DGHS, partially methylated DGHS derivatives, and the end product DGTS. In Fig. 6, the pH-activity profile and the determination of the AdoMet K_m for the overall reaction of BTA1_{Cr} are shown. The relative stoichiometries of the methylation products change with increasing pHs (Fig. 6A), indicating a difference in the pH optima between the BtaA-like and the BtaB-like domains. Figure 6B shows the quantification of the total counts in each lane of Fig. 6A, plotted against the pH; this analysis indicates that the pH optimum of the overall reaction is ~7.2. Figure 6C shows a plot of the rate of overall product formation against substrate concentration; this analysis was used to determine, by direct fitting of a hyperbola to the experimental data, that the apparent K_m for AdoMet was 16 μM.

Site-directed mutagenesis of AdoMet binding domains of BTA1_{Cr}. To corroborate the relatedness of the two domains of BTA1_{Cr} and the respective bacterial proteins, site-directed mutagenesis was used to change residues predicted to be critical for AdoMet binding in the active sites of the BtaA-like and BtaB-like domains. These were designated BTA1_{Cr}A and BTA1_{Cr}B, respectively (Fig. 3). The BTA1_{Cr}A protein was inactive in the synthesis of DGHS, as shown in Fig. 7, second lane, with only background levels of label in the DGTS or DGHS regions of the chromatogram. BTA1_{Cr}B, however, produced DGHS (Fig. 7, third lane), as confirmed by cochromatography with the product of a strain carrying btaARs (Fig. 3) on a low-copy-number plasmid, which caused the accumulation of DGHS (Fig. 7, fourth lane). Figure 7, fifth lane, shows DGTS accumulating in the pBTA1-containing E. coli strain, and Fig. 7, sixth lane, shows that the BtaB-like methyltransferase domain in pBTA1-μA was still capable of methylating DGHS produced by BtaARs. These results establish the functions of the two domains of BTA1_{Cr} as being comparable to those of the individual bacterial orthologs and corroborate the hypothesis that BTA1_{Cr} is capable of carrying out all reactions necessary for DGTS biosynthesis in Chlamydomonas.

FIG. 4. Identification of a BtaA-BtaB fusion protein encoded by the Chlamydomonas genome. (A) An approximately 6-kb locus (gene model C_570065) encodes a protein with N- and C-terminal domains showing similarity to the bacterial betaine lipid biosynthesis proteins BtaB and BtaA, respectively, 5' and 3' untranslated regions are indicated in grey, and exons and introns are indicated as thick and thin line segments, respectively. (B) Multiple-sequence alignment of the BTA1_{Cr} protein and its bacterial counterparts in R. sphaeroides. The residues marked with arrowheads were targeted for alanine mutagenesis in the BTA1_{Cr}A and BTA1_{Cr}B proteins. The sequences of BtaB and BtaA (GenBank accession numbers AAK53561 and AAK53560, respectively) were treated as a single entity here; the starting methionine of BtaARs is indicated by double underlining. The sequences are indicated by a solid line; the BtaARs protein sequence is indicated by a broken line. Grey shading and black shading indicate conserved and identical residues, respectively; dashes indicate gaps.
DISCUSSION

With the exception of our case study of BTA1_Cr, all of our conclusions are based on an in silico analysis of genome sequences of Chlamydomonas. We present an annotation of glycerolipid biosynthetic genes and a subsequent metabolic pathway reconstruction, which was guided in part by our general knowledge of lipid biosynthesis in Arabidopsis and other plants. Genome comparisons between less well-studied organisms, such as Chlamydomonas, and more well-studied model organisms, such as Arabidopsis, at least with regard to lipid metabolism, provide great opportunities to rapidly assess the evolution or adaptation of, in this case, Chlamydomonas; they also permit one to focus on the essential differences, e.g., the absence of PtdCho and the biosynthesis of DGTS in Chlamydomonas. However, one should not forget that annotation by an in silico analysis primarily serves to build hypotheses, which subsequently need to be rigorously corroborated to gain generally accepted knowledge. For example, the absence of a certain enzyme or pathway does not mean that alternative pathways do not exist. Likewise, predicting the subcellular localizations of proteins from signal sequences is possible only with limitations. Keeping these caveats in mind, we cautiously draw the conclusions discussed below, which hopefully will stimulate and guide further membrane lipid research with Chlamydomonas.

Chlamydomonas lipid metabolism is less complex than that of flowering plants. During the course of bioinformatic analysis and annotation, several themes became apparent. First, the core pathways of fatty acid biosynthesis and glycerolipid assembly in Chlamydomonas appear to be simpler than those in Arabidopsis, reflected both in the presence and/or in the absence of certain pathways and in the apparent sizes of the gene families that represent the various activities. A case in point is that of galactolipid synthesis. In the Chlamydomonas genome, we have identified only one MGDG synthase gene (MGD1) and one DGDG synthase gene (DGD1), as opposed to three MGD1 isoform-encoding genes (3) and two DGD1 isoform-encoding genes (26) in the Arabidopsis genome. This phenomenon may be thought of as being parallel to the lack of PtdCho and its presumed replacement by the nonphosphorous analog DGTS in Chlamydomonas. In Arabidopsis, phosphate deprivation has been shown to up-regulate the expression of MGD2, MGD3, and DGD2, concomitant with a loss of PtdCho and an accumulation of DGDG in extraplastidic membranes (10). 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 (2). 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 apparently has no need to replace PtdCho with DGDG due to the presence of DGTS, the phosphate-inducible pathway of DGDG biosynthesis may be expendable and has been lost during the course of evolution, or it may simply have evolved only in flowering plants.

Potential for dual targeting of proteins involved in lipid metabolism. A second theme is apparent in the biosynthesis of fatty acids, in that some of the core components of this process may be dually targeted to both the plastid and the mitochondrion. Several lines of evidence support this hypothesis. First, it is known from studies of isolated organelles of flowering plants that fatty acid synthesis occurs in both plastids and mitochondria (54), the former being responsible for the majority of fatty acid biosynthesis to supply precursors for mem-

FIG. 5. BTA1_Cr is sufficient to catalyze the biosynthesis of DGTS. (A) E. coli harboring pBTA1 was grown and expressed as described in Materials and Methods, and lipids were separated by TLC and visualized with iodine vapor. A culture containing empty vector pQE-31 served as a negative control. CL, cardiolipin. (B) Fast-atom bombardment–mass spectrometry in the positive-ion mode was used to confirm the identity of the putative DGTS spot on the TLC plates. Peaks at m/z 710.7 and m/z 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, respectively.
brane lipid synthesis and the latter being a function of the biosynthesis of lipoic acid, a critical prosthetic group for certain mitochondrial enzymes (49). Several genes encoding enzymes that are central to fatty acid synthesis, e.g., components of the multimeric acetyl-CoA carboxylase and the type II fatty acid synthase, as well as ACPs, are potentially represented by only one gene (two genes in the case of ACPs) in the Chlamydomonas genome. Since the activities are present in multiple compartments but may be the products of a single gene, these proteins may be imported by both plastids and mitochondria; this phenomenon has a growing number of precedents, especially for proteins involved in core metabolic processes, such as nucleotide biosynthesis and DNA replication (7, 55).

In agreement with these observations, 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 dually targeted multifunctional acetyl-CoA carboxylase in grasses (15) also lends credence to this hypothesis. Additionally, Beisson et al. (4) 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 both Arabidopsis and Chlamydomonas, although direct experimental evidence is currently lacking.

BTA1cr is a bifunctional enzyme involved in DGTS biosynthesis. BTA1cr was identified based on similarities to the bacterial betaine lipid synthesis proteins BtaAR and BtaBR, encoding the presumed AdoMet:DAG 3-amino-3-carboxypropyltransferase and the DGHS methylase, respectively (Fig. 3). To experimentally verify the predicted enzymatic activities of BTA1cr, we expressed the BTA1cr cDNA (GenBank AY656806) in E. coli. This procedure resulted in DGTS biosynthesis from the expression of a single protein, thus confirming that BTA1cr catalyzes all of the reactions specific to the pathway. The production of the BtaA-like portion of BTA1cr, by itself in E. coli was expected to result in DGHS accumulation, as was shown for BtaAR (Fig. 7, fourth lane); however, we were unable to show that the truncated protein has DGHS synthesis activity in E. coli (data not shown).

As an alternative way of revealing the functions of the two domains of BTA1cr, we altered residues in the predicted AdoMet binding sites of the BtaA-like and BtaB-like domains. The results of this experiment were informative on several
levels. First, the fact that the BTA1Cr V340A-D341A mutant (BTA1Cr,µA) was inactive in the synthesis of DGHS and DGTS lends credence to the model that the BtaA-like domain binds AdoMet in a methyltransferase fold. Given the structure of the cognate binding site of methyltransferases, V340 is expected to make favorable hydrophobic interactions with the adenine moiety of AdoMet, and D341 is proposed to form hydrogen bonds to the 2′ and 3′ hydroxyls of the ribose portion. Mutation of these residues to alanine rendered the DGHS synthase function of BTA1Cr inactive or at least compromised it to below the limits of detection. The DGHS trimethylase function was left unaffected, given that the BTA1Cr,µA protein was still capable of methylating DGHS produced by simultaneous expression of the bacterial btaA/Rd gene (Fig. 7, sixth lane). This finding provided an additional piece of information regarding the sequence of reactions, showing that the BtaB-like domain of BTA1Cr is able to take DGHS from the membrane as a substrate, rather than that it is transferred directly from the active site of the BtaA-like domain, as would be the case for substrate channeling. These data are in agreement with the findings of in vitro assays conducted with isolated C. reinhardtii membranes (29) as well as the results presented in Fig. 6A, showing an accumulation of a transient pool of DGHS in in vitro assays.

Enzyme mechanisms for DGTS biosynthesis are conserved across kingdoms. BTA1Cr activity was measured in a cell-free system, relying on endogenously formed DAG, the presumed lipid substrate, and exogenous radiolabeled AdoMet. The analysis was somewhat complicated by the fact that DGHS synthase and methyltransferase activities were present on the same polypeptide; thus, multiple products were formed. However, measurement of all products on the same TLC plate (Fig. 6A) made this a trivial point, in that the sum of DGHS and its methylated products gave the total flux through the BtaA-like domain, and this quantity was used in the analyses shown in Fig. 6B and C. In fact, Fig. 6A 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 that the final methylation (forming DGTS) is favored at an even higher pH (∼8). The pH dependence of these subsequent methylation reactions may reflect a change in the pKₐ values of amine species with higher degrees of methylation. The apparent Kₚ for recombinant BTA1Cr activity is presumably directly comparable to that for the activity that was measured in C. reinhardtii membranes (29). However, the value for the recombinant enzyme (∼16 µM) was about fivefold lower than that for the native enzyme, indicating that the membrane composition and environment of the enzyme may affect the affinity for AdoMet of the DGHS synthase module. This scenario 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 Kₚ of the BTA1Cr domain for AdoMet might be lower. The question of the true Kₚ remains unanswered given our current inability to reconstitute BTA1Cr 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 exception that the eukaryotic activities are fused into a single polypeptide. This latter phenomenon is common among metabolic enzymes, such as urease, which consists of three separate structural components in Klebsiella aerogenes that are fused together on a single polypeptide in legumes (20), and acetyl-CoA carboxylase, which is present as a multimeric complex in bacteria but consists of a single multifunctional polypeptide in eukaryotes (30). Apparently, the same regulatory advantages that are in play in those systems are important in DGTS biosynthesis as well; i.e., having one gene to encode the machinery for the entire pathway presumably alleviates the coordination required for the proper expression of multiple individual genes.

Conclusions. In this study, we identified a set of genes encoding products that are likely to be involved in membrane glycerolipid biosynthesis in Chlamydomonas. However, the assembly of a biological membrane is more than 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 situation of a lipid being synthesized in one membrane and transported to another must be accounted for when one is devising a mechanistic description of membrane biogenesis. Likewise, simply knowing the genes involved in PtdEtn and DGTS biosynthesis (or that of any other set of lipids in a given membrane) implies nothing about how the final stoichiometric ratio between them is regulated so as to form a functional membrane with the correct biophysical properties. Given the apparent lack of redundancy in the pathways described above, the absence of complicating factors, such as tissue specificity, and the potential for functional analysis by gene silencing, 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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References
1. Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25:3389-3402.
2. Anderson, M. X., M. H. Stridh, K. E. Larsson, C. Liljenberg, and A. S. Sandelinus. 2003. Phosphate-deficient oat replaces a major portion of the plasma membrane phospholipids with the galactolipid digalactosyldiacylglycerol. FEBS Lett. 537:126-132.
3. Arai, K., E. Marechal, M. A. Block, D. Brun, T. Masuda, H. Shimada, K. Takamiya, H. Ohta, and J. Joyard. 2001. Two types of MGDG synthase genes, found widely in both 16:3 and 18:3 plants, differentially mediate galactolipid synthesis in photosynthetic and nonphotosynthetic tissues in Arabidopsis thaliana. Proc. Natl. Acad. Sci. USA 98:10960-10965.
4. Beisson, F., A. J. Koo, S. Runksa, J. Schwender, M. Pollard, J. J. Thelen, T. Paddock, J. J. Salas, L. Savage, A. Milcamps, V. B. Mhaske, Y. Cho, and J. B. Ohlrogge. 2003. Arabidopsis genes involved in acyl lipid metabolism. A 2003 census of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based database. Plant Physiol. 132:681-697.
5. Bolognese, C. Z. H. Huang, and D. A. Gage. 1995. Accumulation of a novel glycolipid and a betaine lipid in cells of Rhodobacter sphaeroides grown under phosphate limitation. Arch. Biochem. Biophys. 317:103-111.
6. Bolognese, C. P., and P. McGraw. 2000. The isolation and characterization...
in yeast of a gene for Arabidopsis S-adenosylmethionine:phosphoethanolamine-N-methyltransferase. Plant Physiol. 124:1800–1813.

7. Chabregas, S. M., D. D. Luche, L. P. Farias, A. F. Ribeiro, M. A. van Sluys, C. F. M. Menck, and M. C. Silva. 2001. Dual targeting properties of the N-terminal signal sequence of Arabidopsis thaliana THI1 protein to mitochondria and chloroplasts. Plant Mol. Biol. 46:639–650.

8. Davies, J. P., F. H. Yildiz, and A. Grossman. 1996. SacI, a putative regulator that is critical for survival of Chlamydomonas reinhardtii during sulfur deprivation. EMBO J. 15:2193–2199.

9. Dörmann, P., I. Balbo, and C. Benning. 1999. Arabidopsis galactolipid bio-synthesis and lipid trafficking mediated by DGDI. Science 284:2181–2184.

10. Dörmann, P., and C. Benning. 2002. Galactolipid role in seed trends. Plant Physiol. 129:112–118.

11. El Manani, A., G. Dubertret, M. J. Delrieu, O. Roche, and A. Trompieres. 1998. Mutants of Chlamydomonas reinhardtii affected in phosphatidylglycerol metabolism and thylakoid biogenesis. Plant Physiol. Biochem. 36:609–619.

12. Emanuelsson, O., H. Nielsen, and G. von Heijne. 1998. ChloroP, a neural network at road method for predicting chloroplast transit peptides and their cleavage sites. Proc. Natl. Acad. Sci. USA 95:5910–5915.

13. Emanuelsson, O., H. Nielsen, and G. von Heijne. 1999. Update on the Commission on Plant Gene Nomenclature biosynthesis of diacylglycerol-O-4-sulfolipid head precursor UDP-sulfoquinovose in vitro. J. Biol. Chem. 274:3941–3946.

14. Fransen, W., R. L. Jones, D. J. Bues, W. Riekhof, J. E. Froehlich, and C. Benning. 2003. Recombinant Arabidopsis SQD1 converts UDP-glucose and sulfoquinovose 1,125:1501–1507.

15. Fransen, W., R. L. Jones, D. J. Bues, W. Riekhof, J. E. Froehlich, and C. Benning. 2003. Recombinant Arabidopsis SQD1 converts UDP-glucose and sulfoquinovose in vitro. J. Biol. Chem. 274:3941–3946.

16. Fransen, W., R. L. Jones, D. J. Bues, W. Riekhof, J. E. Froehlich, and C. Benning. 2003. Recombinant Arabidopsis SQD1 converts UDP-glucose and sulfoquinovose in vitro. J. Biol. Chem. 274:3941–3946.

17. Fransen, W., R. L. Jones, D. J. Bues, W. Riekhof, J. E. Froehlich, and C. Benning. 2003. Recombinant Arabidopsis SQD1 converts UDP-glucose and sulfoquinovose in vitro. J. Biol. Chem. 274:3941–3946.

18. Fransen, W., R. L. Jones, D. J. Bues, W. Riekhof, J. E. Froehlich, and C. Benning. 2003. Recombinant Arabidopsis SQD1 converts UDP-glucose and sulfoquinovose in vitro. J. Biol. Chem. 274:3941–3946.