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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

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Abstract

When we think of extremophiles, organisms adapted to extreme environments, prokaryotes come to mind first. However, the unicellular red micro-alga *Galdieria sulphuraria* (Cyanidiales) is a eukaryote that can represent up to 90% of the biomass in extreme habitats such as hot sulfur springs with pH values of 0–4 and temperatures of up to 56 °C. This red alga thrives autotrophically as well as heterotrophically on more than 50 different carbon sources, including a number of rare sugars and sugar alcohols. This biochemical versatility suggests a large repertoire of metabolic enzymes, rivaled by few organisms and a potentially rich source of thermo-stable enzymes for biotechnology. The temperatures under which this organism carries out photosynthesis are at the high end of the range for this process, making *G. sulphuraria* a valuable model for physical studies on the photosynthetic apparatus. In addition, the gene sequences of this living fossil reveal much about the evolution of modern eukaryotes. Finally, the alga tolerates high concentrations of toxic metal ions such as cadmium, mercury, aluminum, and nickel, suggesting potential application in bioremediation. To begin to explore the unique biology of *G. sulphuraria,* 5270 expressed sequence tags from two different cDNA libraries have been sequenced and annotated. Particular emphasis has been placed on the reconstruction of metabolic pathways present in this organism. For example, we provide evidence for (i) a complete pathway for lipid A biosynthesis; (ii) export of triose-phosphates from rhodoplasts; (iii) and absence of eukaryotic hexokinases. Sequence data and additional information are available at http://genomics.msu.edu/galdieria.

Abbreviations: ACCase, acetyl-coA carboxylase; ADP-Glc, ADP-glucose; AGPase, UDP-glucose pyrophosphorylase; CDP-Cho, CDP-choline; DGDG, digalactosyldiacylglycerol; DHAP, dihydroxyacetone phosphate; FA, fatty acid; FAS, fatty acid synthase; Glc 6-P, glucose-6-phosphate; MGDG, monogalactosyldiacylglycerol; PEP, phosphoenolpyruvate; 2PGA, 2-phosphoglyceric acid; 3PGA, 3-phosphoglyceric

[†]Deceased. Wolfgang's contribution was critical to this project. His knowledge and personality will be greatly missed.

acid; PGP, phosphatidylglycerophosphate; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdFGro, phosphatidylglycerol; Ptdlns, phosphatidylinositol; RubisCO, ribulose-l,5-bisphosphate carboxylase/oxygenase; SQDG, sulfoquinovosyldiaclyglycerol; TPT, triose phosphate/phosphate translocator; UDP-Glc, UDP-glucose; UGPase, UDP-glucose pyrophosphorylase

Introduction

Most extremophiles that survive at high temperatures (95–113 °C) are microorganisms from archaeal or bacterial origins. In contrast, the presently known upper temperature limit for eukaryotic microorganisms is 62 °C, and most metazoans (multicellular eukaryotes) are unable to grow above 50 °C (Tansey and Brock, 1972; Rothschild and Mancinelli, 2001). Surprisingly, photosynthetic prokaryotes, such as the cyanobacteria, are completely absent from hot acidic waters (Brock, 1973). Instead, thermo-acidophilic photosynthetic unicellular red algae of the Cyanidiales are the principal photosynthetic organisms found in these ecological niches (Doemel and Brock, 1970; Rothschild and Mancinelli, 2001; Donachie *et al*., 2002). The three species belonging to the Cyanidiales (*Cyanidium, Cyanidioschyzon,* and *Galdieria*) can grow at pH 0 and temperatures up to 57 °C (Doemel and Brock, 1971; Brock, 1978) and they are found globally in hot, acidic habitats.

The taxonomic positions of *Cyanidium, Cyanidioschyzon* and *Galdieria* have been recently updated (Albertano *et al*., 2000). Previously, more than one organism has been referred to under the name *Cyanidium caldarium*. Based on detailed studies of morphology and the modes of cell division, three independent species have been established: *Galdieria sulphuraria, Cyanidium caldarium,* and *Cyanidioschyzon merolae* (De Luca *et al*., 1978; Merola *et al*., 1981). Moreover, the strains *C. caldarium* (Allen), *C. caldarium* M-8, and *C. caldarium* Forma B are considered to be identical with *G. sulphuraria*, while *C. caldarium* RK-1 and *C. caldarium* Forma A correspond to C. *caldarium*. This proposed taxonomy is also in concordance with recent molecular evidence (Cozzolino *et al*., 2000; Oliveira and Bhattacharya, 2000).

G. sulphuraria naturally inhabits volcanic environments such as hot sulfur springs and solfatara soils (pH 0.05–4 and temperatures up to 57 °C), but also thrives in equally hostile environments originating from human activities such as strip or opencast mining. In these ecological niches, it frequently represents up to 90% of the total biomass and almost 100% of the eukaryotic biomass. Cells thrive in pure CO_2 (Seckbach and Libby, 1970; Seckbach *et al*., 1970) and at elevated atmospheric pressure. *G. sulphuraria* is also able to grow photoautotrophically, mixotrophically, and heterotrophically (Gross, 1999). It thrives on more than 50 different carbon sources such as sugars, sugar alcohols and amino acids (Rigano *et al*., 1976; Gross and Schnarrenberger, 1995a,b; Gross, 1999; Oesterhelt *et al*., 1999), a metabolic flexibility matched by only few other organisms. However, *G. sulphuraria* has also adapted to less hostile environments, as a non-thermophilic strain was recently isolated in the Czech Republic (Gross *et al*., 2002).

Acidic environments are frequently characterized by high concentrations of iron, cadmium, aluminum, nickel, and other metals that are highly toxic to almost all organisms. *G. sulphuraria* is resistant to high levels of toxic metal ions such as 200 mM aluminum. The resistance is likely due to active secretion of metal ions (Yoshimura *et al*., 1999, 2000; Nagasaka *et al*., 2002), but such a mechanism has yet to be confirmed. In addition, *G. sulphuraria* tolerates up to 10% (w/v) salinity (Albertano *et al*., 2000).

Remarkably, *G. sulphuraria* can maintain a proton gradient of 1:1 million across its plasma membrane during several months of continuous darkness (Gross, 2000), a feat which acidophilic green algae such as *Dunaliella acidophila* cannot match. The mechanism by which this temporary impermeability to protons is achieved is unknown. However, *G. sulphuraria* possesses a very rigid protein-rich cell wall (Bailey and Staehelin, 1968; Staehelin, 1968), and it has been hypothesized, based on studies with archaebacteria, that the incorporation of sterols, saturated fatty acids, bipolar tetraether lipids, and proteins could account for this impermeability (Benz and Cros,

1978; Benz *et al*., 1980; Elferink *et al*., 1992; Komatsu and Chong, 1998). Very long chain dicarboxylic acids are thought to have similar roles in some eubacteria (Jung *et al*., 1993, 1994; Burdette *et al*., 2002).

Using microspectrophotometry, a genome size of 10.8 Mbp was estimated for *G. sulphuraria* 074W (Muravenko *et al*., 2001). Genome sizes ranging between 9.8 Mbp (*G. sulphuraria* 19.71) and 14.2 Mbp (*Galdieria spec*. isolates, Rio Tinto, Spain) were determined using pulsed-field gel electrophoresis (Moreira *et al*., 1994). A genome of 13.4–14.2 Mbp was reported for the related species *C. merolae* (Takahashi *et al*., 1995). The lower range of these estimates, together with *Ostreococcus tauri* (*Prasinophyceae*) (Derelle *et al*., 2002), are among the smallest genomes reported for free-living eukaryotes (yeast: 12.1 Mbp). A genome size of 12 Mbp represents approximately 1/10th the size of the A. *thaliana* genome (AGI, 2000), indicating an unusually high gene density, small or absent introns, and very few repetitive sequences. Little information is available on the intron–exon structure of *Galdieria* genes. The gene encoding thylakoid membrane light harvesting complex (LHC) contains five introns of 50–74 bp length. Four are typical spliceosomal introns with GT-AC borders, the fifth starts with GC (Marquardt *et al*., 2000).

Very limited sequence information is currently available from red algae in general, and from thermo-acidophilic red algae in particular. As of December 2003, the only large-scale EST-dataset for red algae stems from sporophytes and gametophytes of the marine red alga *Porphyra yezoensis*. A search of GenBank for the keywords *Galdieria, Cyanidium,* and *Cyanidioschyzon* revealed 83 entries for *Galdieria*, 68 entries for *Cyanidium*, and 52 for *Cyanidioschyzon*.

A rapid method for generating sequence information and for identifying genes of a particular organism is the random sequencing of cDNAs to produce expressed sequence tags (ESTs). We have employed this method to the unicellular, thermo-acidophilic red alga *Galdieria sulphuraria* and we report here the sequencing of approximately 6000 ESTs from this organism. An EST-catalogue from this alga is useful for functional and phylogenetic comparisons with other organisms, and for the reconstruction of unique metabolic pathways. Moreover, the extreme habitat of *G. sulphuraria* likely requires specific adaptations to abiotic stresses such as high temperature, low pH , and toxic metal ions. The sequence information reported here, in conjunction with the accompanying website, provides molecular access to this enigmatic microalga.

Materials and methods

Strains and media

Autotrophic growth conditions

Galdieria sulphuraria strain 074G was cultivated axenically in mineral medium at 30 °C in a 12 h light/12 h dark photoperiod (80 μM m^{-2} s^{-1}) as described previously (Gross and Schnarrenberger, 1995a,b). Photoautotrophic cultures were aerated with 100% CO_2.

Heterotrophic growth conditions

G. sulphuraria 074G was cultivated axenically in mineral medium supplemented with 25 mM glucose at 37 °C in the dark as described previously (Gross and Schnarrenberger, 1995a,b).

Construction of cDNA libraries

Cells equivalent to 10 g fresh weight were disrupted in a bead beater (1 min) in extraction buffer (50 mM Tris–Cl pH 7.5, 50 mM EDTA, 1% (w/v) SDS, 0.5 mg ml^{-1} proteinase K). After centrifugation (20 min, 12 000 × *g*) the supernatant was extracted twice with phenol:chloroform:isoamylalcohol (24:24:1 v/v) and DNA and RNA was precipitated by the addition of 2 vol isopropanol. The pellet was dissolved in DEPC-treated ddH_2O and RNA was precipitated by adding 1 vol 4 M LiCl. The RNA pellet was washed twice with 70% (v/v) ethanol, dried, and dissolved in ddH_2O. Poly (A) mRNA was isolated from total RNA using a commercial kit (Qiagen, Hilden, Germany). cDNA was synthesized from 5 μg of mRNA using a cDNA synthesis kit (Stratagene, La Jolla, CA) and was directionally cloned into the UNI-Zap XR vector (Stratagene). Phage DNA was packaged into *in vitro* assembled phage heads using the Gigapack III Gold Cloning kit (Stratagene). The resulting cDNA libraries contained 3×10^5 (heterotrophic library) and 2×10^6 (photoautotrophic library) independent clones. The libraries were

amplified to $>10^9$ pfu ml^{-1} according to the manufacturer's recommendations.

Isolation of plasmid DNA, DNA sequencing, and data storage

Phage libraries were converted into plasmid libraries by *in vivo* mass excision according to the manufacturer's recommendations (ExAssist system, Stratagene). Colonies were randomly picked using a GeneMachines Mantis Colony and Plaque Picker (GeneMachines, San Carlos, CA) and plasmid DNA was prepared from overnight cultures using a Qiagen 3000 robot (Qiagen). DNA sequences were determined by cycle-sequencing and sequence analysis using an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). All sequence data and chromatograms were stored on a Geospiza Finch server (Geospiza, Seattle, WA).

Reconstitution of phosphate translocators

Spinach chloroplast envelope membranes were prepared as described previously (Joyard *et al.*, 1982). *G. sulphuraria* cells were homogenized in a bead beater with acid-washed glass beads (0.5 mm diameter) for 3 min in a buffer consisting of 50 mM Tricine-KOH (pH 7.6), 2 mM EDTA, 2 mM Na-ascorbate, and 1 mM PMSF. The homogenate was diluted by addition of 10 volumes homogenization buffer, centrifuged at 5000 × g for 5 min to remove intact cells and debris, and subsequently centrifuged at 120 000 × g for 30 min to collect the membrane fraction. The bright-blue supernatant was discarded, and the pellet was resuspended in homogenization buffer and subjected to an additional centrifugation as described above to remove remaining soluble proteins. The membrane pellet was frozen in liquid nitrogen and stored at −80 °C until reconstitution. Reconstitution of membranes into liposomes and transport experiments were carried out as described previously (Flügge and Weber, 1994; Kammerer *et al.*, 1998).

Degenerate primers specific for hexokinase

The sequences of the degenerate oligonucleotide primers derived from an alignment of the amino acid sequences of hexokinases from plants, fungi, and mammals were as follows: Hxklfw [5′-d[TIG GIGGIACIAAYTTYMGIGT-3′]; Hxk2fw [5′-d[TIGGITTYACITTYWSITTYCC]-3′];Hxk3rev-[5′-d[ARIGTICCIACIGTRTCRTT]-3′]; Hxk4rev [5′-d[ARIGCIGCICCIAYICCISWICC]-3′]. PCR was performed with the primer combinations Hxk2fw/Hxk3rev and Hxklfw/Hxk4rev, respectively, using 5–10 μg of genomic DNA and a cycling program of 2 min at 94 °C followed by 35 amplification cycles (30 s, 94 °C; 30 s, 56 °C, 60 s, 72 °C) and a subsequent extension step at 72 °C for 5 min. All PCR products were sub-cloned and sequenced to confirm their identity.

Results and discussion

Sequence analysis

Two cDNA libraries, generated from photoautotrophically and heterotrophically grown *G. sulphuraria* cells, respectively, were used in this study (see Materials and methods for details). A total of 5915 cDNA inserts were sequenced from their 5′-ends, of which 3323 represented the autotrophic library, and 2592 represented the heterotrophic library. Base calls were screened for vector and *E. coli* contamination, and quality values were assigned to each base using the *phred* algorithm (Ewing and Green, 1998, 2000; Ewing *et al.*, 1998). A total of 5270 sequence reads (89% of the total) passed the filter, and the final dataset consisted of 3024 sequences generated from the autotrophic library and 2246 sequences generated from the heterotrophic library. The average *phred* Q20 (Ewing *et al.*, 1998) read length was 563 bases. The 5270 passing sequences were clustered using the stackPACK software package (South African National Bioinformatics Institute, University of the Western Cape, Republic of South Africa), resulting in 3047 contigs (1.7 Mbp) of unique, non-redundant sequence. Given that the *G. sulphuraria* genome is approximately 10–12 Mbp in size (Moreira *et al.*, 1994; Muravenko *et al.*, 2001), 15–17% of the genome would be covered by the sequence data reported here.

Of these 3047 contigs, 1746 and 1276 consisted of ESTs from only the autotrophic or heterotrophic libraries, respectively. Surprisingly little overlap was found between both libraries, only 34 contigs contained sequence information derived

from both libraries. This is most likely due to two reasons: (i) gene expression levels are expected to be significantly different under both conditions, hence each library represents a snapshot of the expression profile under a specific culture condition rather than a comprehensive collection of cDNA clones; (ii) during the early phases of random sequencing projects such as EST sequencing, a relatively high 'novelty rate' is obtained whereas redundancy massively increases in the later stages (Wendl *et al*., 2001). Two thousand four hundred and thirty one ESTs (80%) were unique sequences, whereas only 20% of the 3047 contigs contained more than one EST sequence. This indicates that we did not reach saturation; hence only little overlap between both libraries is to be expected.

The 3047 individual contigs were compared to NCBI's NR and Arabidopsis GenBank databases (Benson *et al*., 2003), respectively, using the BLASTX algorithm (Altschul *et al*., 1990, 1997). Of the contigs, 1691 (55%) displayed similarities to the NR dataset with E-values equal or smaller than $1e^{-5}$, and 1376 contigs (44%) displayed similarities equal or smaller than $1e^{-5}$ with the Arabidopsis dataset. Hence, approximately 45% of contigs represent novel sequences that have not previously been identified in other organisms. Based on the similarities to genes of known function, putative functions could be assigned to 45% of the ESTs, whereas 55% encode proteins of unknown function or for which only a general function prediction was possible (Table 1).

A comprehensive, searchable list of BLASTX results can be found at http://genomics.msu.edu/galdieria. In addition, a more detailed breakdown of functional categories can be found at the website.

Pathway analysis

Since the ESTs are accessible through the accompanying website and because of space limitations, the EST analysis presented here is focused on the reconstruction of central metabolic pathways and a survey of putative solute transporters in the red

Table 1. Functional grouping of EST sequences.

	Information storage and processing (aut/het/both/total)
Translation, ribosomal structure and biogenesis	110/53/3/166
Transcription	79/64/0/143
DNA replication, recombination, repair	43/21/1/65
	Cellular processes
Cell division and chromosome partitioning	48/22/3/73
Posttranslational modification, protein turnover, chaperones	87/57/2/146
Cell envelope biogenesis, outer membrane, cell wall	9/5/2/16
Cell motility and sécrétion	10/5/0/15
Inorganic ion transport and metabolism	32/26/1/59
Signal transduction mechanisms	69/51/1/121
	Metabolism
Energy production and conversion	47/45/4/126
Carbohydrate transport and metabolism	78/80/1/159
Amino acid transport and metabolism	67/31/0/98
Nucleotide transport and metabolism	9/13/0/22
Coenzyme metabolism	18/27/1/46
Lipid metabolism	38/25/1/64
Secondary metabolites biosynthesis, transport and catabolism	36/20/0/56
	Poorly characterized
Function unknown	798/590/11/1399
General function prédiction only	127/101/3/231

alga. Of the ESTs, 525 encode metabolic enzymes, and 152 encode putative transport proteins.

Photosynthetic and respiratory carbon and energy metabolism

As expected for a photoautotrophic organism, cDNAs encoding all enzymes of the Calvin cycle, with the sole exception of ribose-5-phosphate isomerase, were present among the sequenced ESTs. cDNAs encoding the entry enzyme of the oxidative pentose-phosphate pathway, glucose-6-phosphate dehydrogenase, were identified, whereas no EST with similarities to the OPPP-specific enzyme transaldolase were found. With the exception of fumarase, the mitochondrial tricarboxylic acid cycle is represented in the dataset and components of all five complexes of the mitochondrial electron transport chain, as well as an alternative oxidase, were identified.

EST-analysis provides no evidence for the presence of hexokinase in G. sulphuraria

All enzymes of the glycolytic pathway downstream of fructose-6-phosphate are represented by ESTs. Surprisingly, no ESTs encoding phosphoglucomutase, phosphoglucoisomerase, hexokinase, fructokinase or glucokinase were found, while the activity of these enzymes was previously demonstrated in *G. sulphuraria* cell extracts (Oesterhelt *et al*., 1996; Heilmann *et al*., 1997; W. Gross, unpublished data). The absence of cDNAs encoding hexokinase may be due to under-representation of corresponding ESTs in the libraries, or to very low expression levels of the gene. To further explore the presence or absence of hexokinase-related sequences in *G. sulphuraria,* we employed PCR using degenerate oligonucleotide primers, and screening of cDNA libraries under low-stringency conditions. Based on aligned hexokinase amino acid sequences from animals, fungi, and plants, two sets of degenerate oligonucleotide primers were designed (see Materials and methods) and tested on genomic DNAs from yeast and *Arabidopsis*. In each case, specific products were amplified (Figure 1), suggesting that these primers can generally be used to amplify hexokinase-related sequences from different kingdoms.

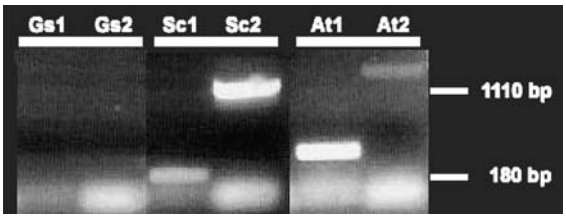

Figure 1. Detection of hexokinase-related sequences by PCR using degenerate primers derived from hexokinase sequences from animals, plants, and fungi. Two sets of degenerate primers were used to amplify hexokinase-related DNA sequences from genomic DNA of Arabidopsis (Atl, At2), yeast (Scl, Sc2), and *Galdieria* (Gsl, Gs2).

However, when the primer sets were applied to genomic DNA of *G. sulphuraria,* no specific products were amplified. In addition, using different plant hexokinase cDNAs as hybridization probes, the autotrophic and the heterotrophic cDNA libraries were screened under low-stringency conditions for hexokinase-related cDNA clones from *G. sulphuraria*. No positive clones were isolated by this approach. It should be noted that, using this approach, we have been able to identify a novel, distantly related hexokinase-like cDNA from potato (GenBank acc. no. AFl18134; 58% amino acid identity with hexokinase 1 from spinach), demonstrating that the screening conditions permit the isolation of less conserved cDNAs. The absence of hexokinase-related DNA sequences in the EST-collection and the failure of the PCR-based approach, indicates that *G. sulphuraria* does not possess a typical eukaryotic hexokinase.

Further support for the absence of a eukaryote-type hexokinase in *G. sulphuraria* comes from the recently finished genome sequence of the closely related species *C. merolae* (Matsuzaki *et al*., 2004). The *C. merolae* genome does not encode a eukaryotic hexokinase but a glucokinase (CMO276C) that is typically found in bacteria but not in eukaryotic organisms. The *C. merolae* glucokinase shows 36% amino acid identity with glucokinase from *Synechocystis* PCC603 (e-value $3e^{-47}$) and 30% amino acid identity with the corresponding protein from *E. coli*. Interestingly, the red algal glucokinase is characterized by a C-terminal 73 amino acid extension that is not found in prokaryotic forms of the enzyme. The C-terminus contains a highly hydrophobic domain that very likely represents a transmembrane helix, indicating

that the *C. merolae* glucokinase is anchored in a cellular membrane. Although the primary structure of red algal glucokinase is not related to hexokinases from green plants, the membrane anchor that is found in several plant hexokinases but not in other eukaryotic hexokinases (Wiese *et al.*, 1999) is also present in the *C. merolae* enzyme. In contrast to the green plant enzyme that carries the membrane anchor at its N-terminus, the red algal enzyme, however, has a C-terminal membrane anchor.

Identification of candidate genes in starch and floridoside metabolism

In contrast to green plants, red algae do not store starch (floridean starch) in the plastid stroma, but instead in the cytosolic compartment (Viola *et al.*, 2001). Whereas starch in most green plants consists of 15–30% of the linear β-1,4 glucan amylose and 70–85% of the branched chain β-1,4 and β-1,6 glucan amylopectin, floridean starch consists exclusively of amylopectin (Viola *et al.*, 2001; Yu *et al.*, 2002). The precursor for starch biosynthesis in green plants is ADP-Glc (Martin and Smith, 1995; Smith *et al.*, 1997; Smith, 1999), whereas both UDP-Glc and ADP-Glc have been reported as precursors for starch biosynthesis in red alga (Viola *et al.*, 2001). UDP-glucose is synthesized from glucose-1-phosphate and UTP by UDP-glucose pyrophosphorylase (UGPase) and ADP-glucose is synthesized from ATP and glucose-1-phosphate by ADP-glucose pyrophosphorylase (AGPase). Our data do not provide evidence for the presence of AGPase in *G. sulphuraria,* whereas UGPase could be clearly detected (A4_36F11). It displays highest similarity with UGPase from the red alga *G. gracilaris* (66% identity over a stretch of 203 amino acids, e-value $1e^{-73}$), followed by several UGPases from mammals and fungi (human: 63% identity over a stretch of 204 amino acids, e-value $4e^{-68}$). UGPases from vascular plants were more distantly related, the highest similarity was detected to UGPase from barley (59% identities over a stretch of 196 amino acids, e-value $3e^{-60}$).

One EST (HET_25C03) showed significant similarities with β-glucan synthases from fungi and granule-bound starch synthases from pea and bean. The deduced amino acid sequence closely matches the E-X_7-E motif (Figure 2) that is found in eukaryotic glycosyltransferase family 3 proteins such as glycogen synthase (Cid *et al.*, 2000, 2002). However, similar motifs are found in four other families of retaining glycosyltransferases family members. Hence, it cannot be excluded that EST HET_25C03 encodes a protein with a function that is not related to glycogen synthesis.

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HET_25C03                PSLFEPGGIVQHEFFVAGT
                         ||  || |    |  | |
Animal/fungal consensus PSYYEPWGYTPAECTVMGI
                           E--X7---E
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Figure 2. Alignment of EST HET_25C03 with the consensus motif of eukaryotic family 3 glycosyltransferases.

Several ESTs with similarities to starch phosphorylases (cytosolic isoform H), isoamylases, and β-amylases were identified. In *G. sulphuraria,* these enzymes are most likely involved in the breakdown of floridean starch. In contrast to higher plants, red algae frequently use the soluble sugars floridoside (2-β-D-galactosylglycerol) and isofloridoside (1-β-D-galactosylglycerol) instead of sucrose. Floridoside is synthesized from UDP-galactose and glycerol-3-phosphate by the concerted actions of floridoside-phosphate synthase (EC 2.4.1.137) and and floridoside-phosphate phosphatase (EC 2.4.1.96) (Kremer and Kirst, 1981). UDP-galactose is synthesized from UDG-Glc and galactose-1-phosphate by galactose-1-phosphate uridylytransferase (EC 2.7.712). This enzyme is most likely not present in higher plants but its activity has recently been reported in *G. sulphuraria* (Gross and Schnarrenberger, 1995a,b) and a corresponding cDNA has been described from G. *gracilis* (Lluisma and Ragan, 1998). At least two ESTs from *G. sulphuraria* show high similarity to known galactose-1-phosphate uridilyltransferases (HET_ 36B03, HET_11G09), which is highly suggestive for the presence of this enzyme in the thermo-acidophilic red alga. In many vascular plants, animals, fungi, and bacteria, UDP-galactose is synthesized from UDP-Glc by UDG-Glc epimerase (EC 5.1.3.2) and UDP-Glc epimerase activity was also reported for *G. sulphuraria* (Prosselkov *et al.*, 1996). However, no ESTs showing similarity to UDG-Glc epimerase were found.

Floridoside-phosphate synthase and floridoside-phosphate phosphatase have not been characterized at the molecular level, therefore no corresponding ESTs could be identified. However, several ESTs with similarities to putative

galactosyltransferases were found, and it is tempting to speculate that the corresponding proteins may be involved in floridoside biosynthesis. Two ESTs (A4_22C06, HET_24G12) with high similarity (e-values $3e^{-66}$ and $2e^{-34}$, respectively) to α-galactosidases (EC 3.2.1.22) were present. This enzyme has been described for a number of fungal and bacterial species, but not yet for a member of the plant kingdom.

EST analysis indicates the presence of a complete photorespiratory pathway in G. sulphuraria

Photosynthesis at high temperatures is challenging because of the increased oxygenation to carboxylation ratio of RubisCO under these conditions. Moreover, the growth in silica-covered endolithic mats reduces gas exchange with ambient air, and leads to low CO_2-partial pressures, which promote the oxygenation reaction (Gross *et al*., 1998). Although RubisCO from the closely related organism *G. partita* was shown to have the highest specificity in the direction of the carboxylation reaction of all analyzed RubisCO enzymes (Uemura *et al*., 1997; Sugawara *et al*., 1999; Okano *et al*., 2002), a notable production of phosphoglycolate is to be expected. Some algal species are able to excrete glycolate (Urbach and Gimmler, 1968; Beudeker and Tabita, 1983; Goyal and Tolbert, 1996; Goyal, 2002), however this adaptation has not been reported for *G. sulphuraria*. Based on the EST dataset, several enzymes involved in the photorespiratory pathway such as phosphoglycolate phosphatase (HET_43H04), glycolate oxidase (A4_38D07, HET_37H12), catalase (A4_33F10), serine-glyoxylate aminotransferase (A4_23A05), serine hydroxymethyltransferase (A4_32A08, A4_36C10, A4_10D04, A4_39-E01), and the P (A4_04D06), T (A4_05B09, A4_34F04), and H-proteins (A4_06E09) of glycine decarboxylase were identified in *G. sulphuraria*. In addition, several ESTs representing glutamine synthetase were detected (A4_33A10, A4_33F03, HET_10AO, HET_24C05). Glutamate synthase (GOGAT) is encoded by the plastid genomes of the closely related species *Cyanidium caldarium* (Glöckner *et al*., 2000) and *Cyanidioschyzon merolae* (Ohta *et al*., 2003); therefore the absence of GOGAT from the EST collection is not surprising. No ESTs with similarities to phosphoglycolate phosphatase or glycerate kinase were found, presumably because the expression of the respective genes is low in *G. sulphuraria*.

Analysis of fatty acid and lipid metabolism related ESTs indicates a potential for lipid a biosynthesis in G. sulphuraria

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 multiprotein 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 members of the green lineage, most likely synthesizes fatty acid in the plastid via bacterial/plastidic type multimeric ACCase and FAS complexes, and then incorporates some of these FA into plastidic membrane lipids, and exports a portion of these fatty acids 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 hydratatase 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 phosphatidylethanol-

amine biosynthesis were identified, consisting of phosphatidylserine decarboxylase (HET_44E08) and the CDP-ethanolamine pathway (ethanolamine kinase, HET_10E09; ethanolamine phosphate cytidylyltransferase, A4_10G06). Phospha-tidylcholine is also apparently synthesized by two pathways in which either phosphoethanolamine is methylated to phosphocholine (phosphoethanolamine methyl-transferase, 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, two 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 Ptdlns 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-D4 desaturase (GS00710, two ESTs) were present.

Plastidial lipids consist of four species: mono- and digalactosyldiacylglycerol (MGDG and DGDG), sulfoquinovosyldiaclyglycerol (SQDG), and PtdGro. MGDG synthase (HET_20D10) and SQD1 (HETJ20F10) 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 lipid A has never been unequivocally identified in seed plants. Here, we provide for the first time evidence for a complete lipid A biosynthetic pathway in plants. In *Galdieria,* the first two steps (LpxA, LpxC) in the pathway are likely to be encoded in the plastid genome by analogy *with Cyanidioschyzon merolae* (Ohta *et al*., 2003) and *Cyanidium caldarium* RK1 (Glöckner *et al*., 2000). Later steps in the pathway (LpxD, LpxB, LpxK, WaaA) appear to be nuclear encoded and corresponding ESTs are present in our collection.

The function of lipid A in plants (if present) is mysterious, and there are no published reports of lipid A or derivatives being detected in seed plants. It is tempting to posit that, given the extreme environment in which these unicellular 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. Given the recent report of chloroplast transformation in *Porphyridium* (Lapidot *et al*., 2002), it would be interesting to create a genetic null mutant of LpxA to study the function of lipid A in unicellular red algae.

Solute transporters in G. sulphuraria

The metabolic flexibility of *G. sulphuraria* and its resistance to abiotic stressors such as high salinity and toxic metal ions makes this organism an interesting object for the study of membrane transport processes. Analysis of the EST-dataset revealed 152 cDNAs encoding putative solute transporters. Of particular interest is an EST with similarity to a Na^+/H^+ symporter from *Deinococcus radiourans* (A4_06B09, 32% identity over a stretch of 164 amino acids, e-value $6e^{-12}$) that may be involved in the excretion of sodium ions from *Galdieria* cells. Similar transporters have not previously been described in eukaryotes. Another transporter new to eukaryotes is related to the magnesium uptake system mgtE from *Clostridium tetani* and other bacteria (A4_34G04, 30% identity over a stretch of 214 amino acids, e-value $2e^{-31}$). Many other solute transporters are more closely related to those from eukaryotic organisms, such as putative members of the mitochondrial carrier family, ammonium and amino acid transporters, and a number of ABC-transporters. Because of their potential importance for heterotrophic and mixotrophic growth, we focused our analysis on a family of putative monosaccharide transporters. In addition, because the pathway for the export of recently assimilated carbon from rhodoplasts is unknown, we analyzed the dataset for putative plastidic phosphate-translocators.

The G. sulphuraria *genome encodes at least nine distinct putative monosaccharide transporters*

Seed plants possess three distinct families of sugar transporters: (i) disaccharide transporters that primarily catalyze sucrose transport; (ii) monosaccharide transporters mediate the transport of a variable range of monosaccharides (Büttner and Sauer, 2000; Williams *et al*., 2000); and (iii) a maltose transporter that represents a novel type of sugar transporter (Niittyla *et al*., 2004). In seed plants, transporters play crucial roles in the intra- and intercellular and long-distance transport of sugars throughout the plant. *Galdieria,* however, is a unicellular organism. Hence, there is no need for cell-to-cell and long-distance transport of sugars. Nevertheless, a search for *G. sulphuraria* ESTs encoding putative sugar transporters identified 12 orthologs of well-described monosaccharide and polyol transporters from other organisms. Nine contigs are exclusively represented in the heterotrophic library whereas three are confined to the autotrophic library (Table 2).

None of the ESTs were present in both libraries, and the nine contigs presumably define nine independent genes. Direct amino acid sequence alignments corroborate this hypothesis (Figure 3). The remaining three ESTs presumably also represent distinct genes, however, due to the lack of sequence overlap this assumption could not be further verified.

Galdieria is able to thrive on 27 different sugars and sugar alcohols (Gross and Schnarrenberger, 1995a, b) and uptake studies demonstrated that at least 14 different sugar transporters are involved in the uptake of carbohydrates from the environment (Oesterhelt *et al*., 1999; Oesterhelt and Gross, 2002). The molecular evidence presented here clearly corroborates the conclusions derived from the physiological studies. Based on the EST-dataset, *Galdieria* possesses at least nine distinct, and likely 13 different monosaccharide transporters. In addition, one putative disaccharide transporter was identified (HET_32B04).

While the uptake of radiolabelled sugars in autotrophically grown cells has previously been reported as barely detectable (Oesterhelt *et al*., 1999), the EST data suggest that at least three distinct sugar transporters are expressed in autotrophic cells. These might be involved in the sensing of substrates in a similar manner to the transporter-paralogs Snf3 and Rgt2 in yeast (Özcan *et al*., 1996, 1998). However, the structure and function of the corresponding proteins in *G. sulphuraria* still awaits analysis.

A major physiological difference between *G. sulphuraria* and the closely related species *C. merolae* is that the latter is unable to grow heterotrophically. Accordingly, the *C. merolae* genome (Matsuzaki *et al*., 2004) encodes only a single putative monosaccharide transporter (putative arabinose permease; CMK066C), indicating that the relatively large number of putative

Table 2. Putative monosaccharide transporters in *Galdieria sulphuraria*. ESTs isolated from the heterotrophic library are indicated by het and those isolated from the photoautotrophic library are indicated by aut.

Contig	Length (bp)	Library	No. of ESTs in contig	Closest otholog in *Arabidopsis*
06540	1676	het	6	At2g 18480
02210	1809	aut	6	At2g 18480
23470	839	het	1	At3gl8830
30210	657	het	1	At3gl8830
23420	600	het	1	At2gl6120
00780	771	aut	2	At2gl6120
29150	463	het	1	At4g36670
26980	662	het	1	At4g02050
26590	557	het	1	At4g 16480
24560	650	het	1	At4g 16480
07820	652	aut	1	At2g43330
29810	591	het	1	At2g35740

The closest *Arabidopsis* orthologs were determined by BLASTX searches against the AGI protein database.

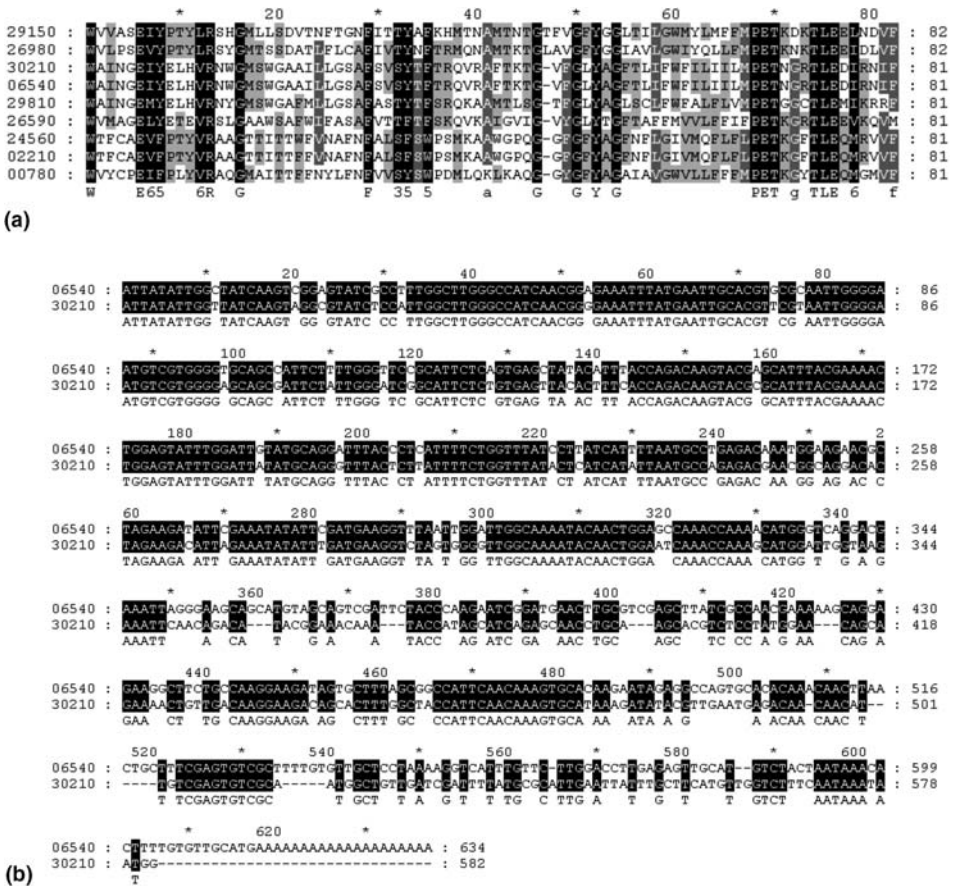

Figure 3. Alignment of the overlapping regions of nine contigs that show homology to monosaccharide transporters from other organisms. (A) Amino acid alignment. Based on the homology to related transporters from other organisms, the aligned region represents the C-terminus of the putative monosaccharide transporters from *G. sulphuraria*. Please note that the deduced amino acid sequences of contigs 30210 and 06540 are identical in the depicted region. (B) Nucleic acid sequence alignment of contigs 30210 and 06540. Although the deduced amino acids sequences of contigs 30210 and 06540 are identical over a stretch of amino acids, the corresponding cDNA sequences show only 69% identity, indicating that both putative transporters are encoded by distinct genes.

monosaccharide transporters in *G. sulphuraria* is related to its metabolic versatility.

Identification of plastidic phosphate translocators from G. sulphuraria

During the day, the path for carbon export from chloroplasts in green plants is via the plastidic triose phosphate/phosphate translocator (Flügge, 1999). Up to 50% of the carbon dioxide assimilated during the day is stored as transitory starch within chloroplasts and exported to the cytosol during the night in the form of glucose and maltose (Niittyla *et al*., 2004; Weise *et al*., 2004). However, as outlined above, red algae store starch in the cytosol and produce floridosides as soluble sugars. Hence, recently assimilated carbon must be exported from the rhodoplast to drive the biosynthesis of carbohydrates in the cytosol. The day-path for carbon export from the rhodoplast is unknown, and the question arises as to whether red algae use a TPT-related transport system. To answer this question, we analyzed reconstituted membranes from *G. sulphuraria* for the characteristic transport signature of plastidic phosphate translocators, and we searched the EST-database for cDNAs encoding proteins that are related to plastidic phosphate translocators from higher plants.

Plant total membrane fractions can be reliably tested for the presence of plastidic phosphate transporter activities by reconstitution of the membrane fraction into liposomes preloaded with

specific counter-exchange substrates (Flügge and Weber, 1994). Reconstituted *G. sulphuraria* membranes showed high activity of phosphate/phosphate and phosphate/dihydroxyacetone phosphate (DHAP) counter-exchange, whereas 3PGA, 2PGA, PEP, and Glc 6-P transport rates were similar to those of the negative control substrate potassium gluconate (Figure 4). Reconstituted spinach chloroplast envelope membranes also catalyzed the transport of 3PGA, and, to a minor extent, of PEP and 2PGA (Figure 4).

These results demonstrate that (i) *G. sulphuraria* has a triose-phosphate/phosphate translocator, and (ii) that this phosphate translocator is likely to have a very narrow substrate specificity. This result was further corroborated by the identification of four ESTs with similarity to plastidic phosphate translocators from higher plants. The deduced amino acid sequences were added to the dataset previously used by Knappe *et al.* (2003) and a phylogenetic tree was calculated using the neighbor-joining method (Figure 5). Two phosphate translocator-related sequences from *Galdieria* (A4_14H8, HET_39C12) formed a distinct clade within the plastidic phosphate translocator family as defined by Knappe *et al.* (2003) and thus clearly represent plastidic phosphate translocators. One EST (A4_13D9) clustered with phosphate-translocator related proteins from yeast, plants, and mammals that are most likely localized in the endomembrane system of these organisms (Knappe *et al.*, 2003). One additional EST (A4_16F5) was basal to the whole family and could not be grouped with any of the previously defined PT-like protein families.

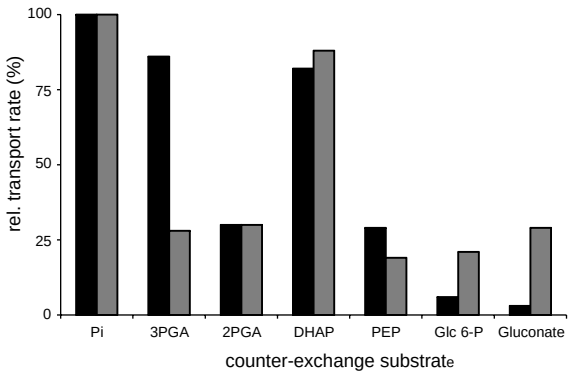

Figure 4. Substrate specificities of reconstituted phosphate-translocators from spinach chloroplast envelope membranes and total *Galdieria sulphuraria* membranes. Spinach chloroplast envelope membranes (closed bars) or a *G. sulphuraria* total membrane fraction (hatched bars), respectively, were reconstituted into liposomes preloaded with 20 mM counter-exchange substrate as indicated. Transport experiments were started by adding radiolabeled *ortho*-phosphate and uptake was stopped after 1 min by loading the reaction mixture on anion exchange columns. Phosphate uptake was quantified by scintillation counting. Transport rates were normalized to phosphate/phosphate exchange. Data represent the mean values of three independent experiments.

Taken together, the experimental and the EST-data indicate that plastidic triose phosphate translocators are a general feature of both the red and the green linage of eukaryotic photosynthetic organisms. Along this line, nine phosphate translocator-related proteins are encoded by the *C. merolae* genome. Related proteins, most likely residing in the endomembrane system can be identified in all characterized eukaryotic genomes (Knappe *et al.*, 2003) but not in prokaryotes. This is in stark contrast to the non-mitochondrial adenylate translocator family, which can be found only plants, prokaryotic obligate intracellular parasites, and in the obligate intracellular parasitic fungus *Encephalitozoon cuniculi* (Linka *et al.*, 2003).

Since red algae do not store transitory starch in plastids but synthesize starch in the cytosol, it can be assumed that the night-path for carbon export from plastids as outlined above does not exist in red algae. In support of this hypothesis, we did not find any ESTs with similarity to the plastidic maltose transporter MEX1 (Niittyla *et al.*, 2004), and none of the 13 ESTs encoding putative monosaccharide transporters was closely related to the plastidic glucose translocator pGlcT (Weber *et al.*, 2000). Furthermore, the export of glucose from plastids is likely to require the activity of hexokinase (Wiese *et al.*, 1999; Weber *et al.*, 2000), and antisense repression of hexokinase in potato plants caused a starch excess phenotype (Veramendi *et al.*, 1999). However, as outlined above, we did not find evidence for a eukaryote-type hexokinase in *Galdieria,* further corroborating the hypothesis that a green plant type night-path for carbon export is absent in *Galdieria,* and possibly in red algae in general.

Conclusions

The adaptation of the red micro-alga *G. sulphuraria* to its extreme habitat and its biochemical and

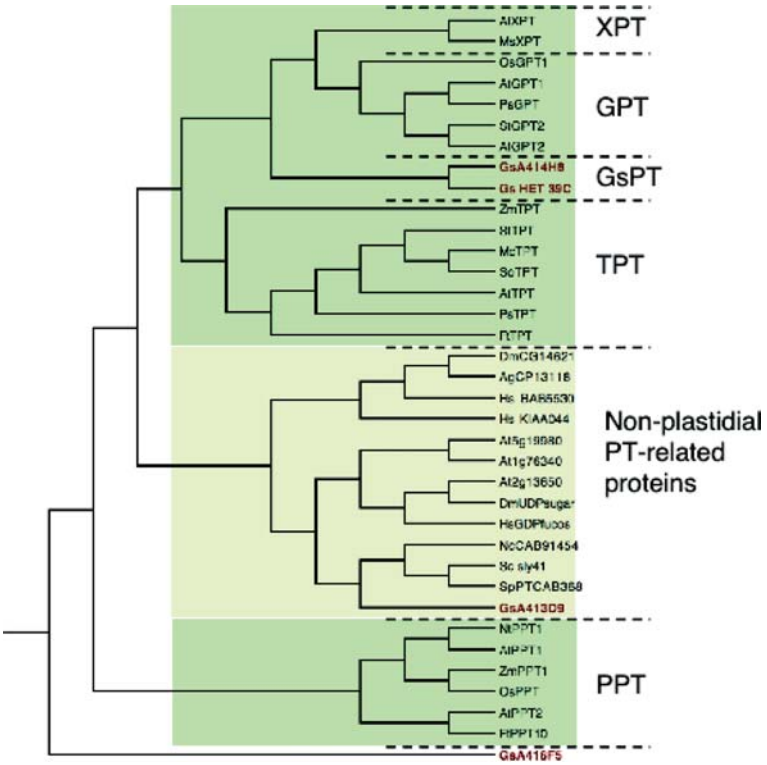

Figure 5. Phylogenetic tree of phosphate translocators from plants and related proteins from other kingdoms. Phosphate translocator-related sequences from *G. sulphuraria* were added to the dataset used by Knappe *et al.* (2003). All sequences were trimmed to an overlapping region with the ESTs from *G. sulphuraria* and a phylogenetic tree was calculated using the neighbor-joining method. Phosphate translocator-related sequences form a distinct clade most closely related to PPTs and XPTs from higher plants. TPT, triose-phosphate/phosphate translocator; PPT, phosphoenolpyruvate/phosphate translocator; XPT, xylulose 5-phosphate/phosphate translocator; GPT, glucose 6-phosphate/phosphate translocator; GsPT, phosphate translocator-related sequences from *G. sulphuraria*.

physiological versatility are highly interesting biological phenomena. However, the study of the underlying molecular mechanism was hampered by a lack of sequence information. To initiate an exploration of the molecular biology of *G. sulphuraria,* we constructed two cDNA libraries from autotrophically and heterotrophically grown cultures, respectively, and sequenced approx. 3000 cDNAs from each library.

EST-analysis of *Galdieria sulphuraria* revealed the potential for lipid A biosynthesis, the pathway for carbon export from rhodoplasts by a plastidic phosphate translocator, a surprising number of putative monosaccharide transporters, and the likely absence of a eukaryotic-type hexokinase from the red alga. Due to space constraints many other interesting features, such as the presence of polyphosphate kinase and glycerol kinase, which are absent from known plant genomes, and evidence for selenocysteine metabolism and a methylmalonate pathway could not be discussed. The datas*et al*so represents a useful resource for phylogenetic and evolutionary studies. Of particular interest will be comparative genomics of *G. sulphuraria* and *C. merolae*. These phylogentically closely related microalga have very different physiologies and comparative genomics will certainly unravel the underlying molecular differences. The accompanying website provides tools for mining the EST-dataset, and will continuously be updated with sequence information from the ongoing *Galdieria* genome project.

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