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UNDERSTANDING THE ORIGIN AND FUNCTION OF ORGANELLAR METABOLITE TRANSPORT PROTEINS IN PHOTOSYNTHETIC EUKARYOTES: *GALDIERIA SULPHURARIA* AND *ARABIDOPSIS THALIANA* AS MODEL SYSTEMS

presented by

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has been accepted towards fulfillment
of the requirements for the

Ph.D. degree in Genetics

Major Professor's Signature

12/05/2008

Date

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By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics

2008

ABSTRACT

UNDERSTANDING THE ORIGIN AND FUNCTION OF ORGANELLAR METABOLITE TRANSPORT PROTEINS IN PHOTOSYNTHETIC EUKARYOTES: *GALDIERIA SULPHURARIA* AND *ARABIDOPSIS THALIANA* AS MODEL SYSTEMS

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Membrane-bound compartments, especially the organelles mitochondria and plastids, are a hallmark of eukaryotic cells. Organellar metabolite transport proteins facilitate the exchange of metabolites across membranes in a cell and are crucial for connecting biochemical pathways that operate in separate compartments. Of particular interest to plant scientists is the plastid organelle of photosynthetic eukaryotes. Plastids synthesize and deliver major biological molecule classes such as carbohydrates, fatty acids, amino acids, and nucleic acids to the rest of the cell and thus the plastid has to be extensively connected to the cytosol. Plastids originated from an endosymbiotic cyanobacteria-like ancestor about 1.6 billion years ago and in this thesis the differences between plastid metabolite transporters of the eukaryotic model plant *Arabidopsis thaliana* and prokaryotic cyanobacteria were investigated. A phylogenomic analysis of 83 predicted plastid metabolite transporters from *Arabidopsis thaliana* has been conducted in collaboration with Dr. Debashish Bhattacharya. These studies allowed the conclusion that the majority of the transport proteins in extant plastids are absent from free-living cyanobacteria and originated from eukaryotic host genes. They represent true innovations associated with organelle evolution. Transporters became likely targeted to the endosymbiont via the endoplasmic reticulum of the host early in its evolution. Furthermore, the results suggest that export of photosynthates from the plastid in form of

sugar-phosphates has been a selective advantage to set-up a permanent endosymbiosis between the host and the endosymbiont. While these sugar-phosphate transport proteins are conserved in all photosynthetic eukaryotes, their biochemical properties co-evolved to meet the specific metabolic requirements in the distinct groups of the eukaryotic kingdom. As reported in Chapter 3, further studies have shown that in contrast to higher plants, the red alga *Galdieria sulphuraria* has a high affinity export system for triose-phosphates and lacks hexose-phosphates transport across the plastid envelope membrane. This reflects an adaptation for an efficient export of photosynthates from the organelle due to an absence of a plastidic starch pool in red algae.

Metabolite carriers facilitate also the transport of compounds in a single, highly compartmentalized cellular pathway. A prime example is the photorespiratory pathway. 2-phosphoglycolate is produced by the oxygenase reaction of the enzyme ribulose-1,5-bisphosphate Carboxylase/Oxygenase (RubisCO) and subsequently recycled to 3-phosphoglycerate in the compartments chloroplast, cytosol, peroxisome, and mitochondrion. A reverse genetic approach was used to identify the transport proteins involved in photorespiration and 32 candidates have been designated. Five of these were genetically analyzed to test their role in the recycling of 2-phosphoglycolate, leading to the discovery of a novel transporter required for a functional photorespiratory pathway. This protein is localized to the inner envelope membrane of mitochondria and the transporter most likely imports a cofactor from the cytosol, which is required for the mitochondrial glycine decarboxylase enzyme complex.

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Andreas Weber for this one-time opportunity to join his research group and complete my graduate studies here at Michigan State University. Under his supervision I developed my confidence and capabilities to inquire into the phenomena on my daily scientific endeavor. He allowed me to be a student, an instructor, a teacher and an independent thinker. He always encouraged me to broaden my scientific knowledge and to enjoy research. I would also like to thank the Director Dr. Barbara Sears and Associate Director Dr. David Arnosti, the faculty and Jeannine Lee of the Genetics Graduate Program for organizing such a great educational program at Michigan State University. The fulfillment of the requirements during the first three years of my graduate studies was of paramount importance for my scientific progress and will be for my future goals. My committee members Dr. Robert Larkin, Dr. John Ohlrogge, Dr. Steve van Nocker, and Dr. Richard Allison deserve a special note. They contributed by discussion and opinions to my progress and allowed the transfer to Germany without any concern during my last year. I would also like to thank all graduate students, postdocs and faculty members for their interaction and discussion at seminars, in the laboratories, and off-campus. In addition, these acknowledgements would not be complete if I did not mention the former and recent members of "The Weber Lab". Thanks for your support and your tremendous scientific and non-scientific help. Most importantly, I would like to thank my wife Nicole. You are the sunshine of my life. I thank my parents, for their support to leave "Old Europe Into The West". But I have to confess that there are places on this Earth, which are even farther away than Michigan.

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KEY TO ABBREVIATIONS

2-PG: 2-phospoglycolate

3-PGA: 3-phosphoglycerate

α-KG: alpha-ketoglutarate

ADP: adenine diphosphate

AMP: adenine monophosphate

ATP: adenine triphosphate

At: *Arabidopsis thaliana*

A.thaliana: Arabidopsis thaliana

CaCl2: calcium chloride

CCM: carbon concentrating mechanism

cDNA: complementatry DNA

cMDH: chloroplastic malate dehydrogenase

CO2: carbon dioxide

DCT: dicarboxylate transporter

DHAP: dihydroxyacetone phosphate

DiT: dicarboxylate transporter

DNA: deoxyribonucleic acid

EGT: endosymbiont gene transfer

E. coli: Escherichia coli

ER: endoplasmic Reticulum

EST: expressed sequence tag

E-value: expectation-value

FAD: flavin adenine dinucleotide

FBPase: fructose-1,6-bisphosphatase

Fd: ferredoxin

Fd-GOGAT: glutamate synthase

Frc1,6BP: fructose-1,6-bisphosphate

Frc6P: fructose-6-phosphate

Gal: galactose

Gal1P: galactose-1-phosphate

GAPDH: glyceraldehydes-3-phosphate dehydrogenase

GDC: glycine decarboxylase

gDNA: genomic DNA

GFP: green fluorescent protein

GGT: glutamate:glyoxylate aminotransferase

Glc: glucose

Glc1P: glucose-1-phosphate

Glc6P: glucose-6-phosphate

Glu: glutamate

Gluc6P: gluconate-6-phosphate

Gly3P: glycerol-3-phosphate

GLYK: glycerate kinase

GOGAT: glutamate synthase

GOX: glycolate oxidase

GPT: glucose-6-phosphate/phosphate translocator

Gs: *Galdieria sulphuraria*

G. sulphuraria: Galdieria sulphuraria

GS: glutamine synthetase

H+: proton

H2O: water

H2O2: hydrogen peroxide

HGT: horizontal gene transfer

HPR: hydoxypyruvate reductase

IPTG: isopropyl β-D-1-thiogalactopyranoside

Ki: inhibitory constant

KM: Michaelis-Menten constant

Li: lithium

LiAc: Lithium acetic acid

Mal: malate

MCF: mitochondrial carrier family

MgCl2: Magnesium chloride

mMDH: mitochondrial malate dehydrogenase

MP: maximum parsimony

NAD(P): nicotinamide adenine dinucleotide (phosphate)

NAD(P)H: reduced nicotinamide adenine dinucleotide (phosphate)

NJ: neighbor joining

NDP: nucleotide diphosphate

NH3: ammonia

NH4+: ammonium ion

O2: oxygen

OAA: oxaloacetic acid

OPPP: oxidative pentose phosphate pathway

pABA: para-aminobenzoic acid

PCR: polymerase chain reaction

PGK: 3-phosphoglycerate kinase

PEP: phosphoenolpyruvate

PGP: phosphoglycolate phosphatase

Pi: orthophosphoric acid

PLP: pyridoxal-phosphate

pMDH: peroxisomal malate dehydrogenase

pPT: plastid phosphate translocator

PPT: phosphoenolpyruvate/phosphate translocator

PR: photorespiration

Pyr: pyruvic acid

Rib5P: ribose-5-phosphate

RNA: ribonucleic acid

RubisCO: ribulose-1,5-bisphosphate carboxylase/oxygenase

RuBP: ribulose-1,5-bisphosphate

S. cerevisiae: Saccharomyces cerevisiae

SGT: serine:glyoxylate aminotransferase

SHMT: serine hydoxymethyltransferase

TCA: tricarboxylic acid

THF: tetrahydrofolate

Tic: translocon at the inner chloroplast envelope

Toc: translocon at the outer chloroplast envelope

TP: triose-phosphate

TPT: triose-phosphate/phosphate translocator

UDP: uridine diphosphate

UMP: uridine monophosphate

XPT xylulose-5-phosphate/phosphate translocator

INTRODUCTION

PLASTID EVOLUTION FROM THE PERSPECTIVE OF METABOLITE TRANSPORT PROTEINS

INTRODUCTION

Approximately three billion years ago, an ancestor of the extant free-living cyanobacteria introduced oxygenic photosynthesis to a literally oxygen-free atmosphere (Blankenship and Hartman, 1998). Descendants of these early oxygenic photosynthetic organisms are sustaining the oxygen concentration in the present Earth's atmosphere (Blankenship, 1992; Cavalier-Smith, 2006; Falkowski and Godfrey, 2008). Sunlight is absorbed and utilized by the photosynthetic apparatus for withdrawing electrons from water, thereby generating a proton gradient and oxygen (O2) as a byproduct. The electrons and protons are stored as usable chemical energy to assimilate inorganic nutrients (i.e., C, N, P and S) into organic matter. Thus, autotrophic organisms are of central importance for the Earth, providing the essential O2 for cellular respiration of aerobic organisms, and are the primary producers at the base of almost every food chain (Field et al., 1998).

More than one billion years ago it is likely that a phagotrophic eukaryote gained the ability to conduct photosynthesis by engulfing a cyanobacterium-like prokaryote, thus changing from a heterotrophic to a mixotrophic and subsequently autotrophic lifestyle (Yoon et al., 2004; Cavalier-Smith, 2006). This permitted the invasion of and thriving in new habitats. Over millions of years the endosymbiotic cyanobacterium evolved into the organelle plastid (Reyes-Prieto et al., 2007). Today's photoautotrophic organisms exist ubiquitously on the Earth with a remarkable diversity of species and appearance (Bhattacharya et al., 2004). The smallest and largest photosynthetic eukaryotic organisms described to date are the green micro-alga *Ostreococcus tauri* (Lanier et al., 2008) and the Giant Sequoia tree, respectively. The red alga *Galdieria sulphuraria* and green alga *Chlamydomonas* species adapted to the upper (56°C) (Gross, 2000) and lower

temperature limits (Morgan-Kiss et al., 2006) of eukaryotic life. Phototrophic organisms recently attracted much attention as renewable and sustainable sources for biomass and energy production and as alternative bioreactors to the established eubacteria, yeasts and human cell lines (Gordon and Polle, 2007; Field et al., 2008; Raja et al., 2008; Rubin, 2008). Plastids are the site of carbon dioxide, nitrite, and sulfur assimilation (Calvin, 1962; Crawford, 1995; Kopriva, 2006). They partially or fully conduct the biosynthesis of various metabolite classes, such as fatty acids, starch, amino acids, nucleic acids, isoprenoides (chlorophyll, carotenoids, gibberelline) or phenylpropanoids (cell wall components, pesticides, anthocyane) (Weber et al., 2005). To understand and fully utilize the potential of photosynthetic eukaryotes, it is helpful to reconstruct the events that led to plastid origin and decipher the common features and specific adaptations in extant, distantly related photosynthetic organisms.

Plastid origin from a cyanobacterial-like prokaryote was postulated already at the turn of the 19th and 20th century by microscopists Sachs, Altmann, and Schimper (reviewed in Kutschera and Niklas, 2005) and led in 1971 to the "Theory of Endosymbiosis" by Lynn Margulis (Margulis and Nealson, 1989). Based on numerous single and multi-gene phylogenetic analyses from diverse plastid genomes and nuclear-encoded plastid-targeted genes (Martin et al., 2002; Reyes-Prieto and Bhattacharya, 2007a), it is now commonly accepted that the primary endosymbiosis had been a single event in eukaryotic evolution and all photosynthetic species containing primary plastids have been recently summarized in the supergroup *Archaeplastida* (Adl, 2005). Within approximately 150 million years, the unicellular primordial alga (i.e., the protoalga) had been established, and it gave rise to the three major lineages of the *Archaeplastida*:

Glaucophyta (glaucophyte algae), *Rhodophyceae* (red algae) and *Chloroplastida* (green alga/plants) (Yoon et al., 2004; Adl, 2005; Reyes-Prieto and Bhattacharya, 2007b). Multicellularity evolved independently in the *Rhodophyceae* and in the *Chloroplastida*, whereas only the *Chloroplastida* mastered the colonization of the land. This was accompanied by an extraordinary radiation of the existing species (Bhattacharya et al., 2004). Heterotrophic eukaryotes later captured green or red algae, respectively, transforming them to secondary plastids of green algal or red algal origin (Gould et al., 2008). The process of secondary endosymbiosis gave rise to a plethora of new species mainly micro- and macro-algae, dominating aquatic environments as major primary producers (Field et al., 1998). Serial secondary endosymbiosis by replacing a secondary plastid by another one and even tertiary endosymbiosis via an engulfment of algae harboring a secondary plastid by another heterotrophic eukaryote are evident (Gould et al., 2008). Also of interest is the complete or partial loss of plastid functions in several species (Fast et al., 2001). Noteworthy is also continuing evolution of novel photosynthetic organisms of primary endosymbiotic origin, as it has been recently reported for the host species *Paulinella* (Nowack et al., 2008).

The antiquity of the last common ancestor, the recurrent acquisition of the plastid and species radiation has scrambled the evolutionary signal of plastid origin. In the last decade, plastid-, EST- and complete genome sequence projects from a diverse group of photosynthetic organisms were initiated by the research community to clarify the complex host-endosymbiont partnership. The green lineage has been followed from the unicellular and multi-cellular green algae to mosses (Cove, 2005; Merchant et al., 2007), gymnosperms and several mono- and dicotyledonous plants (Arabidopsis Genome

Initiative, 2000; Goff et al., 2002; Tuskan et al., 2006; Pavy et al., 2007). A catalog of sequences from diverse micro- and macro-algae with primary, secondary and even tertiary plastids was initiated to compile red algae evolution (Matsuzaki et al., 2004). The genome of the glaucophyte alga *Cyanophora paradoxa* is currently sequenced to complement the available sequence resources with the third major lineage of the *Archaeplastida* (Reyes-Prieto et al., 2006; Loeffelhardt, 2007). Besides all the diversity, common themes and specialization in plastid evolution can be now validated and addressed:

Endosymbiont gene transfer (EGT) to the host nucleus massively reduced the plastid genome size (Timmis et al., 2004). The model-plant *Arabidopsis thaliana* has a chloroplast genome of ~155 kb with 87 protein-coding genes (Sato et al., 1999), which is just a small fraction of extant cyanobacterial genomes. On the other hand, bioinformatic and proteomic predictions assume nearly 4,500 proteins in the organelle (Martin et al., 2002; Sun et al., 2008). Expression and re-import of these nucleus-encoded plastid targeted proteins has been predicted for a long time to be a hallmark for a successful organelle establishment (Dyall et al., 2004). An intriguing question is to what extent and for what purpose cyanobacterial genes had been transferred and maintained in the host nucleus. According to Sato et al. (2005) and Martin et al. (2002), the *A. thaliana* nuclear genome harbors between 1,200 – 4,500 genes of cyanobacterial origin, respectively. The majority of these cyanobacterial genes have non-plastid function (Martin et al., 2002), hence, the present plastid proteome evolved from multiple sources including cyanobacterial, host proteins and eubacterial genes acquired by horizontal gene transfer. Data from glaucophytes and red algae suggest a much smaller portion of transferred

cyanobacterial genes and in contrary to *A. thaliana*, the majority of these candidates are plastid targeted (Reyes-Prieto et al., 2006). Although these findings are not in full agreement, they still demonstrate a significant and complex contribution of EGT to the evolution of the host cell. Host genes and endosymbiont genes have been replaced by one another, duplicated, diverged and several times redirected to a new compartment or even existing homologous genes recombined with each other. A challenge will be to follow up these rearrangements in numerous model organisms and assemble the tree of photosynthetic eukaryotes.

Prerequisites for the gene transfer to the nucleus were (i) the regaining transcriptional and translational activation and (ii) the retargeting of plastid-needed proteins (Martin, 2003). Recent studies demonstrated the transfer of plastid-encoded antibiotic resistance genes that were introduced by plastome transformation to the nucleus, including gene activation, within a short timeframe (Huang et al., 2003; Stegemann et al., 2003). More elaborate must have been the evolution of the plastid protein import machinery. The sophisticated Toc (Translocon at the outer chloroplast envelope) and Tic (Translocon at the inner chloroplast envelope) system evolved not only to import the majority of the proteins across both envelope membranes, but also to distribute them to one of the six possible destinations (outer and inner envelope membrane, intermembrane space, plastid stroma, thylakoid membrane and lumen) (Inaba and Schnell, 2008). Comparative analysis of the Toc and Tic apparatus across many photosynthetic eukaryotes points again to a monophyly of plastids and suggests its early establishment, likely already in the protoalga. Core parts of the cyanobacterial protein

transport machinery have been recruited and were functionally replaced or improved with new elements, which are absent in the cyanobacterial membrane (Gould et al., 2008).

Another major control point in plastid biogenesis was the establishment of coordinated division of endosymbiont and host cell (Miyagishima, 2005). Unrestricted growth of the endosymbiont would harm the host and inhibiting its division would ultimately lead to its loss. The plastid division apparatus follows the same evolutionary path described earlier for the protein import apparatus (Miyagishima, 2005). The organelle division is based on ancient bacterial binary fission and several genes with cyanobacterial and other eubacterial origin are still conserved in extant plastids. The nuclear genome took control over the expression of most of the genes (Glynn et al., 2007). In land plants, the transfer is completed and none of the proteins are encoded on the organelle genomes sequenced to date, but some algal genomes sporadically still encode division factors (Miyagishima, 2005; Glynn et al., 2007). Analogous genes replaced or had been added as division components originated from eukaryotic and eubacterial sources. Even after the split of the *Archaeplastida*, lineage-specific remodeling occurred. This makes it difficult to provide a universally valid plastid division model and in particular challenging to find new division related genes via sequence-based comparative analysis (Glynn et al., 2007).

From the engulfment of the plastid ancestor to the split into the *Glaucophyta*, red algae and green algae/plants, a period of 150 million years has been estimated (Yoon et al., 2004). All above-mentioned major events in plastid origin are results of a complex multi-gene acquisition and build the foundation of all descendants of the proto-alga. To date, it is not satisfactorily answered how the two organisms managed to coexist long

enough to provide sufficient time for the transformation of the endosymbiont into an organelle.

In the following paragraph and in chapters one and two I will outline the potential of metabolite exchange and integrating metabolisms to set-up a strong mutual symbiosis, which ultimately led to an enslavement of the cyanobacterium by the protist. This initial metabolic interaction might have provided the time needed for the emergence of a full-fledged organelle, including large scale EGT and establishment of effective protein import apparatus and division machinery. My aim was to use comparative genomics to unravel the differences of the metabolite transport protein composition between the plastid inner envelope membrane and the plasma membrane of free-living cyanobacteria. In this work, I could show that recruitment of host-derived metabolite transporters to the plastid envelope membrane was very likely a driving force in the transition of a mutualistic endosymbiosis to the proto-alga. Chapters one and two had been prepared in collaboration with the laboratory of Debashish Bhattacharya (University of Iowa) and were published in two different scientific journals.

In the proposed scenario, a complete cyanobacterium was engulfed by a phagotrophic eukaryote and escaped digestion in the food vacuole. Eventually, the membrane surrounding the cyanobacterium was lost or merged with the cyanobacterial outer membrane (Cavalier-Smith, 2000), resulting in two membranes marking the barrier to the new environment: the cytosol of the host. The composition of the outer membrane changed considerably over evolutionary time scales (Block et al., 2007). Eubacterial lipoproteins and lipopolysaccharides are absent from higher plant chloroplasts and the phospholipid phosphatidylcholine was specifically introduced into the outer envelope

membrane from the endomembrane system of the host (Douce and Joyard, 1990; Cavalier-Smith, 2000). Extant *Archaeplastida* display an intimate connection between the Endoplasmic Reticulum (ER) and the envelopes of the plastid. Lipids are synthesized in the plastid stroma and a sophisticated traffic system has been established between plastid and ER in both directions for the biogenesis of plastidial and extra-plastidial membranes (Benning, 2008). As a conserved feature of extant primary plastids, the Tic/Toc protein import apparatus recognizes most nuclear-encoded genes via a N-terminal leader sequence of 25 - 125 amino acids (Soll and Schleiff, 2004). Still, some plastid-targeted proteins do not exhibit an obvious leader sequence and an alternative protein translocation route via the ER and Golgi vesicle sorting system has been recently discovered (Villarejo et al., 2005). The host-plastid interaction was repeatedly established through endosymbiosis in secondary plastid evolution. Three to four envelope membranes surround secondary plastids (Bhattacharya et al., 2004) and nuclear-encoded proteins are delivered by a multi-partite leader sequence through the concerted action of the ER sorting system and the Tic/Toc related import machinery of the organelle (Kilian and Kroth, 2003).

These direct contacts between ER and plastid support the idea that vesicle transport to and from the plastid was initiated at an early stage of endosymbiosis. ER or Golgi-derived vesicles initially might have fused randomly with the outer membrane of the cyanobacterium, releasing their contents into the periplasmatic space and the proteins were inserted into the cyanobacterial plasma membrane or taken up by the cyanobiont. Thus, even if insertion of host metabolite transporters into the cyanobiont's plasma membrane were initially not very efficient, it would have given the host the opportunity

to connect the cyanobiont with its cytosol and tap into photosynthates from the cyanobacterium. This could have become a selective advantage for the phagotrophic eukaryote and the endosymbiont and favored the establishment of a permanent partnership.

In chapter one we address the phylogenetic origin of the plastid-targeted triose-phosphate/phosphate transporter (TPT). As a starting point, I used the available EST database from the unicellular red alga *G. sulphuraria* to annotate and assemble the homologous plastid phosphate translocator (pPT) genes. This work was part of the publication: "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" (Weber et al., 2004). The results led us to hypothesize that the ancestor protein of the TPT has evolved from a host-derived endomembrane transporter, which was "mistargeted" to the cyanobacterial membrane and thus enabled a metabolic connection between the endosymbiont and the host. The TPT as a member of the pPT family was the first solute transport protein identified on a molecular basis in the inner envelope membrane in higher plants (Flügge, 1999). This transporter is of particular interest, because it is the major route for export of assimilated carbon from the plastid into the cytosol during the day in seed plants. Our analysis in chapter one revealed that homologous TPT genes are widespread in the red algal lineage including chromalveolate algae with secondary plastids, but related genes are absent from cyanobacteria and other prokaryotes. Export of triose-phosphate is truly an innovation of plastid evolution.

The pPT family originated from nucleotide-sugar transporter present in the endomembrane system of extant eukaryotes. However, none of the extant pPT members

do transport nucleotide sugars, thus leading to the question how a nucleotide sugar transporter might have functioned in the initial establishment of a metabolic connection between cyanobiont and host. Nucleotide-sugars are the substrate for literally all glycosylation reactions in a cell. They are incorporated into disaccharides, oligo- and polysaccharides, which are utilized as compounds for transport, storage, structural integrity and post-translational modifications in the cell. Recently, (Deschamps et al., 2008) highlighted the importance of the nucleotide-sugar transporters for merging the storage polysaccharide metabolism in host and cyanobiont. According to their hypothesis, both organisms initially harbored complete and independent sets of enzymes to synthesize, store and mobilize carbon in their respective compartments. The insertion of an NDP-sugar transporter would have allowed the host to withdraw the cyanobacterial starch precursor ADP-glucose from the cyanobiont. The key assumption of this hypothesis is that ADP-glucose represents an activated form of organic carbon that has already been committed to storage, thus it can be withdrawn from the evolving plastid without disturbing its primary carbon metabolism. However, ADP-glucose is a poor substrate for the soluble starch synthase in heterotrophic eukaryotes. To counter this argument, it has been argued that the gene encoding soluble starch synthase of cyanobacterial origin has been transferred to the host nucleus at an early stage and was subsequently expressed in the cytosol of the host. Importantly, this would only require the transfer of a single gene to the host. It is not necessary for the gene product to be retargeted to the cyanobacterium and proteins for mobilization of the host α-glucans are already present. Thus, the proposed scenario is a very simple one in evolutionary terms – it does require only a single gene transfer and 'mistargeting' of a single endomembrane-

derived transporter. The scenario would be even simpler if the ancestral host starch or glycogen synthase would have accepted ADP-glucose as precursor, even with low affinity. In this case, the initial connection would not have required EGT or evolutionary innovations.

Nucleotide-sugar transport proteins operate in a strict counter-exchange mode and the transport direction depends on the concentration of the substrates on either sides of the membrane. The export of NDP-sugar is compensated with an import of nucleotide-monophosphates, such as AMP or UMP (Handford et al., 2006; Rollwitz et al., 2006). In the endosymbiosis context, this counter-exchange mode of transport would have prevented the depletion of the cyanobacterial nucleotide pool. A relatively higher concentration of ADP-glucose at its side of biosynthesis inside the endosymbiont would favor its export and immediate consumption in the cytosol. As outlined above, the transporter was likely inefficiently inserted and only carbon committed to storage was drained, thus it did not affect the viability of the symbiont (Deschamps et al., 2008). However, to date, a paralogous NDP-sugar export system has not been identified in the inner envelope membrane of any extant photosynthetic eukaryote. Identification of such a transporter would represent the missing link that would lend strong support to the "metabolic symbiosis and birth of the Plant kingdom" hypothesis put forward by Deschamps et al. (2008). According to my work presented in chapter one, the pPT gene family with at least three distinct members already evolved in the last common ancestor of the red algae and the green algae and plants, shortly after the initial endosymbiosis was established (Weber et al., 2006). They all are equipped with an N-terminal leader sequence for an efficient retargeting and insertion by the Toc and Tic system. As a result,

the major flux of sugar-phosphates across the membrane of plastid in higher plants was reorganized from an NDP-sugar transporter towards a triose-phosphate (TP)/phosphate, phosphoenolpyruvate (PEP)/phosphate and glucose-6-phosphate (Glc6P)/phosphate shuttle system (Flügge, 1999). The transport systems fit perfectly into metabolic compartmentalization of higher plants: triose-phosphate is exported to the cytosol and is mainly used for sucrose or cell wall biosynthesis. PEP is taken up from the cytosol to drive fatty acid biosynthesis and synthesis of compounds by the shikimate pathway (i.e. aromatic amino acids and a heterogeneous group of phenolics). The glucose-6-phosphate transporter (GPT) provides glucose-6-phosphate (G6P) for starch synthesis and the oxidative pentose phosphate pathway (OPPP) in heterotrophic tissues, or the Ribulose-1,5-bisphosphate Carboxylase/Oxygenase (RubisCO) bypass reaction in mixotrophic tissues.

In summary, the most likely scenario in evolutionary terms is that the NDP-sugar transporter represented the starting point for connecting the previously independent carbon metabolisms of host and cyanobiont. Gene duplications and further radiation of the gene family delivered the genetic material for the evolution of functional diversification, eventually promoting the evolution of a more efficient targeting system to the plasma membrane of the endosymbiont.

The findings about the phylogenetic origin of the plastid phosphate transporter family encouraged me to extend the phylogenomic analysis of the repertoire of plastid metabolite transporters to a broad range of different photosynthetic organisms. In present-day plastids of seed plants, photosynthesis provides the energy for the Calvin-Benson-Cycle and several other anabolic pathways, including the synthesis of starch, fatty acids,

several amino acids, nucleic acids, and the reductive assimilation of inorganic ions like nitrate and sulfate. For each pathway, a number of precursors and intermediates have to be transported across the envelope membrane (Weber et al., 2005). Plastids must be extensively connected to the cytoplasm to distribute the numerous products within the cell. In contrast, the cyanobacterial membranes generally prevent leakage of their primary metabolites to the surrounding. The plasma membrane is mainly configured for the uptake of inorganic and organic nutrients and the excretion of waste products or components for the periplasmic space, the outer membrane, and cell wall (Kaneko et al., 1996).

In chapter two we addressed two questions concerning the transporter evolution of the plastid inner envelope membrane. First, we sought to estimate the contribution of host and symbiont on the transporter repertoire in photosynthetic eukaryotes. Is the pPT the rule or the exception for host proteins, which had been inserted into the cyanobacterial plasma membrane? Second, we determined the differences between the *A. thaliana* transporter set and photosynthetically active prokaryotic and eukaryotic organisms. This would give us for the first time an insight into the conserved and lineage-specific metabolite "permeome" of higher plant plastids. To address these hypotheses, we analyzed the phylogenetic origin and distribution of well-annotated transporter proteins from *A. thaliana*. Transport proteins have characteristic hydrophobic transmembrane spans to reside in the membrane as well as hydrophilic domains to allow interaction and transport of their substrates (Schwacke et al., 2003). *In silico* searches for genes in the *A. thaliana* genome with a predicted plastid target sequence and hydrophobic domains provided an initial catalogue of candidate genes (Weber et al. 2005). Several plastid-

specific proteomic studies confirmed the presence and extended the list of proteins targeted to inner envelope membranes of plastids to approximately ~130 putative metabolite transporter proteins (Sun et al., 2008). This number does not include genes from the Toc, Tic and thylakoid-related protein import machinery and prominent thylakoid-localized electron-transporting proteins of the photosynthetic core complex, such as D1, D2, cytochrome b6, cytochrome f, or proton-coupled ATP-synthase subunits. Our results for the first time revealed that the host has been the major source for metabolite transporter acquisition by the plastid. We also found an unexpectedly high proportion of *Chlamydiae*-derived genes encoding plastid metabolite transporters. The group of Debashish Bhattacharya examined this result in more detail and identified a total of 55 genes in *Plantae* with chlamydial origin (Moustafa et al., 2008). A similar result was demonstrated for the red alga *Cyanidioschyzon merolae* (Huang and Gogarten, 2007). This provides a minor but crucial third genome source for plastid evolution by horizontal gene transfer or even an "endosymbiotic" gene transfer from a parasitic *Chlamydiae* bacterium. Intriguingly, most of the metabolite transport proteins were distributed in both green and red algae and to a much lesser extent present in only one of them. This suggests that the initial phase of the proto-alga, before the split into the *Archaeplastida* lineages, was accompanied by a massive invasion of the envelope membrane with host-proteins.

As mentioned earlier, the extant pPT family evolved from endomembrane NDP-sugar transporters. The three pPT subfamilies do not simply encode differentially expressed or targeted gene products with identical functions. They evolved distinct substrate specificities and define the major flux of carbon across higher plant plastid

envelope membranes (Flügge, 1999). The phylogenetic tree shown in chapter one argues for highly conserved orthologous sugar-phosphate transporters with the preferred substrates Pi, TP, PEP, and Glc6P in the red alga *G. sulphuraria*. This is in contrast to the enormous differences in how starch and soluble sugars are synthesized in the green and the red lineages (Viola et al., 2001). Red algae harbor an UDP-glucose specific starch synthase of ancient eukaryotic origin, which is localized in the cytosol. The soluble starch synthase from green algae and plants is closely related to cyanobacterial genes and incorporates ADP-glucose into the polysaccharide chain in the stroma of the organelle (Deschamps et al., 2008). Probably, UDP- and ADP-glucose dependent starch synthesis was still manifested in the genome of the proto-alga. By the time the common ancestor split up into the *Rhodophyceae* and *Chloroplastida*, the carbon metabolism was far from being established as we find it in extant species. The pathway has been relocated to the plastid stroma in green plants and algae (Deschamps et al., 2008) or stayed and gradually developed in the cytosol of *Rhodophyceae*. In addition, red algae produce the predominant soluble carbohydrate floridoside, composed of a glycerol and galactose moieties (Viola et al., 2001) and plants synthesize the disaccharide sucrose from UDP-glucose and fructose-6-phosphate (Huber and Huber, 1996).

Based on phylogenomic analysis, I developed the hypothesis that transport of glycolytic intermediates, i.e. PEP, TP, and Glc6P, was a successful invention in plastid biogenesis and is conserved in primary photosynthetic eukaryotes. Hence, if the hypothesis is correct, the homologues in *G. sulphuraria* should represent truly orthologous genes with identical substrate specificities as their counterparts in the green lineage. However, the major differences in carbon metabolism between photosynthetic

eukaryotes challenge the hypothesis. Possibly, the ancient NDP-sugar transporters continued to evolve divergently in red algae from the TPT, PPT and GPT of plants. To find an answer to these contradictory hypotheses, I functionally tested the pPT genes from *G. sulphuraria* by recombinant expression and biochemical characterization, and comparison to their counterparts in higher plants. The results are presented in detail in chapter three. I discussed alternative mechanisms in green plants and red algae to organize their primary carbon partitioning between rhodoplasts and cytosol. Prior to this study, carbon assimilation and especially the metabolic fate of the photosynthates in *Rhodophyceae* did not receive great attention compared to green algae and land plants. This does not match with the major role of red algae in the global carbon cycle, since they dominate carbon fixation in aquatic environment, which approximately accounts for half of the global photoautrophic CO_2 assimilation (Field et al., 1998). In chapter three, I demonstrate that the red alga *G. sulphuraria*, in comparison to the *Plantae*, possesses an export system for triose-phosphates with very high specificity and affinity. In red algae, TPT controls the flux of carbon during autotrophic and heterotrophic growth conditions and, in contrast to plants, glucose-6-phosphate transport activity does not exist in the red alga as a major import route for sugar-phosphates. The lower affinity TPT in green plants might represent an adaptation to maintain a relatively high concentration of TP that is required for driving plastidial starch biosynthesis. The TPT protein from *G. sulphuraria* is a good example for the biochemical diversity of known proteins in distantly related photoautotrophic organisms and implies that this gene pool is an underestimated toolbox to engineer plants for applied sciences.

Besides understanding of the origin and contribution of metabolite carriers for plastid evolution, a major challenge is linking the catalogue of uncharacterized transport proteins to their biological function. A defining feature of eukaryotes is their cellular compartmentation. The cytosol is separated from the internal space of mitochondria, plastids, vacuole, peroxisomes, nucleus, and ER by biological membranes. Lipid bilayers create a diffusion barrier for hydrophilic and charged molecules and define separate compartments for enzymatic reactions. In a single cell, pathways can be confined in a specific or even partitioned in more than one compartment. In multi-cellular organisms, several pathways are connected between different cells, tissues or even organs and often separated by more than one membrane. Thus, metabolite transport proteins are required for nutrient and metabolite uptake, secretion of toxic by-products, to sustain and even regulate the metabolic flux through pathways and distribute a single metabolite to distinct compartments at the same time (Kunze et al., 2002). One quarter of the *A. thaliana* genome encodes for proteins with at least one transmembrane span that are predicted to be embedded in one of the cellular membranes (Schwacke et al., 2003; Komatsu et al., 2007). This number includes numerous receptor kinases, electron transport chain or protein import components from mitochondria and chloroplasts. Nevertheless, it is estimated that up to 40% of these membrane-localized proteins could represent metabolite transporters (Ward, 2001). Most of the predicted transporters lack homology to known proteins from other species and less than 20% of the putative carriers are characterized at the biochemical and molecular level (Barbier-Brygoo et al., 2001).

During the day, the major flux across plastid membranes is the export of triose-phosphate, which is catalyzed by the TPT (Flügge, 1999). TP is the product of the

reductive part of the Calvin-Benson-cycle (Calvin, 1962). Carboxylation of ribulose-1,5-bisphosphate (RuBP) by the RubisCO results in two molecules of 3-phosphoglycerate (3-PGA) and the following two enzymatic reactions generate triose-phosphate, consuming ATP and NADPH provided by the photosynthetic light reactions.

Atmospheric oxygen (O2) competes with CO2 for the acceptor molecule RuBP, yielding significant amounts of 2-phosphoglycolate (2-PG) and 3-phosphoglycerate (3-PGA) (Ogren, 1984). In contrast to 3-PGA, 2-PG cannot be metabolized further in the Calvin-Benson-cycle. It is hydrolyzed in the chloroplast to glycolate and exported to the cytosol to be recycled by the photorespiratory pathway (Figure 1) (Douce and Neuburger, 1999). Measurements on chloroplast membranes demonstrated the presence of a transport system, which is able to export glycolate with rates required to cope with the flux through the photorespiratory pathway (Howitz and McCarty, 1991). Two molecules of the C-2 carbon glycolate are converted to one C-3 molecule glycerate in a complex cycle organized in peroxisomes, mitochondria and cytosol. Glycerate is taken up by the chloroplast, phosphorylated and reduced to triose-phosphate (Howitz and McCarty, 1991; Schwarte and Bauwe, 2007). Numerous studies identified the enzymes, which hydrolyze 2-PG to glycolate (Somerville and Ogren, 1979), oxidize and transaminate glycolate to glycine in the peroxisomes (Kendall et al., 1983; Liepman and Olsen, 2001; Igarashi et al., 2003; Queval et al., 2007). Two molecules of glycine are converted to serine in the mitochondria (Somerville and Ogren, 1981; Voll et al., 2006; Engel et al., 2007), and serine is deaminated to hydroxypyruvate and reduced to glycerate in the peroxisomes (Liepman and Olsen, 2001; Igarashi et al., 2003) (Murray et al., 1989). During the 1970s and 1980s, forward genetics screens with mutants of *A. thaliana* and *Hordeum vulgare*

has been a powerful tool to identify many of the photorespiratory genes (Ogren, 1984). Isolated mutants grew normally under elevated carbon dioxide levels, but the enzymes were essential under photorespiratory conditions (Ogren, 1984). Actually, genetic dissection of photorespiration using *Arabidopsis* mutants paved the way for *A. thaliana* as a model organism in plant biochemical genetics and plant physiology (Somerville, 2001). Surprisingly, however, map-based cloning of the photorespiratory *A. thaliana* genes did not reveal a single transporter of the mandatory shuttle systems needed for the described conversion of glycolate to glycerate between chloroplast, peroxisomes, and mitochondria.

The only isolated transport protein linked to the photorespiratory pathway and identified by forward genetic screen is the dicarboxylate transporter DCT1/DiT2.1 in the inner envelope membrane of chloroplasts (Figure 1) (Somerville and Ogren, 1983; Renne et al., 2003). During glycine oxidation in the mitochondria, two molecules of glycine are converted to one molecule of serine, NADH, CO2 and ammonia. The DCT protein is required in the so-called nitrogen cycle of the photorespiratory pathway to re-assimilate the released ammonia (Keys, 2006). The concerted activity of glutamine synthetase (GS) and glutamate synthase (GOGAT) incorporates ammonia into 2-oxoglutarate in the chloroplast yielding glutamate. Export of glutamate depends on the activity of the DCT/DiT2.1 protein. An *A. thaliana* mutant defective in this carrier cannot export glutamate to the cytosol and the amino-donor for the peroxisomal aminotransferase reaction of glyoxylate to glycine is depleted fast (Renne et al., 2003). Similarly, impaired GS or GOGAT enzymes short-circuit the re-assimilation of 2-PG, demonstrating that the

nitrogen cycle is essential for the growth under photorespiratory conditions (Wallsgrove et al., 1987; Coschigano et al., 1998).

With a concentration of 380 ppm CO2 and 210,000 ppm of O2 in the air, the carboxylation to oxygenation ratio of RubisCO is approximately 3:1 in C3 plants without a carbon concentrating mechanism (CCM) (Sharkey, 1988). Hence, a massive flux through the carbon and nitrogen cycle of photorespiration results (Figure 1). The protein-mediated transport of glycerate/glycolate (Howitz and McCarty, 1991), glycolate/glycine/serine/glycerate/glutamate/2-oxoglutarate (Reumann et al., 1998) and glycine/serine (Walker et al., 1982; Yu et al., 1983) is documented on isolated membranes from chloroplast, peroxisomes and mitochondria, respectively. However the genes encoding the corresponding transporters have not yet been identified at the molecular level. Several reasons could explain why the forward genetic screens were unsuccessful to identify additional metabolite transporters. So far, only plants that show a severe decrease in chlorophyll content within four days after shifting from high CO2 to ambient air have been further analyzed (Somerville, 2001). Prolonging the period of photorespiratory conditions could serve to identify weaker alleles for the pale green phenotype. Overlapping substrate specificity of metabolite transporters could mask the phenotype of a single gene mutant. The mitochondrial glycine decarboxylase complex for glycine oxidation is absolutely essential for the fitness of the plant. The *gdc* knockout mutant is lethal at ambient and elevated carbon dioxide and thus demonstrate that not every gene in the photorespiratory pathway is dispensable under elevated CO2 conditions (Engel et al., 2007). It is tempting to speculate that this could be true for other proteins, such as the porin-like channel of the peroxisomes, which facilitates the transport of a

broad range of mono- and dicarboxylate anions (Reumann et al., 1998). A loss of this activity would presumably interfere with the peroxisomal glyoxylate cycle to mobilize fatty acids (Graham, 2008) and the oxaloacetate/malate shuttle to exchange reduction equivalents with the cytosol (Reumann and Weber, 2006).

In chapter four and five I revisited the highly compartmentalized photorespiratory pathway and initiated a search for the long outstanding metabolite transporters. In chapter four I summarize putative alternative pathways leading to ammonia re-assimilation in the organelles mitochondria and chloroplast. I emphasize the required, but not yet identified transport systems. In chapter five I took advantage of publicly available bioinformatics databases to screen for genes with two or more predicted transmembrane spans, which are highly co-expressed with known photorespiratory genes (Schwacke et al., 2003; Steinhauser et al., 2004). I initiated a reverse genetics candidate approach for five of the best 32 co-expressed putative metabolite transporters and report on the discovery of a novel transporter involved in the photorespiratory pathway. The long-term goal will be to identify the transporters involved in the photorespiratory pathway in higher plants.

Figure 1: The photorespiratory pathway

The enzymes involved in photorespiratory pathway are compartmentalized between chloroplasts, peroxisomes, and mitochondria (cMDH, chloroplastic malate dehydrogenase; GDC, glycine decarboxylase; GGT, glutamate:glyoxylate aminotransferase; GLYK, glycerate kinase; GOX, glycolate oxidase; GS/GOGAT, glutamine synthetase/glutamate synthase; HPR, hydoxypyruvate reductase; mMDH, mitochondrial malate dehydrogenase; PGP, phosphoglycolate phosphatase; pMDH, peroxisomal malate dehydrogenase; RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SGT, serine:glyoxylate aminotransferase; SHMT, serine hydoxymethyl transferase). Photorespiratory metabolites are abbreviated as follows: 3-PGA, 3-phosphoglycerate; Glu, glutamate; KG, α-ketoglutarate; Mal, malic acid; OAA, oxaloacetic acid; RuBP, ribulose-bisphosphate; THF, tetrahydofolate. The transport proteins represented in this scheme have been characterized biochemically (white box) or the corresponding genes have been cloned (black box). The scheme was adapted from Reumann and Weber (2006). The figure in this dissertation is presented in color.

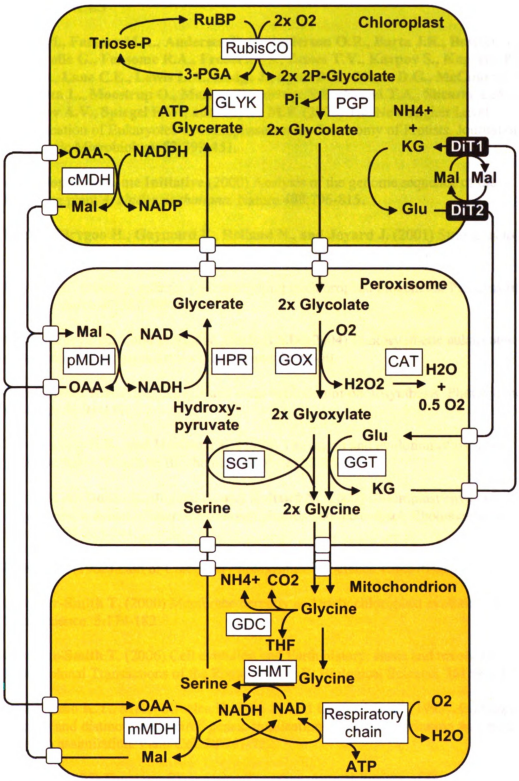

Figure 1: The photorespiratory pathway

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CHAPTER 1

PHYLOGENY OF THE PLASTID PHOSPHATE TRANSLOCATOR FAMILY[1]

[1] This work has been published in **Weber A.P.M., Linka M., and Bhattacharya D.** (2006) Single, ancient origin of a plastid metabolite translocator family in *Plantae* from a endomembrane-derived ancestor. Eukaryotic Cell 5:609-612.

ABSTRACT

Phylogenetic analyses show the single origin of a plastid metabolite translocator family in the *Plantae* from a gene encoding an existing endomembrane–derived protein. Red algal secondary endosymbiosis has spread a translocator gene into the ancestor of the "chromalveolate" protists, where it has diversified into a novel clade of proteins.

INTRODUCTION

The photosynthetic organelle (plastid) in red, green, and glaucophyte algae (*Plantae*) likely had a single origin from a cyanobacterial endosymbiosis (Cavalier-Smith, 1992; Palmer, 2003). This ancient event (ca. 1 – 1.5 billion years ago) (Yoon et al., 2004; Douzery et al., 2004) was followed by the transfer of genetic material from the endosymbiont to the nuclear genome of the host, the evolution of a protein import apparatus for the plastid, and targeting sequences for nucleus-encoded plastid–targeted proteins, and the establishment of genome-plastome intracellular communication and regulation (Brown et al., 2001; Fey et al., 2005; Strand, 2004). Current hypotheses regarding plastid origin and evolution provide plausible explanations for the later stages of organelle establishment (e.g., gene transfer to the nucleus), but do not specifically address the initial formation of the endosymbiosis.

We hypothesize that the insertion of a metabolite antiporter into the ancestral plastid membrane was essential for establishing the primary endosymbiosis, allowing the ancestor of the *Plantae* to profit immediately from cyanobacterial carbon fixation. This antiporter likely evolved from an existing host metabolite translocator with either

mitochondrial function (Bullerwell and Gray, 2004; Gray et al., 2004) or the endomembrane system. In contrast, autotrophic free-living cyanobacteria have no obvious need for such antiporters (i.e., bacterial translocators are not detectable with BLAST searches). Members of the nucleotide-sugar/triose phosphate translocator gene family (Knappe et al., 2003) are likely ancestors to plastid translocators because (i) genes encoding these proteins are found in all sequenced eukaryotic genomes, (ii) orthologous proteins are absent from prokaryotes, and (iii) some gene family members are targeted to plant plastids.

MATERIAL AND METHODS

To address plastid translocator origin, we gathered available sequences (genome and expressed sequence tag data) from the NCBI (http://www.ncbi.nlm.nih.gov/), DOE Joint Genome Institute (http://www.jgi.doe.gov/), Michigan State University Galdieria Database (http://genomics.msu.edu/galdieria/ sequence_data.html), *Porphyra yezoensis* expressed sequence tag index (http://www.kazusa.or.jp/en/plant/porphyra/EST/), and the *Cyanidioschyzon merolae* Genome Project (http://merolae.biol.s.u-tokyo.ac.jp/). Homologs were identified using BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST/) with an E-value cut-off of ≤ 0.0001. A translocator phylogeny was inferred using protein maximum-likelihood (ML) method (PHYML V2.4.3 (http://atgc.lirmm.fr/phyml/), neighbor joining (NJ) (PHYLIP V3.63, http://evolution.genetics.washington.edu/phylip.html), maximum parsimony (MP) (PAUP*V4.0b10, http://paup.csit.fsu.edu/), and Bayesian inference (MrBayes V3.0b4,

http://mrbayes.csit.fsu.edu/index.php) (for details of phylogenetic approach, see Hackett et al., 2004). A total of 250 amino acids from 65 plastid phosphate translocators from red and "chromalveolate" (i.e., chlorophyll *c*-containing) algae and land plants and their non-plastid homologs were included in these analyses.

RESULTS AND DISCUSSION

The resulting tree (Figure 1.1) shows that plastid translocators are monophyletic and had a single origin from an existing endomembrane translocator (bootstrap support: ML, 100%; NJ, 98%; MP, 93%), consistent with our model. Each major group of plant plastid translocators (i.e., TPT, triose-phosphate/phosphate translocator; GPT/XPT, glucose-6-phosphate and xylulose-5-phosphate/phosphate translocator, respectively; PPT, phosphoenolpyruvate/phosphate translocator, shown in green text in Figure 1.1) form robustly supported lineages (ML and MP bootstrap values > 90%) that are sister to homologs in the red algae (Figure 1.1, shown in red text). This result supports the monophyly of red and green algae and land plants (e.g., Rodriguez-Ezpeleta et al., 2005) and is consistent with the origin of the different plastid translocators in their common ancestor; i.e., all plastid translocators are monophyletic and each is divided into sister groups comprised of the red and green clades. Presumably, these *Plantae* translocators diversified because of the selective advantage they offer; i.e., to harvest the different fixed carbon products of the cyanobacterial primary endosymbiont. This phylogeny provides strong evidence for a single primary endosymbiosis in the studied *Plantae*, reflecting a critical and early step in plastid evolution. The monophyly of GPTs and

XPTs has previously been reported (Knappe et al., 2003) and likely reflects a more recent plant-specific gene duplication event.

The chromalveolates are a taxonomically diverse assemblage of protists comprised of alveolates (dinoflagellate algae, ciliates, and parasitic apicomplexans) and chromists (cryptophytes, haptophytes, and stramenopiles) that are thought to have ancestrally contained a plastid of red algal origin (Cavalier-Smith, 1999). This group is yet to be substantiated in a global eukaryotic phylogeny. It is therefore of interest that the available (i.e., apicomplexan, haptophyte, and stramenopile) chromalveolate sequences are monophyletic in our tree (Figure 1.1) and solidly associated (ML, 80%, NJ, 95%) with one clade of red algal proteins (i.e., *C. merolae* CMK114C, *G. sulphuraria* HET39C12). The addition of cryptophyte and dinoflagellate plastid translocators is needed to verify this result. Interestingly, preliminary biochemical analysis of the *G. sulphuraria* HET39C12 translocator suggests that it is a TPT (Marc Linka unpublished data); therefore, the ancestral chromalveolate plastid translocator was likely of this type.

The topology of the chromalveolate subtree supports a single origin of the plastid translocator gene in the common ancestor of these species (i.e., supporting their monophyly) from the nucleus of the red algal endosymbiont. It is noteworthy that PPT and GPT/XPT from *Plantae* are absent from the chromalveolates. In organisms containing plastids of secondary endosymbiotic origin with three or four plastid envelope membranes, transfer of a plastid phosphate translocator-related gene to the host nucleus and retargeting to the inner plastid membrane alone are not sufficient for the export of photosynthates to the host. Additional translocators with identical transport properties would be required in the third, the remnant of the endosymbiont plasma membrane, and

the fourth membranes, the plastid endoplasmic reticulum, to connect the metabolism of the host and the endosymbiont. This essential metabolic connection has been accomplished in the ancestor of the chromalveolates in our tree with the red algal gene undergoing diversification through duplication and divergence giving rise to the complex branching pattern shown in Figure 1.1. The specific functions and the detailed membrane localizations of the different chromalveolate translocators remain to be determined.

We analyzed chromalveolate translocators with significant N-terminal extensions to determine if they are putatively plastid (or apicoplast) targeted. Using PATS (http://gecco.org.chemie.uni-frankfurt.de/pats/pats-index.php) and PlasmoAP (http://www.plasmodb.org/restricted/PlasmoAPcgi.shtml), apicoplast targeting is supported for the apicomplexan *Plasmodium* spp. (e.g., *P. falciparum* NP_703643; PATS probability = 0.954, PlasmoAP = 5/5 tests positive). Similarly, the N-terminal extension in the diatom (stramenopile) *Thalassiosira pseudonana* Sc15 (gene located on Scaffold 15) sequence contains a typical bipartite plastid-targeting sequence (Lang et al., 1998) comprised of a signal sequence using SignalP (http://www.cbs.dtu.dk/services/SignalP/, probability = 0.997; predicted length is 18 amino acids) followed by a transit peptide (using TargetP, probability = 0.740). These results suggest that the chromalveolate clade in our tree includes plastid-targeted translocators, although it is possible that others in this group may have lost this function. For example, the translocator from *Theileria annulata* apparently does not encode a N-terminal extension, but is clearly related to the other apicomplexan sequences. Although we do not have glaucophyte translocators in the tree, the signature activity of plastidic phosphate translocators has been detected in isolated cyanelles of *Cyanophora paradoxa*, indicating the presence of plastidic phosphate

translocators (Schlichting and Bothe, 1993). We therefore hypothesize that the insertion of a phosphate translocator into the plasma membrane of the endosymbiont occurred before the split of the *Plantae* and was probably a critical step in rendering the association between the cyanobacterium and the mitochondriate eukaryote irreversible. The proposed sequence of events for plastid establishment (Figure 1.2 and Figure 1.3) does not involve major evolutionary innovations. The basic components of a plastid envelope protein import apparatus were present in the cyanobacterium (Reumann et al., 2005) and it is reasonable to assume that this machinery was capable of importing proteins from the host cell cytosol, albeit with low efficiency; i.e., the outer leaflet of the plastid outer envelope membrane consists mainly of ER-derived phospholipids (Douce and Joyard, 1981; Cavalier-Smith, 2000), indicating a close interaction between plastid envelope membranes and the host endomembrane system. It is likely that the cyanobacterium was engulfed as a prey item (as often occurs today) through phagocytosis, an event that likely occurred countless times and in some of these cells, the prey was not digested in the food vacuole but rather maintained as an endosymbiont. A descendant of these cells gave rise to the *Plantae*. The origin (or replacement) of plastids through cell (or organelle) engulfment has occurred several times in evolution and can be found in taxa such as the filose amoeba *Paulinella chromatophora* (Bhattacharya et al., 1995) and in several dinoflagellate lineages (Schnepf and Elbrächter, 1999; Saldarriaga et al., 2001; Hackett et al., 2004). The position of the *Plantae* in the tree of life (Baldauf, 2003; Rodriguez-Ezpeleta et al., 2005) suggests that its common ancestor was a highly developed flagellate (or had flagellated stages) that most likely had the capacity for phagocytosis and endomembrane formation (Cavalier-Smith, 2000).

Figure 1.1. Maximum-likelihood phylogeny of endomembrane and plastid translocators ML bootstrap values (200 replications) are shown above the branches on the left of the slash mark, whereas the values to the right are from a NJ analysis (100 replications). Only bootstrap values $\geq$ 60% are shown. The different plastid translocators are GPT (glucose-6-phosphate/phosphate translocator), PPT (phosphoenolpyruvate/phosphate translocator), TPT (triose-phosphate/phosphate translocator), and XPT (xylulose-5-phosphate translocator). Apicomplexans: *Theileria annulata*, *Plasmodium falciparum*, *Toxoplasma gondii*; Haptophytes:Emiliana huxleyi; Stramenopiles: *Thallassiosira pseudonana*. The numbers in the circles indicate translocators to the right that resulted from primary (1) and secondary (2) endosymbiosis. *T. pseudonana: Thallassiosira pseudonana; A. thaliana: Arabidopsis thaliana; C. merolae: Cyanidioschyzon merolae*

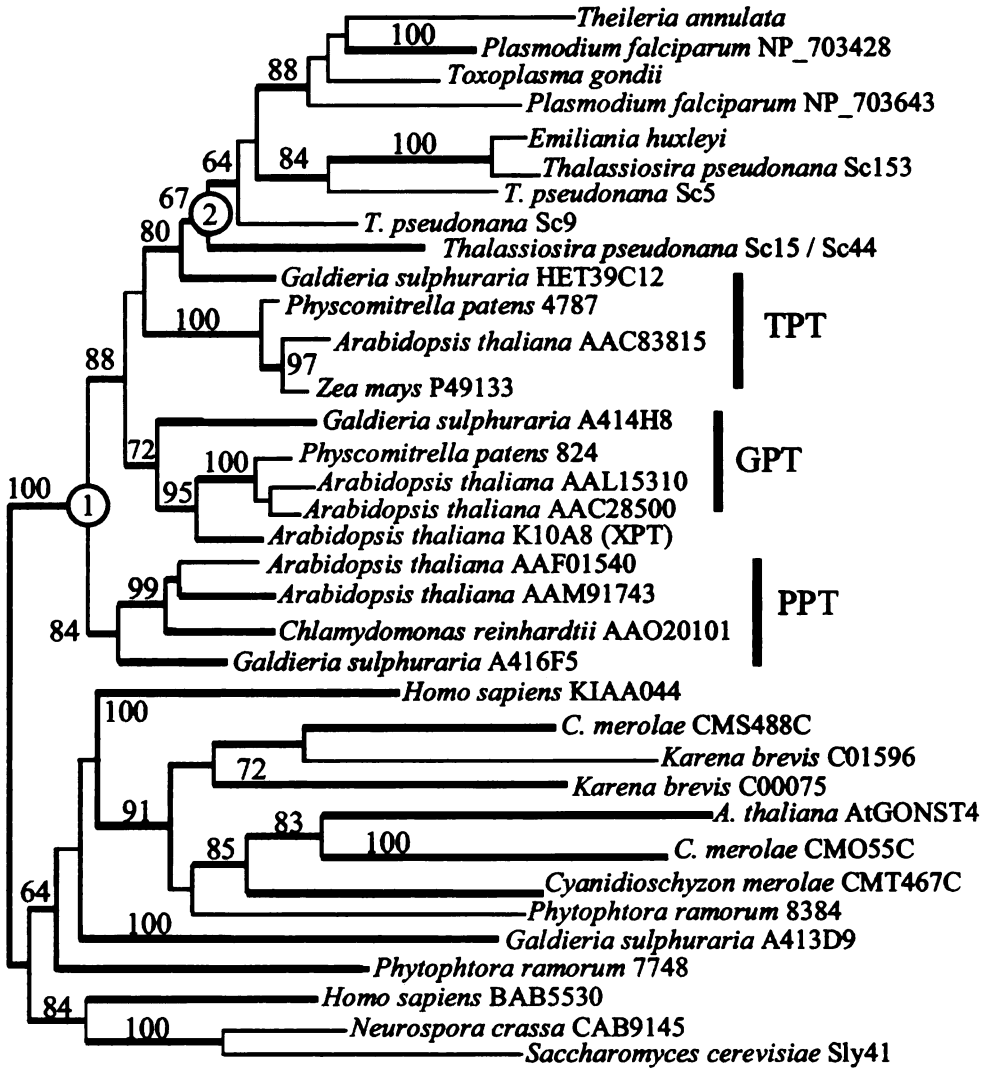

Figure 1.1. Maximum likelihood phylogeny of endomembrane and plastid translocators

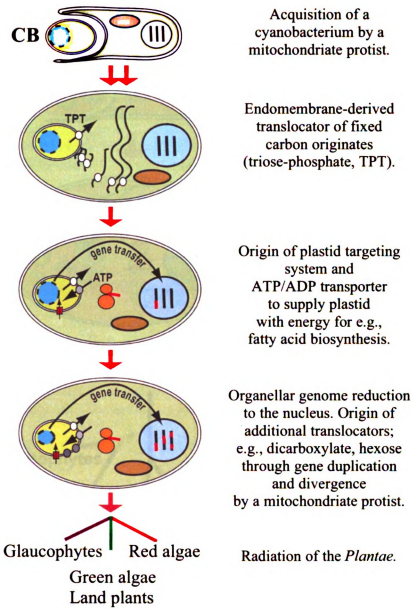

Figure 1.2. Model for plastid and translocator origin in the common ancestor of the *Plantae* during primary endosymbiosis

CB: cyanobacterium; ATP: Adenine triphosphate; TPT: Triosephosphate/phosphate translocator. The figure in this dissertation is presented in color

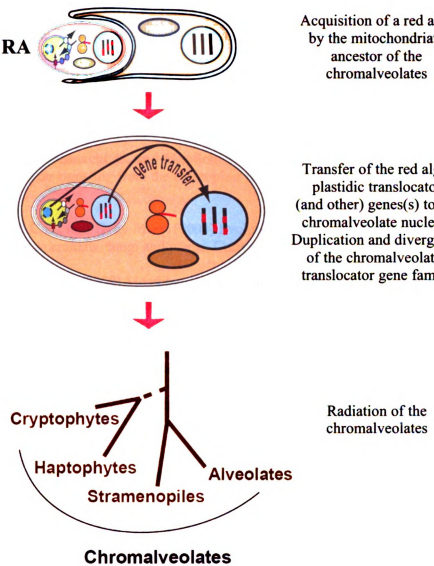

Figure 1.3. Model for plastid and translocator origin in the common ancestor of the chromalveolates during secondary endosymbiosis

RA is red alga. The chromalveolate tree reflects the present understanding of the phylogeny of this group (e.g., Bhattacharya et al., 2004; Harper et al., 2005). The figure in this dissertation is presented in color.

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CHAPTER 2

PHYLOGENY AND ORIGIN OF PLASTID METABOLITE TRANSPORT PROTEINS FROM *ARABIDOPSIS THALIANA*[2]

[2] This work has been published in **Tyra H.M., Linka M., Weber A.P.M., and Bhattacharya D.,** (2007) Host origin of plastid solute transporters in the first photosynthetic eukaryotes. Genome Biology **8**:R212-224. Taira H.M. and I contributed equally to this work. Taira H.M. wrote the initial draft of the manuscript. Dr. D. Bhattacharya was responsible for the final phylogenetic trees.

ABSTRACT

Background: It is generally accepted that a single primary endosymbiosis in the *Plantae* (red, green (including land plants), and glaucophyte algae) a common ancestor gave rise to the ancestral photosynthetic organelle (plastid). Plastid establishment necessitated many steps, including the transfer and activation of endosymbiont genes that were relocated to the nuclear genome of the 'host' followed by import of the encoded proteins into the organelle. These innovations are, however, highly complex and could not have driven the initial formation of the endosymbiosis. We postulate that the re-targeting of existing host solute transporters to the plastid fore-runner was critical for the early success of the primary endosymbiosis, allowing the host to harvest endosymbiont primary production.

Results: We tested this model of transporter evolution by conducting a comprehensive analysis of the plastid permeome in *Arabidopsis thaliana*. Of 137 well-annotated transporter proteins that were initially considered, 83 that are broadly distributed in *Plantae* were submitted to phylogenetic analysis. Consistent with our hypothesis, we find that 58% of *A. thaliana* transporters, including all carbohydrate transporters, are of host origin, whereas only 12% arose from the cyanobacterial endosymbiont. Four transporter genes are derived from a *Chlamydia*-like source, suggesting that establishment of the primary plastid likely involved contributions from at least two prokaryotic sources.

Conclusion: Our results indicate that the existing plastid solute transport system shared by *Plantae* is derived primarily from host genes. Important contributions also came from the cyanobacterial endosymbiont and *Chlamydia*-like bacteria likely co-resident in the first algae.

INTRODUCTION

Plastids in eukaryotes that contain chlorophyll are capable of carrying out photosynthesis, a process that converts light energy, carbon dioxide, and water into organic compounds. The evolutionary history of this organelle unfolded over a billion years ago when a previously non-photosynthetic protist engulfed and maintained a free-living cyanobacterium in its cytoplasm (Yoon et al., 2004). It is hard to over-state the importance of this ancient and extraordinarily rare primary endosymbiosis because plastids allowed the evolution of algae and the plants that form the base of the food chain for many ecosystems on Earth. Current data suggest that the primary endosymbiosis occurred once in the common ancestor of the red, green (including land plants), glaucophyte algae, the *Plantae* (Rodriguez-Ezpelata et al., 2005; Weber et al., 2006; Reyes-Prieto and Bhattacharya, 2007), with the original plastid and the nuclear-encoded machinery for running the organelle spreading in subsequent cell captures to other branches of the eukaryotic tree (Cavalier-Smith et al., 1994; Mc Fadden, 1999; Bhattacharya et al., 2004). The only other known case of a potential *bona fide* cyanobacterial primary endosymbiosis occurred relatively recently in the thecate amoeba *Paulinella chromatophora* (Marin et al., 2005; Yoon et al., 2006).

The gradualist view of evolution through mutation-selection suggests that it would have taken millions of years for the captured prokaryote to become fully integrated into the 'host' eukaryote, ultimately becoming the site not only for carbon fixation but also for other complex functions, such as lipid, isoprenoid, and amino acid biosynthesis (Weber et al., 2005). These processes were associated with the migration of much of the cyanobacterial genome to the host nucleus and development of the complex protein

import system that are key shared features among all canonical plastids (Timmis et al., 2004; Weber et al., 2006; Gutensohn et al., 2006). A remarkable exception to the view that endosymbiosis was a gradual process of integration is offered by the katablepharid protist 'Hatena', which undergoes large-scale morphological changes following the engulfment of a green alga (Okamato and Inouye, 2005).

Regardless of whether the ancient primary endosymbiosis fostered an accelerated rate of morphological evolution in the *Plantae* ancestor or whether general cell morphology was unchanged as in the *Paulinella* example (Johnson et al., 1919), one thing is clear - in the absence of rapid benefits to the host it is unlikely that the endosymbiosis would long have been sustained. Given the need for short-term survival, a key feature of early success for the endosymbiosis must have been the integration of the metabolism of the two cells. A solution for this process would have been solute transporters that regulate the flux of metabolites (for example, ATP, phosphate, sugars and sugar phosphates, metal ions, and other important ions) across the organelle membranes. Controlled exchange in response to environmental factors such as changes in light intensity and trace metal availability (Raven et al., 1999; Renne et al., 2003; Reiser et al., 2004) is decisive because the unregulated flux of metabolites would have had detrimental effects and, thereby, lowered the evolutionary fitness of the endosymbiosis. A complex system of solute transporters is in place today in extant plastids that provides the link between this organelle and the surrounding cytosol (Ferro et al., 2003; Weber, 2004; Weber and Fischer, 2007). Here we focus on the evolutionary history of these plastid metabolite transporters to infer early events in plastid evolution.

We make two assumptions in this study. First, a system of metabolite transporters was a critical and early development in plastid evolution to supply the endosymbiont with essential nutrients and to enable the host to reap immediate benefit from photosynthetic primary production. It is unclear why the cyanobacterium that was destined to become the plastid escaped digestion in the host but this scenario has also played out in 'Hatena' and in *Paulinella*. Second, whereas the genome of the previously free-living cyanobacterium encoded all the transport systems required for the uptake of essential inorganic nutrients, it most likely did not harbor genes encoding transporters for the export of organic solutes to the host - this would have served no obvious pre-existing purpose in the prokaryote. Precisely how the plastid solute transport system was established is unknown. One possible model involves a primarily cyanobacterial origin, in which the plastid continued to utilize its own original cyanobacterial solute transporters with their evolution over time into proteins that perform most or all currently known plastid permeome functions. An alternative model involves a host-driven solute transport system, likely derived from the vacuolar envelope that initially surrounded the endosymbiont after its engulfment (Weber et al., 2006). And finally, both of the new partners could have contributed proteins equally to this machinery, resulting in a chimeric system composed of the most beneficial combination possible of prokaryotic and eukaryotic transporters. To determine which of these competing hypotheses best explains plastid transporter evolution, we undertook an initial bioinformatics analysis of 137 *A. thaliana* solute transporters and then a detailed phylogenetic analysis of a subset of 83 conserved proteins that included available data from other *Plantae*. The *A. thaliana* transporters are either predicted or have been shown to be chloroplast targeted and are

ideal for tracking plastid permeome evolution. Using these data we demonstrate that over one-half of *Plantae* plastid targeted transporters are putatively of host origin whereas less than a quarter arose from the cyanobacterial endosymbiont. This suggests that primarily host genes made the lasting contribution to the *Plantae* host-endosymbiont relationship with regard to the plastid solute transport system. We also find evidence for the origin of four transporter genes or gene families from a *Chlamydia*-like source. This latter result raises the possibility that establishment of the ancient primary plastid may have involved contributions from at least two prokaryotic sources, perhaps explaining its singular nature. This hypothesis received substantial support from the recent finding of at least 21 genes of *Chlamydia*-like origin in the nuclear genome of the extremophilic red alga *Cyanidioschyzon merolae* (Huang and Gogarten et al., 2007).

MATERIAL AND METHODS

Initial transporter analyses

As a starting point for the compilation of a conservative set of predicted or confirmed plastid envelope membrane transporters, we used a previously published list of 137 plastid-targeted membrane proteins that was based on predicted plastid localization and classification by the transporter classification system (Weber et al., 2005). This list was manually curated to remove proteins from the list if published evidence indicated that they were localized to a cellular location other than chloroplasts, if they represented membrane-bound enzymes, or if they were annotated as components of the Tic/Toc protein import apparatus, the photosynthetic machinery of the thylakoid membrane, or

the Sec or Tat protein targeting pathways. This curated list of candidate genes was updated and amended with recently published chloroplast envelope membrane transporters, such as AtFOLT1, a plastid localized transporter belonging to the mitochondrial carrier family that does not contain a plastid targeting signal (Bedhomme et al., 2005) and was thus not included in previous lists. The final list contained 83 *A. thaliana* predicted or confirmed chloroplast solute transporters.

The sequence for each protein was obtained from The *Arabidopsis* Information Resource website (http://www.arabidopsis.org/index.jsp). These protein sequences were used as queries in the BLAST program "blastp" and "tblastn" searches of the NCBI Database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the plant and algal genomes available through the Joint Genome Institute Eukaryotic Genomics Website (http://genome.jgi-psf.org/euk_home.html), the *Cyanidioschyzon merolae* Genome Project website (http://merolae.biol.s.u-tokyo.ac.jp/), the *Galdieria sulphuraria* Genome Project website (http://genomics.msu.edu/galdieria/), and Dragonblast V2.1 (Ruemmele SE, unpublished data), a web based database in Bhattacharya's lab that contains EST datasets for several chromalveolates, *Plantae*, excavates, Rhizaria, and Amoebozoa. We used the predicted protein sequences for the following species for our analysis whenever available: *Arabidopsis thaliana*, *Oryza sativa*, *Physcomitrella patens*, *Chlamydomonas reinhardtii*, *Ostreococcus tauri*, *Ostreococcus lucimarinus*, *Cyanidioschyzon merolae*, *Galdieria sulphuraria*, *Cyanophora paradoxa*, *Dictyostelium discoideum*, *Strongylocentrotus purpuratus*, *Xenopus laevis*, *Danio rerio*, *Mus musculus*, *Canis familiaris*, and *Homo sapiens*. In addition, we included at least one insect, three fungal species, and a broad range of *Bacteria* and *Archaea* in our analysis. The BLAST searches used an E-value cut-

off < 0.00001. If a translated EST sequence was not available, the nucleotide sequence was translated over six frames using the EXPASY translate tool (http://ca.expasy.org/tools/dna.html). The resulting protein sequences were used in a BLAST search against the NCBI protein database to ensure the correct translation was obtained.

We used the ClustalW feature included with BioEdit V7.0.5.3 to generate protein alignments (Hall, 1999). Alignments were visually inspected and manually corrected if necessary. Trees were generated under maximum likelihood using PHYML V2.4.4 utilizing the WAG model of amino acid substitution and estimating both the proportion of invariable sites and the alpha parameter (that is, WAG + I + Γ) (Guindon and Gascuel, 2003). We performed non-parametric bootstrap analysis with 100 replicates for each PHYML analysis. The resulting trees were analyzed to determine the origin of the transporter in *A. thaliana* and other *Plantae*.

The designation of gene origin was done as follows. When the *Plantae* solute transporter formed a well-supported (usually > 70% bootstrap support) monophyletic group with homologs in opisthokonts (that is, animals and fungi) and secondarily with other eukaryotes such as excavates and chromalveolates (if present), then it was classified as having a 'Host' origin. Under this scheme, no bacterial sequences interrupted the eukaryotic domain. 'Cyanobacterial' or '*Chlamydia*-like' origin was inferred if the *Plantae* sequence formed a monophyletic group with protein sequences from either of these lineages with strong bootstrap support. Other bacterial or eukaryotic sequences could (not necessarily) be in these trees but there had to be a robust separation of the *Plantae* and cyanobacteria or *Chlamydia*-like clade from all other homologs. We had two other

categories of gene origin that likely reflected a lack of phylogenetic resolution or pervasive horizontal gene transfer (HGT) among taxa that defied a clear inference of origin. The first was the 'Other' category in which the *Plantae* transporter formed a well-supported monophyletic clade but its position relative to available bacterial data was unresolved, thereby not allowing us to identify the donor taxon. The second, '*Plantae*-specific', was for transporters that had no significant hits to sequences in GenBank or other databases and appeared to be limited to the *Plantae*. The protein alignments are available in the download section of the Bhattacharya Lab website (http://www.biology.uiowa.edu/debweb/downloads/index.php).

Detailed phylogenetic analyses

For eight representative transporters from the five categories described above we inferred a maximum likelihood phylogeny using RAxML (RAxML-VI-HPC, v2.2.1) (Stamatakis et al., 2005) and the WAG + Γ evolutionary model. The specific transporters were: 'Host' - At4g39460 (SAMT, S-adenosylmethionine transporter, 236 amino acids), At5g66380 (AtFOLT1, *Arabidopsis thaliana* folate transporter 1, 268 amino acids); 'Cyanobacterial' - At1g19800 (TGD1, trigalactosyldiacylglycerol 1, permease-like protein for lipid transport, 234 amino acids), At5g64940 (ABC transporter protein, 487 amino acids); '*Chlamydia*-like' - At1g15500 (plastidic ATP/ADP transporter 2 (AtNTT2), 436 amino acids), At4g37270 (HMA1, copper exporting ATPase, 444 amino acids); 'Other' - At1g32080 (putative membrane protein, 211 amino acids); '*Plantae*-specific' - At5g24690 (hypothetical expressed protein, 274 amino acids). These detailed analyses used a random starting tree (one round of taxon addition) and the rapid hill-climbing

algorithm (that is, option -f d in RAxML). To generate bootstrap values for these phylogenies, we used RAxML with the same settings and 100 replications. In addition, we used Bayesian inference (MrBayes V3.0b4) (Huelsenbeck and Ronquist, 2001) with each of the eight data sets using the WAG + I + Γ model to calculate posterior probabilities for nodes in the RAxML trees. Metropolis-coupled Markov chain Monte Carlo from a random starting tree was used in this analysis with two independent runs and 1 cold and 3 heated chains. The Bayesian analyses were run for two million generations each with trees sampled every 100th generation. To increase the probability of chain convergence, we sampled trees after the standard deviation values of the two runs were < 0.01 to calculate the posterior probabilities. We also ran the Bayesian analysis for the remaining two putative '*Chlamydia*-like' genes in *Plantae* (dicarboxylate translocators DiT1, DiT2.1, and DiT2.2, and the phosphate transporter PHT2;1) to assess the topologies. We incorporated a representative diversity of available sequences in all of these trees.

RESULTS AND DISCUSSION

Distribution of transporters within *Plantae*

Phylogenetic analysis of the best-annotated transporter data that are currently available from *Arabidopsis* was used to identify and putatively annotate homologs from other *Plantae*. Of 137 transporter proteins that were initially considered, BLAST and phylogenetic analyses and manual curation of recently available data led to the identification of 83 proteins that were of sufficient conservation and broad distribution

among *Plantae* to be used for further analyses. Each of these 83 proteins that included gene families (that is, representing 63 distinct, ancestral genes; Table 2.1) was used as input in BLAST and PHYML bootstrap analyses to infer the trees. This approach identified 41 proteins that are present in both red and green algae (including land plants) and, therefore, were likely found in the *Plantae* ancestor (glaucophyte homologs were found for some of these genes; for example, ADP/ATP translocase, hypothetical protein At3g45890). Eleven proteins were restricted to green algae and land plants, seven were plant-specific, and two were limited to red algae and land plants. The distribution of these proteins with respect to their putative origin in *Plantae* is shown in Figure 2.1(A). Given the lack of evidence for widespread horizontal gene transfer in extant *Plantae*, which most likely lost the capacity for phagotrophy early in its evolution (Archibald et al., 2003; Reyes-Prieto et al., 2007), we postulate that the patchy distribution for many plastid targeted transporters primarily reflects differential gene loss over the greater than one billion years that has passed since the primary endosymbiosis (Yoon et al., 2004). Under this interpretation, the large set of shared transporters among *Plantae* lineages provides resounding support for the monophyly of this supergroup (Hackett et al., 2007).

Table 2.1. List of *Arabidopsis thaliana* chloroplast solute transporters analyzed in this study and their putative evolutionary origins

***Arabidopsis thaliana* solute transporters**	
Putative evolutionary origin: Host	
AGI code	Gene Annotation
At1g05580	Cation/hydrogen exchanger
At1g54320	Ligand-effect modulator 3 (LEM3) family
At1g59870	ABC transporter
At1g61800	Glucose-6-phosphate/phosphate translocator 2 (GPT2)
At1g64150	Expressed protein
At1g66950	ABC transporter
At1g70610	Transporter associated with antigen processing protein 1 (AtTAP1)
At1g79450	Ligand-effect modulator 3 (LEM3) family
At2g04620	Cation efflux family protein
At2g13100	Glycerol-3-phosphate transporter
At2g27810	Xanthine/uracil permease
At2g28070	ABC transporter
At2g29650	Na+-dependent inorganic phosphate cotransporter
At2g38060	Na+-dependent inorganic phosphate cotransporter
At2g38330	Multi antimicrobial extrusion (MATE Efflux) protein
At2g40420	Amino acid transporter
At3g01550	Phosphoenolpyruvate/phosphate translocator 2 (PPT2)
At3g12740	Ligand-effect modulator 3 LEM3 family
At3g17690	Cyclic nucleotide-binding transporter 2
At3g17700	Cyclic nucleotide-binding transporter 1
At3g45890	Expressed protein
At3g52310	ABC transporter
At4g00370	Anion transporter 2 (ANTR2)
At4g13590	Expressed protein
At4g17340	Major intrinsic family protein
At4g25750	ABC transporter
At4g32400	Adenine nucleotide uniporter
At4g32650	Arabidopsis thaliana K^+ rectifying channel 1 (ATKC1)
At4g38380	Multi antimicrobial extrusion (MATE Efflux) protein
At4g39460	S-adenosylmethionine carrier 2 (SAMT)

Table 2.1. (cont'd).

***Arabidopsis thaliana* solute transporters**	
Putative evolutionary origin: Host	
AGI code	Gene Annotation
At5g04770	Amino acid permease
At5g05630	Amino acid permease
At5g13550	Sulfate transporter
At5g14040	Mitochondrial phosphate transporter
At5g16150	Hexose transporter
At5g17630	Xyluose-5-phosphate/phosphate transporter 1 (XPT)
At5g19410	ABC transporter (White)
At5g19600	Sulfate transporter
At5g22830	CorA-like magnesium transporter
At5g26820	Ferroportin-related protein
At5g33320	Phosphoenolpyruvate/phosphate translocator (PPT1)
At5g42130	Mitochondrial substrate carrier family
At5g45450	Iron transporter-related
At5g46110	Triose phosphate/phosphate translocator (TPT)
At5g52860	ABC transporter (White)
At5g54800	Glucose-6-phosphate/phosphate transporter 1 (GPT1)
At5g59250	Sugar transporter
At5g66380	Folate transporter 1 (AtFOLT1)
Putative evolutionary origin: Cyanobacteria	
AGI code	Gene Annotation
At1g04570	Integral membrane family protein
At1g08640	Expressed protein
At1g19800	Trigalactosyldiacylglycerol 1, permease-like protein (TGD1)
At1g78620	Integral membrane family protein
At2g32040	Folate monoglutamate transporter, FT
At3g51140	Expressed protein
At3g60590	Expressed protein
At4g33520	Metal-transporting P-type ATPase (PAA1)
At5g12470	Expressed protein
At5g64940	ABC1-family protein

Table 2.1. (cont'd).

***Arabidopsis thaliana* solute transporters**	
Putative evolutionary origin: '*Chlamydia*-like'	
AGI code	Gene Annotation
At1g15500	Adenine nucleotide translocase 2 (AtNTT2)
At1g80300	Adenine nucleotide translocase 1 (AtNTT1)
At3g26570	Low affinity phosphate transporter (PHT2;1)
At4g37270	Heavy metal ATPase HMA1
At5g12860	Dicarboxylate translocator 1 (DiT1)
At5g64280	Dicarboxylate translocator 2.2 (DiT2.2)
At5g64290	Dicarboxylate translocator 2.1 (DiT2.1)
Putative evolutionary origin: 'Other'	
AGI code	Gene Annotation
At1g01790	Potassium transporter
At1g32080	Membrane protein
At1g44920	Expressed protein
At1g54350	ABC transporter
At1g78560	Bile acid:sodium symporter
At2g02590	Expressed protein (Putative small multi-drug export)
At2g21340	Enhanced disease susceptibility protein
At2g26900	Bile acid:sodium symporter
At3g25410	Bile acid:sodium symporter
At4g30580	1-Acylglycerol-3-phosphate O-acyltransferase (ATS2)
At5g03555	Cytosine/purines, uracil, thiamine, allantoin family permease
At5g13720	Expressed protein
At5g52540	Expressed protein
At5g62720	Integral membrane HPP family protein
Putative evolutionary origin: '*Plantae*-specific'	
AGI code	Gene Annotation
At2g38550	Expressed protein
At3g57280	Expressed protein
At5g17520	Maltose transporter (MEX1)
At5g24690	Expressed protein

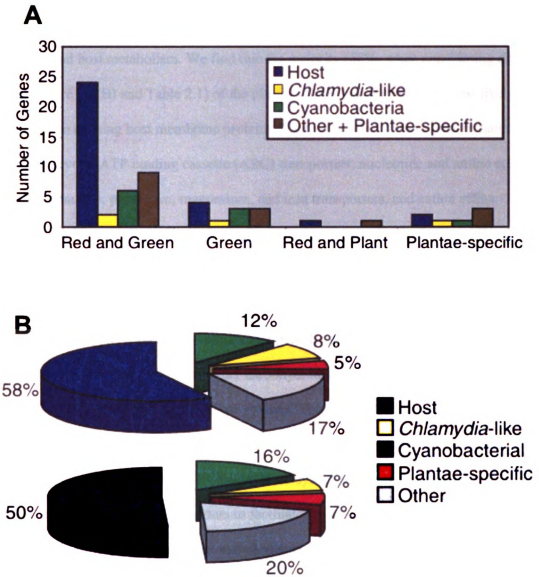

Figure 2.1. Origin of plastid targeted solute transporters in *Plantae*

(A) Gene distribution among *Plantae* and gene origin for 63 distinct transporters considered in this study. **(B)** Summary pie charts showing the origin of all the 83 transporters (top chart) and the 63 distinct genes (lower chart) considered in this study. The figure in this dissertation is presented in color.

Most proteins of the plastid envelope permeome are host-derived

Analysis of the phylogenetic data supports the notion that the host drove the integration of plastid and host metabolism. We find that the majority (58%, when considering all 83 genes; Figure 2.1(B) and Table 2.1) of the plastid solute transporters were most likely derived from existing host membrane proteins. These 48 proteins are diverse in nature, including several ATP binding cassette (ABC) transporters, nucleotide and amino acid permeases, sulfate, potassium, magnesium, and iron transporters, and cation efflux proteins (see Figure 2.2 (A) for S-adenosylmethionine transporter (SAMT) and Figure 2.2 (B) for *A. thaliana* folate transporter 1 (AtFOLT1) as representative trees).

Of particular interest is the finding that in addition to the members of the nucleotide-sugar/triose phosphate translocator gene family previously reported to be of host origin (Weber et al., 2006), all other carbohydrate transporters included in our analysis were derived from existing host proteins. This result strongly suggests that the host utilized existing eukaryotic transport proteins pre-adapted to this function to 'tap' into the photosynthates produced by the captured cyanobacterium. In addition, the *Plantae* host also provided transporters to facilitate the movement of valuable nutrients such as magnesium, potassium, iron, and phosphate into the captured prokaryote. The replacement of pre-existing cyanobacterial anion and cation transporters with host derived proteins again suggests that there was strong selection to rapidly establish control over and utilize the endosymbiont. This process was most likely accomplished by using transporters derived from the host vacuolar envelope (Weber et al., 2006).

Figure 2.2. Plastid targeted solute transporters of putative 'host' origin in *Plantae*

These are RAxML trees with the numbers above the branches inferred from a RAxML bootstrap analysis and the thick branches showing significant ($P > 0.95$) support from a Bayesian phylogenetic inference. Only bootstrap values ≥ 60% are shown. Branch lengths are proportional to the number of substitutions per site (see scale bars). The filled magenta circle shows the node that unites the *Plantae* taxa within the eukaryotic domain. The different algal groups are shown in different text colors: red for red algae, green for green algae and land plants, and brown for chromalveolates. The inclusion of chromalveolates within the *Plantae* is believed to reflect horizontal or endosymbiotic gene transfer events (for example, Li et al., 2006). The two transporters are: **(A)** SAMT, S-adenosylmethionine carrier transporter; and **(B)** AtFOLT1, *A. thaliana* folate transporter 1. The name of the *A. thaliana* solute transporter used for the query is indicated for both trees shown in this figure. The figure in this dissertation is presented in color.

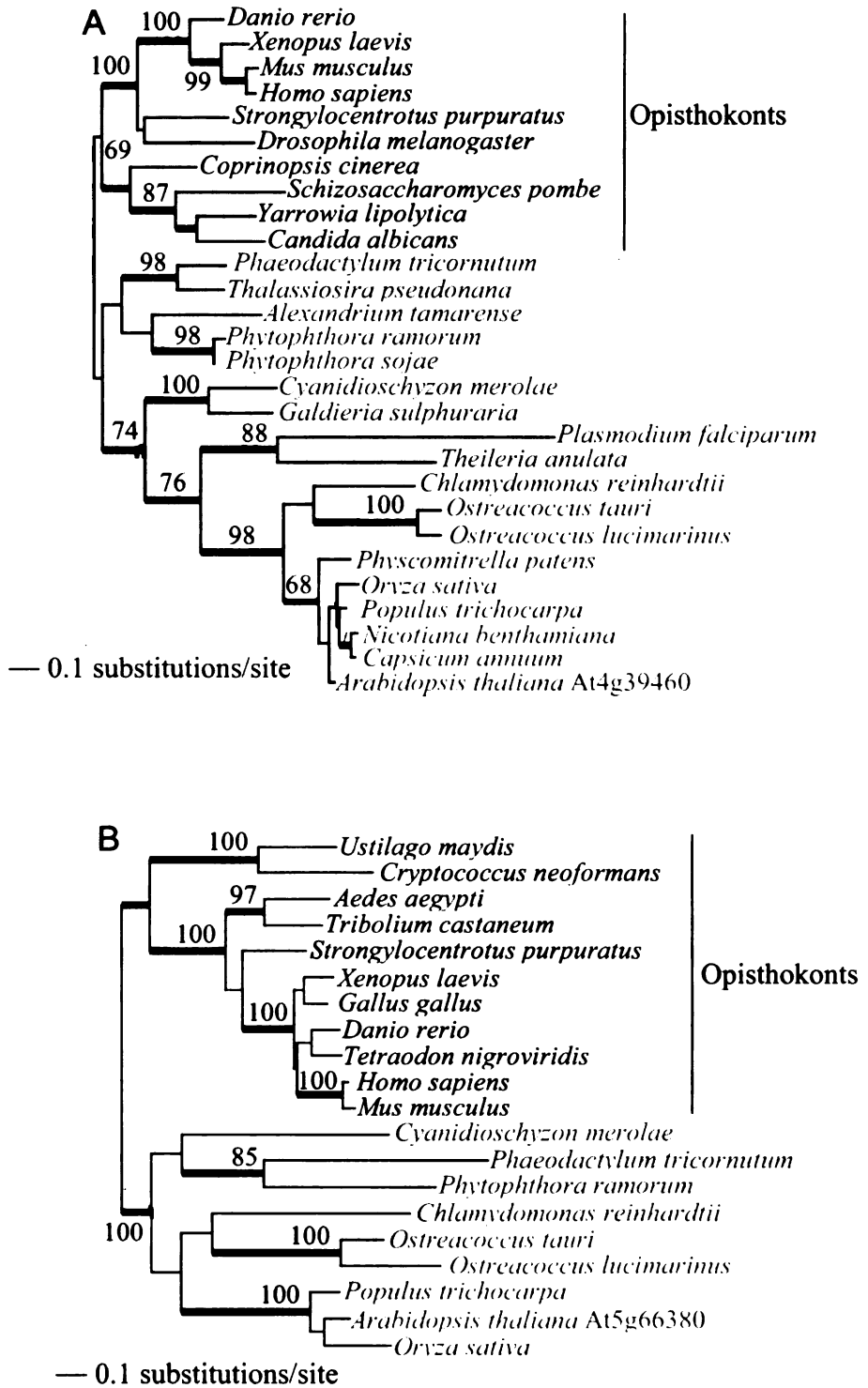

Figure 2.2. Plastid targeted solute transporters of putative 'host' origin in *Plantae*

The cyanobacterial contribution

The cyanobacterial endosymbiont putatively contributed ten solute transporters to the plastid transport system (Table 2.1). These proteins include permease-like protein TGD1 (trigalactosyldiacylglycerol 1, Figure 2.3), which is required for integrating the prokaryotic (that is, cyanobacterial) with the eukaryotic (that is, endoplasmic reticulum) pathway for lipid biosynthesis (Xu et al., 2003; Awai et al., 2006; Benning et al., 2006), the metal-transporting P-type ATPase PAA1 (Shikanai et al., Abdel-Ghany et al., 2005), and a transporter required for folate/biopterin biosynthesis (Klaus et al., 2006). The remaining seven proteins of unknown function that are localized to the chloroplast inner membrane were included in the cyanobacterial group. Whereas the predicted secondary structure of most of these proteins indicates they represent transporters (that is, they contain at least four transmembrane domains that are connected by short loops), some, such as the ABC1-family protein At5g64940 (Figure 2.3) contain only one or two predicted transmembrane domains and may thus have functions other than metabolite transport. It is also intriguing that with the exception of the PAA1 copper transporter the only cyanobacterial transport proteins apparently retained by *Arabidopsis* are those for which the host lacked a suitable replacement. For example, the initial steps of folic acid biosynthesis in plants are confined to the chloroplast; the final steps are localized in the cytosol and in mitochondria (Sahr et al., 2006; Basset et al., 2004a; Basset et al., 2004b). Plastids thus depend on an external folate supply and require an uptake system for this important metabolite. Interestingly, redundant systems for folate uptake exist in *Arabidopsis* chloroplasts, consisting of the cyanobacterial-derived folate transporter FT (Klaus et al., 2005) and the host-derived transporter AtFOLT1 (Bedhomme et al., 2005).

Figure 2.3. Plastid targeted solute transporters of putative 'cyanobacterial'/'plastid endosymbiont' origin in *Plantae*

For details of tree building see Figure 2.2. The filled magenta circle shows the node that unites the *Plantae* taxa as sister to cyanobacteria. The different photosynthetic groups are shown in different text colors: blue for cyanobacteria, red for red algae, green for green algae and land plants, and brown for chromalveolates. The inclusion of chromalveolates or *Euglenozoa* (Eugl.) within the *Plantae* is believed to reflect horizontal or endosymbiotic gene transfer events (for example, Li et al., 2006). The two transporters are: **(A)** TGD1, trigalactosyl-diacylglycerol 1, lipid transporter; and **(B)** ABC1-family transporter protein. The name of the *A. thaliana* solute transporter used for the query is indicated for both trees shown in this figure. The figure in this dissertation is presented in color.

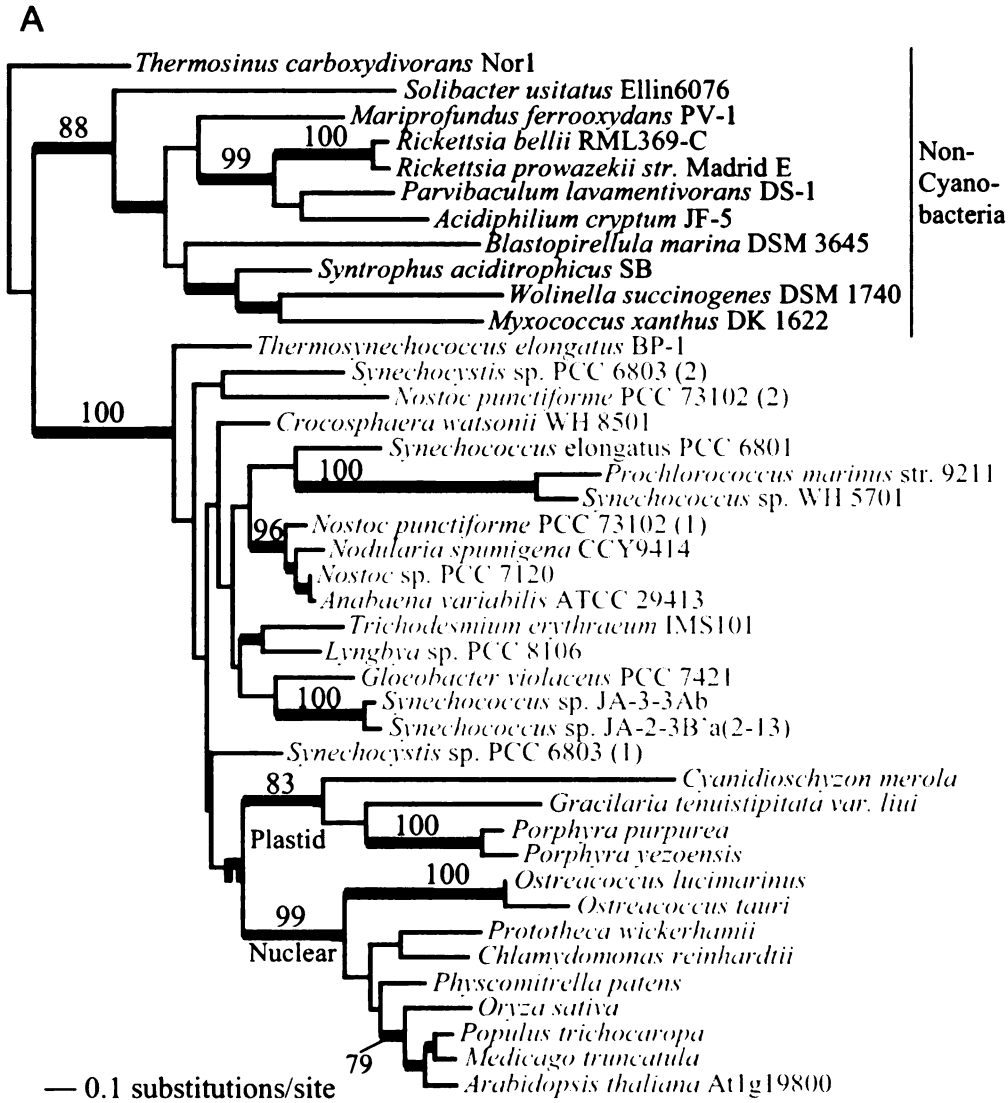

Figure 2.3. Plastid targeted solute transporters of putative 'cyanobacterial'/'plastid endosymbiont' origin in *Plantae*

Figure 2.3 continues on the following page.

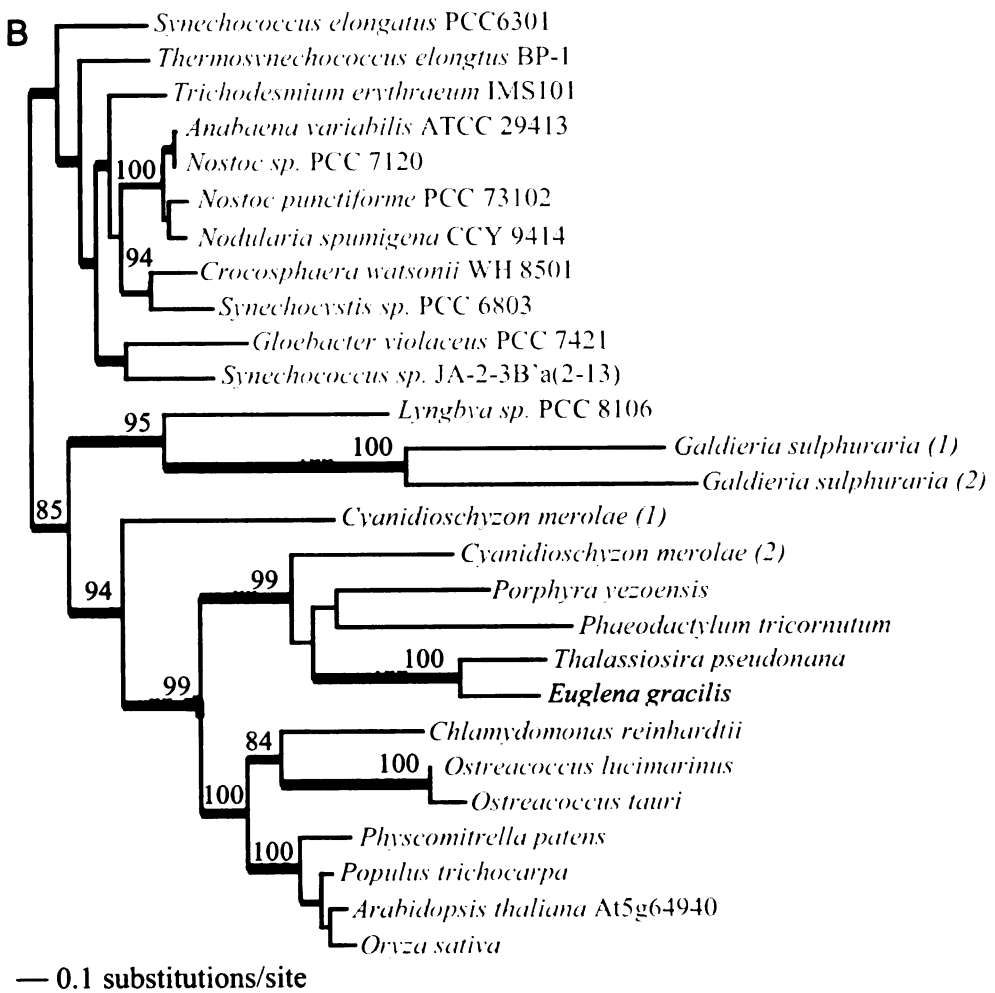

Figure 2.3. (cont'd).

'*Chlamydia*-like' transporters

In addition to the host and cyanobacteria, a third significant contributor to the *Plantae* plastid solute transport system is the *Chlamydiae*. A surprisingly high number (four) of plastid envelope membrane transporters have been contributed by these prokaryotes. The presence of plant-like genes in *Chlamydia* has been noted in the past, sparking debate over whether their presence indicated a transfer from the ancestral plant to *Chlamydia*, an evolutionary relationship between cyanobacteria and *Chlamydia*, or a horizontal gene transfer (HGT) from a chlamydial parasite to the plant ancestor (Brinkman et al., 2002; Wolf et al., 1999; Schmitz-Esser et al., 2004). Phylogenetic analysis of plastid, *Chlamydiae*, and *Rickettsiae* ADP/ATP translocases (Schmitz-Esser et al., 2004) supports an ancient *Chlamydia*-to-*Plantae* direction of transfer. This explanation for the origin of the ADP/ATP translocase gene (and other *Chlamydial*-like genes) in *Plantae* was strongly supported by the phylogenomic analysis of Huang and Gogarten (2007). We found a monophyletic relationship between the AtNTT1 and AtNTT2 (the *A. thaliana* plastid ADP/ATP translocases 1 and 2) and *Chlamydiae* ATP/ADP translocases (Figure 2.4) (Greub and Raoult, 2003; Linka et al., 2003). In addition, the copper transporter heavy metal ATPase 1 (HMA1; Figure 2.4), the dicarboxylate translocators (DiT) DiT1, DiT2.1, and DiT2.2 (data not shown), and the low affinity phosphate transporter PHT2;1 (data not shown) apparently has a chlamydial origin in *Plantae* (Huang and Gogarten, 2007). All of these trees provide bootstrap (except for the DiT tree) support for the monophyly of the '*Chlamydia*-like' and plastid transporters. In the case of HMA1 there are two ancient paralogs in plants, one of cyanobacterial likely endosymbiotic origin and one from a *Chlamydia*-like source that is shared with red and green algae. The DiTs

(Weber and Flügge, 2002) are present only in green algae, plants, and bacteria (that is, not in red algae). Whereas genomic data for glaucophytes are not yet available, transport experiments using isolated *Cyanophora* cyanelles showed that this glaucophyte uses a transport system for glutamine and 2-oxoglutarate that is distinct from green plant DiTs (Kloos et al., 1993). Taken together, these data indicate that '*Chlamydia*-like' dicarboxylate translocators have likely been lost from red algae and glaucophytes. An alternative explanation is that the gene was acquired by the green lineage after the split of *Chlorophyta* and *Rhodophyta.* A DiT2 gene was also found in the dinoflagellates *Amphidinium carterae* and *Heterocapsa triquetra*, which likely originated from an independent horizontal gene transfer (HGT). Several 'green' genes have been found in dinoflagellates and other chromalveolates that could have either originated from multiple independent HGTs or an ancient green algal endosymbiosis (for discussion, see Nosenko et al., 2006).

In summary, it is surprising that bacteria not putatively involved in the endosymbiosis contributed 8% of the transporters that we have identified. When one considers the functions of these transporters, the chlamydial contribution becomes more important. HMA1 increases copper and/or zinc transport into the plastid under conditions of high light, facilitating the production of copper/zinc superoxide dismutase (CuZnSOD), which protects the plant from superoxide radicals produced under high light conditions (Seigneurin-Berny et al., 2006, Lee et al., 2007). PHT2;1, a phosphate transporter, controls phosphate allocation under conditions of phosphate-starvation (Versaw and Harrison, 2002). The DiT transporters are involved in assimilating nitrogen and recovering carbon lost to photorespiration, a process that is initiated by the

oxygenation reaction of RubisCO that primarily occurs under conditions when a high O_2:CO_2 ratio is present in the vicinity of RubisCO. Mutants lacking these transporters are unable to survive in ambient CO_2 concentrations (Renne et al., 2003; Reumann and Weber, 2006; Schneidereit et al., 2006). Finally, the AtNTT1 and AtNTT2 transporters are required for ATP import into the plastid during the dark (that is, in the absence of photosynthetic ATP production), particularly during lipid and chlorophyll biosynthesis. Although AtNTT2 mutants are still capable of producing lipids, indicating that the plastid has an alternative method for generating the ATP required for lipid biosynthesis, the production is significantly reduced and mutant plants have a sharply reduced growth rate Reiser et al., 2004). *Arabidopsis* mutants deficient in both AtNTT1 and AtNTT2 develop necrotic lesions when grown under short days, accumulate H_2O_2, and, strikingly, show constitutive expression of CuZnSOD2 and ascorbate peroxidase (Reinhold et al., 2007). The phenotype of the mutant was linked to reduced magnesium chelatase activity and it was concluded that ATP import into plastids in the dark is required for chlorophyll biosynthesis and for preventing photooxidative damage (Reinhold et al., 2007). The import of ATP into plastids in the dark is thus clearly a case in which the endosymbiont benefits from host metabolism. The ancient origin of these transporters in the tree of photosynthetic eukaryotes (Figure 2.4) is indicative of an essential role of this uptake system in the formation of the endosymbiosis. With the exception of the DiT translocators, each of these transporters appear to perform somewhat redundant functions (that is, copper and phosphate transport) but in a way that permits the plant to adapt to stresses involved in life on the land (that is, high light and O_2 levels or low phosphate

availability). This may explain why the genes encoding these four plastid transporters have been retained in the *Arabidopsis* genome.

How the '*Chlamydia*-like' genes entered into the *Plantae* ancestor is unclear but it is possible that both the cyanobacterial endosymbiont and chlamydial parasites may have co-existed in the cell. Many environmental *Chlamydia* are known today that are broadly distributed in animals and protists (Molmeret et al., 2005). The co-existence of these two distinct prokaryotes may have provided the genetic 'toolkit' to make permanent the endosymbiosis with gene transfer from each cell providing essential functions for endosymbiont utilization. An alternative explanation is that the cyanobacterial endosymbiont was itself highly chimeric (that is, the 'fluid chromosome model') (Embley and Martin, 2006) and contained genes of chlamydial origin that had been gathered through HGT. Although possible, this scenario seems less plausible because it invokes, for example, the presence of an ATP/ADP translocator (a gene typical for 'energy parasites' such as *Rickettsiae*) in the genome of an oxygenic photosynthetic cell that is unlikely to encounter high concentrations of ATP in the surrounding environment; To this end, a '*Chlamydia*-like' ATP/ADP translocator is absent from all studied cyanobacteria.

Figure 2.4. Plastid targeted solute transporters of putative '*Chlamydia*-like' origin in *Plantae*

For details of tree building see Figure 2.2. The filled magenta circle shows the node that unites chlamydial taxa with plastid targeted *Plantae* transporters. The different photosynthetic groups are shown in different text colors: blue for cyanobacteria, red for red algae, green for green algae and land plants, magenta for glaucophytes, and brown for chromalveolates. The inclusion of chromalveolates within the *Plantae* is believed to reflect horizontal or endosymbiotic gene transfer events (for example, Li et al., 2006). The two transporters are: **(A)** ATP/ADP translocator (NTT); and **(B)** Heavy metal ATPase (HMA1) copper transporter. The name of the *A. thaliana* solute transporter used for the query is indicated for both trees shown in this figure. The figure in this dissertation is presented in color.

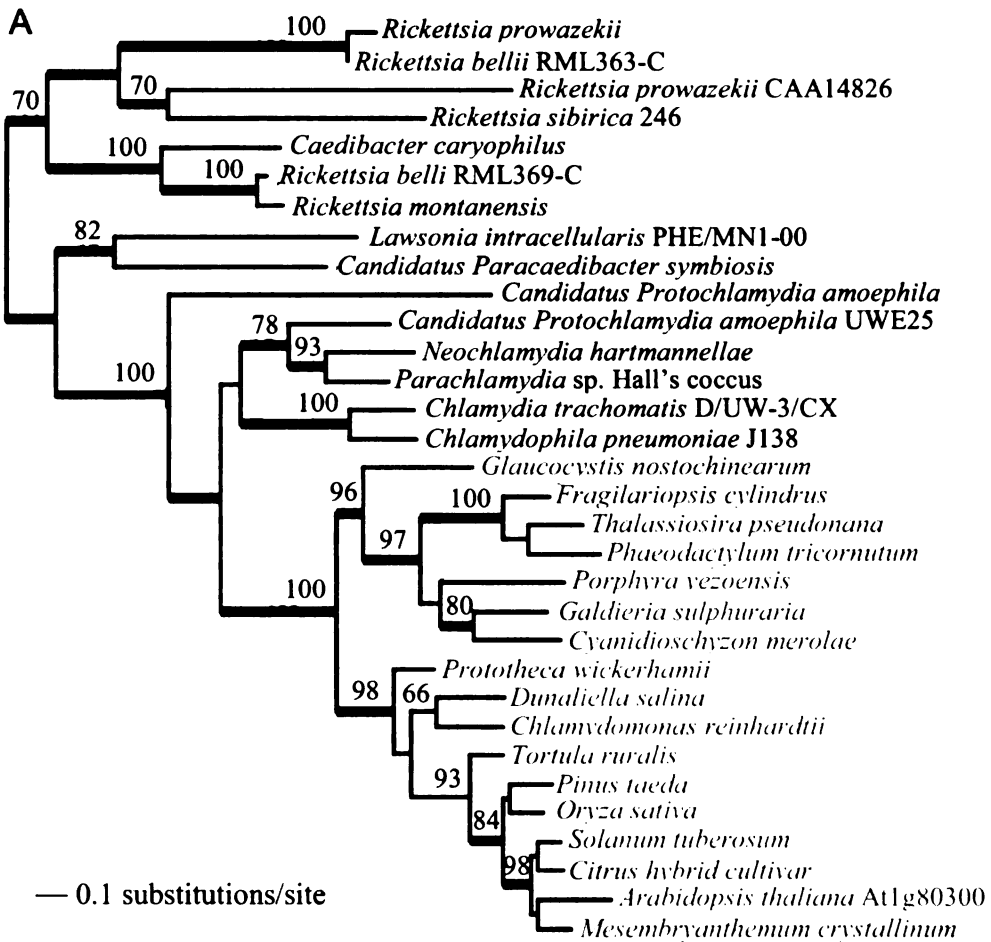

Figure 2.4. Plastid targeted solute transporters of putative '*Chlamydia*-like' origin in *Plantae*

Figure 2.4 continues on the following page.

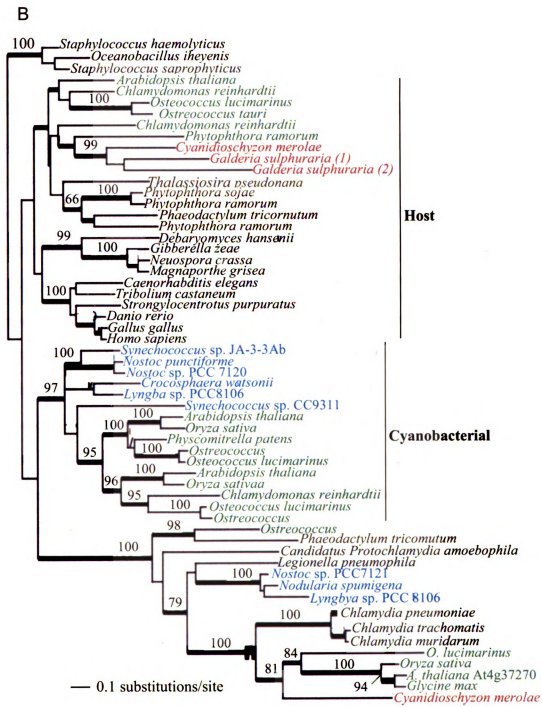

Figure 2.4. (cont'd).

'Other' and '*Plantae*-specific' transporters

We were unable to conclusively determine the origin of 18 transport proteins. Fourteen of these data sets resulted in PHYML trees in which the *Plantae* transporters were rooted within prokaryotes but without bootstrap support for a specific affiliation. An excellent example is provided by At1g32080 (Figure 2.5), which is a putative membrane protein conserved among *Plantae*, chromalveolates, and a diverse set of Eubacteria and Archaea (that is, the *Thermococcus* and *Pyrococcus* clade). Although the prokaryotic source of this gene in *Plantae* is unclear with the available data, the eukaryotic clade is clearly monophyletic, which is consistent with a single gene origin in the *Plantae* ancestor and, thereafter, transfer to chromalveolates (for example, diatoms in this tree) via secondary endosymbiotic gene transfer (Li et al., 2006). The unresolved provenance of At1g32080 and the 'Other' set of transporters in *Plantae* can be explained by pervasive HGT followed by full or partial gene replacement or differential gene loss among prokaryotes that has erased the ancient phylogenetic signal. Alternatively, these results may indicate erratic rates of sequence divergence that make it impossible to model protein evolution for these sequences. Given the growing evidence, however, for recurring HGT among bacteria (Dagan and Martin, 2007), it is likely that genes in the 'Other' category have reticulate evolutionary histories. In this regard it is noteworthy that the likely frequent HGTs seen in Figure 2.5 (A) among prokaryotes and other genes in this category contrasts starkly with the apparent single origin and vertical inheritance in *Plantae*. This result suggests a clear difference in rates of HGT for these genes with elevated rates in prokaryotes relative to eukaryotes.

Of the remaining transporters, four fell in the '*Plantae*-specific' category because they lacked identifiable homologs outside of this supergroup and may simply be too divergent to determine their origin. This includes At5g24690 (Figure 2.5 (B), a hypothetical expressed protein) and the plastidic maltose exporter 1 (MEX1; data not shown). The latter is required for export of maltose resulting from starch breakdown from plastids at night in green plants. Storage of starch inside the chloroplast is exclusively found in the green linage. Therefore, MEX1 has likely co-evolved with plastid-based starch biosynthesis and breakdown since it can be detected only in members of the *Viridiplantae* with one gene found in the dinoflagellate *Karlodinium micrum*, which, as described above for DiT2, likely has resulted from a HGT.

Figure 2.5. Plastid targeted solute transporters of 'Other' or *'Plantae*-specific' origin in *Plantae*

For details of tree building see Figure 2.2. The filled magenta circle shows the node that unites the *Plantae* taxa. The different algal groups are shown in different text colors: red for red algae, green for green algae and land plants, magenta for glaucophytes, and brown for chromalveolates. The inclusion of chromalveolates within the *Plantae* is believed to reflect horizontal or endosymbiotic gene transfer events (Li et al., 2006). **(A)** *A. thaliana* solute transporter in the 'Other' category: putative membrane protein (At1g32080) **(B)** *A. thaliana* solute transporter in the *'Plantae*-specific' category: hypothetical expressed protein (At5g24690). The figure in this dissertation is presented in color.

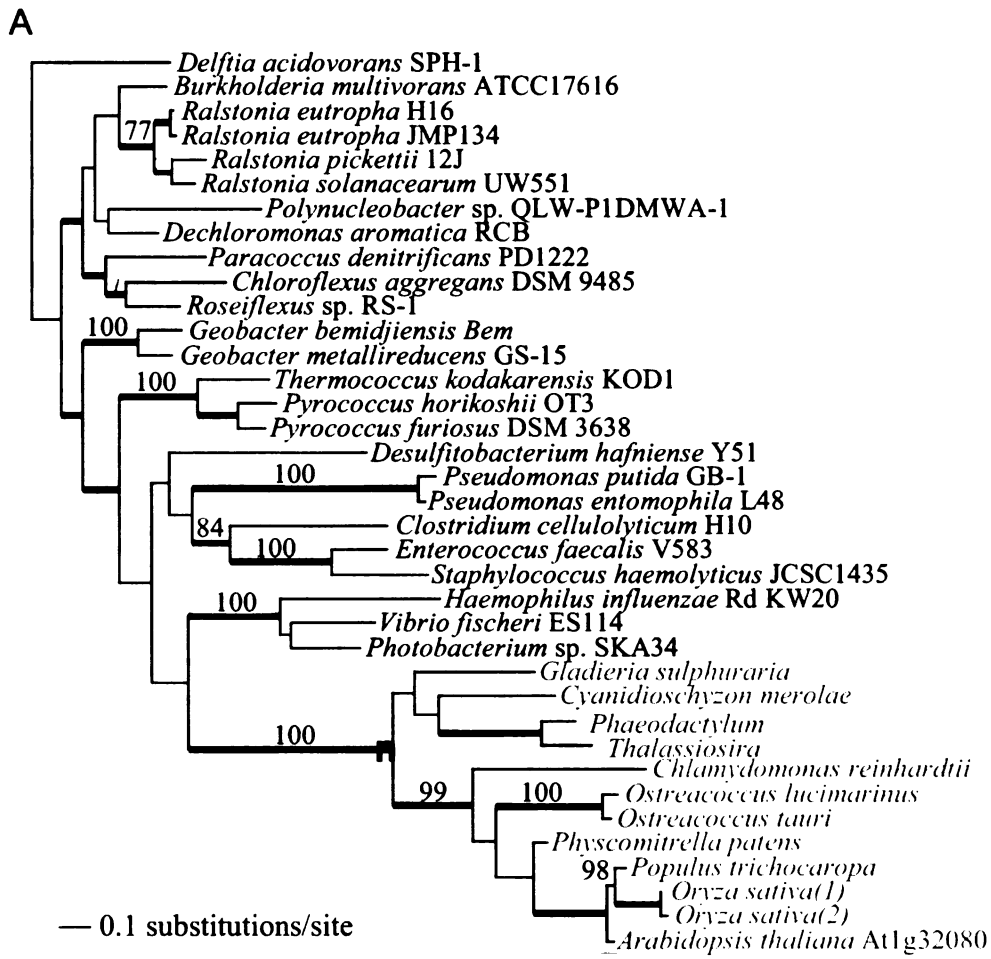

Figure 2.5. Plastid targeted solute transporters of 'Other' or *'Plantae*-specific' origin in *Plantae*

Figure 2.5 continues on the following page.

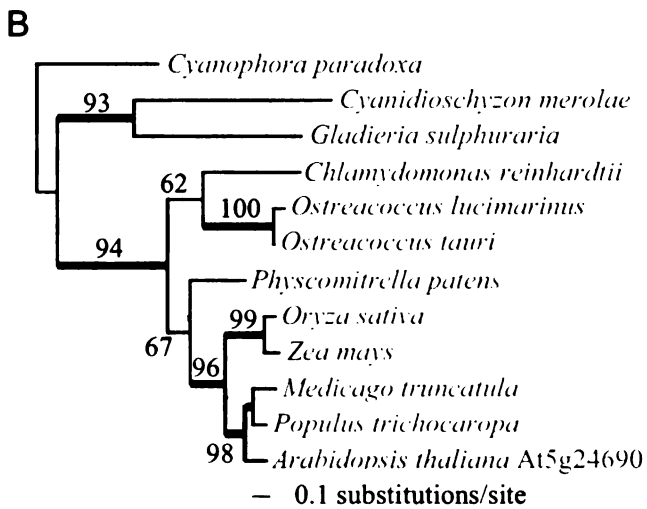

Figure 2.5. Plastid targeted solute transporters of 'Other' or *'Plantae*-specific' origin in *Plantae*

Conclusion

Here we determined the phylogeny of 83 *Arabidopsis* plastid solute transporters to determine whether they are of endosymbiotic origin from the captured cyanobacterium, of host origin, or of a 'mixed' origin from both of these sources. Our analysis has afforded a rare look at early, critical events in primary plastid evolution and support the notion that integration of plastid-host metabolism was primarily driven by host-derived transporters with important contributions coming from the cyanobacterial endosymbiont and *Chlamydia*-like bacteria. Another class of proteins of currently unknown origin included plant specific transporters such as MEX1. Despite the power of our comparative approach, our work has some important limitations. One is that because we used the *Arabidopsis* transporter set, we most certainly have missed a number of *Plantae* transporters that are specific to red or green algae and have been lost from the *Arabidopsis* genome. In addition, we lack significant data from glaucophytes, but the upcoming *Cyanophora paradoxa* (glaucophyte) nuclear genome sequence (http://www.biology.uiowa.edu/cyanophora/) will allow us to incorporate this lineage into future inferences about transporter evolution. It is reasonable to assume, however, given the wealth of data supporting *Plantae* monophyly (McFadden, 1999; Rodriguez-Ezpeleta et al., 2005; Weber et al., 2006; Reyes-Prieto and Bhattacharya, 2007), that our inferences regarding the red and green lineages also apply to their glaucophyte sisters. Despite these limitations and the fact that phylogenetic signal is imperfectly maintained over a billion years of evolution, our comprehensive analysis of the chloroplast solute transport system will likely hold up and can be further tested as other genome sequences become available.

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CHAPTER 3

ANALYSIS OF THE PLASTID PHOSPHATE TRANSLOCATOR FAMILY FROM THE RED ALGA *GALDIERIA SULPHURARIA*[3]

[3] This work has been published in **Linka M., Jamai A., Weber A.P.M.** (2008) Functional characterization of the plastidic phosphate translocator gene family from the thermo-acidophilic red alga *Galdieria sulphuraria* reveals specific adaptations of primary carbon partitioning in green plants and red algae. Plant Physiology **148**: 1487-1496. I performed all of the experiments shown here.

ABSTRACT

In chloroplasts of green plants and algae, CO2 is assimilated into triose-phosphates; a large part of these triose-phosphates is exported to the cytosol by a triose-phosphate/phosphate translocator (TPT) whereas some is stored in the plastid as starch. Plastidial phosphate translocators (pPT) have evolved from transport proteins of the host endomembrane system shortly after the origin of chloroplasts by endosymbiosis. The red microalga *Galdieria sulphuraria* shares three conserved putative orthologous transport proteins with the distantly related seed plants and green algae. However, red algae, in contrast to green plants, store starch in their cytosol, not inside plastids. Hence, due to the lack of a plastidic starch pool, a larger share of recently assimilated CO2 needs to be exported to the cytosol. We thus hypothesized that red algal transporters have distinct substrate specificity in comparison to their green orthologs. This hypothesis was tested by expression of the red algal genes in yeast and assessment of their substrate specificities and kinetic constants. Indeed, two of the three red algal pPT candidate orthologs have clearly distinct substrate specificities when compared to their green homologs. GsTPT displays very narrow substrate specificity and high affinity; in contrast to green plant TPTs, 3-phosphoglyceric acid (3-PGA) is poorly transported and thus not able to serve as a triose phosphate/3-PGA redox shuttle *in vivo*. Apparently, the specific features of red algal primary carbon metabolism promoted the evolution of a highly efficient export system with high affinities for its substrates. The low-affinity TPT of plants maintains triose phosphate levels sufficient for starch biosynthesis inside of chloroplasts, whereas the red algal TPT is optimized for efficient export of triose phosphate from the chloroplast.

INTRODUCTION

In plants, the photosynthetic light reactions provide the energy for major plastid localized pathways, such as CO2 assimilation, the synthesis of starch, fatty acids, several amino acids, nucleic acids, and the reductive assimilation of inorganic ions like nitrate and sulfate (Weber et al., 2005; Zrenner et al., 2006). To supply the cell and the organism with these primary metabolites, a large number of precursors, end products, and intermediates have to be transported across the organelle envelope membrane and therefore present-day plastids are extensively connected to the cytoplasm by metabolite transporters that reside in the envelope membranes (Weber and Fischer, 2007; Tegeder and Weber, 2006).

Chloroplasts originated approximately 1.6 billion years ago through a single primary endosymbiosis between a non-photosynthetic primitive mitochondriate eukaryote and a cyanobacterium (Yoon et al., 2004; Bhattacharya et al., 2007; Reyes-Prieto et al., 2007). Within a period of 0.15 billion years, establishment of the plastid and divergence of the three major lineages of the *Archaeplastida* (Adl et al., 2005), that is the red algae (*Rhodophyceae*), green algae/land plants (*Chloroplastida*), and glaucophytes (*Glaucophyta*), began (Bhattacharya et al., 2004; Yoon et al., 2004). Establishment of the chloroplast within the host cell required massive remodeling of its membrane proteome; novel transport proteins to connect its metabolism with the metabolic network of the host cell had to be acquired (Bhattacharya et al., 2007; Weber and Fischer, 2007). Phylogenetic and phylogenomics analyses recently revealed that a large portion of these plastid-resident transporters is host-derived, indicating that integration of the chloroplast with host metabolism was predominantly a host-driven process (Tyra et al., 2007). Genes

encoding these transporters are not present in extant cyanobacterial genomes but most are conserved throughout the *Archaeplastida*, indicating they have been established at an early stage during formation of endosymbiosis, likely already at the stage of the protoalga (Tyra et al., 2007).

Of particular importance for establishment of chloroplasts was an efficient and controlled export of photoassimilates from the endosymbiont to its host cell (Weber et al., 2006). In the *Chloroplastida*, a triose-phosphate/phosphate transporter (TPT) exports a significant amount of the dihydroxyacetone phosphate (DHAP) that is generated by the Calvin-Benson cycle to the cytosol; there it mainly serves as precursor for sucrose and cell wall biosynthesis (Flügge, 1999; Reiter, 2002). However, part of the triose phosphate is retained in the plastid stroma and used to fuel starch biosynthesis and other intraplastidial biosynthetic pathways (Zeeman et al., 2007). TPT candidate orthologs are highly conserved throughout the photosynthetic eukaryotes (Weber et al., 2006) and transport experiments with isolated organelles or reconstituted organellar membranes demonstrated triose-phosphate transport activity in the red algae and glaucophytes lineages, respectively (Schlichting and Bothe, 1993; Weber et al., 2004). Apparently, triose-phosphate (TP) already served as the main export photoassimilate at the stage of the proto-alga (Weber et al., 2006). In the plant model organism *Arabidopsis thaliana*, additional sugar phosphate transporters with specialized functions have been characterized (Flügge, 1999). Import of phosphoenolpyruvic acid (PEP) from the cytosol by the PEP/phosphate transporter (PPT) drives fatty acid biosynthesis and synthesis of compounds by the shikimate pathway (i.e. aromatic amino acids) (Fischer et al., 1997; Knappe et al., 2003a; Voll et al., 2003) and a glucose-6-phosphate/phosphate transporter

(GPT) provides glucose-6-phosphate (Glc6P) for starch synthesis in heterotrophic plastids (Kammerer et al., 1998; Niewiadomski et al., 2005). Lastly, a protein closely related to the GPT, the pentose-phosphate/phosphate transporter (XPT) connects the oxidative pentose phosphate pathways (OPPP) in cytosol and plastid (Eicks et al., 2002; Flügge and Gao, 2005).

Phylogenetic analysis showed that candidate orthologs for the PPT and the GPT/XPT type of translocators are present in the genome of the ancient red microalga *G. sulphuraria,* which is separated from green plants by an evolutionary distance of at least one billion years (Yoon et al., 2004). However, reconstituted membrane fractions from *G. sulphuraria* showed neither significant PEP nor Glc6P transport activity (Weber et al., 2004). This raises the intriguing question as to whether the genetic repertoire present in the last common ancestor of *Rhodophyceae* and *Chloroplastida* was functionally maintained or whether it diverged after separation of the two phyla. To address this question, we used *G. sulphuraria* as a model for the *Rhodophyceae*. The draft genome of *G. sulphuraria* is publicly available and the unicellular organism shares core features of carbon metabolism with other red algae (Viola et al., 2001; Barbier et al., 2005a). In contrast to higher plants, starch in red algae is produced in the cytosolic compartment, using UDP-Glucose (UDPGlc) as precursor (Coppin et al., 2005; Patron and Keeling, 2005). Cell wall polysaccharides as well as the major soluble carbohydrate floridoside (α-D-galactopyranosyl-1-2'-glycerol) are produced in the same compartment. Notably, in addition to photoautotrophic growth, *G. sulphuraria* is also able to grow mixo- or heterotrophically on more than 50 different carbon sources (Gross and Schnarrenberger, 1995; Barbier et al., 2005b). Carbon partitioning thus has to be coordinated not only

during alternating light and dark periods but also under continuous heterotrophic growth conditions. It is not known in detail how the carbon allocation between the plastid and the cytosol is accomplished in red algae. Based on phylogenetic data, the plastid phosphate translocators presumably are the major routes for metabolite exchange. They have to balance a high demand for photoassimilates in the cytosol with maintaining sufficient levels of triose-phosphates for rhodoplast-localized pathways. Here we report the heterologous expression and biochemical characterization of the plastid phosphate transporter family from *G. sulphuraria.* With regard to higher plants we will discuss an alternative strategy to fine tune carbon flux across the plastid membrane in photosynthetic eukaryotes.

MATERIAL AND METHODS

Growth and sampling of *G. sulphuraria* cells

Galdieria sulphuraria strain 074W (Gross et al., 1999) was cultured autotrophically in a minimal salt medium at 37°C with a light intensity of 80 µmol photons per square meter and second. The culture was grown at pH 2.0 in Erlenmeyer flasks under vigorous shaking at ambient air conditions. Heterotrophic growth of the algal cells was performed at 37°C in culture flasks in complete darkness in the identical salt medium containing 25 mM glucose as sole carbon source. Cells were harvested at the late logarithmic phase by centrifugation (3,000 g, 5 min, 4°C), washed with 1xTE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA), frozen in liquid nitrogen and stored at -80°C or directly used for nucleic acid extraction (Barbier et al., 2005) or total membrane enrichment.

Isolation of genomic DNA and total RNA from *G. sulphuraria*

Isolation of genomic DNA was performed as previously described (Barbier et al., 2005). Briefly, cells were ground in liquid nitrogen and proteins were removed by Phenol/Chloroform/Isoamylalcohol (PCI) extraction. Nucleic acids were precipitated by ethanol and RNA was removed by a DNAse-free RNAse treatment and purified by an additional PCI and ethanol precipitation step. Total RNA was isolated with an acid guanidium isothiocyanate-phenol/chloroform solution as described by (Chomczynski and Sacchi, 1987).

Enrichment of total membrane fractions from *G. sulphuraria*

Harvested cells were resuspended in breaking buffer (100 mM HEPES/KOH, pH 7.6, 1 mM EDTA, 5% glycerol, 5 mM ascorbic acid, 5 mM DTT, 1 mM PMSF) to yield an OD600 of ~100 (0.5 mL) and an equal volume of acid-washed glass beads (0.4-0.6 mm size, Sigma Aldrich) were added. Cells were lysed with a mixer mill (MM301, Retsch GmbH, Germany) for 4 minutes. Broken cells were diluted with 10 mL breaking buffer and centrifuged (2,000 g, 1 min, 4°C). The supernatant contained the total membrane fractions. Membranes were pelleted from the supernatant by ultracentrifugation (100,000 g, 50 min, 4°C), resuspended in 0.4 mL 10 mM HEPES/KOH (pH 7.6), 1 mM MgCl2, and 0.05 mL aliquots were reconstituted into liposomes.

Expression of GsPTs in *S. cerevisiae*

The coding sequences of all three GsPT were amplified from *G. sulphuraria* cDNA by PCR (Platinum P*fx* polymerase, Invitrogen, Carlsbad, CA) using the primer combinations

listed in Table 3.1. PCR products were subcloned into the pGEM-T Easy vector system (Promega, Madison, WI) and sequenced. Forward and reverse primers for the genes GsHET39C12 and GsA16F5 were designed with BamHI and XhoI restriction recognition sites, respectively. The GsA14H8 specific forward primer had a KpnI and the reverse primer an XhoI restriction site. Each cDNA was ligated in frame with an N-terminal polyhistidine-tag into the yeast expression vector pYES2/NT (Invitrogen). Standard molecular methods were applied for DNA restriction and cloning (Sambrook et al., 1995). The resulting constructs were transformed into competent *Saccharomyces cerevisiae* INVSc1 cells (Invitrogen). Selection, maintenance of the transformants, and galactose-inducible expression of the recombinant proteins was done according to the manufacturer's instructions (pYES2/NT expression system, Invitrogen). Preparation of yeast membranes containing heterologously expressed GsHET3912, GsA16F5, and GsA14H8 proteins, respectively, was done as described previously (Bouvier et al., 2006), except that the expression was induced at an OD600 of 0.6 in the presence of 2% galactose and cells had been cultured for eight hours at 30°C. Presence of recombinant proteins was verified by standard SDS-PAGE and immunoblot analysis (Sambrook et al., 1995) using an anti-penta-His antibody (Qiagen, Hilden, Germany) and a secondary anti-mouse IgG antibody conjugated with an alkaline phosphatase (Promega).

Reconstitution into liposomes and transport activity assays

Acetone-washed L-α-phosphatidylcholine (PC) (Sigma-Aldrich, St. Louis, MO) had been sonicated (5 min on ice, Branson Sonicator 250, output 2, duty cycle 30%) to a final concentration of 2% (w/v) in 120 mM Tricine-KOH (pH 7.5) and 30 mM internal

substrate. 50 µL yeast membrane fraction was reconstituted with 950 µL liposome suspension by the freeze-thaw procedure (Kasahara and Hinkle, 1977). After thawing, the proteoliposomes were pulsed 20 times on ice to yield unilamellar vesicles. PD10-desalting columns (GE Healthcare) were pre-equilibrated with 150 mM Tricine-KOH (pH 7.5) and used to remove non-incorporated substrate from the external medium. If not stated otherwise, all transport studies were initiated with radiolabeled [32P]-orthophosphate at a final concentration of 0.25 mM. For each data point, 190 µL proteoliposomes were terminated with 10 µL 10x inhibitor stop solution (200 mM PLP, 20 mM DIDS, 100 mM Tricine-KOH, pH 8.0). Control experiments with membranes from yeast cells harboring the empty vector were performed in parallel. Adding of inhibitor mix before addition of radiolabeled substrate was used to monitor unspecific binding. External phosphate was removed by strong anion-exchange chromatography with AG-1 X8 resin (Acetate form, pre-equilibrated with 150 mM sodium acetate, BioRad, Hercules, CA). The flow-through of the anion-exchange columns containing the proteoliposomes with the imported radiolabeled phosphate was quantified by a liquid scintillation counting. All substrates used for uptake studies were purchased from Sigma-Aldrich. Kinetic constants were determined by measuring the initial velocity of each experiment. Michaelis-Menten constant (KM) has been analyzed with at least six external Pi concentrations ranging between 0.05 mM – 10 mM and competitive inhibition of Pi transport was assessed by the inhibitor constant Ki as described by Dixon (Dixon, 1953). GraphPad-Prism Software was used for non-linear regression fitting of all enzyme kinetic data.

RT-PCR gene expression study

DNAse-treated RNA was used for first strand cDNA synthesis (Superscript II First-Strand Synthesis System, Invitrogen). Oligonucleotide primers for expression studies of the *G. sulphuraria* genes are summarized in Table 3.1.

Other methods

Protein concentrations were determined with a standard Bradford assay. Proteins in the membrane fractions were delipidated prior to determination (Shultz et al., 2005).

Table 3.1. Oligonucleotide primers designed in this study
Amplified *Galdieria sulphuraria* gene products were used for cloning heterologous expression (HE) constructs or Reverse-Transcriptase PCR (RT-PCR) expression analysis. Underlined nucleotides highlight restrictions sites for cloning into expression vector system pYES. Fwd, forward primer; Rev, Reverse primer.

Primer	Sequence (5'–3')	Direction	Purpose
Gs39C12			
ML58	TAGGATCCATGAACCTTCTAAAGAATCCATCA	fwd	HE
ML59	ATCTCGAGATGAACCCAGAGTCTACTTTATTT	rev	HE
ML137	AGCACTTGAACCCTTTGTCG	fwd	RT-PCR
ML138	TGCATTGGCAGGAGTGATAG	rev	RT-PCR
Gs16F5			
ML56	TAGGATCCCAAGGAAACTCACTATTCGTC	fwd	HE
ML66	GTCTCGAGTCTATTCTATTTTCTCCTTCTTCTTGG	rev	HE
ML135	CAACCATGGCTACCTTTCGT	fwd	RT-PCR
ML136	CGTCAAAAAGCCAATCCAAT	rev	RT-PCR
Gs14H8			
ML94	TGGTACCCAAAGGAGAAAAGGATATCATAAGAGC	fwd	HE
ML95	ATCTCGAGACTACTGCTTTTCTCGCTTCG	rev	HE
ML133	AGAGTGGTGGCTCTCCTCAA	fwd	RT-PCR
ML134	GATGAATCCGGTCCAGGTAA	rev	RT-PCR
GsActin			
ML132	CACAGCATCTGGCCAAACTT	fwd	RT-PCR
ML131	GACCCACATTTGTTGGAAGG	rev	RT-PCR

RESULTS

Molecular features of the plastidic phosphate translocator (pPT) homologues from *G. sulphuraria*

Three genes encoding proteins with significant similarity to higher plant pPT have previously been identified in the genome of the red alga *G. sulphuraria* (Weber et al., 2006). Based on detailed phylogenetic analysis, GsHET39C12 is the candidate ortholog to the plastid triose-phosphate/phosphate translocator (TPT) and will thus be called GsTPT. GsA14H8 and GsA16F5 cluster with the functionally characterized green plant plastid glucose-6-phosphate/phosphate transport proteins (GPT), and phosphoenolpyruvate/phosphate translocator proteins (PPT), respectively. They were thus assigned with the acronyms GsGPT and GsPPT. The coding sequences of *GsTPT* and *GsPPT* contain 1224 nucleotides, corresponding to 407 amino acid residues and computed molecular masses of 45.83 kDa (TPT) and 44.98 kDa (PPT). GsGPT has a calculated molecular mass of 45.45 kDa, consisting of 410 amino acid residues that are encoded by 1233 nucleotides. The N-terminal regions of all three proteins represent putative plastid target sequences that display only low sequence similarity with the pPTs from higher plants (Figure 3.1) and the red alga *Cyanidioschyzon merolae*. The *G. sulphuraria* proteins show an average sequence identity of 37% and a 55% similarity with their *A. thaliana* homologs and a slightly higher identity of 48% to the corresponding *C. merolae* proteins (Table 3.2). Six lysine and two arginine residues that are invariantly embedded in conserved motifs in all functionally characterized pPT proteins to date (Knappe et al., 2003a), are also found in all three *G. sulphuraria* homologues. Relative to the GsTPT sequence, the positively charged amino acids are at

positions K123, K199, R261, K266, K267, K361, R362, and K399 (Figure 3.1). All three putative transport proteins are highly hydrophobic proteins that are predicted by ConPred II (Arai et al., 2004) to contain nine to ten membrane spanning alpha-helices. The *GsTPT* and *GsGPT* genes contain one intron that separates a short first exon from the residual coding sequence (Table 3.2). *GsPPT* has a total of four exons. The last exon covers approximately 50% of the CDS. All three sequences have been submitted to GeneBank and can be also found at the MSU Galdieria genome database (http://genomics.msu.edu/galdieria). Accession numbers and annotations are listed in Table 3.2.

Table 3.2. Molecular characteristics of the plastidic phosphate transporter GsTPT, GsPPT and GsGPT from *Galdieria sulphuraria*.

	GsTPT	**GsPPT**	**GsGPT**
Accession number	EU853171	EU853172	EU853173
Galdieria Genome Browser Build 3.0	Stig_15 Gs21660	Stig_53 Gs53050	Stig_45 Gs48050
Amino acid statisitc report [%]			
Identity to AtTPT	33	30	32
Similarity to AtTPT	53	51	53
Identity to AtPPT	31	41	31
Similarity to AtPPT	52	57	52
Identity to AtGPT	33	33	37
Similarity to AtGPT	56	51	55
# of Exons	2	4	2
Exon length (nt)	119 1105	244 174 191 615	112 1121
# of Introns	1	3	1
Exon length (nt)	53	55 47 70	50
# of transmembrane spans	9	10	9

Figure 3.1. Protein alignment of plastidic phosphate transporters (pPT) from *Arabidopsis thaliana* (At) and *Galdieria sulphuraria* (Gs)

Multiple sequence alignment program ClustalW2 (Larkin et al., 2007) at EMBL-EBI lined up the sequence similarities and the tool GeneDoc (http://www.nrbsc.org/gfx/genedoc/index.html) visualized and edited the higher plant pPT proteins and their red algal homologues Gs39C12 (GsTPT), Gs16F5 (GsPPT) and Gs14H8 (GsGPT). AtTPT, triose-phosphate/phosphate translocator, AGI genome code At5g46110; AtGPT1, glucose-6-phosphate/phosphate translocator, At5g54800; AtPPT1, phosphoenolpyruvate/phosphate translocator, At5g33320.

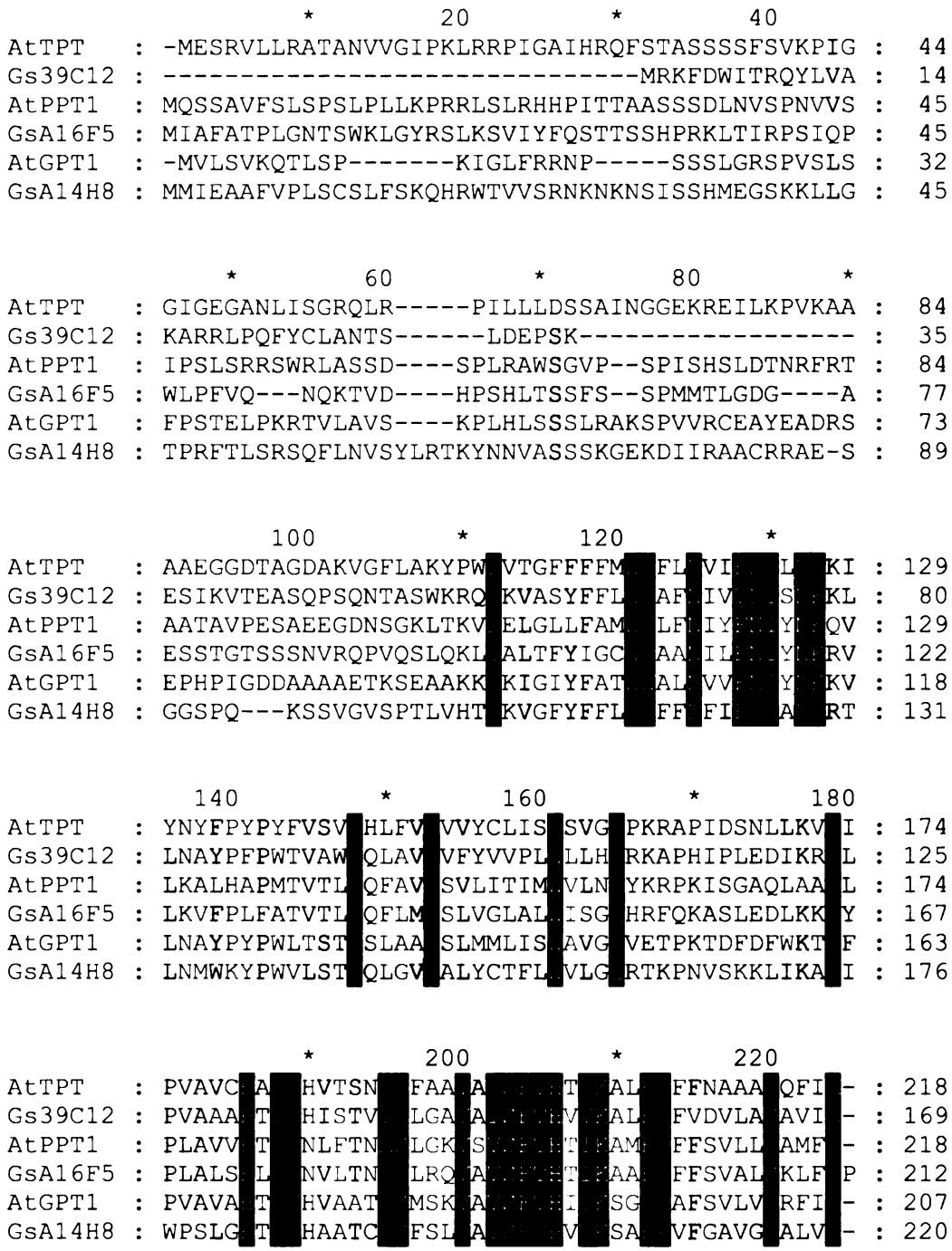

Figure 3.1. Protein alignment of plastidic phosphate transporters from *Arabidopsis thaliana* (At) and *Galdieria sulphuraria (Gs)*

The Figure 3.1 continues on the following page.

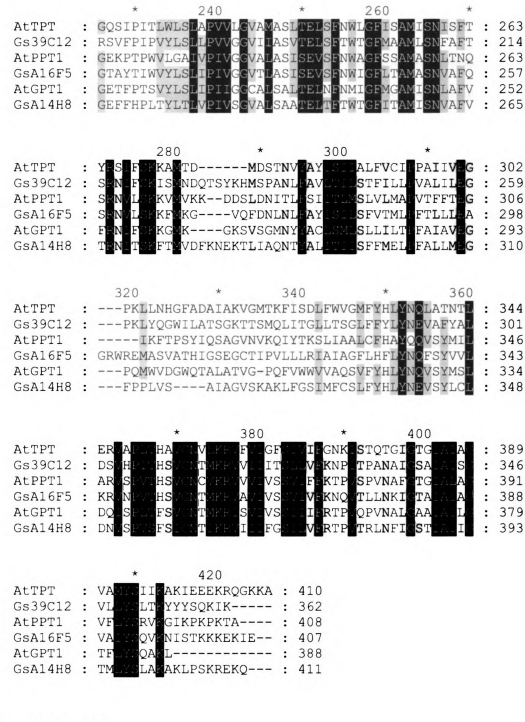

Figure 3.1. (cont'd).

Heterologous expression of GsTPT, GsPPT, and GsGPT

To assess the substrate specificities of the *G. sulphuraria* pPTs, we cloned the corresponding cDNAs into the yeast expression vector pYES/NT under control of the galactose-inducible GAL4 promoter. The regions of the cDNAs encoding for the putative target sequences of each protein were removed and instead fused to an N-terminal hexa-histidine tag. After transformation of the corresponding constructs into the yeast strain INVSc1, all three pPT homologues could be successfully expressed and accumulated in the membrane fraction (Figure 3.2). Immunoblot analysis with an anti-polyhistidine tag antibody verified the galactose-inducible accumulation of the pPT proteins (Figure 3.2, lane 2-4) compared to controls, which maintained the empty expression vector (Figure 3.2, lane 1). The calculated molecular masses of the N-terminal His-tagged proteins were 41.6 kDa, 45.2 kDa and 41.2 kDa for GsTPT, GsPPT and GsGPT, respectively. The presence of recombinant protein was verified for all biological replicates by Western blot prior to reconstitution.

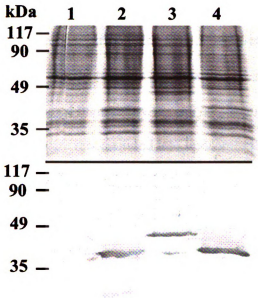

Figure 3.2. Expression of plastid phosphate transporter (pPT) homologues from *Galdieria sulphuraria* (Gs) in yeast

Upper part: Membrane proteins from galactose-induced *S. cerevisiae* cells transformed either with an unmodified expression vector (Lane 1) or with constructs for GsTPT (lane 2), GsPPT (lane 3) and GsGPT (lane 4) were separated by SDS-PAGE and stained with Coomassie Blue R250. **Lower part:** Immunoblot analysis of an identical SDS-polyacrylamide-gel as described above. N-terminal poly-histidine tagged proteins were colormetrically verified with anti-His antibody and anti-mouse-IgG antibody linked to an alkaline phosphatase. TPT, triose-phosphate/phosphate translocator; GPT, Glucose-6-Phosphate-Translocator; PPT, phosphoenolpyruvate/phosphate translocator; kDa, kilo Dalton.

Functional analysis of three putative transport proteins from *G. sulphuraria*

All functionally characterized plastidic phosphate translocators of higher plants catalyze, in addition to their characteristic substrates, also a strict homo-exchange of *ortho*-phosphate (Flügge, 1999). To test whether the yeast-expressed, recombinant GsPTs are functional, we thus first examined their ability to catalyze the signature Pi homo-exchange. To this end, they were reconstituted into liposomes that were preloaded with 30 mM Pi (i.e., the liposomes contained 30 mM Pi inside). Then radiolabeled [32P]-phosphate was added to the liposomes and the uptake kinetics were recorded. In GsTPT and GsPPT containing proteoliposomes, protein-mediated isotope equilibrium was reached within ten minutes, using an external Pi concentration of 0.25 mM (Figure 3.3). GsGPT mediated Pi uptake equilibrated after 15 minutes with a four times higher external [32P]-Pi concentration of 1 mM. *Ortho*-phosphate exchange followed a first-order kinetic with an equilibrium plateau (Vmax) of 400±13.74, 187.3±3.31 and 237.1±7.02 nmol per mg protein and initial rates of 60.69±2.48, 30.94±0.43 and 23.23±1.27 nmol per mg protein and minute for GsTPT, GsPPT, and GsGPT, respectively. Control membranes from yeast cells transformed with an empty pYES/NT vector or boiled recombinant proteins showed negligible accumulation rates over time (data not shown). PLP and DIDS also effectively inhibited GspPT mediated import of [32P]-phosphate (data not shown). Marginal uptake of radiolabeled [32P]-phosphate compared to control experiments was measured when vesicles did not contain Pi as a counter substrate (Figure 3.3). In summary, all putative phosphate transport proteins from *G. sulphuraria* were functionally expressed in yeast and are able to catalyze the signature Pi homo-exchange activity, similar to their higher plants homologs.

Figure 3.3. Time-kinetics of pPT homologues from *Galdieria sulphuraria* (Gs) Liposomes were reconstituted with membrane proteins from *S. cerevisiae* cells expressing GsTPT (panel A), GsPPT (panel B) or GsGPT (panel C). Uptake of *ortho*-Phosphate (Pi) was measured in the presence (●) or absence (○) of intraliposomal Pi (30 mM). Transport was initiated with a final concentration of 0.25 mM [32P]-Pi (panel A & B) or 1 mM [32P]-Pi (panel C). Diagrams represent a typical result of at least three independent studies.

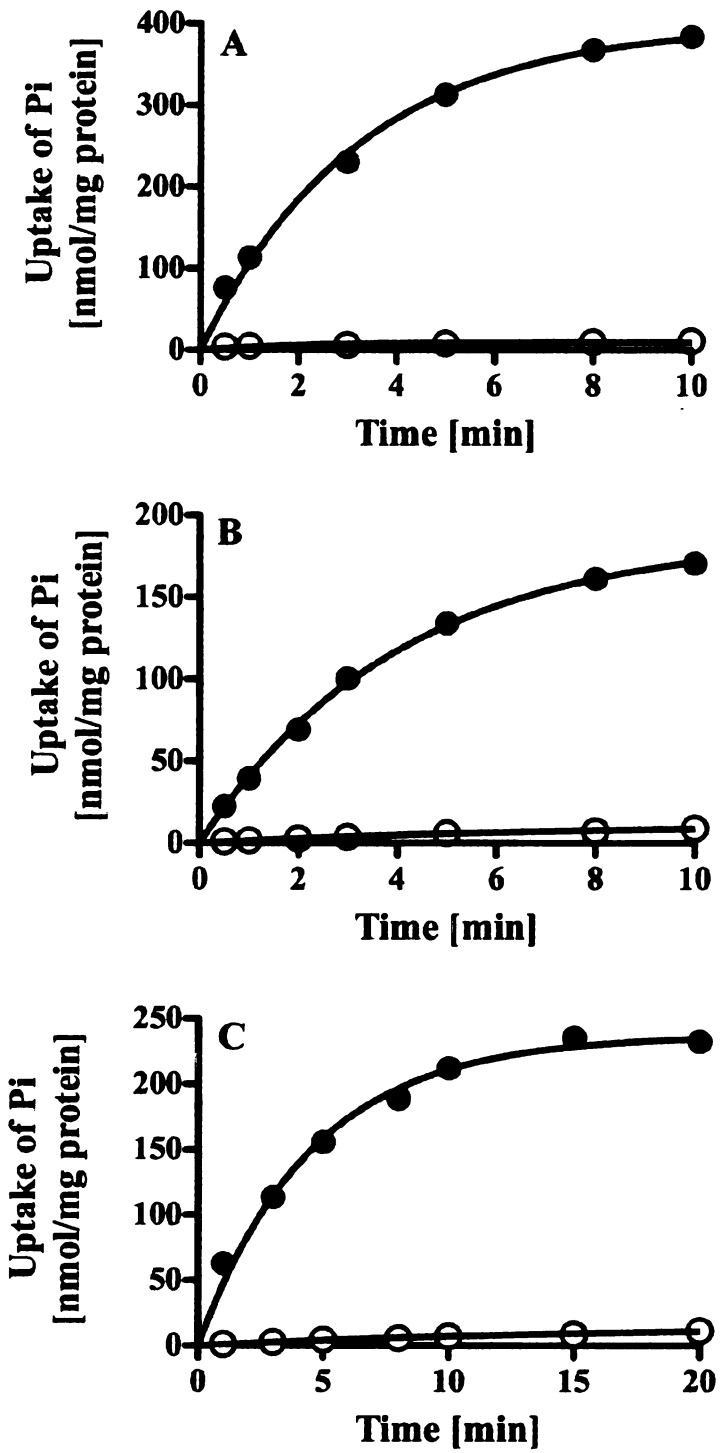

Figure 3.3. Time-kinetics of pPT homologues from *Galdieria sulphuraria* (Gs)

Transport properties of GsTPT, GsPPT and GsGPT

To assess the substrate specificity of the recombinant GspPT proteins, vesicles were preloaded with saturating concentrations (i.e., 30 mM) of various counter-exchange substrates and initial rates of [32P]-Pi uptake were determined.

GsTPT: Relative to the Pi/Pi homo-exchange experiment, the uptake rate of [32P]-Pi into proteoliposomes reconstituted with GsTPT was 86.7±13.3% when proteoliposomes were preloaded with the triose-phosphate DHAP. Uptake rates into liposomes preloaded with 3-phosphoglyceric acid (3-PGA), phospho*enol*pyruvic acid (PEP), or glucose-6-phosphate (Glc6P), respectively, were low (Figure 3.4A). Uptake was also negligible when GsTPT liposomes were preloaded with glucose-1-phosphate (Glc1P), fructose-6-phosphate (Frc6P), galactose-1-phosphate (Gal1P), glycerol-3-phosphate (Gly3P) (Figure 3.4A), adenosine-monophosphate (AMP), uridine-monophosphate (UMP), gluconate-6-phosphate (Gluc6P), ribose-5-phosphate (Rib5P), ADP-glucose (ADP-Glc), UDP-glucose (UDP-Glc), UDP-galactose (UDP-Gal), alpha-ketoglutaric acid (α-KG), malic acid (Mal), pyruvic acid (Pyr), or oxaloacetic acid (OAA) (data not shown). GsTPT has an apparent *K*M value of 0.33±0.07 mM for Pi and DHAP competitively inhibited Pi uptake with a *K*i value of 0.5±0.04 mM (Table 3.3). 3-PGA and PEP inhibited [32P]-Pi uptake only at non-physiological concentrations of 8 and 9 mM, respectively, and Glc6P did not show significant inhibition of Pi transport at any of the tested concentrations.

GsPPT: Reconstituted GsPPT protein efficiently used Pi and PEP as a counter substrate for the import of radiolabelled [32P]-phosphate (Figure 3.4B). 18 additional substrates, as listed above for the GsTPT, showed marginal initial Pi uptake rates of less

than 30% of the Pi/Pi homo-exchange rate (Figure 3.4B and data not shown). GsPPT has an apparent *K*M value of 0.76±0.075 mM for Pi and a *K*i value of 0.36±0.04 mM for PEP. Compared to PEP, 11-times higher 3-PGA and 22-times higher DHAP levels, respectively, were needed to inhibit Pi uptake by 50% (Table 3.3). Glc6P had no affinity for the Pi binding site.

GsGPT: Vesicles reconstituted with the GPT homologue from *G. sulphuraria* were preloaded with the same 20 substrates as given above; in all cases the [32P]-Pi uptake was much lower than for liposomes preloaded with 30 mM Pi (Figure 3.4C). GsGPT has rather low affinity to *ortho*-phosphate (*K*M: 5.07±0.4 mM) (Table 3.3). Compared to non-preloaded proteoliposomes (13.03±5.9%), approximately three- to fourfold higher uptake rates were measured when liposomes were preloaded with either 3-PGA or DHAP. Importantly, however, even high concentrations of 3-PGA and DHAP did not significantly inhibit Pi homo-exchange (Table 3.3). Glc6P and PEP are non-relevant substrates for the GsGPT protein.

Figure 3.4. Internal substrate dependence on the transport activities of GsTPT, GsPPT, and GsGPT

Liposomes were preloaded with various substrates (30 mM) and reconstituted with membrane proteins from yeast cells expressing GsTPT (panel A), GsPPT (panel B) or GsGPT (panel C). Uptake experiments were initiated with 0.25 mM [32P]-Pi (panel A & B) or 1 mM [32P]-Pi (panel C) and terminated after 1.5 min (GsTPT), 2 min (GsPPT) or 4 min (GsGPT). As negative control, proteoliposomes were preincubated (2 min) with inhibitor stop solution to calculate net uptake activity. Relative uptake activities were compared to the Pi/Pi counter-exchange experiment, which was set to 100%. Data are summarized as the arithmetic mean ± SE of at least three independent experiments. Pi, ortho-phosphate; DHAP, dihydroxyacetone phosphate; 3-PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvic acid; Glc6P, glucose-6-phosphate; Glc1P, glucose-1-Phosphate; Frc6P, fructose-6-phosphate; Gal1P, galactose-1-phosphate, Gly3P, glycerol-3-phosphate.

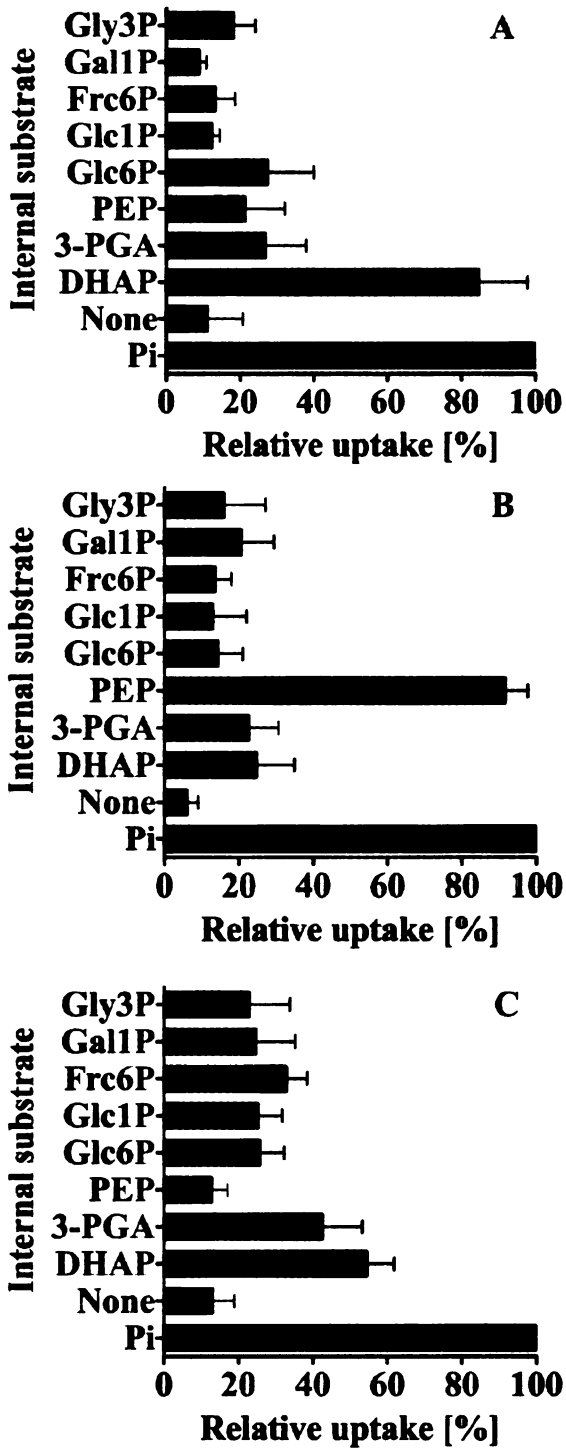

Figure 3.4. Internal substrate dependence on the transport activities of GsTPT, GsPPT and GsGPT

Table 3.3. Comparison of kinetic constants from *G. sulphuraria* and higher plant pPT homologues
The Michaelis-Menten constant (KM) for Pi was determined using various external [32P]-Pi concentrations (0.05 mM - 10 mM). The competitive inhibition constant (Ki) of [32P]-Pi uptake was assayed at two different external Pi concentrations with increasing inhibitor concentrations (0.05 mM – 20 mM) (Dixon, 1953). All proteoliposomes were preloaded with 30 mM Pi. Data represent the arithmetic mean ± SE of at least three independent experiments. Recombinant higher plant pPT data are taken from Flügge (1999). "None" refers to: No competitive inhibitory constant could be measured under the given experimental conditions. Abbreviations are listed in Figure 3.4.

Kinetic Constants	TPT homologue [mM]		PPT homologue [mM]		GPT homologue [mM]	
	Plants	Gs	Plants	Gs	Plants	Gs
KM (Pi)	1.0	0.33 ± 0.07	0.8	0.76 ± 0.08	1.1	5.07 ± 0.4
Ki (DHAP)	1.0	0.5 ± 0.04	8.0	7.93 ± 0.23	0.6	None
Ki (3-PGA)	1.0	7.71 ± 0.47	4.6	3.95 ± 0.1	1.8	None
Ki (PEP)	3.3	8.75 ± 0.40	0.3	0.36 ± 0.04	2.9	None
Ki (Glc-6P)	None	None	None	None	1.1	None

Transcript levels and protein activity of the plastidic phosphate translocator homologues in *G. sulphuraria* cells

In land plants, TPT expression is confined to photosynthetic tissues whereas GPT expression is highest in heterotrophic tissues (Kammerer et al., 1998; Flügge, 1999; Niewiadomski et al., 2005). PPT-genes are expressed at low levels in various tissues (Knappe et al., 2003b). To determine the expression pattern of GspPTs, semi-quantitative RT-PCR was used. Figure 3.5 shows that *GsTPT*, *GsPPT*, and *GsGPT* transcripts are clearly detectable and abundant under both, autotrophic and heterotrophic growth conditions. The steady state transcript levels of all three genes were higher than those of the reference gene actin (Gs15600; Galdieria Genome Browser Build 3.0). Liposomes

reconstituted with total membrane fractions exhibit high [32P]-Pi import using DHAP as a counter substrate under both culturing regimes (Figure 3.5). Exchange rates with PEP and 3-PGA as counter-exchange substrates were much lower as compared to DHAP. Exchange of glucose-6-phosphate with Pi was close to the background values of unloaded vesicles (Figure 3.5).

Figure 3.5. Transcript abundance and protein activity of GsTPT, GsPPT, and GsGPT in *G. sulphuraria* cells

Upper part: Total RNA from auto- and heterotrophically cultured *G. sulphuraria* cells was isolated, treated with DNase and used for first strand cDNA synthesis. Expression of GsTPT, GsPPT, GsGPT and actin transcripts was amplified by PCR with 25, 30 or 35 elongation cycles and visualized on an ethidium bromide-stained 1.25% (w/v) agarose-gel. **Lower part:** Relative [32P]-Pi uptake rates into liposomes reconstituted with total *G. sulphuraria* membranes isolated from heterotrophically (black bar) or autotrophically (white bar) grown cells, respectively. Membrane fractions were reconstituted into liposomes preloaded with 30 mM various substrates. Experiment was performed and abbreviations were used as given in the legend to Figure 3.4.

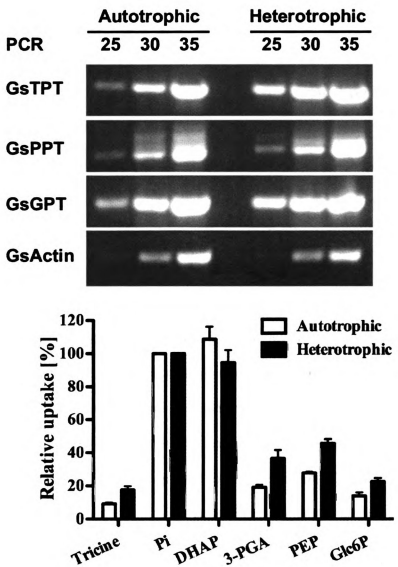

Figure 3.5. Transcript abundance and protein activity of GsTPT, GsPPT, and GsGPT in *G. sulphuraria* cells

DISCUSSION

In this study, we report the molecular and biochemical characteristics of the plastidic phosphate translocator family from the unicellular red alga *G. sulphuraria*. All three members of the *GsPT* gene family were heterologously expressed in *S. cerevisiae,* functionally reconstituted into liposomes (Figure 3.2 and Figure 3.3), and their substrate specificities and kinetic constants were assessed (Figure 3.4 and Table 3.3).

The *G. sulphuraria* genome encodes a high-affinity TPT with narrow substrate specificity

Recombinant *G. sulphuraria* TPT mediates a strict counter-exchange of the triose-phosphate DHAP with *ortho*-phosphate (Figure 3.4). Interestingly, the kinetic constants of GsTPT considerably differ from those of the green plant TPT (Table 3.3). The red algal transporter exhibits a three times higher affinity for Pi and, as indicated by its low *K*i value, the substrate DHAP has a twofold higher affinity to the Pi binding site. Further, GsTPT is highly specific for DHAP. In contrast to the green plant ortholog, 3-PGA poorly acts as a counter substrate, even under saturating conditions (Figure 3.4A). In addition, 3-PGA is a weak competitive inhibitor of Pi import with an eightfold and fifteen-fold higher Ki value as compared to the TPT from plants and the Ki (DHAP) constant from GsTPT, respectively (Table 3.3). These results argue strongly against 3-PGA as a physiological relevant substrate of GsTPT. PEP and Glc6P were even less efficient exchange substrates and inhibitors of Pi transport (Figure 3.4 and Table 3.3). Very likely, the distinct kinetic properties of GsTPT represent an important adaptation to red algal carbon metabolism. In contrast to the *Chloroplastida*, red algae store an

insoluble starch-like polymer called floridean starch and synthesize cell wall building blocks and the main soluble carbohydrate floridoside, composed of UDP-Gal and Gly3P moieties in their cytosol (Viola et al., 2001; Collen et al., 2004; Barbier et al., 2005a). Hence, primary carbon partitioning in red algae is predominantly organized within the cytosol and GsTPT enables solely the export of triose-phosphates, even at low stromal concentrations, to cope with the massive cytosolic demand of recently fixed carbon (Figure 3.6). On the other hand, GsTPT is highly expressed and active in heterotrophically cultured *G. sulphuraria* cells (Figure 3.5). Under these conditions, GsTPT presumably mediates TP import into the plastid, thus mediating carbon flux between cytosol and non-photosynthetic rhodoplasts. In contrast, land plants export TP from photosynthetic chloroplasts via TPT and import Glc6P into heterotrophic plastids via GPT. The TPT is an integral part of the photoassimilate partitioning in plants (Häusler et al., 2000; Smith and Stitt, 2007). Accumulation of sucrose and hexose-phosphates in the cytosol sequesters cytosolic Pi and thus slows down the export of triose-phosphate. A resulting increased stromal 3-PGA/Pi ratio allosterically activates ADP-glucose pyrophosphorylase, which is the committing step of plastidic starch biosynthesis (Ballicora et al., 2004). Plants have to maintain sufficiently high levels of TP in the plastid stroma to enable both the regeneration of the CO2 acceptor ribulose-1,5-bisphosphate and starch biosynthesis during the light period (Zeeman et al., 2007). The apparent lower affinity of the plant transporter for Pi and TP thus ensures higher steady state levels of TP in the stroma to drive these reactions.

G. sulphuraria has a PPT with similar properties as its green plant ortholog

PEP uptake from the cytosol is required for stroma localized fatty acid and shikimate biosynthesis (Flügge, 1999). Previously published results have been ambiguous about the existence of a putative PEP/Pi transporter (PPT) in *G. sulphuraria*. Phylogenetic analysis revealed an orthologous candidate gene (Weber et al., 2006), although isolated membrane fractions did not exhibit pronounced PEP uptake activity (Weber et al., 2004). Here, we show that EST GsA16F5 (Weber et al., 2006) represents a highly conserved PPT with almost identical kinetic constants in both the green and red lineages (Table 3.3). Both transporters are highly specific for Pi and PEP and poorly accept 3-PGA, DHAP, or any other of the 16 tested metabolites as a substrate (Figure 3.4B, data not shown). In addition, we now employed an improved reconstitution method for total membranes from autotrophic and heterotrophic cell cultures that yielded lower background and a weak but clearly detectable PEP/Pi counter-exchange activity (Figure 3.5). The low PEP transport capacity (Figure 3.5) mirrors published data from chloroplasts of C3 land plants (Flügge and Weber, 1994). TPT is by far the most abundant transport protein in C3 chloroplasts and accounts for a high DHAP exchange capacity (Flügge, 1999). Fatty acid biosynthesis and the shikimate pathway in red algae are predicted to be similar to that of seed plants and are presumably localized in the plastid, based on phylogenetic analyses and N-terminal target sequence predictions (Weber et al., 2004; Richards et al., 2006). A question that cannot be conclusively answered at the moment is whether rhodoplasts, analogous to chloroplasts of green plants, also have negligible activities of plastid phosphoglyceromutase and enolase to produce PEP from plastidial 3-PGA (Trimming and Emes, 1993). The *G. sulphuraria* genome encodes for two phosphoglyceromutase

isozymes (Gs04140 and Gs52680) and a single enolase (Gs21490), which are all three computationally predicted as cytosolic enzymes (Nielsen et al., 1997) without any N-terminal targeting sequences, when compared with their homologous genes from *A. thaliana* (phosphoglyceromutase: At4g09520, At3g08590, At1g09780; enolase: At2g36530, At2g29560, At1g74030) (Friso et al., 2004; Larkin et al., 2007). Alternatively, PEP could be generated from pyruvic acid via the pyruvate orthophosphate dikinase (PPDK) reaction. The annotated PPDK gene Gs42070 is computationally predicted to be localized in the cytosol, exhibiting a high similarity with cyanobacterial PPDK genes, and is not phylogenetically related to the dual targeted enzyme from *A. thaliana* (At4g15530) (Parsley and Hibberd, 2006). While computational targeting predictions have to be taken with a grain of salt, bioinformatics analysis indicates that conversion of TP to PEP is not possible in *G. sulphuraria* and rhodoplasts thus depend on PEP import from the cytosol to drive PEP-depended reactions in the stroma (Figure 3.6).

G. sulphuraria does not possess a plastidic hexose phosphate importer

Reconstituted recombinant putative GsGPT mediated a strict Pi/Pi homo-exchange that followed a first order rate kinetics (Figure 3.3). An unusually high *K*M value of 5 mM for Pi (Table 3.3) indicates that GsGPT under physiological conditions is not able to compete with GsTPT and GsPPT for the common substrate Pi. While liposomes preloaded with high (i.e., saturating) concentrations of DHAP or 3-PGA (i.e., 30 mM internal substrate concentration) showed Pi uptake rates of approximately 50% of Pi/Pi homo-exchange, it is important to notice that neither 3-PGA nor DHAP significantly inhibited Pi/Pi homo-exchange either at physiological concentrations or at 100-fold excess (Table 3.3). The

prime substrate candidate glucose-6-phosphate was poorly accepted under saturating conditions (Figure 3.4) and it did not inhibit Pi import in the Pi/Pi homo-exchange setup (Table 3.3). These results strongly indicate that 3-PGA, DHAP, and Glc6P are not physiologically relevant substrates of the putative GsGPT. We also tested various additional metabolites, such as nucleotide-sugars, mononucleotides, hexose-phosphates (Glc1P, Frc6P, and Gal1P), pentose-phosphates and a precursor of floridoside biosynthesis, glycerol-3-phosphate. All of these are poorly exchanged with [32P]-Pi and the physiological substrate of GsGPT thus remains unidentified. In non-green plastids of plants, Glc6P is the preferred precursor for starch synthesis and NADPH generation via the oxidative pentose phosphate pathway (OPPP) due to the absence of a fructose-1,6-bisphosphatase (FBPase) activity (Flügge, 1999). In photosynthetic tissues, plastidic FBPase is inactivated at night and thus hexose phosphates cannot be generated from triose phosphates (and *vice versa*). In contrast, red algal starch biosynthesis is cytosolic and plastidic FBPase from *G. sulphuraria* is not subject to a strict redox regulation (Reichert et al., 2003; Oesterhelt et al., 2007). Importing triose-phosphates via the GsTPT could thus sustain the production of hexose-phosphate for carbon and NADPH supply in the rhodoplast during the night or prolonged heterotrophic growth conditions, thus bypassing the requirement for a plastidic hexose phosphate translocator (Figure 3.6). Further work will be required to pinpoint the catalytic activity of this enigmatic protein.

An operative DHAP/3-PGA reduction shuttle is unlikely in *G. sulphuraria*

3-PGA is a major substrate for the TPT in higher plants (Flügge, 1999). In spinach and other model plants, 3-PGA levels in the plastid and the cytosol frequently exceed DHAP

levels by several fold (Gerhardt et al., 1987). However, the affinity of the spinach TPT is identical for both metabolites. It has been proposed that *in vivo* a significant amount of 3-PGA transport occurs across the envelope membrane; the TPT could thus operate as a NAD(P)H reduction equivalent shuttle between stroma and cytosol (Heineke et al., 1991). The substrate specificity and the kinetic constants of recombinant proteins, as discussed in detail earlier, and total membrane fractions from *G. sulphuraria* presumably cannot sustain a physiological relevant exchange rate of 3-PGA across the rhodoplasts envelope membrane. Still, DHAP offers a possibility to export reduction equivalent to the cytosol. The non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (NP-GAPDH) (Rius et al., 2006), which is also present in the draft genome sequence of the red alga (Gs00840) or the combined glycolytic reaction sequence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 3-PGA kinase (PGK) (Plaxton, 1996) generates NADPH, or NADH and ATP, respectively. However, in contrast to green plants, not 3-PGA but Pi must be postulated as the alternative counter-substrate for DHAP.

Impact of subtle differences of TPT in *Chloroplastida* and *Rhodophyta*

Triose-phosphate is the main entry and branching point in the glycolytic network in photosynthetically active cells (Plaxton, 1996). Metabolic flux has to be regulated between generating hexose-phosphates via gluconeogenesis and glycolytic "downstream" products, such as 3-PGA, PEP, and pyruvic acid. In higher plants, the redox transfer by a TP/3-PGA shuttle via (NP)-GAPDH and PGK is proposed to be marginal and GAPDH expression is mainly induced under stress conditions, such as anaerobiosis and heat shock or sucrose feeding (Yang et al., 1993; Bustos et al., 2008; Holtgrefe et al., 2008). On the

other hand, glycolysis has to provide adequate amounts of PEP to replenish TCA intermediates used for biosynthesis of, e.g., amino acids, to produce pyruvic acid for mitochondrial metabolism, or to fuel plastid fatty acid biosynthesis (Plaxton, 1996). Because 3-PGA transport is seemingly a specific adaptation in plants, we hypothesize that 3-PGA transport across the chloroplast envelope membrane might provide a bypass of the complex regulation of the cytosolic triose-phosphate pool that permits supporting cytosolic PEP biosynthesis and serves under stress conditions as an additional redox shuttle system.

Figure 3.6. Flexible transport of triose-phosphate across the plastid membrane is central for the cellular carbon metabolism in the red alga *G. sulphuraria*

In photosynthetically active cells, triose-phosphate is exported via the GsTPT from rhodoplasts to sustain soluble and insoluble carbohydrate synthesis (floridoside and floridean starch) in the cytosol. Under heterotrophic growth conditions the same transporter supplies the rhodoplasts with triose-phosphate for, e.g., hexose-phosphate synthesis. Rhodoplasts are not capable to produce PEP via partial glycolysis and are thus dependent on the import of PEP from the cytosol via the PPT protein for fatty acid and aromatic amino acid biosynthesis. The red algal plastid envelope does not contain any 3-PGA and Glc-6-P transport activities, which is a specific trait of green plants. PPT, PEP/phosphate-translocator; TPT, triose-phosphate/phosphate-translocator; Frc1,6BP, fructose-1,6-bisphosphate; Gal, galactose; Glc, glucose; Ru-1,5BP, ribulose-1,5-bisphosphate; other acronyms are listed in the legend to Figure 3.4. The figure in this dissertation is presented in color.

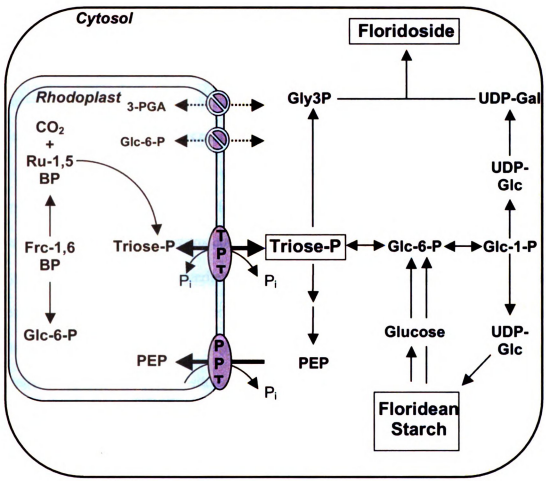

Figure 3.6. Flexible transport of triose-phosphate across the plastid membrane is central for the cellular carbon metabolism in the red alga *G. sulphuraria*

Conclusions

Although the primary structures of plastidic phosphate translocators are highly conserved between green plants and red algae, these proteins have evolved quite distinct biochemical characteristics in the different lineages of the *Archaeplastida*. Comparative biochemical analysis of candidate orthologs from distantly related organisms thus provides novel insights into alternative modes for regulating "ancient" metabolic pathways. While the triose-phosphate/phosphate antiport activity across the plastid envelope membrane is highly conserved across all photosynthetic eukaryotes analyzed to date, the red algal transporters catalyze neither 3-PGA nor Glc6P transport (Figure 3.6).

G. sulphuraria must have evolved alternative mechanisms to distribute triose-phosphate into various pools (i.e. floridean starch, floridoside, fatty acid and amino acid synthesis), a less stringent redox control of Calvin cycle, oxidative pentose phosphate pathway, and glycolysis (Oesterhelt et al., 2007). Plastidial phosphate translocators thus represent crucial components of primary carbon partioning in higher plants and red algae that have evolved to the specific requirements of each lineage through modulation of substrate specificities and kinetic constants.

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CHAPTER 4

PROPOSED METABOLITE TRANSPORT PROCESSES FOR THE RE-ASSIMILATION OF AMMONIA DURING PHOTORESPIRATION[4]

[4] This work has been published in **Linka M., and Weber A.P.M.** (2005) Shuffling ammonia between mitochondria and plastids during photorespiration. Trends in Plant Science **10**:461-465.

ABSTRACT

Surprisingly, it was recently found that glutamine synthetase is dual targeted to chloroplasts and mitochondria in *Arabidopsis thaliana* leaves. Mitochondrial glutamine synthetase likely assimilates ammonia, which is generated in large amounts in mitochondria during photorespiration. However, ammonia assimilation is a two-step process and the second step, catalyzed by glutamate synthase, is exclusively located in plastids. Hence, a shuttle for ammonia, possibly in the form of amino acids, is required between mitochondria and plastids. We discuss two alternative shuttles, an ornithine-citrulline shuttle and a glutamine-glutamate shuttle. Both shuttles allow the safe transport of the toxic metabolite ammonium in the form of amino acids. The ornithine-citrulline shuttle also provides a means for the transport of carbon dioxide from mitochondria to plastids, but this shuttle requires more energy than the alternative glutamate-glutamine shuttle.

RESULTS

Photorespiration is a complex, multi-compartment biochemical pathway

Ribulose-1,5-bisphosphate carboxlyase/oxygenase (RubisCO) is a bifunctional enzyme that catalyzes both the carboxylation of ribulose 1,5-bisphosphate (RuBP) and its oxygenation. Oxygenation of RuBP produces one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PG). 2-PG cannot be directly metabolized inside chloroplasts; instead, two molecules of 2-PG are recycled to one molecule of 3-PGA by the complex, multi-compartment photosynthetic carbon oxidation cycle (Figure 4.1). Because this

pathway leads to consumption of oxygen and production of carbon dioxide in the light, it is also called photorespiration. The photorespiratory pathway involves chloroplasts, peroxisomes, and mitochondria. 2-PG is dephosphorylated by phosphoglycolate phosphatase (PGP) in the chloroplast and the resulting glycolate is shuttled to peroxisomes where it is oxidized to glyoxylate, which is subsequently transaminated to glycine. Glycine is transported to the mitochondria where two molecules of glycine are converted to one molecule each of serine, ammonia, and carbon dioxide by glycine decarboxylase (GDC) and serine hydroxymethyltransferase (SHMT). Serine is transported back to the peroxisomes, converted to glycerate by serine glyoxylate aminotransferase and hydroxypyruvate reductase (HPR) and glycerate is finally shuttled to the chloroplasts where it is phosphorylated to 3-PGA by glycerate kinase (GLYK). The reaction sequence converts two molecules of 2-PG into one molecule of each 3-PGA and carbon dioxide and thus leads to the net loss of one carbon (Wingler et al., 2000; Douce and Neuburger, 1999). The oxidation of glycine by GDC in mitochondria during photorespiration generates massive amounts of the cytotoxic metabolite ammonia, exceeding ammonia production from nitrate via the concerted action of nitrate reductase and nitrite reductase by at least one order of magnitude (Ogren, 1984). It was assumed that photorespiratory ammonia would be either actively or passively transported to plastids to be assimilated into glutamate by the glutamine synthetase 2 (GS2) and Fd-dependent glutamate synthase (Fd-GOGAT) system (Figure 4.1).

Glutamine synthetase is dual targeted to plastids and mitochondria

The surprising discovery of dual targeting of GS2 to both plastids and mitochondria (Taira et al., 2004) has changed this view – the presence of GS2 in mitochondria opens the possibility of ammonia assimilation by GS in the mitochondrial matrix, directly at the site of ammonia generation by GDC. However, to maintain the nitrogen stoichiometry of the photorespiratory pathway, glutamine (Gln) needs to be converted to glutamate (Glu), which serves as amino donor for the peroxisome-localized glyoxylate aminotransferase reaction. The conversion of glutamine and alpha-ketoglutarate (α-KG) to glutamate is catalyzed by Fd-GOGAT and this enzyme is exclusively localized in the plastid stroma (Lancien et al., 2002; Coschigano et al., 1998). Alternatively, it could be envisaged that ammonia would be directly assimilated into glutamate in mitochondria by glutamate dehydrogenase (GDH). However, GDH mutants do not show a photorespiratory phenotype in ambient air (Melo-Oliveira et al., 1996), whereas Fd-GOGAT mutants show a strong photorespiratory phenotype and are not viable in ambient air (Coschigano et al., 1998; Somerville and Ogren, 1980). Hence, Fd-GOGAT is essential and glutamine (or ammonia) needs to be shuttled from mitochondria to chloroplasts to close the photorespiratory nitrogen cycle.

Figure 4.1. Simplified scheme of the current textbook photorespiratory cycle

The oxygenation of ribulose-1,5-bisphosphate (RuBP) leads to the production of 2-phosphoglycolate (2-PG) which is dephosphorylated to glycolate (Glt). After transfer to peroxisomes, glycolate is oxidized to glyoxylate (Gox) and aminated to glycine (Gly), which is then transported to the mitochondria. Gly is oxidatively decarboxylated by glycine decarboxylase (GDC) in the mitochondrial matrix, yielding Methylen-THF, CO2, and ammonia. Ammonia and CO2 arrive back in the chloroplasts, presumably by diffusion, to be re-assimilated by glutamine synthetase (GS) and glutamate synthase (GOGAT) and RubisCO, respectively. Glutamate, the amino donor for the amination of Gox in the peroxisomes and the reaction product alpha-ketoglutarate (α-KG) are shuttled between plastids and peroxisomes by a plastidic malate-coupled two-translocator system (Weber and Flügge, 2002; Weber et al., 2004) and unknown transporters in the peroxisomal membrane. For sake of simplicity, the conversion of glycine to serine, and the downstream reaction sequence leading to the conversion of serine to 3-phosphoglycerate is omitted. (1) plastidic glycolate transporter; (2) peroxisomal glycolate transporter; (3) plastidic glutamate/malate translocator; (4) plastidic α-KG/malate translocator; (5) peroxisomal glutamate transporter; (6) peroxisomal α-KG transporter; (7) peroxisomal glycine transporter; (8) mitochondrial glycine transporter. The figure in this dissertation is presented in color.

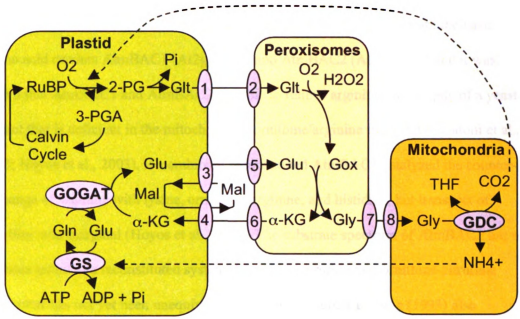

Figure 4.1. Simplified scheme of the current textbook photorespiratory cycle

A citrulline-ornithine shuttle between plastids and mitochondria

To couple mitochondrial ammonia assimilation with plastidic glutamate biosynthesis, Taira et al. (2004) have suggested a citrulline-ornithine shuttle between chloroplasts and mitochondria (Figure 4.2) and have supported this concept by demonstrating that isolated leaf mitochondria are able to catalyze the glutamine- and ATP-dependent conversion of ornithine to citrulline. Ornithine-citrulline transporters are well characterized in yeast and mammals. For example, two closely related human ornithine-citrulline transporters have been cloned and functionally characterized; they transport arginine, lysine, ornithine, and citrulline by exchange and unidirectional mechanisms (Fiermonte et al., 2003). Less is

known about mitochondrial ornithine-citrulline transporters in plants. The closest relatives of mammalian ornithine-citrulline transporters in *Arabidopsis* are the basic amino acid carriers AtmBAC1 (At2g33820) and AtmBAC2 (At1g79900) and it was shown that AtmBAC1 and AtmBAC2 are able to relieve arginine-auxotrophy of a yeast mutant that is deficient in the mitochondrial ornithine/arginine transporter (Catoni et al., 2003; Hoyos et al., 2003). Recombinant reconstituted AtmBAC1 catalyzed the counter-exchange of arginine with lysine, ornithine, arginine, and histidine, but transport of citrulline was marginal (Hoyos et al., 2003). The substrate spectrum of AtmBAC2 has not yet been tested in a reconstituted system. Hence, the *Arabidopsis* ornithine-citrulline transporter has not yet been unequivocally identified. Robert Ludwig (1993) also previously showed that isolated *Arabidopsis* chloroplasts are able to convert externally supplied citrulline into ammonium, bicarbonate, ornithine, and ATP. This indicates the presence of a citrulline transporter in the chloroplast envelope membrane, although uptake of citrulline into chloroplasts has not been directly shown by, e.g., the silicon-oil filtration-centrifugation technique. In summary, evidence derived from experiments with isolated chloroplasts and mitochondria indicates that the components of the proposed citrulline-ornithine shuttle seem to be present and active in leaves but the corresponding proteins remain to be identified. Importantly, the ornithine-citrulline shuttle would not only (indirectly) transfer ammonia from mitochondria to plastids, but also simultaneously carbon dioxide, and thus both products of the glycine decarboxylase reaction that need to be re-assimilated in the chloroplast stroma. Although this concept is appealing, it comes at a price: Whereas fixing one molecule of O2 by RubisCO and salvaging of the resulting 2-phosphoglycerate (2-PG) by a textbook C2-cycle (not including re-assimilation of CO2

that is lost during the process) costs approximately 9.5 ATP (Canvin and Salon, 1997), two additional ATP would be required in the proposed citrulline-ornithine cycle due to mitochondrial carbamoylphosphate synthase activity (the additional ATP required by GS2 in mitochondria is offset by production of one ATP by carbamate kinase in plastids), thus raising the overall ATP-consumption by 20% to 11.5 ATP.

Figure 4.2. A citrulline/ornithine shuttle between mitochondria and plastids

Glycine (Gly), produced in peroxisomes , is decarboxylated and deaminated by GDC in the mitochondrial matrix. In contrast to the scheme shown in Figure 4.1, GS assimilates ammonia into glutamate (Glu) directly within the mitochondrial matrix, yielding glutamine (Gln). Carbamoylphosphate (CarbP) is synthesized from Gln and CO2 by carbamoylphosphate synthetase (CPS), thereby regenerating the ammonia acceptor Glu. Citrulline (Cit) is generated from ornithine (Orn) and CarbP by Orn carbamoyltransferase (OCT) and Cit is shuttled to plastids were it is converted back to Orn and CarP by catabolic OCT. Carbamate kinase (CK) generates CO_2, ammonia, and ATP from CarbP. For clarity, the generation of glycine, its conversion of glycine to serine, and the downstream reaction sequence leading to the conversion of serine to 3-PGA is omitted. (8) mitochondrial glycine transporter; (9) mitochondrial ornithine/citrulline translocator; (10) plastidic ornithine/citrulline translocator. The figure in this dissertation is presented in color.

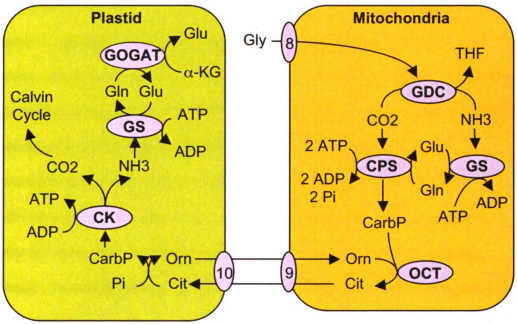

Figure 4.2. A citrulline/ornithine shuttle between mitochondria and plastids

A less costly alternative – the glutamate-glutamine shuttle

Alternatively, a glutamate/glutamine shuttle between mitochondria and plastids can be envisaged that is compatible with ammonia assimilation by GS2 in mitochondria and that does not require more ATP than a textbook C2-cycle (Figures 4.1 and Figure 4.3). In this cycle, ammonia would be assimilated by GS2 in mitochondria, yielding glutamine. Glutamine is exported from mitochondria, imported into plastids, and the amido group from glutamine is transferred to α-KG by GOGAT, yielding two molecules of glutamate. One molecule of glutamate is shuttled back to mitochondria where it serves as substrate for the GS-reaction. The ATP expense for ammonia assimilation would thus be shifted from plastids to mitochondria, but would remain otherwise unchanged. This hypothetical

glutamine/glutamate shuttle between mitochondria and chloroplasts would require the presence of glutamine and glutamate transporters in both organelles. The activity of a glutamine-glutamate antiporter has been described in isolated chloroplasts (Yu and Woo, 1988); hence chloroplasts are able to take up glutamate in counter-exchange with export of glutamine. Relatively little is known about amino acid transporters of plant mitochondria. It was shown that glutamate enters beetroot and cauliflower bud mitochondria; the uptake required the presence of both phosphate and a permeable dicarboxylic anion (Day and Wiskich, 1977). The activity of a glutamate-aspartate exchanger was demonstrated in isolated pea mitochondria, the partially purified protein was constituted into liposomes, and capable to facilitate the exchange of glutamate for both aspartate and glutamate (Vivekananda and Oliver, 1989). Mammalian mitochondria also possess a mitochondrial glutamate-aspartate exchanger (Palmieri et al., 2001), and in addition related proteins that catalyze the co-transport of glutamate with H+ (or counter-exchange with a hydroxyl ion) (Fiermonte et al., 2002). The mitochondrial aspartate-glutamate carrier from *Saccharomyces cerevisiae*, unlike its human orthologs, catalyzes both aspartate-glutamate exchange and substrate uniport (Cavero et al., 2003). It is not known whether the plant glutamate-aspartate carrier is able to catalyze glutamate uniport or whether plant mitochondria possess a glutamate carrier. It is also not known whether glutamine can be transported across the membrane of plant mitochondria; however, in analogy to mammalian mitochondria, it is likely that plant mitochondria possess a transport system for glutamine. For example, rat liver mitochondria have a glutamine-transporter that is able to catalyze both the unidirectional flux of glutamine and glutamine-glutamine counter-transport (Indiveri et al., 1998). After reconstitution into

liposomes, this protein also catalyzes the counter-exchange of glutamine with glutamate or aspartate, albeit this activity was one order of magnitude lower than glutamine-glutamine counter-transport (Indiveri et al., 1998). In summary, with the exception of the mitochondrial glutamine transporter, all components of a glutamate/glutamine shuttle between mitochondria and plastids have been experimentally verified in leaf cells. In contrast to the above-described Orn/Cit shuttle, though, the Glu/Gln shuttle only provides a vehicle the transport of NH3 between both organelles, whereas the transport of carbon dioxide would be dependent on passive diffusion. On the other hand, shuffling NH3 between mitochondria and chloroplasts by the Glu/Gln shuttle requires two ATP less than by the Orn/Cit shuttle.

Safety first – a bucket chain for intracellular ammonium transport

Assimilation of photorespiratory ammonia in mitochondria by GS requires a shuttle system for ammonia transport between mitochondria and plastids. Both, an ornithine/citrulline shuttle (Figure 4.2) or a glutamate/glutamine shuttle (Figure 4.3) could fulfill this function. In both cases, no transport of free ammonia would occur; instead, this toxic metabolite would be transiently stored as amino acids and would be transported safely in this form. Future research will be required to examine whether either of the two alternative shuttles is physiologically relevant (if not both). Likely, the photorespiratory cycle just became more complex, involving even more metabolite transporters than previously assumed (Weber, 2005).

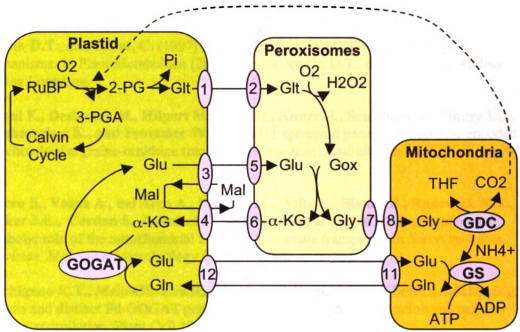

Figure 4.3. A glutamate/glutamine shuttle between mitochondria and plastids

In contrast to scheme depicted in Figure 4.1, ammonia generated by GDC in mitochondria is not assimilated into Glu in the plastids, but by mitochondrial GS and the resulting Gln is shuttled to the plastid stroma were it is used for Glu biosynthesis from α-KG by GOGAT. The ammonia acceptor Glu is returned to mitochondria by the same shuttle system. For clarity, the conversion of glycine to serine, and the downstream reaction sequence leading to the conversion of serine to 3-PGA is omitted. (1) plastidic glycolate transporter; (2) peroxisomal glycolate transporter; (3) plastidic glutamate/malate translocator; (4) plastidic α-KG/malate translocator; (5) peroxisomal glutamate transporter; (6) peroxisomal α-KG transporter; (7) peroxisomal glycine transporter; (8) mitochondrial glycine transporter; (11) mitochondrial Glu/Gln translocator; (12) plastidic glutamate/glutamine translocator. The figure in this dissertation is presented in color.

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CHAPTER 5

A REVERSE GENETIC APPROACH TO IDENTIFY THE PROPOSED METABOLITE TRANSPORT PROTEINS IN THE PHOTORESPIRATORY PATHWAY[5]

[5] Dr. Nicole Linka and Andrea Bräutigam contributed to Table 5.3 and Dr. David Gagneul provided the metabolite data for Figures 5.4, 5.5, and 5.7.

ABSTRACT

In C3 plants, the oxygenation reaction of Ribulose-1,5-bisphosphate Carboxylase/ Oxygenase (RubisCO) produces a significant amount of 2-phosphoglycolate, which must be converted to 3-phosphoglycerate in the photorespiratory pathway. The enzymatic steps of this pathway are remarkably coordinated although they are distributed in the three compartments plastids, peroxisomes, and mitochondria. To cope with the high flux of the intermediates between these compartments during the day, a significant number of metabolite transporters are required. To date, forward genetic screens have identified only a single plastidial transporter DiT2.1/DCT1 (dicarboxylate transporter) as a classical photorespiratory mutant. The mutant is non-viable under ambient air conditions and indistinguishable from wild type under CO2 enriched air. To identify additional metabolite transporters, publicly available microarray datasets were screened for putative transporter genes that are co-expressed with fifteen known photorespiratory enzymes. We identified 32 candidate genes and present the initial genetic analysis of five genes. One mutant shows a delayed pale green phenotype after 14 days in ambient air. The corresponding gene was previously identified as a putative mitochondrial carnitine carrier, designated BOU (Bout de Souffle). However, our results indicate that instead of carnitine, BOU presumably imports Vitamin B6 as an essential cofactor required for the function of the photorespiratory enzyme glycine decarboxylase.

INTRODUCTION

Photorespiration (PR) and the Calvin-Benson-Cycle are closely linked pathways via the bifunctional enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) (Ogren, 1984; Tolbert, 1997). Under ambient atmospheric conditions, oxygen (O2) competes with carbon dioxide (CO2) for the reaction with ribulose-1,5-bisphosphate (RuBP) catalyzed by RubisCO. Carboxylation of ribulose-1,5-bisphosphate yields two molecules of 3-phosphoglyerate (3-PGA) that are converted to triose phosphates (TP) using chemical energy in the form of ATP and NADPH provided by the photosynthetic light reactions (Calvin, 1962). However, oxygenation yields only one molecule of 3-PGA and one molecule of 2-phosphoglycolate (2-PG). The latter molecule represents a metabolic dead end in the chloroplast stroma. It must thus be removed from the chloroplast and recycled to 3-PGA in a complex, highly compartmentalized pathway named photorespiration. In photorespiration, two molecules of 2-PG are recycled to one molecule of 3-PGA performed in a series of enzymatic steps, which are distributed in three different subcellular compartments (Figure 1; Reumann and Weber, 2006). 2-PG is hydrolyzed to glycolate in the chloroplast (Somerville and Ogren, 1979; Schwarte and Bauwe, 2007), and metabolized to glyoxylate and further to glycine in the peroxisomes (Volokita and Somerville, 1987; Igarashi et al., 2003). Glycine oxidation and serine synthesis takes place in the mitochondria (Somerville and Ogren, 1981; Voll et al., 2006; Engel et al., 2007) and serine is shuttled back to the peroxisomes to be deaminated and reduced to glycerate (Murray et al., 1989; Liepman and Olsen, 2001). Glycerate is imported and finally phosphorylated to 3-PGA in the chloroplast (Boldt et al., 2005). The resulting 3-PGA is then further metabolized in the Calvin-Benson-Cycle. In summary,

two molecules of 2-PG are converted to one molecule each of, 3-PGA, CO2, and NH3. The pathway leads to a net loss of CO2 and ammonia and therefore, in addition to a carbon cycle, photorespiration is intertwined with a nitrogen cycle (Ogren, 1984; Keys, 2006). Ammonia (NH3) is released during glycine oxidation in the mitochondria (Keys et al., 1978). It has been widely accepted that NH3 enters the chloroplast and is re-assimilated by the concerted action of glutamine synthetase (GS) and glutamate synthase (GOGAT) (Somerville and Ogren, 1980; Coschigano et al., 1998; Wallsgrove et al., 1987). The product glutamate is guided to the peroxisomes and directly utilized to produce glycine from glyoxylate – In this way, a new round of nitrogen cycling is initiated. The flux of NH3 re-assimilation exceeds by far the rate of *de novo* ammonium ion production and has been demonstrated to be absolutely required under photorespiratory conditions to circumvent toxic glyoxylate accumulation (Sharkey, 1988; Keys, 2006).

It has been recently demonstrated that GS is dually targeted to chloroplasts and mitochondria (Taira et al., 2004). This would allow the re-assimilation of the NH3 into glutamine on location. Still, the enzyme GOGAT is exclusively active in the chloroplast (Coschigano et al., 1998) and glutamine instead of ammonia has to be transported (Linka and Weber, 2005). In another scenario, ammonia could be incorporated into carbamoyl phosphate and subsequently into ornithine yielding citrulline in the mitochondria. Citrulline is broken down in the chloroplast to ornithine and ammonia enters the GS/GOGAT cycle (Ludwig, 1993; Linka and Weber, 2005).

The identification of enzymes that are required for conversion of 2-PG to 3-PGA has been one of the earliest and, in retrospect, most successful forward genetic screens in

the *Arabidopsis thaliana* research field (Somerville and Ogren, 1979; Somerville, 2001). Classical photorespiratory mutants are indistinguishable from wild-type plants in CO_2 enriched air but not viable under ambient air conditions (Somerville and Ogren, 1979). PR mutants significantly contributed to establishing *A. thaliana* as a model organism for plant biochemical geneticists and plant physiologists (Meyerowitz and Pruitt, 1985). Almost three decades later, the enzyme glycerate-3-kinase as the last enzymatic step in the series was identified on the molecular level and published as the "last unknown enzyme in the photorespiratory cycle" (Boldt et al., 2005). As outlined above, photorespiration is a prime example for pathway compartmentalization in eukaryotic cells, since enzymes for the carbon and nitrogen cycle are partitioned almost in equal parts in chloroplasts, peroxisomes and mitochondria (Kunze et al., 2002). Despite the excellent progress in understanding the photorespiratory pathway, none of the metabolite transporters navigating the photorespiratory intermediates through the cell are mapped on the genome of *Arabidopsis thaliana* (Reumann and Weber, 2006). Forward genetic screens identified only one affiliated transport system: the malate/glutamate transporter DiT2.1/DCT1 (dicarboxylate transporter; Somerville and Ogren, 1983; Renne et al., 2003). This exports the amino-donor glutamate from the chloroplast for the glyoxylate:glutamate aminotransferase (GGT) reaction in the peroxisomes as described earlier. The released product α-ketoglutarate (α-KG) has to be shuttled back to the chloroplast and converted to glutamate by the GOGAT reactions (Weber and Flügge, 2002).

The list of *A. thaliana* photorespiratory mutants has been continuously expanded. For example the peroxisomal catalase detoxifies H2O2 released by the glycolate oxidase

(Queval et al., 2007) and malate dehydrogenases sustain the NADH pool via an oxaloacetate/malate redox-system for the reduction of hydroxypyruvate to glycerate (Cousins et al., 2008). The mitochondrial glycine decarboxylase is a multienzyme complex consisting of four different subunits (P protein, H protein, T protein, and L protein) and several cofactors [lipoic acid, tetrahydrofolate (THF), NAD, FAD, and pyridoxal-phosphate (PLP)] (Douce et al., 2001). A null mutant for the P protein subunit is inviable and reduced GDC activities result in classical PR phenotypes (Heineke et al., 2001; Engel et al., 2007). It has been demonstrated that impaired biosynthesis of the cofactor lipoic acid (Ewald et al., 2007) or THF re-cycling in mitochondria causes a decrease in GDC activity (Collakova et al., 2008). Import of malonic acid and pyruvate is a prerequisite for lipoic acid synthesis and the three precursors glutamate, pABA (para-aminobenzoic acid) and pterin have to be provided for THF synthesis inside the mitochondria (Wada et al., 1997; Rebeille et al., 2007).

Apparently, primary photorespiratory metabolites as well as a great number of additional compounds have to be distributed within the cell to cope with the high photorespiratory rates during the day. In this paper we attempted to establish a catalog of metabolite transporter candidates by a reverse genetic approach. Routine transcriptional profiling is providing an enormous data set, which is becoming a valuable tool to associate proteins of unknown function to known pathways. We used the comprehensive system-biology database (CSB.DB) to identify the best 5% of transcripts co-regulated with a set of 15 highly co-expressed photorespiratory genes. Candidates, which are co-expressed with several PR genes and contain at least two predicted hydrophobic

transmembrane spans, were selected for genetic analysis to identify classical photorespiratory mutants.

MATERIAL AND METHODS

Chemicals

The radiolabeled compounds [14C(U)]-arginine, [14C(U)]-glutamic acid, [14C(U)]-glycine, [14C(U)]-L-serine, [N-methyl-14C]-L-carnitine, [2-14C]-malonic acid and [1-14C]-L-ornithine were purchased from Moravek Radiochemicals and Biochemicals (Brea, CA, USA). Sigma-Aldrich (St. Louis, MO, USA) supplied the chemicals and the company Bio-Rad (Hercules, CA, USA) provided the Bio-Beads SM-2 hydrophobic adsorbent and the ion exchange resins Dowex AG1-X8 and AG50-X8. Reagents and enzymes for nucleic acid amplification, purification and cloning were purchased from Promega (Madison, WI, USA), Qiagen (Hilden, Germany) and Invitrogen (Carlsbad, CA, USA).

Plant Material and Growth Conditions

Seeds of the *A. thaliana* ecotype Columbia-0 and T-DNA insertion lines for At1g54350 (SALK003891 & SALK069087), At2g42770 (SALK118636), At2g48070 (SALK066383) and At3g26710 (SALK055566) were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at Ohio State University (USA). The *A. thaliana* GABI-Kat line 079D12 was ordered from the Center for Biotechnology (CeBiTec), Bielefeld University (Germany) and the SHMT (*shm1*) line was previously established in

our lab (Voll et al., 2006). Surface-sterilized seeds were germinated on petri dishes containing half-concentrated MS medium supplemented with 1% (w/v) sucrose and 0.8% agar and plantlets were transferred on soil after two weeks and watered regularly with Hoagland solution. Unless stated otherwise, the plants were kept under a 12 h-light (120 μEinstein per square meter and minute)/12 h-dark regime at 22°C in growth chambers with elevated CO2 concentrations (0.3%) throughout their complete life cycle. Selection of the transgenic *A. thaliana* plants was performed with kanamycin (50 μg/mL) or hygromycin (30 μg/mL).

Transcript co-response analyses

The publicly available comprehensive system-biology database (CSB.DB) (Steinhauser et al., 2004) was used to identify genes transcript, co-expressed with 15 genes known to be involved in the photorespiratory pathway in *A. thaliana*. AtGenExpress matrices *atge0100* and *atge0200* comprised of 63 experiments with 12,200 genes of the developmental expression series, and the abiotic stress series (aboveground samples) with 13,197 genes, respectively (Schmid et al., 2005), were analyzed. The NASC Arrays database (Nottingham Arabidopsis Stock Centre) with microarray experiments from the matrix *nasc0271* was also included (Craigon et al., 2004). Based on the Spearman's correlation, the best 5% of co-responding genes from each single gene query were further analyzed with the ARAMEMNON database to identify putative membrane proteins with at least two predicted alpha helix transmembrane spans (Schwacke et al., 2003; Steinhauser et al., 2004).

Phylogenetic tree analysis

The putative mitochondrial carnitine carrier, designated BOU (Bout de Souffle), belongs to the eukaryotic mitochondrial carrier family (MCF). Homologous sequences of the MCF from yeast and human and *Arabidopsis thaliana* were obtained from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The BLAST program at NCBI (Altschul et al., 1990) identified homologous genes in the genome of the red alga *Galdieria sulphuraria* (http://genomics.msu.edu/galdieria/) using the human and yeast proteins as a query with a cut-off expectation value (E-value) of ≤ 0.0001. The phylogenetic analysis was performed with full-length amino acid sequences of 208 proteins according to Weber et al., 2006.

Isolation of homozygous T-DNA insertion lines

Genomic DNA was isolated and T-DNA insertions were determined by standard PCR with the following primers: T-DNA specific primer LBa1 and ML116 were used to amplify from the T-DNA left border in SALK lines and the GABI-Kat line, respectively, and the At3g18780 (AtACT2)-specific primer pair ML40/ML41 served as a quality control. At1g54350 gene-specific primer pair ML102/ML103, At2g42770 gene-specific primer pair ML104/ML105, At3g26710 gene-specific primer pair ML108/ML109, At2g48070 gene-specific primer pair ML107/ML120, and At5g46800 gene-specific primer pair ML114/ML115 were designed to analyze the transgenic lines. Primer sequences are shown in Table 5.1. Amplified PCR fragments were cloned into the pGEM-T Easy vector (Promega) and the T-DNA insertion sites were verified by sequencing (Research Technology Support Facility, Michigan State University, USA).

Homozygotes were further propagated and heterozygous mutants were self-pollinated and analyzed again for homozygous T-DNA insertion.

Isolation of total RNA and RT-PCR analysis

The method by Chomczynski and Sacchi (1987) was used to extract total RNA from 6-weeks old *A. thaliana* rosettes. After DNAse treatment, cDNA was synthesized (Superscript II First-Strand Synthesis System, Invitrogen). The transcript levels were examined from the putative metabolite transporter BOU (At5g46800) and the control gene AtACT7 (At5g09810) by PCR analysis. The PCR reaction was an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 35 s, 58°C for 30 s, 72°C for 2 min, and final extension of 72°C for 3 min and products were visualized on an ethidium bromide-stained, 1% agarose gel.

Table 5.1. Primer sequences used in Chapter 5
Primers were used for cloning of heterologous expression (HE) constructs, Reverse-Transcriptase PCR (RT-PCR) expression analyses, complementation studies (COMP) or T-DNA insertion line screens (TDNA). Underlined nucleotides highlight restrictions sites used for cloning.

Primer	Sequence (5'–3')	Purpose
LBa1	TGGTTCACGTAGTGGGCCATCG	TDNA
ML 40	GTTGGGATGAACCAGAAGGA	TDNA
ML 41	GAACCACCGATCCAGACACT	TDNA
ML 90	TATAGGATCCAAATGGCGGATGCGTGGAAG	HE
ML 91	ATATCTCGAGTTTATCCCAAGCTTGACCT	HE
ML 92	TATAGGTACCACCATGGCGGATGCGTGGAA	HE
ML 93	ATATTCTAGATCCCAAGCTTGACCTTGTCA	HE

Table 5.1. (cont'd).

Primer	Sequence (5'–3')	Purpose
ML 102	TAGCTCTTCTGATCCACGGAG	TDNA
ML 103	TCAATGGTGGCATTAACAAGC	TDNA
ML 104	CCCAACACCCACAAGTAGTTG	TDNA
ML 105	TTTCCTTTGAAGCAAGCTGTG	TDNA
ML 107	AGAGAAAGAAACCAACCTGCC	TDNA
ML 108	TTCGACCTGAACATCATCTCC	TDNA
ML 109	TTGGTCTGATTTTATGTCGGG	TDNA
ML 114	CAAAGGTTTTGATTTTGGTTTG	RTPCR
ML 115	CAAACCCCTTAGCGAACTCTT	TDNA
ML 116	CCCATTTGGACGTGAATGTAGACAC	TDNA
ML 120	TTGCCTTAAACCCACGTACC	TDNA
ML 143	CGCACAATCCCACTATCCTT	TDNA
ML 164	CAATGCGGTTTTGTTCACTG	RT-PCR
ML 165	CCCAAGCTTGACCTTGTCAT	RT-PCR
ML 166	AAAAGAAGCACCAGCGACTC	RT-PCR
ML 167	TTCAATGTCCCTGCCATGTA	RT-PCR
ML 168	TGAACAATCGATGGACCTGA	RT-PCR
ML 175	TACCATGGCGGATGCGTGGA	COMP
ML 176	ATGCTAGC TCCCAAGCTTGACCTTGTC	COMP
ML 180	CACACTCTAGAAGCCGGATCTCAGTG	HE
ML 181	CACACGAGATATACCCATGGCGGATG	HE

Complementation of the GABI-Kat line 079D12

For the molecular complementation of the T-DNA insertion line GABI-Kat 079D12 (*bou1)* the full-length cDNA of BOU (At5g46800) was amplified by PCR with the primer pairs ML175 and ML176 (Table 5.1), cloned into pGEM-T Easy (Promega) and verified by sequencing. The *BOU* gene was excised with the designed restriction sites Nhe I and Nco I and ligated into the plant expression vector pCAMBIA 1302 after removal of the GFP reporter gene with the same restriction enzymes. Homozygous *bou1* plants were transformed by the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998) and several independent lines were selected on MS agar plates containing 20 μg/mL hygromycin. The 35S:BOU construct in the T1 generation was confirmed on the genomic DNA level by PCR with the primers ML143/ML165 and *BOU* transcripts were analyzed with the primers ML164/ML165 and ML114/ML166. The control transcripts *AtACT7* were amplified with the primer ML167 and ML168 (Table 5.1). T2 generations were screened by segregation analysis and homozygous lines were established in the following generation. The standard PCR reaction was an initial denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 35 s, 58°C for 30 s, 72°C for 2 min, and final extension of 72°C for 3 min and products were visualized on an ethidium bromide-stained, 1% agarose gel.

Antibody production and protein analysis

To use BOU as an antigen and for biochemical characterization, the protein was expressed in *Escherichia coli* strain BL21 Codon Plus (Stratagene, La Jolla, USA). Coding sequence was amplified with the primers ML180/ML181 (Table 5.1) by PCR

from cDNA templates. The fragment was subcloned into pGEM-T Easy (Promega) and via Nde I and Bam HI restriction sites introduced into the pET16b vector (Merck Chemicals Ltd., Darmstadt, Germany). Expression was induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) at an OD600 of 0.6 and cells were harvested after two hours. Inclusion bodies containing recombinant BOU protein translationally fused with an N-terminal hexahistidine tag were isolated according to Schroers et al., 1997, and further purified using nickel-affinity chromatography under denaturing conditions (Qiagen). In detail, 100 mL cultures of *E. coli* cells were harvested, resuspended in 10 mL Buffer A (20 mM TRIS/HCl pH 7.8, 1 mM EDTA, 1 mM PMSF), incubated with lysozyme (1 mg/mL) and burst by a subsequent sonication step. The insoluble inclusion bodies were pelleted by centrifugation (12,000 g, 10 min, 4°C), dissolved by sonication in buffer A and centrifuged again at 1,100 g for 5 minutes. The supernatant (containing the inclusion bodies) was transferred to a new tube, and inclusion bodies were collected by centrifugtion (12,000 g, 5 min, 4°C), and washed three times with a buffer containing 2% (v/v) Triton X-100, 20 mM TRIS/HCl pH 7.8, 0.1 mM EDTA. The inclusion bodies were solubilized in 400 µL SLS buffer [1.67 % (w/v) sodium lauroylsarcosine, 20 mM TRIS/HCl pH 7.8, 0.1 mM EDTA] and diluted with 800 µL deionized water. The expression and purification steps were colorimetrically confirmed by Coomassie-stained SDS-PAGE and by immunoblot analysis using a monoclonal anti-penta-His antibody (Qiagen, Germany) combined with an alkaline phosphatase (AP)-conjugated secondary antibody (Promega). Cocalico Biologicals Inc. (Reamstown, PA, USA) produced the polyclonal antibodies against the purified BOU in rabbits. Polyclonal antibodies raised against lipoic acid, H- and P-protein subunits of

glycine decarboxylase (GDC), and serine-hydroxymethyl-transferase (SHMT) were kindly provided by Prof. Bauwe from the University of Rostock (Germany). For the protein analysis, 5 g of 4-5 weeks old *A. thaliana* rosettes were harvested and mitochondrial fractions were prepared as described by Keech et al., 2005. Fifty micrograms of enriched mitochondrial proteins were resolved on a 15% SDS-PAGE, immobilized on PVDF membranes (Bio-Rad) and analyzed with the Amersham ECL (Enhanced Chemiluminescence) Western Blotting detection system with a secondary anti-rabbit antibody conjugated to HRP (horseradish peroxidase, GE Healthcare UK Limited, Buckinghamshire, UK). Protein concentrations were determined with the Pierce BCA protein assay (Pierce Biotechnology, Rockford, IL, USA).

Metabolite analysis

For time course experiments, mutant and wild type plants were grown for four weeks under high CO2 concentrations (0.3% CO2) and transferred to ambient air (0.038% CO2). Entire rosettes were harvested prior to and two, four and seven days after the shift. Rosette leaves from control plants, which were kept at 0.3% CO2, were sampled in parallel. Experiments were set up in a completely randomized manner. Start of the experiment and sampling were done at midday. Shoots were collected, immediately frozen, and subsequently powdered in liquid nitrogen, and stored at -80°C until further processing. Glyoxylate and ammonium were extracted and quantified according to Bräutigam et al., 2007. For proteinogenic and non-proteinogenic amino acid analyses, 100 mg fresh tissue were extracted with 700 µL pre-chilled 100% methanol (-10 to -20°C) supplemented with phenylalanine-d5 20 µM (internal standard for amino acid

profile normalization). Samples were shaken at 70°C for 15 min. After cooling on ice, 700 µL de-ionized water was added to the mixture. Subsequently, 375 µL pre-chilled chloroform was added and the samples were shaken again for 30 min at 4°C. The samples were centrifuged for 10 min at 14,000 g, and the upper, aqueous phase was used for metabolite analysis. Amino acids were characterized and quantified by LC-MS/MS using a Waters Symmetry C18 column (Milford, MA) with a Shimadzu LC-20AD HPLC system (Columbia, MD) coupled to a Waters Quattro micro mass spectrometer (Gu et al., 2007). Amino acid concentrations were calculated by making reference to external calibration curves, and recovery of successive procedures was assessed using the internal standard phenylalanine-d5.

Measurement of glycine decarboxylase activity

Mitochondria from wild type and *bou1* mutant plants were isolated as described above (Keech et al., 2005) except that the final mitochondria pellet was resuspended in 500 µL of 50 mM TES/KOH (pH 7.5), 2 mM EDTA, 5 mM MgCl2, 30% (v/v) glycerol and 0.1% (v/v) Triton X-100 to permeabilize the membrane and release the matrix proteins. The 100 µL activity assay contained 50 µL mitochondrial preparation and final concentrations of 50 mM TES/KOH (pH 7.5), 1 mM EDTA, 2.5 mM MgCl2, 15% (v/v) glycerol and 0.01% (v/v) Triton X-100, 2 mM β-mercaptoethanol, 0.03 mM pyridoxal-phosphate (PLP), 0.5 mM tetra-hydrofolate (THF), 1 mM NAD^+ and 0.2 mM [14C(U)]-glycine. The reactions were conducted in an airtight vial. After 15 min the reaction was terminated by the addition of 1 M sulfuric acid (0.5 mL). GDC activity was linear for at least 30 minutes. The released [14C]-CO2 was transferred with a stream of nitrogen gas

into a new vial filled with 5 mL of a 1 M KOH solution to trap the carbon dioxide as hydrogen bicarbonate (Walker and Oliver, 1986). The released [14C]-CO2 was quantified by liquid scintillation counting (LSC) with a Beckman LS6000 counter (Beckman Coulter Inc., Fullerton, CA).

Heterologous expression of BOU in *Saccharomyces cerevisiae*

To clone BOU into the yeast expression system pYES2/NT (Invitrogen) the full-length sequence was amplified from cDNA by PCR as described earlier. The primer pairs ML90/ML91 and ML92/93 (Table 5.1) were used to yield recombinant BOU protein with an N- and C-terminal hexa-histidine epitope. The fragment obtained with the first primer set was digested with Bam HI and Xho I and cloned into the pYES/NT-A vector. For the C-terminal His-tag fusion, the plasmid pYES/NT-A was digested with Hind III, treated with T4 polymerase (Promega) and subsequently incubated with Xba I. The BOU fragment was processed consecutively with the enzymes Kpn I, T4 polymerase and Xba I and ligated with the prepared pYES/NT-A vector. Transformed INVSc1 yeast (Invitrogen) cells were selected and the recombinant protein was expressed and partially purified as described by Linka et al., 2008. Production of the BOU protein was confirmed by immuoblot analysis using an anti-His antibody (Qiagen, Germany) and an AP-conjugated secondary antibody (Promega).

Reconstitution into liposomes

Two methods were used in this study to functionally reconstitute recombinant BOU protein into liposomes. For the freeze-thaw-sonicate procedure (Palmieri et al., 1995), a

2.5% (w/v) L-α-phosphatidylcholine (PC) suspension was prepared in 120 mM TRIS/HCl (pH 8.0) with 30 mM of the substrate or in 150 mM TRIS/HCl (pH8.0). The phospholipids were dissolved by sonication (Branson Sonicator 250, output 2, duty cycle 30%) on ice for three minutes. 50 µL of the yeast extract or 30 µg of the recombinant, solubilized BOU protein were mixed with the liposome suspension to a final volume of 1 mL. The protein-phospholipid mixture was frozen in liquid nitrogen and thawed at room temperature. To obtain unilamellar and sealed vesicles, the suspension was sonicated with 25 pulses (output 2, duty cycle 30%). Alternatively, in the "cyclic detergent removal procedure" (Krämer and Heberger, 1986), 30-50 µg of the recombinant BOU protein was mixed with 110 µL 10% Triton X-100. A 10% (w/v) PC suspension was prepared in deionized water and 160 µL was added to the protein/detergent mixture. The concentrated micelles were diluted to a volume of 1 mL with TRIS/HCl (pH 7.8) and the putative substrate to a final concentration of 120 mM and 30 mM, respectively. After an incubation of 15 min on ice, the detergent was gradually removed by passing the mixture twelve times over a column filled with 1 g of a hydrophobic absorbent (Bio-Beads SM-2, Bio-Rad). External substrate was removed by an additional chromatography step using a desalting Sephadex PD-10 column (GE Healthcare), previously equilibrated with 150 mM TRIS/HCl (pH 7.8).

Transport assay

Carnitine transport assay: Uptake of 0.1 mM [N-methyl-14C]-L-carnitine into proteoliposomes was tested with and without preloading (L-carnitine; 30 mM). After

defined time intervals (up to 30 minutes) the vesicles were passed through a PD-10 column to separate the external radiolabeled substrate from the vesicles. Liposomes were collected from the flow-through and the imported [N-methyl-14C]-L-carnitine was quantified by LSC.

Glutamate and malonic acid transport assay: Liposomes were prepared with 30 mM glutamate, glutamine or malonic acid and tested for the uptake of radiolabeled [14C(U)]-glutamic acid (0.75 mM) or [2-14C]-malonic acid (0.25 mM), respectively. External radiolabeled substrate was removed by binding to an anion-exchange column (Dowex AG1-X8 Resin acetate form, 100-200 mesh, Bio-Rad), which had been pre-equilibrated with 10 mM TRIS/HCl (pH 7.8) and 140 mM sorbitol. The proteoliposomes-specific radioactivity was quantified by LSC.

Arginine and ornithine transport assay: Putative antiport and uniport transport activities of BOU with the substrates arginine, citrulline and ornithine were investigated. In both cases the experiments were initiated with 0.25 mM [14C]-radiolabeled substrate and stopped by cation-exchange chromatography (AG50W-X8 Resin, 100-200 mesh, BioRad). Prior to the experiment, the hydrogen ion activated resin columns were converted to its lithium form by washing the resin with five volumes of 1 M lithium acetic acid (LiAc). Due to the greater affinity of arginine and ornithine compared to lithium-ions, both substrates could be efficiently removed from the external medium.

Glycine and serine transport assay: As described above, proteoliposomes were produced in the presence of 150 mM Tris/KOH (pH 7.8) or with 120 mM Tris/KOH (pH 7.8) and either 30 mM glycine or serine. A concentration of 0.25 mM [14C(U)]-L-serine and [14C(U)]-glycine were used to initiate the transport assay. External radiolabeled serine

and glycine were removed from the uptake medium by anion-exchange chromatography. Because the affinity of the resin was rather low for these substrates, the hydroxyl ion of the anion-exchange resin AG1W-X8 was replaced with the buffer molecule tricine. Liposomes were eluted from the column with 10 mM Tris/HCl (pH 7.8) and 140 mM sorbitol and quantified by LSC. To study the dependence of glycine and serine import on an electrochemical gradient across the membrane, artificial pH and potassium (K+) gradients were created in the presence of the ionophores nigericin (0.1 μg/mg PC) and valinomycin (1.5 μg/mg PC), respectively, as described by Indiveri et al., 1997. Alternatively, internal or external pH values were adjusted with the buffer compound MES/KOH (pH 6.0) to analyze the effect of serine and glycine transport by a pH gradient across the liposomal membrane.

Giant liposomes preparation and Patch-Clamp recording

Recombinant BOU protein was produced in *E. coli* and inclusion bodies purified as described above. 100 μL of solubilized protein was diluted with 1.4 mL resin buffer [0.1 M sodium phosphate buffer (pH 8), 0.3 mM sodium chloride, 0.01 mM imidazole, 2% (v/v) Triton X-100]. Nickel-NTA agarose (Qiagen) was added (100 μL) and gently shaken for one hour at 4°C. The agarose beads were pelleted (1,000 g, 0.5 min, 4°C), the supernatant discarded, and the beads were washed five times with resin buffer. The protein was eluted with 1 mL resin buffer containing 250 mM imidazole. The initial mixture for functional reconstitution contained 10 μg protein, 0.25% (v/v) Triton X-100, 0.35% (w/v) PC, 0.1 M KCl and 0.02 M MOPS/KOH (pH 8). Proteoliposomes were prepared by hydrophobic chromatography (Krämer and Heberger, 1986). To yield giant

liposomes suitable for patch-clamp measurements, the vesicles were centrifuged at 100,000x g for 1 hour at 4°C and resuspended in 40 μL MOPS/KOH (pH 7.4) containing 5% (v/v) ethylene glycol. Half of the liposomes were partially dehydrated on a microscope slide in a desiccator containing anhydrous CaCl2. After 3 hours the proteoliposomes were rehydrated with 20 μL 0.1 M KCl in a humidified Petri dish overnight to form giant liposomes (Criado and Keller, 1987; Keller et al., 1988; Delcour et al., 1989; Riquelme et al., 1990). Patch-pipettes were prepared from 1.5 mm borosilicate glass with a P-87 Flaming/Brown mircopipette puller (Sutter Instrument Company, Novato, CA). A Nikon Eclipse TE2000-S microscope (Nikon Instruments Europe B.V., Amstelveen, The Netherlands) and the Eppendorf PatchMan NP2 micromanipulator (Eppendorf AG, Hamburg, Germany) was used to achieve gigaohm seal formation with the giant liposomes. Patch-clamp recording was performed using the HEKA EPC10 patch clamp amplifier combined with the PatchMaster software (HEKA Instruments Inc. Bellmore, NY).

RESULTS

Putative metabolite transporter co-expressed with the photorespiratory pathway

The publicly available Comprehensive System-Biology DataBase (CSB.DB) (Steinhauser et al., 2004) provides a statistical tool to scan transcript profiles from the NASC's Affymetrix facility (matrix *nasc0271*; Craigon et al., 2004) and from the AtGenExpress consortium (matrix *atge0100* and *atge0200*, Schmid et al., 2005) for co-expressed genes in the model organism *A. thaliana*. In particular, we were interested in the enzymes

participating in the recycling of 2-phosphoglycolate to 3-phosphoglycerate. We showed that with the exception of glycerate kinase (GLYK), catalase (CAT2), and the subunits L1 and H2 of the glycine decarboxylase, all known photorespiratory genes form a robust cluster of 15 transcripts (data not shown) and thus represent a highly co-regulated metabolic pathway. We used the CSB.DB platform to retrieve additional candidates that co-respond with the strong gene cluster. Based on the Spearman's rank correlation coefficient (SRCC) the best 5% of each of the 15 individual gene queries from each of the three microarray matrices (*atge0100*, *atge0200*, and *nasc0271*) were screened for hydrophobic membrane proteins with at least two putative alpha helix transmembrane regions using the plant membrane database ARAMEMNON (Schwacke et al., 2003). These proteins represent candidate metabolite transporters, which could enable the high flux of metabolites between the subcellular compartments during photorespiration. Initially, 162 different hydrophobic proteins were identified in the top 5% of positively co-regulated transcripts. 32 out of the 162 hydrophobic genes show at least 30 out of the 45 possible co-responses with the fifteen query genes (Table 5.2) in the three microarray matrices, and are summarized in Table 5.3.

Table 5.2. Co-expressed photorespiratory enzymes from *Arabidopsis thaliana* Fifteen out of twenty known photorespiratory genes are highly co-regulated.

AGI	Gene Description	Localization	Reference
At5g36700	2-Phosphoglycolate Phosphatase (PGLP1)	Chloroplast	Schwarte and Bauwe (2007)
At5g35630	Glutamine synthetase (GS2/GLN2)	Chloroplast	Somerville et al. (1980)
At5g12860	2-Oxoglutarate:malate antiporter (DiT1/OMT1)	Chloroplast	Tanigushi et al. (2002)
At5g64290	Glutamate:malate antiporter (DiT2.1/DCT1)	Chloroplast	Renne et al. (2003)
At3g14420	Glycolate oxidase (GOX2)	Peroxisomes	Volokita and Somerville (1987)
At1g23310	Glu:glyoxylate aminotransferase (AOAT1/GGAT)	Peroxisomes	Igarashi et al. (2003)
At2g13360	Ala/Ser:glyoxylate aminotransferase (AGT1/SGT1)	Peroxisomes	Liepman et al. (2001)
At1g68010	Hydroxypyruvate reductase (HPR)	Peroxisomes	Murray et al. (1989)
At2g35370	Glycine decarboxylase H protein (GDH1)	Mitochondria	Bauwe et al. (2003)
At1g32470	Glycine decarboxylase H protein (GDH3)	Mitochondria	Bauwe et al. (2003)
At1g48030	Glycine decarboxylase L protein (LPD2)	Mitochondria	Bauwe et al. (2003)
At4g33010	Glycine decarboxylase P protein (GDP1)	Mitochondria	Engel et al. (2007)
At2g26080	Glycine decarboxylase P protein (GDP2)	Mitochondria	Engel et al. (2007)
At1g11860	Glycine decarboxylase T protein (GDT1)	Mitochondria	Bauwe et al. (2003)
At4g37930	Serine hydroxymethyltransferae (SHMT1)	Mitochondria	Voll et al. (2006)

Table 5.3. Putative transporter genes, which are transcriptionally co-regulated with the photorespiratory pathway

The comprehensive system-biology database was used to identify the top 5% of genes, which are co-expressed with 15 genes of the photorespiratory pathway (Table 5.2). Putative membrane proteins and transmembrane spans were extracted from this list using the ARAMEMNON database (Schwacke et al., 2003). Genes highlighted in bold were further tested in this study. Subcellular localization was experimentally demonstrated (Sun et al., 2008; Haezlewood et al., 2004) for those proteins with an asterisk (*).

	Candidate gene	Gene annotation	Putative transmembrane spans	Predicted subcellular localization
1	At1g10830	Sodium symporter-related	6	Chloroplast/ Mitochondria
2	At1g22850	Hypothetical protein	4 -5	Chloroplast
3	At1g32080	putative membrane protein (MEP1)	12	Chloroplast* (Envelope)
4	At1g44920	Hypothetical protein	4	Chloroplast*
5	At1g50730	Hypothetical protein	2	Mitochondria
6	At1g52870	Putative peroxisomal PMP22-type protein of unknown function	2	Chloroplast/ Mitochondria
7	**At1g54350**	Putative plastidial ABC-type transporter (AtPMP1)	5	Chloroplast* (Envelope)
8	At1g78180	Putative mitochondrial carrier protein	3-5	Chloroplast/ Mitochondria
9	**At2g42770**	Putative peroxisomal PMP22-type protein of unknown function	2	Chloroplast* (Envelope)
10	**At2g48070**	Protein of unknown function	3-4	Chloroplast
11	At3g02690	Membrane protein of unknown function	10	Chloroplast
12	At3g21580	Hypothetical protein	6	Chloroplast
13	At3g25805	Hypothetical protein	7	Chloroplast/ Mitochondria
14	**At3g26710**	Hypothetical protein	4	Chloroplast* (Thylakoid)
15	At3g48200	Hypothetical protein	9	Secretory pathway
16	At3g50685	Hypothetical protein	3	Chloroplast
17	At3g55250	Hypothetical protein	2	Chloroplast/ Mitochondria
18	At3g56160	Putative bile acid:sodium symporter	7	Chloroplast

Table 5.3. (cont'd).

	Candidate gene	Gene annotation	Putative transmembrane spans	Predicted subcellular localization
19	At3g59780	Protein of unknown function	3	Chloroplast/ Nucleus
20	At3g61870	Hypothetical protein	5	Chloroplast
21	At4g02920	Hypothetical protein	2	Unknown
22	At4g16410	Hypothetical protein	3	Chloroplast/ Mitochondria
23	At4g24090	Hypothetical protein	3	Chloroplast
24	At4g24460	Protein of unknown function	10	Chloroplast
25	At4g35760	Hypothetical protein	4	Chloroplast
26	At5g13720	Hypothetical protein	3	Chloroplast
27	At5g27290	Hypothetical protein	3	Chloroplast/ Mitochondria
28	At5g38660	APE1 (Acclimation of Photosynthesis to environment) – unknown function	2	Chloroplast*
29	At5g42130	Putative mitochondrial carrier protein	3-6	Chloroplast* (Envelope)
30	**At5g46800**	Putative mitochondrial carrier protein (BOU)	3-6	Mitochondria*
31	At5g57345	Protein of unknown function	2-3	Chloroplast/ Mitochondria
32	At5g64970	Putative mitochondrial carrier protein	3-6	Mitochondria* + chloroplast*

Phenotype of T-DNA insertion lines from candidate genes

Classical mutants in the photorespiratory pathway grow asymptomatically under elevated levels of carbon dioxide (CO2); however, they turn chlorotic under ambient air conditions (Somerville, 2001). To test the hypothesis that the 32 candidate genes are involved in the photorespiratory pathway, T-DNA insertion lines for several candidate genes were established and their ability to grow under ambient CO2 levels were evaluated. To this end, homozygous T-DNA exon insertion lines were established for the putative plastidial ABC-type transporter At1g54350 (*pmp1-1 & pmp1-2*) and the putative peroxisomal PMP22-type protein of unknown function At2g42770 (SALK118636) (Figure 5.1). Germination under elevated CO2 levels for ten days and shifting the seedlings to ambient air for two weeks induced the photorespiratory phenotype in the known serine-hydroxymethyltransferase (SHMT) mutant *shm1-2,* which was used as a positive control (Figure 5.2; Voll et al., 2006). In contrast, mutant lines *pmp1-1, pmp1-2* and SALK118636 had no obvious growth phenotype compared to *A. thaliana* wild type Columbia-0 plants (Figure 5.2). Seeds had also been germinated under ambient air conditions and in the presence and absence of sucrose as an external carbon source. Under all conditions tested, the three mutant lines were indistinguishable from the wild type (data not shown).

Heterozygous T-DNA insertion lines SALK055566 (At3g26710, hypothetical protein) and SALK066383 (At2g48070, protein of unknown function) were self-fertilized (Figure 5.1) and repeatedly screened for a T-DNA linked homozygous genotype. In both populations, only heterozygous and wild type-like lines germinated and developed normally, independent of the CO2 concentration in the growth chamber (data not shown).

Seedlings from homozygous T-DNA insertions lines of these genes developed white cotyledons and did not establish any new true leaves (Figure 5.2). An external carbon source in the MS medium or different CO2 levels in the growth chamber did not rescue the lethal phenotype (data not shown).

The homozygous T-DNA knockout line *bou1* (Figure 5.1) showed a typical photorespiratory phenotype (Figure 5.3). In ambient air, two week-old *bou1* seedlings displayed a pale green phenotype and were significantly smaller than the wild type Col-0 plants. However, *bou1* seedlings were indistinguishable from wild type when germinated at 0.3% CO2 (Figure 5.3). The CO2 dependent phenotype could also be observed after shifting 6 week old plants to ambient air conditions. After 14 days, *bou1* rosettes displayed a pale green phenotype and enhanced senescence, especially in the older rosette leaves (Figure 5.3). This prompted me to focus on the explanation of the *bou1* phenotype.

Figure 5.1 Isolation of *A. thaliana* T-DNA insertion lines for At1g54350, At2g42770, At2g48070, At3g26710, and At5g46800

Upper part: Gene structure and relative position of T-DNA insertions. A black box refers to an exon. T-DNA insertion sites are indicated by triangles and arrows indicate the relative positions of the primer for PCR analysis. Lower part: Genomic DNA PCR analysis of wild type *A. thaliana* Col-0 plants (WT) and T-DNA insertion lines. Lane G represents gene specific regions surrounding the T-DNA insertion and lane T shows the PCR reaction with primers specific for T-DNA/gene flanking region. Lane C refers always to AtACT2 control gene amplification with primer pair ML40/ML41. **(A)** PCR analysis of the (At1g54350) gene in *pmp1-1* (SALK003891) and *pmp1-2* (SALK069087). Gene specific products with primer pair ML102/ML103 are absent in the homozygous *pmp1-1* and *pmp1-2* mutants and present in WT. Primers LBa1/ML103 were used to amplify the T-DNA specific fragment. **(B)** At2g42770: T-DNA insertion (SALK118636) was verified with primers LBa1/ML105 and absence of the amplified gene fragment At2g42770 in the mutant line (ML104/ML105). **(C)** PCR analysis of the heterozygous T-DNA insertion line SALK055566 (At3g26710). Only plants with amplification of target gene (primer ML108/ML109) and T-DNA flanking region (LBa1/ML109) were isolated. **(D)** PCR analysis of the T-DNA insertion line SALK066383 (At2g48070). GenePrimers ML107/ML120 and T-DNA insertion was verified with primers LBa1/ML107. **(E)** Putative mitochondrial acyl-carnitine carrier protein (BOU) At5g46800. Homozygous T-DNA insertion line *bou1* (GABI Kat line 079D12). ML116/ML114 verified T-DNA specific insertion and primer pair ML115/ML114 were used for genomic bou fragment.

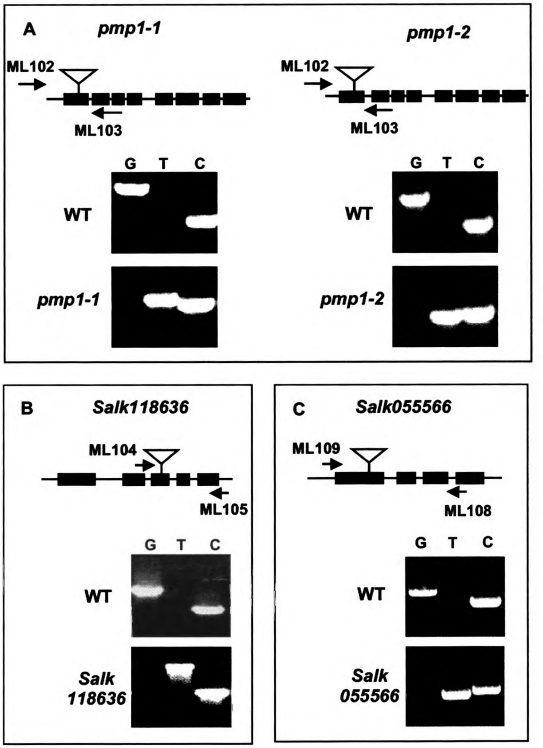

Figure 5.1. Isolation of *A. thaliana* T-DNA insertion lines

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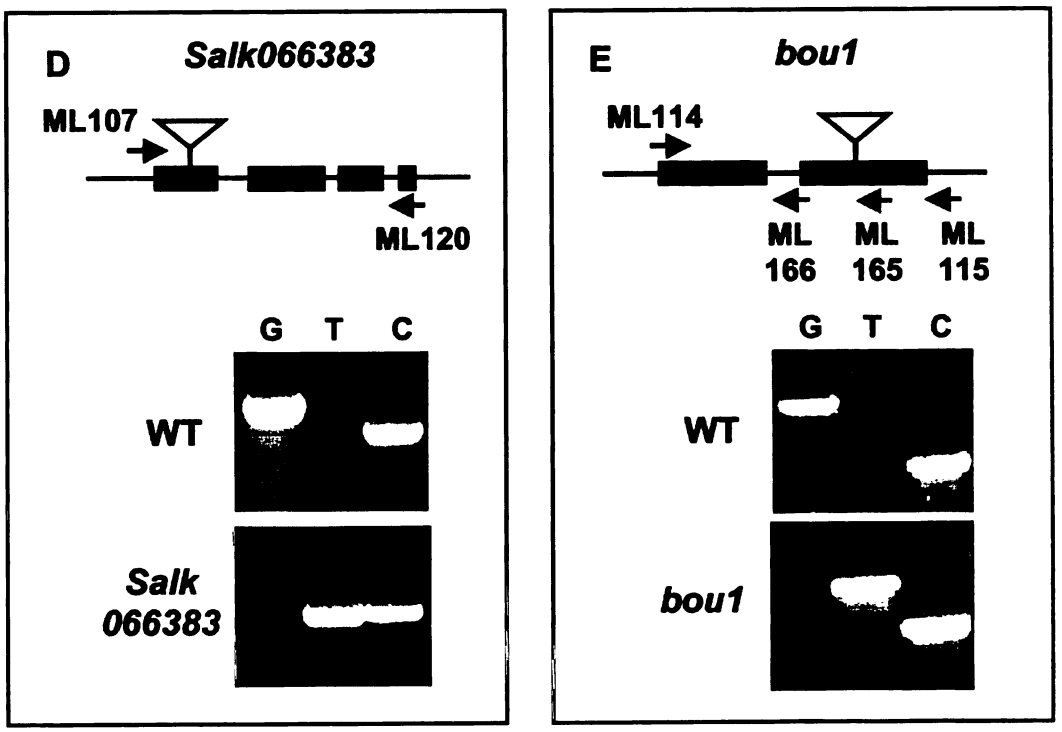

Figure 5.1. (cont'd).

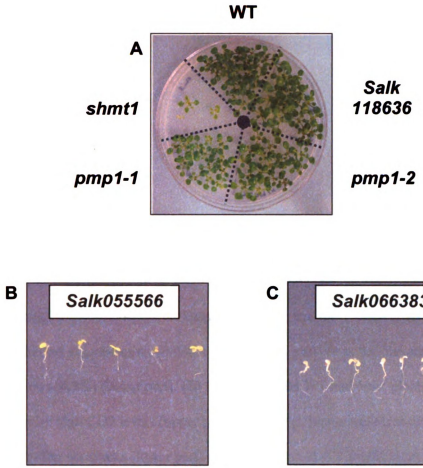

Figure 5.2. Growth phenotype of T-DNA insertion lines from the candidate genes At1g54350, At2g42770, At2g48070, and At3g26710

(A) Seedlings from *A. thaliana* Col-0 (WT), T-DNA insertion lines from At1g54350 (*pmp1-1, pmp1-2*), At2g42770 (SALK118636*)* and the photorespiratory mutant *shmt1-2* (Voll et al. 2006) were germinated under elevated CO2 concentrations (0.3%) for ten days and the picture was taken two weeks after transfer to ambient air (0.038% CO2). **(B)** Homologous T-DNA insertion lines from gene At3g26710 (SALK055566) and **(C)** At2g48070 (SALK066383) were sown under elevated CO2 concentrations and grown for two weeks. The figures in this dissertation are presented in color.

Figure 5.3. Growth phenotype of T-DNA insertion line *bou1*

(A) Seedlings from *Arabidopsis thaliana* Col-0 (WT) and the T-DNA insertion line *bou1* were germinated at ambient air (0.038% CO2) (upper part) or under elevated CO2 concentrations (0.3%) (lower part). **(B)** Eight-week old WT and *bou1* plants had been growing under high CO2 levels (upper part). In parallel, plants were transferred to ambient air after six weeks (lower part) and photographed two weeks later. The figures in this dissertation are presented in color.

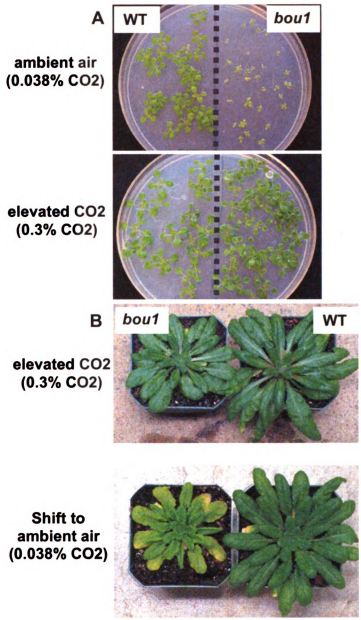

Figure 5.3. Growth phenotype of T-DNA insertion line *bou1*

Rosette leaves of *bou1* accumulate glycine under ambient and elevated CO2 levels

For several photorespiratory mutants it had been demonstrated that one or more intermediates of the pathway accumulate after transfer to ambient air (Somerville, 2001). To corroborate the phenotypic analysis of *bou1*, we quantified photorespiratory marker metabolites, such as glyoxylate, glycine, serine, and NH3 in Col-0 (WT) and *bou1* upon shift from 0.3% CO2 to 0.038% CO2 (Figure 5.4). The most dramatic change was observed for the amino acid glycine. Within 48 hours the glycine concentration in *bou1* rapidly increased from 3.43 ±0.44 μmol per gram fresh weight to 25.64 ±1.76 μmol per gram fresh weight (8-fold). Even more dramatic is the comparison between wild type and *bou1* plants: After two days in ambient air, glycine levels in the mutant were 130-times higher than in the wild type (Figure 5.4). Interestingly, even in high CO2 conditions, the glycine content in the mutant was 25 – 30 times higher than in the wild type. We observed a constant increase of glyoxylate over the time course experiment under ambient air from 31.61 ±4.03 nmol per gram fresh weight to 312.8 ±65.8 nmol per gram fresh weight. This was not observed under elevated CO2 conditions, where glyoxylate is even less abundant compared to WT. The metabolites ammonium ion and serine (Figure 5.4) and glutamate, glutamine and ornithine (Figure 5.5) did not change dramatically during the first five days of the shift experiment in WT and *bou1*, respectively. After seven days of ambient air the compounds serine, glutamine and ornithine are slightly higher in the *bou1* plants. Citrulline levels (Figure 5.5) were always higher in *bou1* plants. However, the 10-fold difference in CO2 concentration between the growth chambers did not have a large effect on the absolute citrulline levels.

Figure 5.4. Concentrations of photorespiratory marker metabolites glyoxylate, ammonium ion, glycine, and serine in *bou1* leaves

Arabidopsis thaliana Col-0 (WT, black bars) and *bou1* (white bars) plants were grown at elevated CO2 concentrations (0.3%) for four weeks. Metabolites were analyzed in plants maintained at elevated CO2 levels (left panel) or moved to ambient air (right panel). Samples were taken prior to and two, four and seven days after shifting to ambient air (d). Results are given as the mean ±SD of three biological replicates in nmol per gram fresh weight for glyoxylate and NH4+ or in µmol per gram fresh weight in the case of glycine and serine. FW, fresh weight; CO2, carbon dioxide.

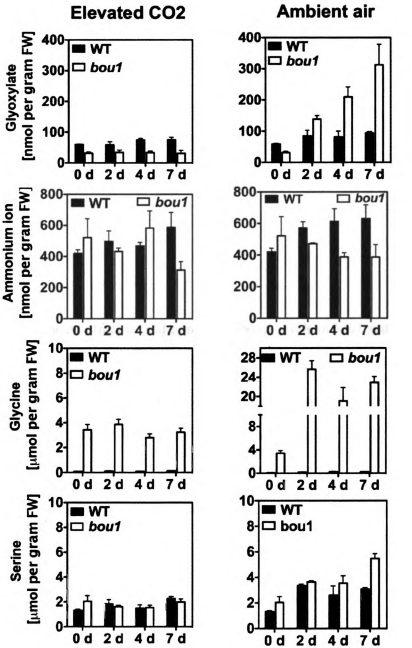

Figure 5.4. Concentrations of photorespiratory marker metabolites glyoxylate, ammonium ion, glycine, and serine in *bou1* leaves

Figure 5.5. CO2-dependent effect on additional metabolite levels in *bou1* and WT

The identical samples as described in Figure 5.4 were analyzed and glutamate, glutamine, ornithine, and citrulline levels were determined. Concentrations are shown as the mean ±SD of three biological replicates in μmol per gram fresh weight (FW). CO2, carbon dioxide.

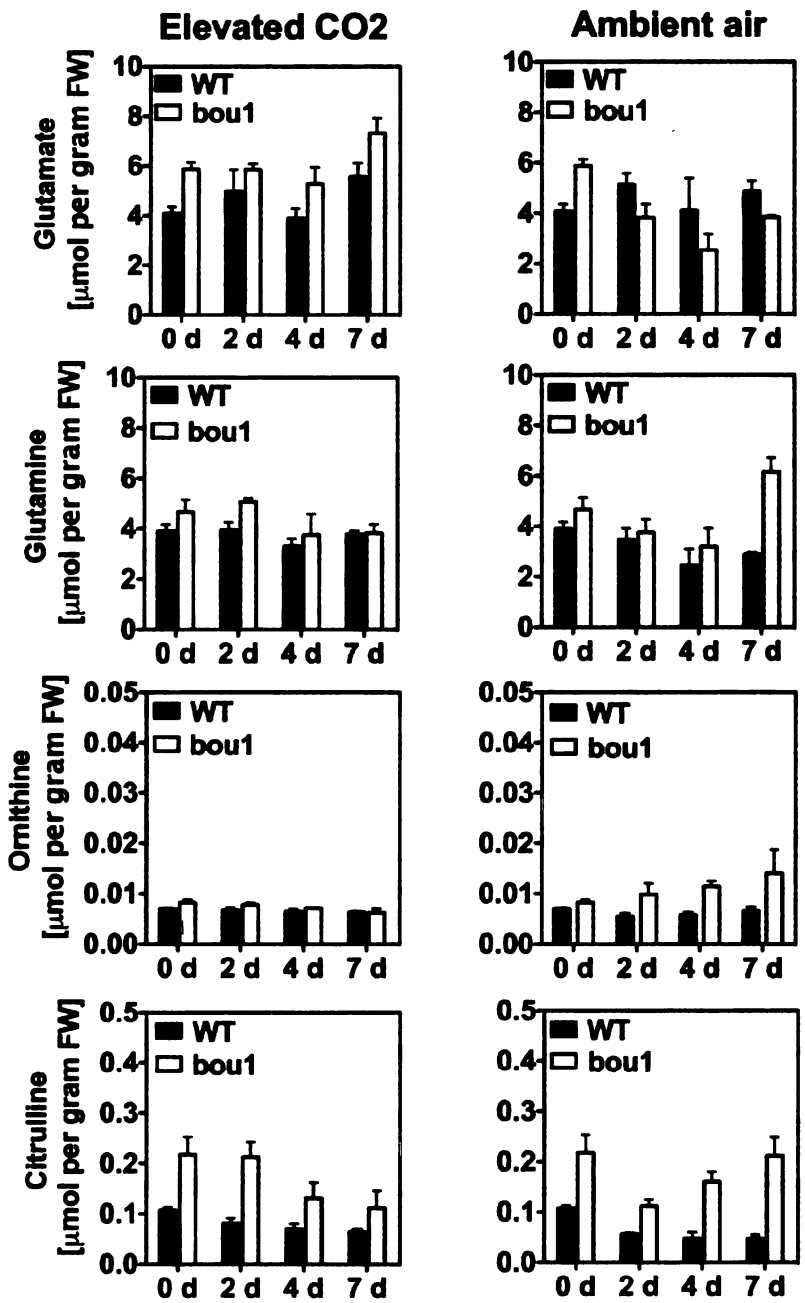

Figure 5.5. CO2-dependent effect on additional metabolite levels in *bou1* and WT

Molecular complementation of the *bou1* mutant

Although a truncated cDNA of the *BOU* gene could still be identified by RT-PCR in *bou1* (Figure 5.6), immunoreactive protein was not detectable in the mitochondrial fraction (Figure 5.9) of *bou1* plants. A *bou1* mutant expressing BOU under the control of the 35S promoter (*bou1:BOU*) (Figure 5.6) germinated and developed normally under ambient air conditions (data not shown) and no significant glycine accumulation was observed (Figure 5.7), showing that the *bou1* mutation causes the CO2-dependent phenotype.

Glycine decarboxylase (GDC) activity is reduced in *bou1*

Elevated levels of glycine and unaltered concentrations of serine and ammonium ions in the *bou1* mutant suggested impaired photorespiratory glycine oxidation due to either a deficiency in glycine uptake into the mitochondria or a lack of GDC activity. Therefore, mitochondria were isolated from high CO2 adapted WT and *bou1* plants, the envelope membranes were permeabilized with detergent, and the rate of [U-14C]-glycine decarboxylation to [14C]-CO2 by GDC was determined (Figure 5.8). In *bou1*, the apparent rate of the GDC reaction was only 15.2 ± 5.3% of that observed for wild type. We further tested the abundance of the GDC and SHMT proteins and the presence of the crucial GDC cofactor lipoic acid (Figure 5.9). The GDC H protein subunit, SHMT and lipoylated proteins were not affected in mitochondrial *bou1* fractions. However, a subtle negative effect on the stability of the PLP-binding GDC subunit P was observed in the *bou1* mutant (Figure 5.9).

Figure 5.6. Molecular complementation of *bou1*

The *bou1* mutant plants were transformed with BOU cDNA driven by the constitutive CaMV 35S promoter. **(A)** PCR analysis of genomic DNA (gDNA) from *A. thaliana* Col-0 (WT) and a representative complementation line *bou1/BOU*. Lane 1: Amplification of an endogenous *BOU* fragment with a *BOU* promoter-specific primer ML114 and primer ML166. Lane 2: Primer pair ML114/ML165 was used to confirm the *bou1* background in the complementation line. Lane 3: 35S-specific primer (ML143) and primer ML166 demonstrate the presence of the complementation construct in the gDNA. **(B)** RT-PCR fragments from *bou1* and a representative complementation line. Lane 1: cDNA amplification with primer pair ML164/ML165 demonstrates restored transcript of *BOU*. Lane 2: Truncated BOU cDNA still present in the *bou1* mutant. RT-PCR was done with primer ML164 and ML166. Lane 3: RT-PCR control with AtACT7 (actin) specific primer set ML167/ML168.

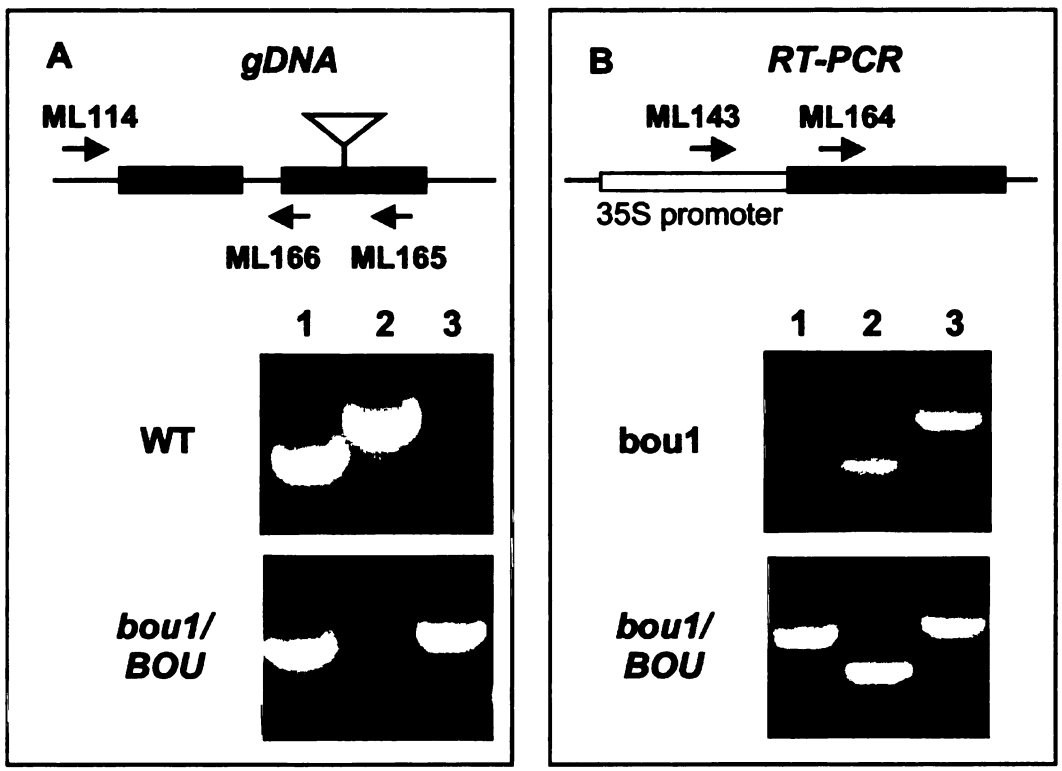

Figure 5.6. Molecular complementation of *bou1*

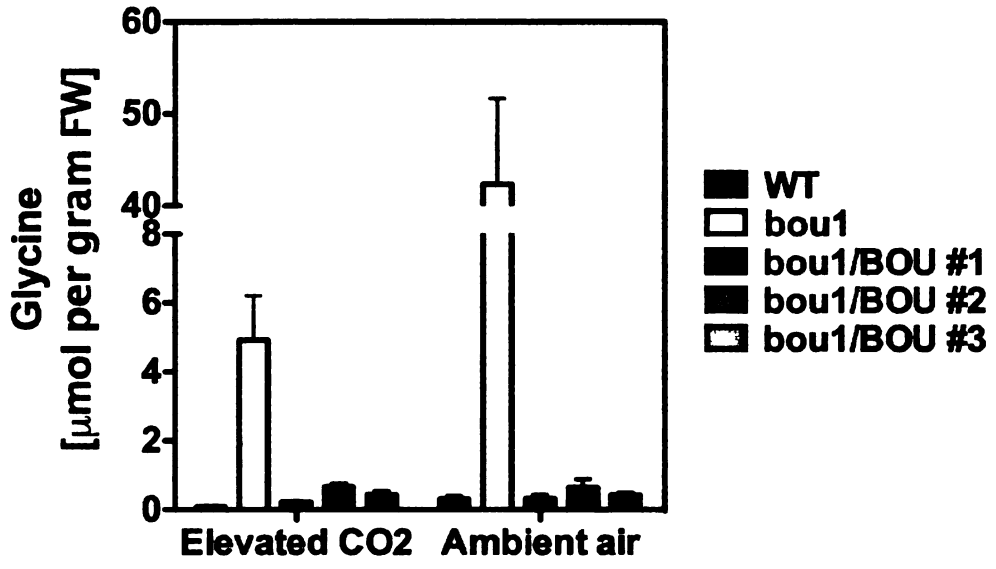

Figure 5.7. Complementation of the *bou1* mutant restores glycine concentrations to wild type levels

A 35S:BOU construct was introduced into the *bou1* mutant line and several independent homozygous complementation lines (*bou1*/BOU) were established. A fraction of high CO2 adapted wild type (WT, black bars), *bou1* (white bars) and bou1/BOU (grey bars) plants were shifted to ambient air and glycine concentrations were determined after seven days. Glycine concentrations are given as the mean ±SD of three technical replicates in µmol per gram fresh weight. CO2, carbon dioxide; FW, fresh weight.

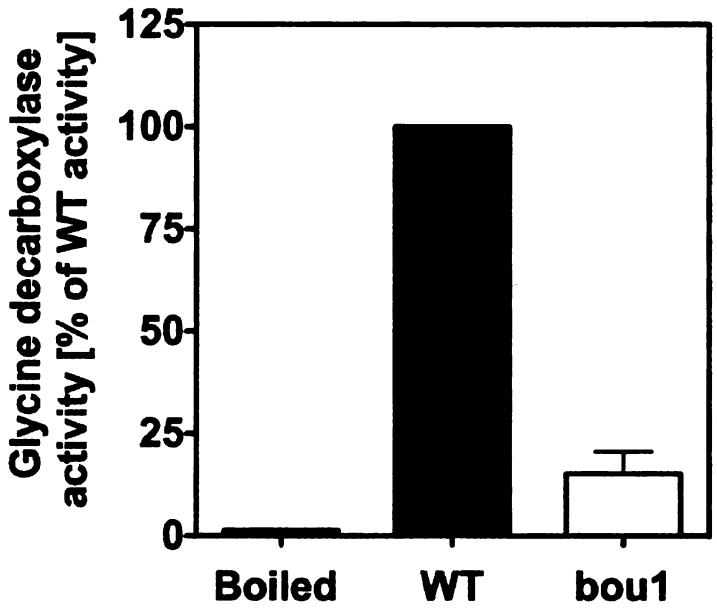

Figure 5.8. Mitochondrial glycine oxidation is impaired in *bou1*

Arabidopsis thaliana Col-0 (WT, black bar) and *bou1* (white bar) plants were grown under elevated CO2 concentrations. Mitochondria were isolated from four week-old leaves and permeabilized with the detergent Triton X-100. An aliquot was incubated with 0.2 mM [U-14C]-glycine for 15 min and the released [14C]-CO2 was determined. As a control, boiled mitochondrial proteins were monitored to rule out non-enzymatic breakdown of glycine. Relative rates are given as mean ± SD. Relative glycine decarboxylase activity (GDC) of 100% refers to 110.29 nmol CO2 per gram protein.

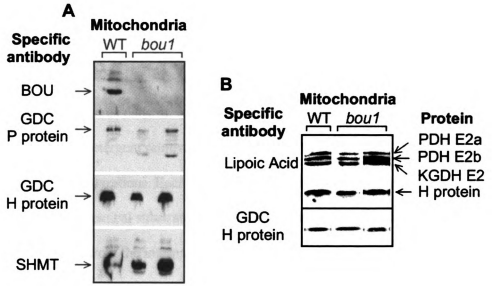

Figure 5.9. Immunoblot analysis of mitochondrial enzymes

(A) A mitochondrial preparation from *A. thaliana* Col-0 (WT) and two preparations of the *bou1* mutant line as described in Figure 6 were analyzed for the subunits P and H of the glycine decarboxylase (GDC), BOU and serine methyltransferease (SHMT). **(B)** A lipoic acid specific antibody analyzed the presence of the lipoylated subunits E2a and 2b from pyruvate dehydrogenase (PDH), the H subunit from GDC, and the E2 subunit of the α-ketoglutarate dehydrogenase (KGDH). Protein extracts were separated on a 15% SDS-PAGE, immobilized on a PVDF membrane and analyzed with the protein-specific antibodies and ECL.

Search for the substrate of the recombinant BOU protein

To investigate the transport activities of the putative carnitine carrier BOU, the protein was expressed in *E. coli* and *S. cerevisiae* (Figure 5.10). The recombinant transport proteins were tagged with a N-terminal 6x His-epitope for detection and purification from the cell extracts. In addition, a C-terminal His-tagged construct was produced in yeast (Figure 5.10). The three constructs were reconstituted by either the freeze/thaw method (FT) or the cyclic detergent removal procedure (CDRP) resulting in six different proteoliposome preparations. Each was tested with a number of putative substrates in a homo-exchange (identical substrate inside and outside), hetero-exchange (external substrate different from the internal), or uniport (no counter-substrate inside) experiment (Table 5.4). Because the substrate carnitine was hypothesized earlier by Lawand et al. (2002), our observation that the *bou1* mutant accumulates glycine (Figure 5.4), and the fact that serine is capable to inhibit glycine uptake into isolated pea mitochondria (Yu et al., 1983), these three substrates were the primary targets of analysis. However, none of these metabolites was taken up into BOU-reconstituted liposomes with significant rates (data not shown). Electrochemical gradients across the liposomal membrane had no positive effect on the negligible glycine or serine transport activity catalyzed by recombinant BOU (Table 5.5).

In a search for other candidates, we next employed a phylogenomics approach. The mitochondrial carrier family (MCF) is widely distributed in eukaryotic organisms. A maximum-likelihood phylogenetic tree was generated with the MCF members from *A. thaliana*, yeast, human, and the red algae *Cyanidioschyzon merolae* and *Galdieria sulphuraria* (Figure 5.11). BOU belongs to a sub-clade of the MCF in which all

functionally tested members transport several amino acids (summarized in Table 5.5). We hence tested uptake of these substrates into liposomes reconstituted with BOU. Neither the proteinogenic amino acids glutamate, glutamine, arginine, nor the non-proteinogenic amino acids ornithine, citrulline were accepted as a substrate. Since GDC activity is diminished in the *bou1* mutant, it is reasonable to hypothesize, that BOU could be involved in the supply of GDC cofactors or precursors for cofactor biosynthesis, which are needed in the mitochondrial matrix. Table 5.6 lists putative substrates, which likely have to be transported across the mitochondrial membranes to provide the biological active cofactors PLP, NAD, FAD, THF and lipoic acid for glycine oxidation (Douce et al., 2001). Except for malonic acid, none of the compounds are available in a radiolabeled form. Thus, flux analysis in a reconstituted liposome system cannot be applied to these substrates. Hence, a method had to be established that permits the screening on non-radiolabeled BOU substrates. A promising approach is the patch-clamp technique, which however requires reconstitution of BOU into giant liposomes that can be visualized in the light microscope. To this end, *E. coli* expressed BOU protein was purified to homogeneity by Nickel-NTA chromatography (Figure 5.10) and reconstituted into liposomes. Giant liposomes were then produced from small, unilamellar liposomes by a dehydration/rehydration step. Using these giant liposomes, pipette-membrane seals could be reproducibly obtained with a resistance of 1-4 giga ohm, which is suitable for patch-clamp recording. Figure 5.12 shows that BOU reconstituted into giant liposomes has voltage-dependent ion channel properties with discrete ion current levels between the applied voltages +100 mV and +200 mV. Seals with resistances of 5-10 GΩ were also achieved with protein-free liposomes, however no voltage-dependent ion channel

activities could be detected between membrane potentials of -140 mV and +200 mV (data not shown). This experimental set-up enables us to test the effect of putative substrates on the channel activity of BOU. The current hypothesis is, that the correct substrate has a high affinity for its binding site and will thus block the voltage-dependent ion channel activity of the BOU transporter, which would be detected by a decreased open probability of the channel. The substrates in Table 5.4 and Table 5.6 are under investigation.

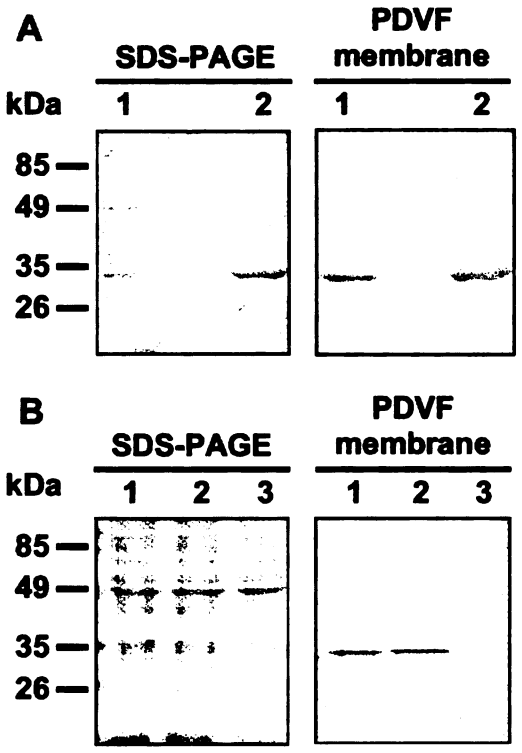

Figure 5.10. Heterologous expression and purification of BOU protein in *E. coli* and *S. cerevisiae*

(A) Inclusion bodies of BOU producing *E. coli* cells (lane 1) and Ni-NTA purified BOU protein (lane 2) were separated by SDS-PAGE and stained with Coomassie Blue. The proteins of an identical gel were blotted on a PDVF membrane and the N-terminal His-tagged BOU was verified with an anti-His antibody. **(B)** Membrane proteins from yeast cells were separated on a 12% SDS-polyacrylamide gel and expression of N-terminal-His-tagged (lane 1) and C-terminal-His-tagged (lane 2) recombinat BOU protein was demonstrated with an anti-His antibody. Protein extracts from yeast cells, which had been transformed with an empty expression vector are shown in lane 3.

Table 5.4. Systematic search for the BOU substrate
N-terminal His-tagged BOU protein was expressed in yeast and *E. coli*. In addition, BOU was also produced with a C-terminal fused His-tag in yeast and tested. The purified protein was reconstituted into liposomes by the freeze/thaw (FT) procedure or by the cyclic detergent removal procedure (CDRP) as described in "Material and Methods".

Radiolabelled external substrates	**Liposomes preloaded with**	**Recombinant protein expressed in**	**Functional reconstitution procedure**
[14C]-Carnitine	Carnitine None	Yeast & *E. coli*	FT & CDRP
[14C]-Glycine	Glycine Serine None	Yeast & *E. coli*	FT & CDRP
[14C]-Serine	Serine Glycine None	Yeast & *E. coli*	FT& CDRP
[14C]-Glutamate	Glutamate Glutamine None	Yeast	FT
[14C]-Glutamine	Glutamine Glutamate None	Yeast	FT
[14C]-Arginine	Arginine Ornithine Citrulline None	Yeast	FT
[14C]-Ornithine	Ornithine Citrulline Arginine None	Yeast	FT
[14C]-Malonic acid	Malonic acid None	Yeast & *E. coli*	FT & CDRP

Table 5.5. Presence of a membrane potential does not stimulate glycine or serine transport by BOU
The ionophore valinomycin induced a potassium (K+) diffusion potential gradient to analyze its influence on glycine or serine transport. To generate an artifical pH gradient with the K+/H+ exchanger nigericin, the internal (in) medium (outward K+ directed gradient) or the external (out) medium (inward K+ directed gradient) was not buffered (NB), respectively.

Liposomes preloaded with	**pH in / pH out**	**K+ (in) / K+ (out) [mM/mM]**	**Valinomycin**	**Nigericin**
Glycine, serine, none	8 / 8	1 / 1	+	
	8 / 8	50 / 1	+	
	8 / 8	1 / 50	+	
Glycine, serine, none	NB / 7	1 / 1		+
	NB / 7	50 / 1		+
Glycine, serine, none	7 / NB	1 / 1		+
	7 / NB	1 / 50		+
Glycine, serine, none	8/6			
	6/8			

Table 5.6. Several cofactors must be provided for the mitochondrial glycine decarboxylase (GDC)
For cofactor biosynthesis inside the mitochondria, precursors have to be imported. If a cofactor biosynthesis occurs outside the mitochondria, the biologically active form must be provided from the cytosol. All steps require protein-mediated transport steps across the inner envelope mitochondrial membrane. Abbreviations: PLP, pyridoxal 5-phosphate; SAM, S-Adenosylmethionine; NaMN, nicotinate mononucleotide; pABA, para-aminobenzoic acid.

GDC subunit	Cofactor	Final step cofactor biosynthesis	Predicted transport processes across mitochondrial membrane
P protein	PLP	Cytosol	PLP Pyridoxine Pyridoxal
H protein	Lipoic acid	Cytosol	Malonic acid Pyruvate "Sulfur" donor (cysteine, SAM) CoenzymeA (Valine, ketopantoate, CoA)
T protein	THF	Mitochondria	Pterin, pABA, glutamate
L protein	FAD	Cytosol	FAD
L protein	NAD	Cytosol / Mitochondria	Quinolinate NaMN NAD

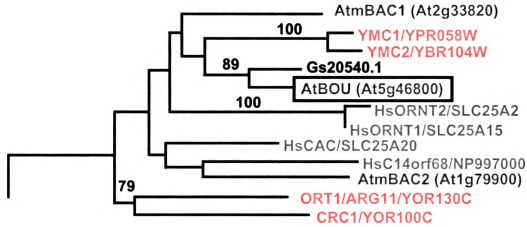

Figure 5.11. BOU relevant branch from the maximum-likelihood unrooted tree of the mitochondrial carrier family (MFC)

MCF sequences from yeast, plants and human were obtained from NCBI (http://www.ncbi.nlm.nih.gov/). The red algae sequences were extracted from the *Galdieria sulphuraria* (http://genomics.msu.edu/galdieria) and *Cyanidioschyzon merolae* (http://merolae.biol.s.u-tokyo.ac.jp) genome project database. Protein annotations from yeast, human, plants and red algae are written in brown, blue, green and red, respectively. Bootstrap values are pointed out at the corresponding nodes. The figure in this dissertation is presented in color.

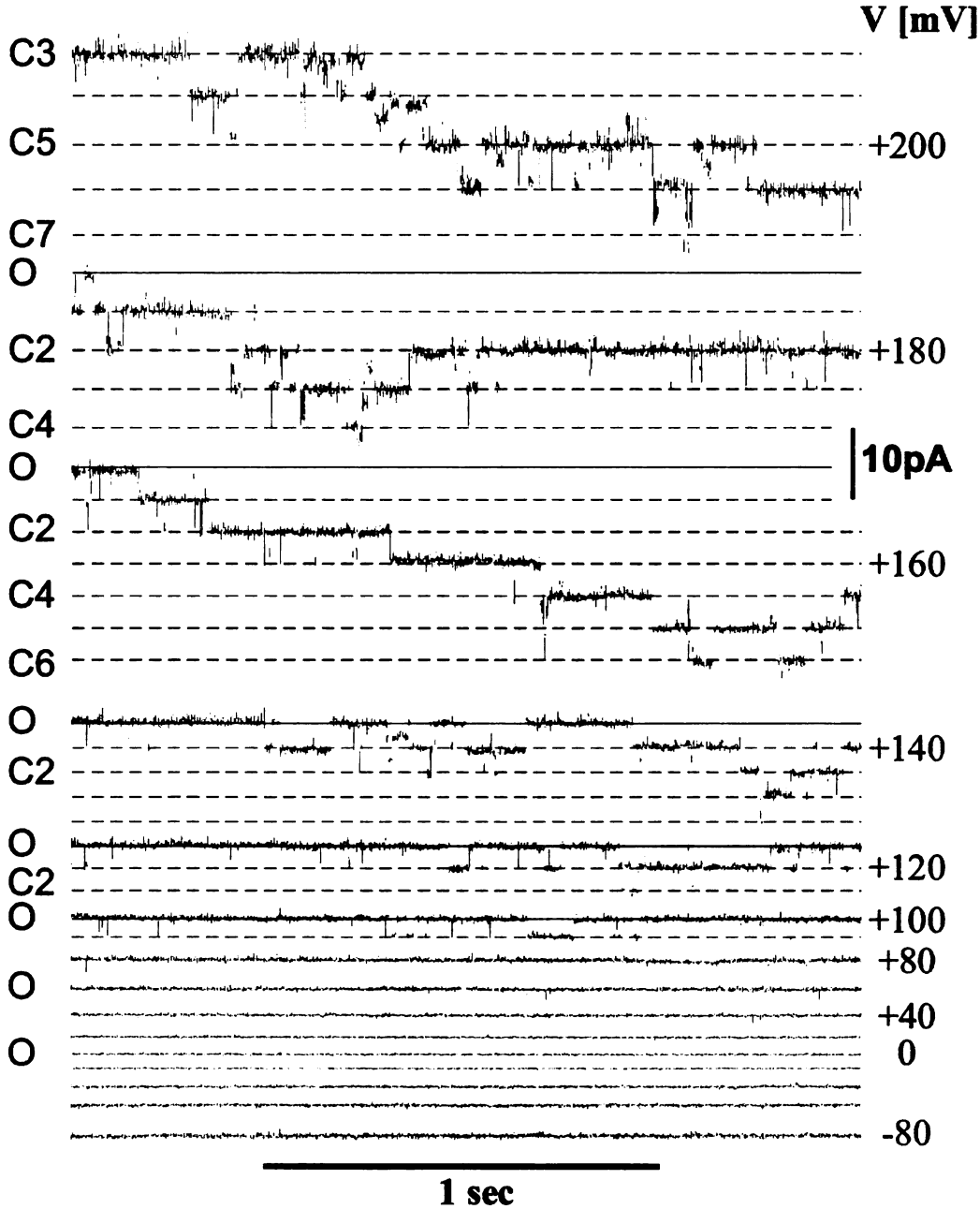

Figure 5.12. Reconstituted BOU protein exhibits ion channel properties

Purified BOU protein was reconstituted into giant liposomes. Single channel currents were recorded in a symmetrical 100 mM KCl buffer between negative (-80 mV) and positive (+200 mV) pipette potentials (V). Currents levels of open state (O) and closed state (C) channels are shown.

DISCUSSION

Identification of membrane proteins co-expressed with photorespiration

The carbon and nitrogen cycles of the photorespiratory pathway are distributed in three different compartments and acting jointly to recycle two molecules 2-phosphoglycolate to 3-PGA (Tolbert, 1997). A prerequisite to facilitate the traffic of the photorespiratory intermediates are numerous shuttles inserted into the plastid, peroxisomal and mitochondrial membranes. As an alternative to identify these transporters by forward genetics, the CSB.DB resource was screened for hydrophobic membrane proteins that are highly co-regulated with known photorespiratory genes (Steinhauser et al., 2004). As commented by Wei et al. (2006) the photorespiration is embedded in the expression pattern of genes from the photosynthetic apparatus and the Calvin-Benson-cycle. Several components of the photosynthetic electron transport chain were indeed found to be repeatedly co-expressed with photorespiration, since the light reaction overlaps temporarily and spatially with the photorespiration and the initial substrate ribulose-1,5-bisphosphate and final product 3-PGA are directly connected to the Calvin-Benson-cycle. The expression profiles of several metabolite transporters were also tested. The Calvin-cycle associated triose-phosphate/phosphate transporter, which exports recently fixed CO2 into the cytosol, was retrieved from our CSB.DB search. Other transporter genes such as the mitochondrial basic amino acid transporter (AtmBAC1) or the peroxisomal nucleotide transporter AtPNC1 do not cluster with the query genes and point out the applicability to screen for photorespiratory candidate transport proteins. To prioritize the candidates for reverse genetic analysis, already characterized carrier proteins and thylakoid-localized membrane proteins were neglected. Despite the progress in proteomic

analysis of all three organelles and GFP studies (Millar et al., 2001; Reumann et al., 2004; Sun et al., 2008), the localization of more than two-third of our candidate genes (23 out of 32) is predicted solely by *in silico* targeting programs. Seven out of the nine candidate proteins were found by *Arabidopsis* chloroplast proteomic studies (Sun et al., 2008), one is postulated to be dual targeted to mitochondria and plastid (At5g64970) (Dunkley et al., 2006) and the remaining candidate is inserted into the mitochondrial membrane (At5g46800) (Lawand et al., 2002).

Photorespiratory transport systems across the plastid inner envelope membrane

Besides the two-translocator shuttle for 2-OG/glutamate recycling in the plastid (Renne et al., 2003), a single transport protein for glycolate/glycerate antiport or their uniport was demonstrated on intact chloroplasts (Howitz and McCarty, 1991). This flexibility in the transport mode (i.e. uniport vs. antiport) is important, because for every recycled and finally imported glycerate two molecules glycolate have to be exported from the plastid (Figure 1). The nitrogen cycle would require a transport system for the ammonium ion, glutamate/glutamine or ornithine/citrulline performed by three to maximal five proteins across the chloroplast inner envelope membrane (Linka and Weber, 2005). Transport of glutamine and citrulline was demonstrated on intact chloroplast - its relevance in photorespiration, however, is speculative (Yu and Woo, 1988; Ludwig, 1993).

Eighteen candidate proteins have a robust plastidial computer-predicted targeting and seven of them were in addition identified by proteomics. T-DNA insertion lines for four plastid-targeted genes did not show a conditional chlorotic phenotype under ambient air conditions (Figure 5.2). Homozygous mutant lines from At2g48070 and At3g26710

lack pigments and seedlings did not develop beyond the cotyledon stage. At2g48070 has been identified by a recent proteomics study as a protein targeted to thylakoid membranes (Zybailov et al., 2008). These results direct its function towards the biogenesis of the photosynthetic apparatus. In the case of SALK line 055566 (At3g26710) only heterozygous mutant lines fully developed. Exon-insertion lines for At1g54350 and At2g42770 grew normally in ambient air (Figure 5.2). At1g54350 (AtPMP1) is annotated as one of 129 ABC transporter proteins in *A. thaliana* (Sanchez-Fernandez et al., 2001). At2g42770 is related to the peroxisomal pore-forming 22 kDa membrane protein PMP22 from human, however, not further characterized yet (Tugal et al., 1999; Visser et al., 2007). Seven additional proteins are annotated as PMP22 or PMP22-related proteins and targeted to plastid, peroxisome and mitochondria in the genome of *A. thaliana* (ARAMEMNON database; Schwacke et al., 2003). At this point it cannot be ruled out that the PMP22 members, AtPMP1 or gene product At3g26710 are involved in photorespiration and it is tempting to speculate that genetic analyses are masked by functional redundancy or by the severity of the loss of function mutant, respectively. The majority of photorespiratory mutants thrive under elevated CO2 concentrations. This is not true for mutants deficient in the enzyme glycine decarboxylase (Engel et al., 2007). A complete loss of the enzyme activity is lethal and alternative strategies such as inducible RNAi targeted gene silencing might be necessary to aim for novel photorespiratory mutants.

The mitochondrial envelope membrane transport protein BOU is required for photorespiratory function

BOU (At5g46800) was the only evident mitochondrial transport candidate that was retrieved from the co-expression data (Table 5.3). The gene belongs to the mitochondrial carrier family (MCF) and was previously published as a putative carnitine/acylcarnitine carrier providing alternatively acetate as a carbon and energy source to the mitochondria (Lawand et al., 2002). Under long-day conditions, the seedlings were not capable to establish photoautotrophic growth. In this study, we isolated a second mutant allele (*bou1*) and demonstrated that "non-photorespiratory" conditions can completely rescue the seedling's development (Figure 5.3). The classical pale green phenotype was not apparent until the 14th day, which might explain why Somerville & Ogren (1979) did not isolated an EMS mutagenized plant with a deficient BOU gene. Their genetic screen concentrated on visible chlorotic effects after four days. Importantly, metabolic analysis of *bou1* demonstrated a massive accumulation of glycine already under 0.3% CO2 and even more dramatic increase at the second day at ambient air (Figure 5.4). This suggests that metabolic profiling of photorespiratory marker metabolites is a powerful tool to identify additional photorespiratory mutants that do not display an apparent phenotype. Glycine import and serine export are the most prominent transport processes during photorespiration in the mitochondrial envelope membrane. Two studies on isolated mitochondria showed that both substrates could interfere with each other for uptake (Walker et al., 1982; Yu et al., 1983). Analogous to the plastid glycolate/glycerate shuttle, a single protein is hypothesized to mediate a glycine/serine transport in a stoichiometry of 2:1. Numerous attempts with distinct experimental conditions failed to

show transport activity of glycine, serine, or carnitine with recombinant BOU (Table 5.4 and Table 5.5). Elevated citrulline levels (Figure 5.5) and the phylogenetic relation to basic amino acids transporters (Figure 5.11) directed the analysis to test the hypothesized ornithine/citrulline and glutamine/glutamate shuttle system without any evident activity. Intriguingly, *bou1* exhibits a similar glycine accumulation in leaves as knockout mutants affected in the provision of cofactors lipoic acid and THF for the glycine decarboxylase complex (Ewald et al., 2007; Collakova et al., 2008). The GDC activity was diminished (Figure 5.8) in *bou1* and the PLP dependent P protein subunit was partially degraded (Figure 5.9). The Vitamin B6 derivative PLP is synthesized in the cytosol (Tambasco-Studart et al., 2005) and GDC is the most abundant soluble protein in leaf mitochondria - therefore a major sink for PLP (Sarojini and Oliver, 1983; Douce et al., 2001; Bauwe and Kolukisaoglu, 2003). Taking both arguments into account, we hypothesize that BOU provides PLP to the mitochondria. Since PLP covalently binds to the P protein subunit and is an integral part of its active site, it is tempting to speculate that a distorted P protein lacking PLP is prone to degradation (Figure 5.9). Since additional enzymes such as amino-transferases are dependent on PLP and a GDC knockout is lethal, an impaired, but not complete lack of PLP provision, has to be proposed at this point. Using the patch-clamp technique we demonstrated that the BOU is functional when reconstituted into liposomes and displays voltage-dependent ion channel activity (Figure 5.12). This suggests that the previously tested metabolites, which were reconstituted with the same procedure, are not the substrates of BOU. Future studies will show if PLP, pyridoxal or pyridoxine can inhibit the voltage-dependent ion channel activity of BOU. Identification of the major mitochondrial glycine import protein will be challenging. The *A. thaliana*

genome predicts at least 53 selective or broad-spectrum amino acids transporters distribute in every plant tissue and in most subcellular compartments including plasma membrane, mitochondria, plastid and peroxisomes (Wipf et al., 2002). Complete loss of glycine import would stall the essential GDC activity and in a broader view the mitochondrial protein synthesis (Engel et al., 2007). For protein synthesis a complete set of amino acids is required and a sophisticated and partial overlapping import system for mitochondria can be postulated. Mutant analysis of promising amino acid transporters combined with metabolite profiling might help to resolve the major route for glycine import.

Photorespiratory transport systems in the peroxisomal membrane

In comparison to mitochondria and plastids, very little is known about peroxisomal membranes (Reumann et al., 2004; Reumann and Weber, 2006). The isolation of pure peroxisomal membrane fraction for proteomic studies is challenging and a target signal of peroxisomal membrane proteins for computer-based analysis is not deciphered yet (Reumann et al., 2004). A porin-like channel is postulated to facilitate the flux of the mono- and dicarboxylates glycolate, oxaloacetate, malate, 2-OG and glycerate (Reumann et al., 1996; Reumann et al., 1998). Transport of amino acids glycine, serine, or glutamate and the bulky cofactors NAD, NADP, or CoenzymeA remains completely unanswered. We could extract from the co-expression analysis a member of the PMP22-related protein family in *A. thaliana*. However, an obvious visible phenotype could not be detected in the corresponding mutant. The twelve PMP22-related genes might be promising targets for metabolic profiling and multiple gene knockout analyses.

OUTLOOK

Despite the overrepresentation of plastid-targeted proteins, co-response analysis is a valuable tool to associate genes of unknown function with photorespiration. BOU was identified as a new player in the photorespiratory network. An initial genetic analysis of the remaining 27 lines is feasible in the model organism *A. thaliana*. Detailed metabolite analysis of the mutant plants and a selective transcript profiling of the candidate genes in wild type plants adapting to high and low CO2 concentration will add important information to identify missing transporters from the photorespiratory network.

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CHAPTER 6

CONCLUSIONS AND PERSPECTIVES

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The long-term goal of studying metabolite transporters is to link the uptake, distribution and secretion or excretion of a metabolite with its particular transport protein. This knowledge is essential for understanding the cellular metabolic networks by providing insights into the control of fluxes of key metabolites between metabolic pathways -from intracellular compartments up to the organismal level. This knowledge is also required for rational engineering of valuable natural products and plant biomass. Proteins, fatty acids, starch, and cellulose have been our central agricultural food and energy resource in the past and will be in future. The importance of solute carriers for the synthesis of the latter metabolites is underestimated mainly due to the limited molecular, biochemical, and structural information on many of these transport processes, as compared to soluble proteins (Xie, 2008). The findings presented in this dissertation validate that transport systems are rather specific enzymes, which are precisely placed in a eukaryotic cell to connect or even regulate the compartmentalized metabolic network, and not simply pore-like structures in biological membranes that are permeable for most of the small molecules.

In chapters one and three I characterized an ancient sugar phosphate transporter system in the plastid envelope membrane of the red alga *G. sulphuraria.* These transporters originated during the endosymbiosis between a photosynthetic cyanobacterium and its eukaryotic host. The insertion of a single pre-existing carrier into the inner membrane of the endosymbiont provided a selective advantage by reallocating photosynthates into the cytosol of the host, thereby directing the symbiosis to a permanent enslavement of the cyanobiont. The bacterial envelope membrane had been

massively altered with transport proteins predominantly derived from the host. Importantly, the plastid phosphate translocator family (pPT) evolved from an ancient nucleotide-sugar transporter to a versatile group of transporters mediating the exchange of Pi, triose-phosphate (TP), phosphoenolpyruvate (PEP), and glucose-6-phosphate (Glc6P) between cytosol and plastid of higher plants (Flügge, 1999). It testifies the dynamic adaptation and the potential of carrier proteins to influence metabolic networks.

The identification of the triose-phosphate/phosphate transporter from *G. sulphuraria* (GsTPT) with a high specificty and high affinity for its substrate triose-phosphate opens up a new tool to investigate carbon partioning into soluble and insoluble sugars in higher plants. The GsTPT has been introduced into the genome of *A. thaliana* wild type and *tpt* knockout plants (Schneider et al., 2002) and stable transformants are currently being established. In green plants, carbon partitioning is primarily organized in the plastid. Recently fixed carbon has three major routes in the Calvin-Benson-cycle. It is exported to the cytosol in form of triose-phosphates, further metabolized to produce hexose-phosphates for starch synthesis, or recycled to the CO_2 receptor ribulose-5-phosphate. The TPT is positioned at the metabolic branch of TP. Triose-phosphate export supplies carbon for sucrose synthesis during the day and on the other hand starch reserves are built up for carbon provision during the night. This assures continuous growth and development of the plant (Smith and Stitt, 2007). Over-expressing a TPT protein in tobacco plants did not significantly alter carbon partitioning, but showed that TPT activity limits the maximal rate of photosynthesis (Häusler et al., 2000a; Häusler et al., 2000b). The GsTPT will allow us to investigate the capacity of TP export to

influence sucrose and starch synthesis. Accumulation of sucrose and hexose-phosphate in the cytosol sequesters cytosolic Pi, which is needed for the counter-exchange with the plastid-localized triose-phosphate. As a result, TP transport rate slows down and 3-PGA levels increase relatively to Pi in the plastid. The altered 3-PGA/Pi ratio activates allosterically the enzyme ADP-glucose pyrophosphorylase (AGP), the committed step in starch synthesis (Ballicora et al., 2004). A three and two-fold higher affinity for Pi and triose-phosphate, respectively, could increase and extend TP export during the day, thereby promoting prolonged sucrose synthesis. TPT is not the solely regulator to control the carbon flux. Thioredoxin and trehalose-6-phosphate or the regulatory molecule fructose-2,6-bisphosphate influence the activity of AGP and sucrose synthesis, respectively (Stitt et al., 1984; Tiessen et al., 2002; Lunn et al., 2006). Monitoring the rate of sucrose and starch accumulation throughout the day and a comprehensive analysis of enzyme activities and levels of soluble sugar and sugar-phosphates will demonstrate if modulating TPT kinetic constants can indeed influence the robust carbon assimilation network. Activities of AGP (Smidansky et al., 2002) or the sucrose degrading enzymes invertase and sucrose synthase in storage organs such as seeds, fruits, and tubers (Herbers and Sonnewald, 1998) contribute to their sink strength and have a effect on organ number, size and dry weight.

The triose-phosphate transport protein from *G. sulphuraria* may be a promising candidate for manipulating the source strength of a crop plant. If this is true, the additive effects of an increased source and sink strength are worth to be evaluated in future. Sink strength is a quantitative trait and an ultimate goal will be to identify the "least gene

alleles combination" to improve the agricultural yield.

Another difference between the TPT proteins of higher plants and the red algae are their affinity to the metabolite 3-PGA. *G. sulphuraria* membranes lost or never evolved the capability for 3-PGA transport. This clearly points towards an important physiological role of 3-PGA transport in higher plant chloroplasts. By comparing the *A. thaliana* wild type and *tpt* knockout plants expressing the red algal protein, we will be able to assess the role of 3-PGA transport across the chloroplast envelope in *A. thaliana* leaves. Based on the results I provided in chapter three, the GsTPT cannot restore the 3-PGA transport activity in the knockout plants. We can use this approach to investigate the proposed TP/3-PGA reduction equivalent shuttle and in addition the postulated provision of 3-PGA for the cytosol to fuel the glycolytic pathway for PEP and pyruvate metabolism. We will focus our investigation on the NADP-dependent malate dehydrogenase in the chloroplast and the glycolytic enzymes in the cytosol. Reduction of oxaloacetate (OAA) to malate regenerates the terminal electron acceptor $NADP^+$ of the photosynthetic system and malate is exported to the cytosol and subsequently oxidized to OAA (Scheibe, 2004). Thus, the redox status of the plastid is tightly connected to the NAD(P)H pool of cytosol and the mitochondria (Raghavendra and Padmasree, 2003). The so-called "malate/OAA valve" and the TP/3-PGA shuttle are the main routes to export reduction equivalent during the day (Heineke et al., 1991). The effect of the impaired TP/3-PGA shuttle on the photosynthetic performance can be studied in detail.

In chapter three I could not assign glucose-6-phosphate as an *in vivo* substrate for

the homologous GPT protein from *G. sulphuraria*. This finding strongly suggests that GsTPT is a versatile exporter and importer of triose-phosphate depending on the autotrophic or heterotrophic growing conditions of the cell, respectively. I tested more than 20 commercially available sugar-phosphates without success. It will be challenging to identify the biological substrate of the "enigmatic" transport protein. Metabolic profiling is currently being pursued in the laboratory and not yet tested metabolites, such as phosphorylated disaccharides, trehalose, floridoside or sulfated soluble sugars might be recognized in the analysis as putative candidate.

The characterization of the pPT family and the comprehensive phylogenetic analysis of the metabolite transport proteins demonstrated the plasticity of plastid organelle evolution. The majority of the plastid metabolite transport systems have been established in the last common ancestor of the *Archaeplastida*. Nevertheless, distantly related photosynthetic organisms evolved alternative solutions to organize and optimize their compartmentalized metabolic network within the last one billion years. Investigation of these homologous proteins offers a great potential to discover a variety of regulatory mechanisms to fine tune the cellular metabolism and increase agricultural productivity and efficiency.

In addition, chapter two provides a new perspective on plastid evolution. Connecting the cytosol of host and cyanobacterium by existing host solute transporters was a key feature for the early success of the endosymbiosis or even reflects a turning point to a permanent partnership of the endosymbiont by the protist. The export of photosynthates was a central element in the integration of plastid-host metabolism.

Importantly, the pPT family was not an exception. Transporters derived primarily from eukaryotic genes and provide a selective advantage for the protist. When this analysis was initiated, several studies investigated the result of the endosymbiotic gene transfer (EGT) on the nuclear genome of photosynthetic eukaryotes (Timmis et al., 2004). Here we demonstrated that the eukaryotic host made a major contribution for the proteome of the organelle. Host genes diversified and were targeted to new locations in the cell with alternative functions as described for cyanobacterial genes by EGT. In essence, we concluded that the initial connection of independent metabolite entities (i.e., cyanobacterium and eukaryotic host cell) by metabolite transport proteins, was a crucial early event in endosymbiosis, providing the selective advantage that permitted the step-wise completion of the process by EGT and establishment of an efficient protein translocation machinery.

Identification of metabolite transporters participating in the photorespiratory pathway

As stated in the first paragraph, the challenge will be to characterize the metabolite carrier families predicted from computer-based analyses of several genomes, membrane-specific proteomics approaches and the transport capacity of different membrane fractions (Barbier-Brygoo et al., 2001). Chapter two, four and five represent typical examples of the discrepancy between the scientific progress made for soluble and insoluble proteins participating in a specific pathway. One limitation of our phylogenomic analysis is the not yet characterized physiological roles of the majority of the transport proteins. By knowing the substrate of each individual carrier we could

functionally categorize the host, cyanobacterial, and chlamydial contribution for the transport of inorganic ions and metabolites across the envelope of the endosymbiont and draw the "ancient plastid-cytosol interaction map" of the proto-alga.

The enzymatic steps to recycle 2-phosphoglycolate in the photorespiratory pathway are described in detail, however, the molecular nature of the transport systems in the plastidial, peroxisomal and mitochondrial membranes are mainly unknown (Reumann and Weber, 2006). A first attempt has been already published to successfully apply the basic knowledge of glycolate metabolism and design an alternative pathway to recycle 2-phosphoglycolate and increase biomass (Kebeish et al., 2007). The glycolate catabolic pathway from *E. coli* was targeted to the plastid in *A. thaliana* to short-circuit the endogenous photorespiratory pathway. Is the photorespiratory pathway and the identification of the transport proteins already "a thing of the past" (Sage and Coleman, 2001)? A detailed investigation of the transporters can contribute to the understanding of the photorespiratory network. For example, if the plastid glycolate exporter would have been identified, a targeted reduction presumably increases the flux through the biotechnologically introduced prokaryotic glycolate pathway. We could challenge the biotechnological approach by testing the capacity of glyoxylate detoxification in the plastid. Is the engineered photorespiratory bypass able to cope with an increased glycolate oxidation and result in an enhanced biomass production?

In chapter four I addressed the nitrogen cycle during photorespiration. The mitochondrial glycine decarboxylase reaction releases CO2 and ammonia. It is the "most inefficient step" in terms of the recycling of glycolate during the photorespiration. Nevertheless, GDC is essential and knockout mutants are lethal even under non-

photorespiratory conditions (Engel et al., 2007). An alternative approach to improve photorespiration could be the re-assimilation of CO2 and ammonia on site. Future experiments have to elucidate, if glutamine synthetase (GS) and carbamoyl-phosphate synthase are required for the assimilation of both gases during the day (Taira et al., 2004; Keys, 2006). To this end, it is still controversial if a glutamine synthetase (GS) is active in the mitochondria. 35S-driven GFP analysis of GS and purified mitochondria exhibit GS activity (Taira et al., 2004). On the other hand, to cope with the significant ammonia release, GS should be an abundant protein in the mitochondrial matrix. However, numerous organelle specific proteomics studies could not identify a mitochondrial GS (Heazlewood et al., 2004; Eubel et al., 2007). Over-expressing GS alone or in combination with the carbamoyl-phosphate synthase in the mitochondria could be an alternative approach to decrease the loss of the ammonia and CO2 gas to the environment.

The classical forward genetic approach identified only a single metabolite transporter linked to photorespiration (Somerville, 2001). The aim in chapter five was to initiate an alternative reverse genetic approach to identify membrane protein candidates mediating the flux through the photorespiratory pathway. We isolated 32 putative metabolite transporters and I initiated the genetic analysis of five of them. Two knockout mutants were indistinguishable from wild type in ambient air. Two insertion lines were lethal and did not develop beyond the cotyledon stage. The phenotype was independent of the CO_2 concentrations in the growth chamber. Lastly, the *boul* mutant showed a delayed classical photorespiratory phenotype. Similar to the GsGPT protein, despite the knowledge gained from genetic, bioinformatic and biochemical analyses of the *boul*

mutant, it has been difficult to assign a substrate for the mitochondrial transporter. The next step will be to demonstrate the vitamin B6 transport by the BOU protein across the mitochondrial membrane. A future goal will be to establish the remaining knockout plants and systematically screen for accumulation of photorespiratory marker metabolites to prioritize the candidate gene list. Essential genes could be targeted with an inducible RNAi approach.

To characterize transport proteins, the genes are heterologous expressed, purified, and functionally reconstituted in artificial vesicles (Palmieri et al., 1995). During my PhD thesis, I established additional systems to functionally characterize transport proteins in the laboratory of Prof. Andreas Weber. We are able to express the proteins in yeast, *E. coli* and in cell-free systems based on extracts of wheat germ and *E. coli*. I established an alternative method, the so called "cyclic detergent removal procedure" (Kraemer and Heberger, 1986), to reconstitute membrane proteins in the laboratory. Transport assays are also limited due to availability of radiolabeled substrates. With the patch-clamp technique it will be possible to test non-radiolabeled substrates. This will allow uptake assays with candidate metabolite, such as vitamin B6 derivatives, which are not commercially available radiolabeled. A combination of all methods will help to overcome the difficulties in transport research to express, preserve the activity of the purified protein and identify the *in vivo* substrate.

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