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Research article

Phylogenetic and Biochemical Evidence Supports the Recruitment of an ADP-Glucose Translocator for the Export of Photosynthate during Plastid Endosymbiosis

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Abstract

The acquisition of photosynthesis by eukaryotic cells through enslavement of a cyanobacterium represents one of the most remarkable turning points in the history of life on Earth. In addition to endosymbiotic gene transfer, the acquisition of a protein import apparatus and the coordination of gene expression between host and endosymbiont genomes, the establishment of a metabolic connection was crucial for a functional endosymbiosis. It was previously hypothesized that the first metabolic connection between both partners of endosymbiosis was achieved through insertion of a host-derived metabolite transporter into the cyanobacterial plasma membrane. Reconstruction of starch metabolism in the common ancestor of photosynthetic eukaryotes suggested that adenosine diphosphoglucose (ADP-Glc), a bacterial-specific metabolite, was likely to be the photosynthate, which was exported from the early cyanobiont. However, extant plastid transporters that have evolved from host-derived endomembrane transporters do not transport ADP-Glc but simple phosphorylated sugars in exchange for orthophosphate. We now show that those eukaryotic nucleotide sugar transporters, which define the closest relatives to the common ancestor of extant plastid envelope carbon translocators, possess an innate ability for transporting ADP-Glc. Such an unexpected ability would have been required to establish plastid endosymbiosis.

Key words: endosymbiosis, nucleotide sugar transporter, plastidic phosphate translocator, starch, cyanobacteria, evolution of photosynthesis.

Introduction

Phylogenetic reconstruction of the ancestral pathway of storage polysaccharide metabolism in the first eukaryotic cells that have acquired photosynthesis through endosymbiosis has suggested that adenosine diphosphoglucose (ADP-Glc) produced by the acquired cyanobiont was exported to the host cytosol, where it was polymerized into starch. The bacterial-specific metabolite, ADP-Glc, was thus hypothesized to define the first metabolic connection between the host and its cyanobacterial endosymbiont (Deschamps, Colleoni, et al. 2008; Deschamps, Haferkamp, et al. 2008; Deschamps, Moreau, et al. 2008). This phylogenetic reconstruction was based on the apparent common unique plastid endosymbiosis that generated the Archaeplastida. It consisted of defining the minimal set of proteins required to explain the present distribution of starch metabolism enzymes that are active either in the cytosol of the Glaucophyta and Rhodophyceae or in the chloroplasts of the Chloroplastida. Although many of the biochemical reactions are common in the three lineages and rely on enzymes displaying a unique phylogenetic origin, the networks differed by the presence in Chloroplastida of enzymes that polymerize starch from ADP-Glc. This nucleotide sugar defines a bacterial-specific metabolite produced by ADP-Glc pyrophosphorylase, an enzyme tightly coupled to the Calvin cycle and photosynthesis through the nature of its substrates and effectors. In agreement with the localization of starch in Chloroplastida, all enzymes of ADP-Glc biosynthesis and utilization displayed a bacterial phylogeny. Yet, both Rhodophyceae and Glaucophyta polymerize starch in the cytosol from uridine diphosphoglucose (UDP-Glc) using an enzyme of common "host" origin (Deschamps, Colleoni, et al. 2008; Plancke et al. 2008; Dauvillée et al. 2009). All the evidence gathered so far strongly indicates that the ancient pathways of starch synthesis and degradation were exclusively cytosolic. In other words, it is highly likely that starch biosynthesis was lost

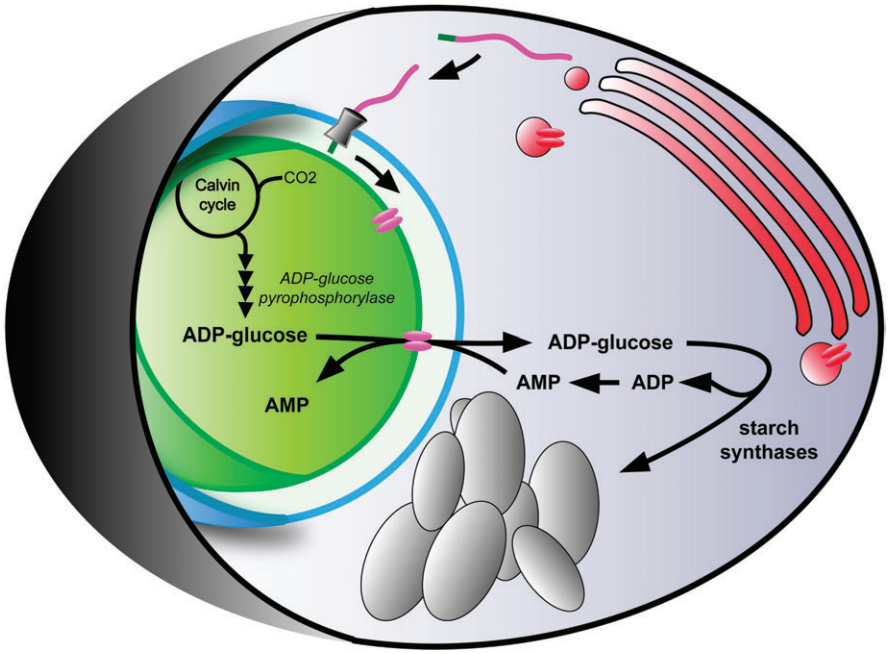

Fig. 1. Endosymbiotic carbon flux. The cyanobiont is displayed in green at the time of endosymbiosis with an emphasis on its inner (solid green line) and outer membranes (solid blue line). The outer membrane displays lower selectivity and will be permeable to relevant metabolites such as glycosyl nucleotides and nucleotides. The carbon fixed by photosynthesis enters the Calvin cycle in the cyanobiont. Partitioning of the flux between storage and the synthesis of novel cellular material occurs within the cyanobiont at the level of ADP-Glc pyrophosphorylase, the carbon within ADP-Glc being committed to storage. As the cyanobiont is unable to perform starch synthesis, ADP-Glc accumulates to high levels. The presence of a host purine NST (represented as a pink dimer) accidently targeted to the inner bacterial membrane will export this excess carbon out to the bacterial periplasm from where it will leak to the cytosol. Within the cytosol, glucose is polymerized into starch by ADP-Glc-specific starch synthases (Deschamps, Colleoni, et al. 2008). The latter are produced thanks to the expression from the host nuclear genome of genes transferred from the cyanobacterial genome (Deschamps, Colleoni, et al. 2008). Starch in the cytosol (in gray) acts as a very powerful carbon sink. The carbon will then be mobilized by a starch degradation machinery of host origin according to host needs (Deschamps, Colleoni, et al. 2008). The host purine NST gene has been duplicated. The original gene product (dark red) is represented as a Golgi membrane protein, whereas the novel copy product (in pink) has accidently acquired a leader-like sequence (in green). Although many different conflicting ideas have been proposed for the ancestral mechanism responsible for the targeting of proteins to the cyanobiont inner membrane, we have drawn the presence of a transit peptide-like sequence active in an ancestral protein translocon complex of the outer membrane (TOC) type of targeting (the ancient TOC is displayed as gray cylinder; see Bodyl et al. 2009 for details on ancestral mechanisms of protein targeting).

from the cyanobiont shortly after the initial endosymbiosis by transfer of storage carbohydrate metabolism to the host cell cytosol. Only in the Chloroplastida was it later regained in the plastid stroma, whereas it remained in the ancestral cytosolic location in the Glaucophyta and Rhodophyceae. A logical consequence of this scenario is that both types of starch biosynthetic enzymes, using either ADP-Glc or UDP-Glc, were present and active in the common ancestor cytosol. However, the enzyme synthesizing ADP-Glc, ADP-Glc pyrophosphorylase, would have remained in the cyanobiont because of its tight coupling with photosynthesis. This scenario clearly requires the presence of an ancient ADP-Glc translocator on the cyanobiont inner membrane responsible for the export of nucleotide sugar to the cytosol for which to date there was no evidence.

It turns out that the metabolic flux (fig. 1) created through this compartmentalization proposal defines an ideal way to establish the first symbiotic link between two entirely unrelated biochemical networks. Indeed, because ADP-Glc is devoted to storage polysaccharide synthesis in bacteria, only the carbon that was designated to escape at least temporarily cyanobacterial metabolism will be exported to the host cytosol. In the cytoplasm, ADP-Glc's only possible fate will consist of polymerization into starch. Because starch catabolism relies on enzymes of host origin, carbon will be made available on demand to the host through the preestablished regulatory networks of host storage polysaccharide catabolism. Starch thus defines an ideal buffer between the asynchronous demand and supply of carbon between the two unrelated partners of endosymbiosis. However, this hypothesis requires that at the time of endosymbiosis, an ADP-Glc translocator was immediately recruited on the inner membranes of the cyanobiont to generate the selective efflux of this nucleotide sugar.

In contrast, although phylogenetic analyses have demonstrated that the carbon exporters of plastids from red and green algae have evolved from nucleotide sugar transporters (NSTs) of the eukaryotic endomembrane system (Weber et al. 2006; Tyra et al. 2007), these modern carbon exporters do not transport ADP-Glc or other nucleotide sugars but hexose phosphates and triose phosphates.

Hence, we are missing the link between the central carbon metabolism of the cyanobiont and its host cell, which is an essential requirement for the evolution of eukaryotic photosynthesis through endosymbiosis. Closing this missing link would require an ancient host-derived NST displaying an innate ability to exchange ADP-Glc for adenosine monophosphate (AMP). Such a transporter could have been recruited as the first major carbon translocator of the cyanobiont, thereby permitting the establishment of endosymbiosis.

At a first glance, this scenario seems far-fetched because ADP-Glc is absent from the eukaryotic world and hence no need exists to evolve a transporter for this metabolite. Moreover, some of these NSTs have been demonstrated to be monospecific (Berninsone et al. 1997; Norambuena et al. 2005), allowing for the transport of a single type of nucleotide sugar, whereas others have been shown to allow the transport of several distinct types (Hong et al. 2000; Berninsone et al. 2001). In all cases, high selectivity was found with respect to the nature of the base rather than the sugar constituting the glycosyl nucleotides (Handford et al. 2006). NSTs can be defined as eukaryotic type III transmembrane solute transporters classified as carriers with an obligatory solute/solute antiport (Saier 2000). NSTs are involved in the transport of nucleotide sugars from the cytosol, where the majority are synthesized into the Golgi apparatus or endoplasmic reticulum (ER) lumen (Handford et al. 2006). The cytosolic nucleotide sugar is exchanged for the corresponding lumenal monophosphate nucleotide. The latter is produced after transfer of the activated sugar by the corresponding glycosyl transferase that releases the nucleoside diphosphate and is subsequently cleaved into inorganic phosphate and the monophosphate nucleotide. In the case of the proposed ancient carbon translocator of plants, ADP-Glc would have been exported from the cyanobiont and activated glucose would have been incorporated into cytosolic starch by glucosyl transferases named starch synthases. A balanced cycle would have therefore implied the counterexchange of ADP-Glc for AMP, whereas orthophosphate would have reentered the cyanobiont through other routes.

However, exchange of ADP-Glc for AMP has not been documented within eukaryotic NSTs. In plants, the only proteins reported to transport this metabolite have evolved only recently within the *Poaceae*. In addition, this latter transporter does not belong to the NST family but rather to a family of mitochondrial adenylate translocators (Kirchberger et al. 2007), which were proven not to show initially any fortuitous ability to transport this substrate (Kirchberger et al. 2008). However, because recruitment of the NST is a prerequisite for establishment of endosymbiosis, the eukaryotic NST had to show an immediate built-in ability to efficiently translocate this metabolite. We therefore investigated biochemically the ability of extant eukaryotic NSTs to fortuitously transport ADP-Glc. We show that extant eukaryotic purine glycosyl nucleotide transporters indeed display this ability and discuss the benefits of recruiting a guanosine diphosphate (GDP) sugar NST from the eukaryotic endomembrane system.

Materials and Methods

Strains and Plasmids

The *Saccharomyces cerevisiae* strain INVSc1 was transformed by the lithium acetate method according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). The expression constructs for Vrg4p and GONST1 under control of constitutive (*TDH3*) and inducible (*GAL1*) promoters, respectively, have been previously described (Xia-Dong and Dean 2001; Handford et al. 2004). The YEpTDH3–Vrg4p–HA_3 was kindly provided by Dr. Neta Dean, State University of New York.

Protein Expression and Embedding in Liposome Membranes

The expression of GONST1 and Vrg4p proteins was induced by diluting the yeast cultures to an OD_{600} of 0.5 in fresh synthetic medium containing 2% of galactose and 2% of glucose, respectively (Linka et al. 2008). Cells were harvested by centrifugation after 8 h at 30 °C. The isolation of total membranes and the preloading step of proteoliposomes with 10 or 30 mM counterexchange substrates were carried out as described (Linka et al. 2008).

Transport Assays

The transport assays were initiated by adding to the uptake medium the [^{14}C]-nucleotide sugar and terminated by loading the proteoliposomes on the anion exchange resin (Resin AG1-X8, Bio-Rad, Hercules, CA) preequilibrated with 150 mM sodium acetate buffer. The eluted proteoliposomes were collected and the imported radiolabelled nucleotide sugar was counted by liquid scintillation counting. Michaelis–Menten constants (K_M) were determined with at least six external nucleotide sugar concentrations. GraphPad Prism software (GraphPad Software Inc., La Jolla, CA) was used for nonlinear regression analysis of all enzyme kinetic data.

Phylogenetic Tree Reconstruction for NST and pPT Proteins; ER Retention Motif Search

Amino acid sequences of AtTPT1 (AT5G43350) and At-GONST1 (AT2G13650) were first used as queries to look for homologous protein sequences using BlastP (Altschul et al. 1997) against a local genome database containing 291 bacterial, 50 archaeal, and 53 eukaryotic complete annotated genomes. All homologous sequences (with an *e* value inferior to 1.0×10^{-05}) were collected from each Blast hit list and aligned together using the Muscle software (Edgar 2004). After automatic gap removal in the resulting multiple alignments, maximum likelihood tree reconstructions were performed using the software FastTree (Price et al. 2009). Both trees were manually inspected to remove possible sequence duplicates and to identify each group/family as previously described by Martinez-Duncker et al. (2003) and Knappe et al. (2003). Both trees were then recomputed with the TREEFINDER software (Jobb et al. 2004) using the Whelan and Goldman + Γ model. Protein

sequences were collected at Genbank (http://www.ncbi.nlm.nih.gov/protein); the Joint Genome Institute (http://www.jgi.doe.gov/); and on genome home project Web sites for *Galdieria sulphuraria* (http://genomics.msu.edu/galdieria/), *Dictyostelium discoideum* (http://dictybase.org/), *Cyanidioschyzon merolae* (http://merolae.biol.s.u-tokyo.ac.jp/), and *Arabidopsis thaliana* (http://www.arabidopsis.org/). The search for ER retention motifs in amino acid sequences was done based on the consensus criteria reported by Jackson et al. (1993), which includes two lysines one of which must be positioned three residues from the C-terminus and the second either four or five residues from the C-terminus.

Results

The Major Carbon Translocators of the Plastidial Membranes of Red and Green Algae Are Monophyletic Sisters to Eukaryotic NSTs

Previous investigations have demonstrated that the major carbon translocators of the inner plastidial membrane of both red and green algae are monophyletic and sisters to nucleotide sugar translocators (NSTs) of eukaryotic endomembranes (Knappe et al. 2003; Martinez-Duncker et al. 2003; Weber et al. 2006). The family of major plastidial carbon translocators that exchange triose-P, hexose-P phosphoenolpyruvate, or xylulose-P for orthophosphate is collectively known as the plastidic phosphate translocator (pPT) family. The distribution of these types of transporters is restricted to Archaeplastida and their secondary endosymbiosis derivatives. We have readdressed the phylogeny and distribution of this family by analyzing a significantly larger set of members and comparing them to the ever-growing list of NSTs. Two phylogenetic trees are displayed, respectively, in figures 2 and 3. Figure 2 focuses mainly on family NST3 as defined by Martinez-Duncker et al. (2003) and displays the relationship of this family with the PTh-KV/A/G and PTh-KD subfamilies (PTh standing for phosphate translocator-homologous according to Knappe et al. 2003). The two transporters characterized in this study are highlighted (GONST1 and Vrg4p, both of type NST3-K). Figure 3 shows the relationship of the pPT family with their closest relatives within the NST3 family: the PTh-KR subfamilies. The relative position of the PTh-KV/A/G and PTh-KD subfamilies detailed in figure 2 is summarized at the bottom of figure 3. The PTh sequences typically lack transit peptides directing them to plastids and often contain signal peptide–like sequences potentially directing them to the endomembrane system. In Arabidopsis, a detailed study based on gene coexpression analysis predicts them to define genes involved in polysaccharide and glycoconjugate biosynthesis (Reyes and Orellana 2008). In some rare cases, some of these PTh sequences were analyzed by functional analysis in liposomes and were indeed demonstrated to be true Golgi NSTs with no ability to exchange sugars for orthophosphate (Rollwitz et al. 2006; Reyes et al. 2010).

Our analyses confirm the previous findings by demonstrating monophyly of the pPT family and that its distribution is restricted to Archaeplastida and secondary endosymbiosis derivatives of the latter (fig. 3). In addition, both trees indicate that eukaryotic NSTs of subfamily M (according to Martinez-Duncker et al. 2003) and PTh of subgroups KD and KV/A/G (and also KT according to Knappe et al. 2003) could be considered as close if not as a unique group (fig. 2). Interestingly, figure 3 indicates that the previously defined eukaryotic group of transporter KR (a third class of PTh; Knappe et al. 2003) should be divided in two subgroups: the first one defining the closest sister group to the plant plastidial pPT and the second one being a relative of the other PTh families. As noted initially by Martinez-Duncker et al. (2003), we confirm the presence of ER retention sequences in a substantial portion of "bona fide" Archaeplastidial pPT transporters (a total of 40 of 82 TPT sequences). The significance of the ER retention signals of the pPT family members is unclear because these proteins contain characteristic transit peptide sequences. Interestingly, when TPT is expressed in yeast cells without its targeting peptide, it is directed to the rough ER and/or mitochondrial membranes (Loddenkötter et al. 1993).

Whenever biochemical evidence is available, all transporters tested that do not belong to the pPT family exchange nucleotide sugars for nucleotide mono- or diphosphates. Interestingly, purine glycosyl nucleotide transporters are widely distributed among eukaryotic NSTs of family 3 including subfamily M and other PThs. They consist of GDP-sugar translocators transporting GDP-mannose (GDP-Man), GDP-arabinose, and GDP-fucose. The phylogenetic evidence coupled to the functional evidence available so far therefore point to the existence of an ancestral plastidial membrane carbon translocator that evolved from a host endomembrane nucleotide sugar translocator. However, the substrate preference of this ancestral translocator and its function in endosymbiosis cannot be deduced from phylogeny alone. Nevertheless, the pPT family is nested within the only family of NSTs known to contain purine nucleotide sugar translocators, a finding that we believe to be relevant to its proposed ancestral function (see below).

Expression of GDP-Man Translocators in Yeast Liposomes

Reconstruction of starch metabolism in the common ancestor Archaeplastida called for the presence of an ancient carbon translocator with the ability to export ADP-Glc to the cytosol where it was polymerized into starch (Deschamps, Colleoni, et al. 2008; Deschamps, Haferkamp, et al. 2008; Deschamps, Moreau, et al. 2008). This transporter was of paramount importance in establishing endosymbiosis because it provided the required symbiotic link by exporting only the photosynthate devoted to storage from the cyanobiont. The finding of a common ancestor to all major plastidial carbon translocators of Archaeplastida suggests that this ancestor is very ancient and thus might have played a role in exporting photosynthate after endosymbiosis (Weber et al. 2006). Furthermore, the relatedness to eukaryotic NSTs suggested to

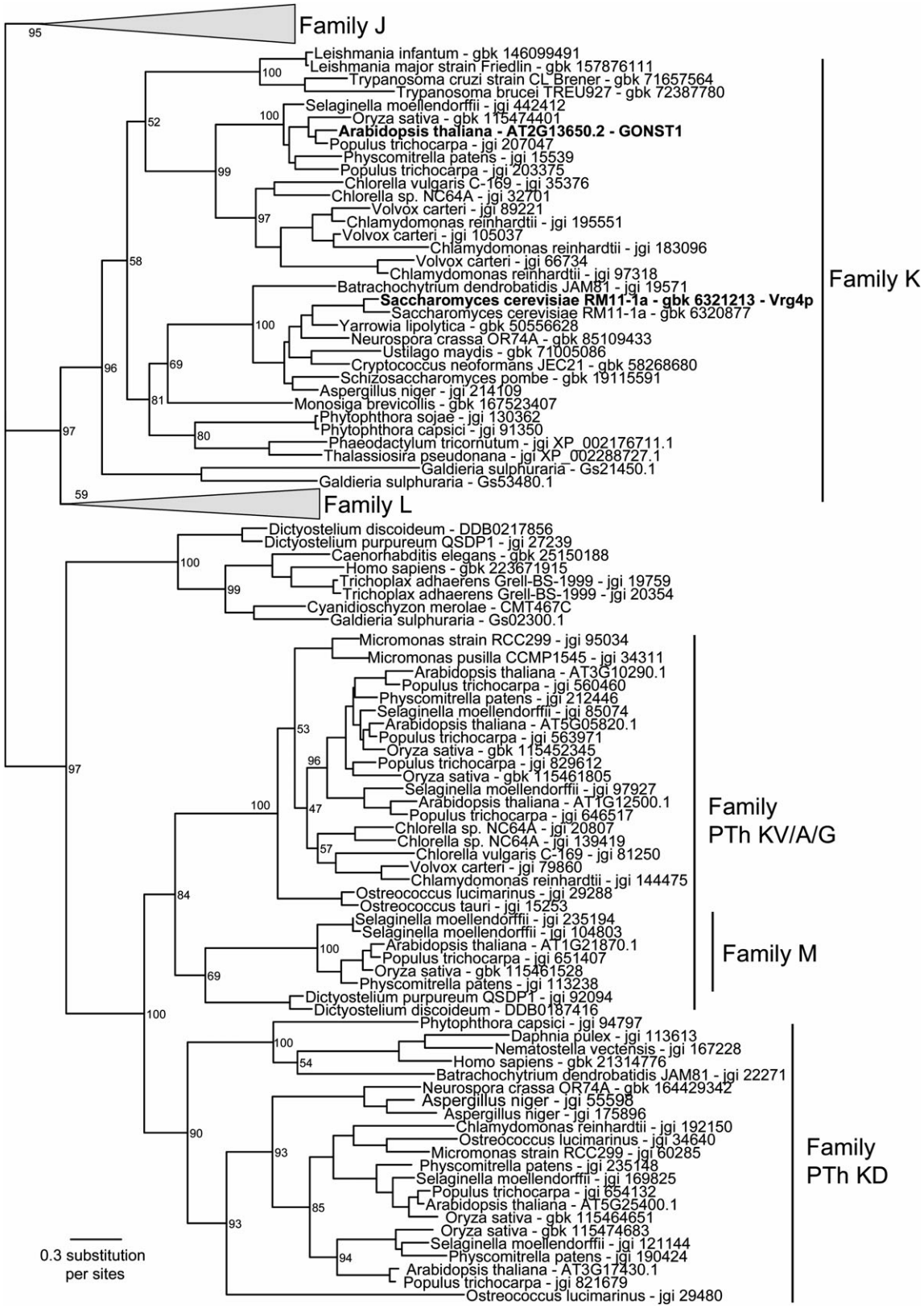

Fig. 2. Maximum likelihood (ML) tree inferred from the amino acid sequences of the NST transporter family 3 and the PTh-KV/A/G and PTh-KD subfamilies. Numbers at nodes are ML bootstrap values. Reference numbers are gbk, genbank gi; jgi, protein ID at the Joint Genome Institute genome portal; other, locus reference at the corresponding genome home Web site (see Materials and Methods).

us that this ancient carbon translocator might have been the ADP-Glc translocator hypothesized by Deschamps, Colleoni, et al. (2008). The recruitment of a eukaryotic NST to establish the endosymbiotic link requires that the latter was instantly able to export ADP-Glc. Indeed, the basic symbiotic link had to be established immediately

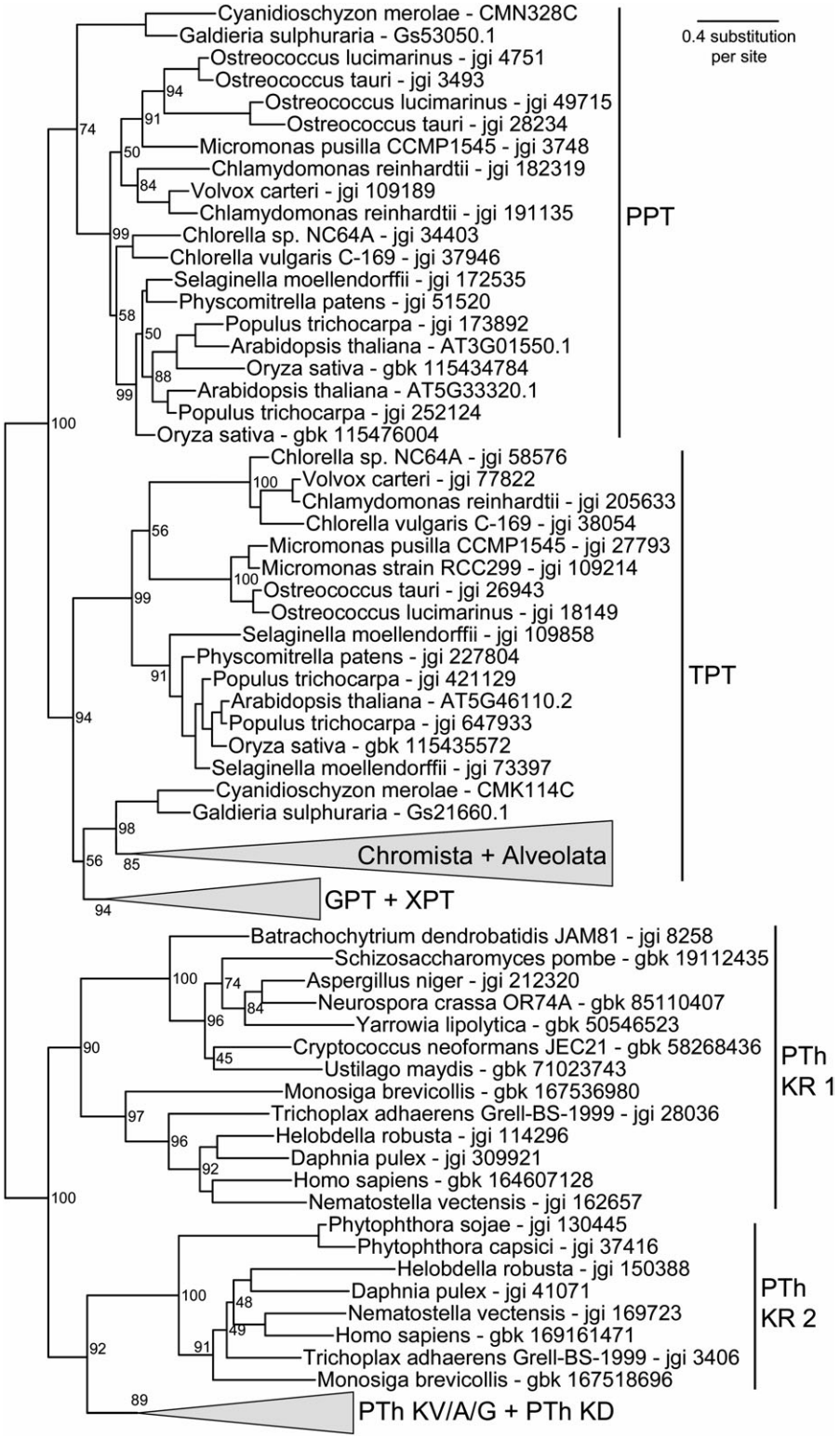

Fig. 3. ML tree inferred from the amino acid sequences of the pTP transporter family and the PTh-KV/A/G and PTh-KD and PTh-KR transporter subfamilies. Numbers at nodes are ML bootstrap values. A putative split of the KR group in two distinct subgroups is proposed with the names "KR part 1" and "KR part 2." Reference numbers are gbk, genbank gi; jgi, protein ID at the Joint Genome Institute genome portal; other, locus reference at the corresponding genome home Web site (see Materials and Methods).

for endosymbiosis to be successful. To test the hypothesis that endomembrane NSTs have an innate ability to transport ADP-Glc, we used a biochemical liposome assay to monitor the activity of such transporters. In this liposome assay, the lumenal and outer metabolite concentrations can be manipulated at will. We have recently adapted such a protocol using yeast-derived membranes that therefore requires the successful expression of

recombinant proteins within such membranes (Linka et al. 2008). Because of the high base selectivity of all NSTs documented to date, we focused on purine rather than pyrimidine-derived glycosyl nucleotide transporters. Among the purine NSTs, only guanosine-derived glycosyl nucleotides are presently known within eukaryotes (Handford et al. 2006) and have been documented within several distinct NST subgroups of family 3. Golgi membrane GDP-Man translocators define an abundant and widely distributed type of NST. In a variety of highly diverse protist lines, such translocators are either known or suspected to be involved in the synthesis of mannosylated cell wall components (Dean et al. 1997; Baldwin et al. 2001; Nishikawa et al. 2002; Segawa et al. 2005; Capul et al. 2007; Jackson-Hayes et al. 2008; Ueki and Nishii 2009). We have chosen the yeast Vrg4p (Dean et al. 1997) and the *A. thaliana* GONST1 (Baldwin et al. 2001) GDP-Man translocators as both have been expressed as active recombinant proteins in yeast membranes (fig. 4*A*). We first demonstrated [^{14}C]-GDP-Man uptake in our vesicular system preloaded with GDP-Man (fig. 4*B and C*). No transport was evidenced either in the absence of preloading or within liposomes prepared from yeast strains transformed with empty vectors.

Biochemical Characterization of the GDP-Man Translocators

In our first series of experiments, we measured the dependence of [^{14}C]-GDP-Man uptake into liposomes preloaded either with nucleotide monophosphates (guanosine monophosphate [GMP], AMP) or nucleotide sugars (GDP-Man, ADP-Glc, UDP-Glc, UDP-galactose; fig. 5*A*). Highly active transport was accomplished with the countersubstrates GDP-Man, GMP, and, surprisingly, AMP, whereas transport of ADP-Glc, uridine monophosphate (UMP), and UDP-sugars fell near the detection limit. Import of labeled [^{14}C]-GDP-Man was measured in the presence of varying concentrations of nonlabeled externally supplied AMP (fig. 6). An apparent inhibitory constant (K_i) for AMP of 0.86 ± 0.1 mM confirmed AMP as a very potent competitive substrate inhibitor for GONST1. Although ADP-Glc was not an efficient countersubstrate for the import of GDP-Man (fig. 5*A*), uptake of [^{14}C]-ADP-Glc was clearly detectable into GONST1-reconstituted vesicles that had been preloaded with either AMP or GMP (figs. 5*C* and 7). Under these conditions, the initial transport rates determined with 0.01 mM [^{14}C]-GDP-Man or 0.5 mM [^{14}C]-ADP-Glc, respectively, are comparable (figs. 5*B, C*, and 7). Thus, the plant-derived NST GONST1 is clearly able to serve as an ADP-Glc/AMP antiporter. The affinity for [^{14}C]-ADP-Glc was rather low compared with GDP-Man (fig. 4*B and C*). With an apparent K_M of 7.1 ± 1.6 mM for ADP-Glc, GONST1 exhibits a 250-fold higher affinity for GDP-Man (K_M 0.026 ± 0.002 mM). In contrast, yeast Vrg4p, although accepting AMP as a counterexchange substrate for GDP-Man (fig. 5*A*), is not able to transport ADP-Glc (not shown).

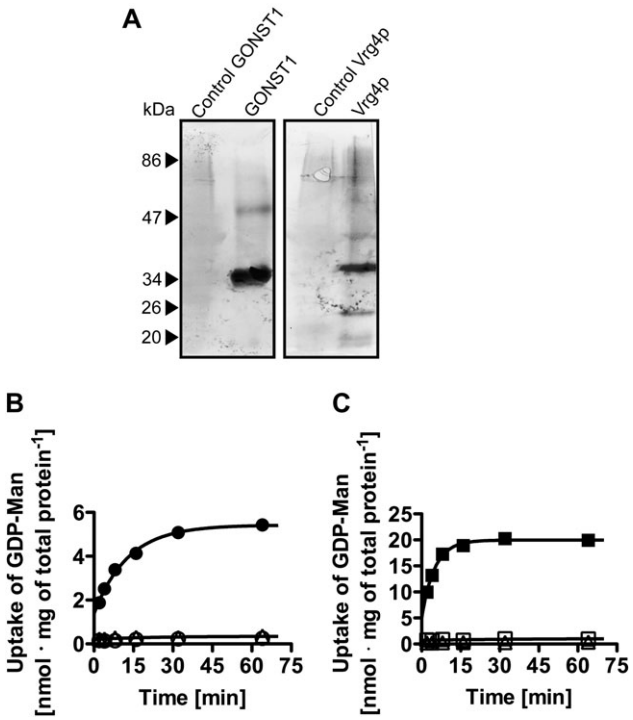

FIG. 4. Expression and GDP-Man import into proteoliposomes reconstituted with Vrg4p and GONST1. (*A*) Total membrane fractions were isolated from control yeast cells transformed with the empty expression vectors (control GONST1 and control Vrg4p) and cells overexpressing the nucleotide sugar transporter GONST1 or Vrg4p. Membrane proteins were resolved by sodium dodecyl suphate polyacrylamide gel electrophoresis and immunoblots were probed with anti-HIS or anti-HA antibodies, which recognize GONST1 (35 kDa) and Vrg4p (36 kDa) proteins, respectively. Time course experiments of GDP-Man transport were performed in proteoliposomes reconstituted with Vrg4p (*B*) or with GONST1 (*C*). The transport of GDP-Man was initiated by adding 0.01 mM of [^{14}C]-GDP-Man to a suspension of proteoliposomes preloaded with 1 mM GDP-Man (closed symbols). No significant GDP-Man transporter activities were measured without GDP-Man preloading (open circles and squares) or with membrane fractions of yeast cells transformed with the empty expression vectors (open triangle). The presented import studies are representative of at least three biologically independent experiments.

Discussion

The Ancient Plastid Endosymbiosis Carbon Translocator Was an Endomembrane nucleotide sugar translocator Related to Extant Family 3 NSTs

The monophyletic nature of the pPTs places these carbon transporters clearly nested within family 3 of the NSTs and more specifically within subfamily M. Interestingly, family 3 is the only family of NSTs that are documented to include purine NSTs (Martinez-Duncker et al. 2003).

Robust monophyly of such a diverse range of pPTs in plants and red algae involved in the transport of hexoses, trioses, phosphoenolpyruvate (PEP), and xylulose has been documented earlier (Knappe et al. 2003; Weber et al. 2006) but was confirmed by our analysis including a much larger set of sequences. We believe this surprising observation to be due on the one hand to the requirement for an

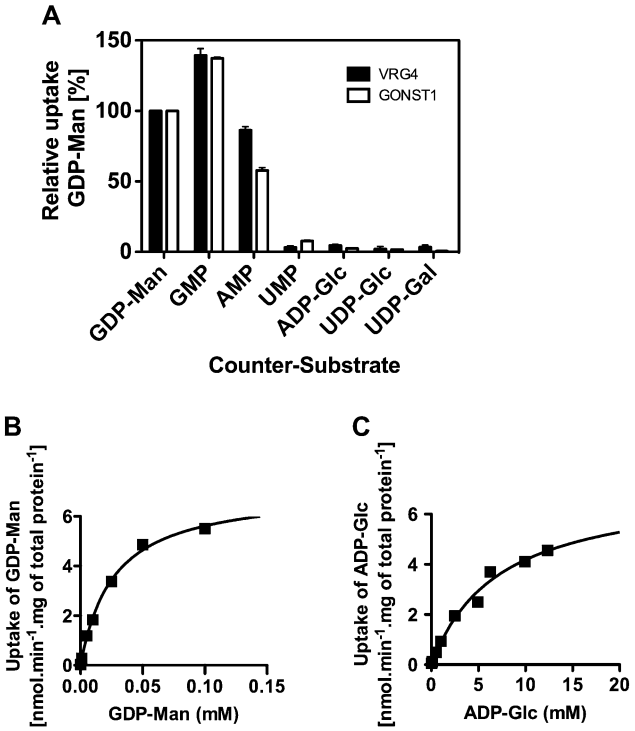

FIG. 5. Kinetic parameters of Vrg4p and GONST1. (*A*) [^{14}C]-GDP-Man uptake dependent on various countersubstrates. Proteoliposomes were reconstituted with membrane proteins from yeast cells expressing GONST1 (white bars) or Vrg4p (black bars) and preloaded with 10 mM of monophosphate nucleotides (GMP, AMP, UMP) or nucleotide sugar (GDP-Man, UDP-Glc, UDP-galactose, or ADP-Glc). The background activity from yeast membranes lacking recombinant Vrg4p and GONST1 was subtracted. The initial uptake rates were compared with [^{14}C]-GDP-Man/GDP-Man counterexchange, which was set to 100%. For Vrg4p and GONST1, the initial rates were 0.21 ± 0.04 and 1.9 ± 0.4 nmol GDP-Man min^{-1} mg total protein^{-1}, respectively. The data are mean ± standard deviation, $n = 3$. The Michaelis–Menten constant (K_M) for GDP-Man (*B*) and ADP-Glc (*C*) of the GONST1 transporter were determined using various external concentrations of [^{14}C]-GDP-Man (0.0001–0.1 mM) and [^{14}C]-ADP-Glc (0.05–12.5 mM). The proteoliposomes were preloaded with 10 mM of GMP. The initial uptake rates of each uptake experiments are displayed as nmol min^{-1} mg total protein^{-1} and plotted against the substrate concentration.

immediately functional ADP-Glc translocator, which undoubtedly favored recruitment of an NST, and on the other hand to the paucity of host proteins targeted to the inner membrane of the cyanobiont.

Our findings do not yet disqualify the idea that the ancestral carbon translocator was a pPT that for instance exchanged triose-P or hexoses for orthophosphate. Indeed, the pPT family is ancient and one can argue that the biochemical properties of such transporters offer more opportunity for integration of metabolism than the NSTs (for review, see Weber et al. 2005). The present weakness in this alternative idea resides in the distribution of pPT-like proteins among eukaryotes. Indeed, their distribution is presently restricted to Archaeplastida and their secondary

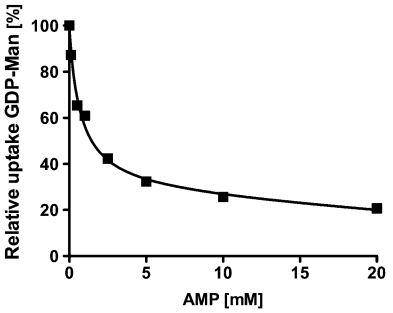

FIG. 6. Inhibitory effect of AMP on GDP-Man transport by GONST1. Proteoliposomes were reconstituted with membrane proteins from yeast cells expressing GONST1 (black squares) and preloaded with 10 mM GMP. At 0 mM AMP, the initial uptake rate [^{14}C]-GDP-Man/GMP was measured at 6.73 ± 0.23 nmol GDP-Man min^{-1} mg total protein^{-1} and was set to 100%. The relative uptake of GDP-Man was determined in the presence of increasing concentrations of AMP in the external uptake medium (0.1–20 mM).

endosymbiosis derivatives. If a host translocator was to be recruited as an ancient carbon translocator, then the most parsimonious scenario is that its activity was simply redirected to the endosymbiont rather than calling for the evolution of a novel type of transporter. Indeed, endosymbiosis relied on immediate establishment of the carbon flux. We therefore argue that there would not have been enough time to evolve a novel transporter and in addition have it immediately targeted to the cyanobiont inner membrane. In other words, the ancient host protein would already have been a phosphate translocator if one is to propose that the common ancestor of pPT displayed such an activity. However, there is presently no evidence for the presence of such an activity in heterotrophic eukaryotes unrelated to primary endosymbiosis. In addition, all PTh proteins examined so far turn out to define NSTs.

Finally, one may wonder why the ancient ADP-Glc translocator was substituted by pPTs in all three lineages. We believe that after evolution of the machinery that enabled

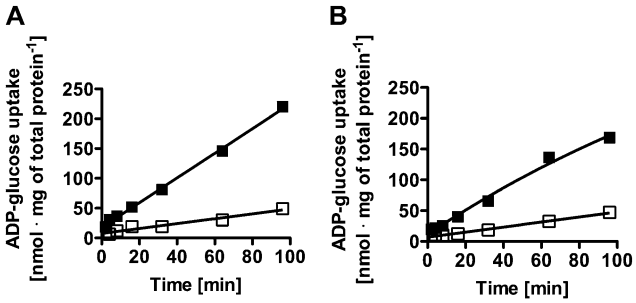

FIG. 7. Time course experiments of ADP-Glc transport in proteoliposomes reconstituted with GONST1. Proteoliposomes were reconstituted with membrane proteins of yeast cells overexpressing GONST1 (closed squares) or without recombinant GONST1 (open squares). Proteoliposomes were preloaded with 30 mM AMP (*A*) or GMP (*B*). The transport of ADP-Glc was initiated by adding 0.5 mM of [^{14}C]-ADP-Glc to proteoliposome suspensions. For AMP- and GMP-preloaded vesicles, the uptake rates were 1.6 ± 0.05 and 1.3 ± 0.1 nmol ADP-Glc min^{-1} mg total protein^{-1}, respectively.

the import of proteins into the stroma, an opportunity arose to multiply the connections between the two previously unrelated networks. This led to optimization of compartmentalization of metabolic pathways that in turn required the evolution of a diversity of transporters. As long as starch was being polymerized from ADP-Glc in the cytosol, the ancient NST and the pPTs generated from it after gene duplication and evolution coexisted. When the ability to polymerize starch from ADP-Glc in the cytosol was either lost or redirected to plastids in Rhodophyceae Glaucophyta and Chloroplastida, respectively (Deschamps, Moreau, et al. 2008), there was no need to maintain this plastidial NST and its function was entirely taken over by the pPTs. Indeed, the evolution of novel substrate specificities in the pPT family is not unheard of. For example, it was recently shown that the apicoplast carbon transporter, which is monophyletic with plant triose phosphate translocators, has acquired the function of a phospho*enol*pyruvate transporter after secondary endosymbiosis (Lim et al. 2010).

The Benefits of the Recruitment of a Host Purine nucleotide sugar translocator

One of the requirements for the establishment of the symbiotic link through ADP-Glc export would be that the recruited NST was immediately effective for export of this nucleotide sugar in exchange for AMP, a function for which it was not designed. The picture that is emerging from the detailed characterizations performed on NSTs is that most are base specific while they show variable degrees of sugar specificity (reviewed in Handford et al. 2006). For this reason, we focused our attention on two purine NSTs. Two GDP-Man NSTs belonging to subfamily *K* of family 3 from both plants and fungi were chosen because they had been successfully expressed in yeast membranes, which defined a prerequisite for the use of our liposome system. Also pPTs are effectively nested within family 3 NSTs in phylogenetic trees. We believe that the very significant inhibition of GDP-Man transport by physiological concentrations of AMP that we now report for both the yeast and the plant NSTs would have defined a very favorable property for recruitment of the corresponding NSTs. Indeed, because on the periplasmic face of the inner cyanobiont membrane the recruited NST experiences substrate concentrations approaching those of the host cytosol, there would have been fierce competition between the nonproductive import of GDP-Man and the desirable exchange of cytosolic AMP for ADP-Glc from within the cyanobiont. The fact that the GDP-Man and other GDP-sugar concentrations within the cytosol are known to be very low (typically below 50 μM) and that high levels of ADP and AMP are produced through polymerization of starch in the cytosol would further increase the ratio of AMP to GDP-Man, thereby favoring entry of AMP into the cyanobiont. That both the GONST1 and the Vrg4p transporters display similar high sensitivities of GDP-Man import to competition by AMP argues that the latter could define a very widely distributed property of family 3 eukaryotic purine NSTs. In addition, on the cyanobiont face of the inner membrane, the purine NST would only export ADP-Glc because the concentration of the latter is expected to outcompete all other potential substrates such as AMP, GMP, or GDP-sugars (see below for a detailed discussion). ADP-Glc seems to be a transported substrate only when using the GONST1 transporter. In effect, transport rates with GONST1 would have been sufficient if the ADP-Glc pool rose to levels equal or slightly above the 1 mM range. We argue that this was indeed the case (see below) and that therefore extant NSTs of family 3 do display the desired biochemical properties. In addition, once a suitable transporter had been targeted to the cyanobiont inner membrane from a duplicated copy of a host endomembrane NST, natural selection would have quickly optimized this transporter with respect to the export of ADP-Glc and counter selected those exchanges that were either nonproductive or selectively taken over by other transporters.

We have previously stressed that the symbiotic flux outlined in figure 1 defines an ideal system by which only the carbon committed to storage is tapped from the cyanobiont and transferred to the eukaryotic cytosol for polymerization into starch that acts as a powerful carbon sink. The mobilization of the starch pools would be governed entirely by host needs thanks to the preexisting host storage polysaccharide catabolism enzymes. This flux buffered through storage defines an optimized connection between two unrelated biochemical networks with no expected cytotoxic impact. At face value, the very high K_M of the GONST1 transporter for ADP-Glc might also be seen as a problem to account for an immediately effective photosynthate export. We argue that in wild-type free-living cyanobacteria in the light, ADP-Glc is abundant and concentrations are likely to range from 10 to 500 μM depending on growth conditions. However, for mutants blocked in ADP-Glc utilization, such as mutants no longer able to synthesize starch, this concentration is likely to rise even further. Indeed, at least three lines of evidence point to a very early loss of starch biosynthesis from the cyanobiont (Deschamps, Colleoni, et al. 2008). In this respect, the cyanobiont can be considered as a mutant blocked for ADP-Glc utilization. It is thus very reasonable to posit an increase of ADP-Glc pools to values approaching or exceeding 1 mM. In this context, the presence of the NST in the cyanobacterial inner membrane is unlikely to exert any kind of cytotoxicity for the cyanobacterium. Indeed, UDP-Glc and all glycosyl nucleotides required for bacterial survival and division such as those involved in peptidoglycan synthesis are pyrimidine nucleotide sugars. The concentrations and nature of such nucleotide sugars are unlikely to have exerted any significant competition with the transport of ADP-Glc. Nevertheless, the use of GDP-Man in bacteria seems to be widespread but restricted to the synthesis of the outer layers such as the lipopolysaccharide (LPS) or capsular polysaccharides, which are dispensable for cell survival. We believe that these layers would have been absent or readily lost by the endosymbiont leading to

a concomitant loss of the ability to synthesize purine nucleotide sugars other than ADP-Glc.

Indeed, strong evidence for the proposed scenario comes from the recent sequencing of the *Paulinella* chromatophore genome, which has evidenced loss of most genes involved in LPS and outer layer polysaccharide metabolism. In particular, all enzymes of mannose metabolism were lost with the exception of GDP-Man pyrophosphorylase leading to the inability of the chromatophore to synthesize purine nucleotide sugars, which are all derived from the latter (Nowack et al. 2008). Thus, the recruited purine NST would have been devoted in the light to the export of ADP-Glc with no effect on the concentrations of other important glycosyl nucleotides. We believe that finding the pPTs nested within the only family of NSTs known to contain purine nucleotide sugar transporters is a highly significant finding in this respect.

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