The Import and Export Business in Plastids: Transport Processes across the Inner Envelope Membrane[1]

Karsten Fischer*

Institute for Arctic and Marine Biology, University of Tromsø, 9037 Tromsø, Norway

Plastids are the characteristic organelles of plant and algae cells and are, like mitochondria, of endosymbiontic origin. They can be traced back to a single cyanobacterial ancestor that was engulfed by a primitive protist about 1.5 billion years ago (Yoon et al., 2004). Higher plants possess different types of plastids, among them undifferentiated proplastids, colorless etioplasts, green photosynthetically active chloroplasts, colored chromoplasts that are responsible for the yellow, orange, and red colors of flowers and fruits, starch-storing amyloplasts, and lipid-storing elaioplasts. The differentiation into these plastid types depends on the tissue and the developmental stage of the plant. Plastids show a tremendous biochemical versatility. They are the site of carbon, sulfur, and nitrogen assimilation, and they synthesize fatty acids, many amino acids, and a plethora of other primary and secondary metabolites. It is, therefore, reasonable to consider plastids as metabolic factories of plant cells. Plastids of red and green algae and of higher plants are enclosed by two membranes, the outer and inner envelope membranes that separate the stroma from the cytosol. The integration of plastids into the metabolism of plant cells is achieved by a large set of proteins transporting numerous inorganic and organic molecules across both membranes. Transport across the outer envelope membrane is enabled by a set of β-barrel proteins that form selective pores (Bölter and Soll, 2001). In contrast, the transport across the inner envelope is catalyzed by a large set of α-helical membrane transporters, which are highly specific for their substrates.

In this Update, I will give a short overview of the current knowledge of transport processes across the plastid inner envelope membrane and of the approaches used for the identification and characterization of plastid membrane transporters. Furthermore, I will discuss the evolution of these proteins and future challenges and opportunities in the field. For more detailed information about particular transporters, the reader is referred to several comprehensive reviews covering plastid transport processes (e.g. Weber and Fischer, 2007; Linka and Weber, 2010).

FROM THE BEGINNINGS OF PLASTID TRANSPORT TOWARD THE PERMEOME OF PLASTIDS

A first direct hint at the transport processes across the inner envelope membrane and especially at the metabolites that are exported from chloroplasts during photosynthesis was obtained from transport experiments with isolated plastids. It could be shown, for example, that chloroplasts export assimilated carbon in the form of triose phosphates (TPs), that they import ATP, and that they have a sophisticated system for the transport of dicarbonic acids. For a detailed and personal overview about the first two decades of transport business I refer the reader to the excellent articles by Heldt (2002) and Walker (2003).

With the cloning of the first cDNA encoding a plastid transporter, the TP/phosphate translocator (TPT)—one member of the family of plastid phosphate translocators (pPTs)—the molecular age of plastid transport began (Flügge et al., 1989). Since then, 35 transporters of the inner envelope membrane have been characterized at the molecular level and the physiological function of most of them have been elucidated. These proteins transport numerous inorganic and organic molecules into and out of plastids (Fig. 1; Table I). A combination of data from in silico analyses and proteomics projects has led to the estimate that as much as 120 to 150 proteins (Weber et al., 2005; Armbruster et al., 2010) might be involved in plastid transport processes. If these estimates are correct, approximately 75% of transporters are yet unknown. Thus, a major goal in future plastid research remains to determine the permeome, the complete set of transport proteins, of chloroplasts and of all other types of plastids.

The Hard Way: Biochemical Approaches

Biochemical approaches were the first used to identify and isolate plastid transporters. They were based on methods for the isolation of chloroplasts developed in the 1960s and on techniques for the isolation of envelope membranes. Both purified organelles and membranes were the starting material for the isolation of transporters by two different strategies. The TPT and later the plastid Glc transporter (pGlcT) were identified by using radiolabeled inhibitors that bind to and inhibit a particular transporter protein. The cor-

---

responding cDNA was subsequently cloned, taking advantage of peptide sequences obtained from labeled proteins (Flügge et al., 1989; Weber et al., 2000). Another strategy that was applied to analyze transporters was the determination of the particular transport activity of a protein by reconstitution of envelope membrane proteins into artificial liposomes. Based on the transport measurements, the transporters were purified to apparent homogeneity by different chromatographic methods. Peptide sequence information was then used to clone cDNAs encoding the transporters. Using these biochemical methods, the dicarboxylate transporter DiT1 and two members of the pPT family, the PPT (transporting phospho*enol*pyruvate [PEP]), and the GPT (transporting Glc 6-P [Glc6P]), were isolated and characterized (Weber et al., 1995; Fischer et al., 1997; Kammerer et al., 1998).

The Mendelian Way: Forward Genetics

One tool is classical or forward genetics, i.e. the identification of mutants with specific phenotypes followed by the identification and analysis of the mutated genes. Primary forward genetics screens, suppressor mutant screens, and other screening strategies have led to the identification of a large number of genes encoding plastid proteins, many of them involved in photosynthesis (Armbruster et al., 2010). The

first transport protein from plastid envelopes was identified almost 30 years ago in a pioneering forward screen aimed at identifying proteins involved in photorespiration or the photorespiratory C2 cycle. The screen was based on the assumption that photorespiratory mutants are unable to survive in air but are viable at high CO_2 concentrations that prevent photorespiration. Besides a number of enzymes of the C2 cycle, an envelope membrane protein was identified that is involved in dicarboxylate transport, a process that is part of the refixation of ammonia released during photorespiration (Somerville and Ogren, 1983). In this case, however, the corresponding gene DiT2 was identified only 20 years later because of its homology to DiT1 and shown to be the Glu/malate transporter that is indeed involved in ammonia refixation (Taniguchi et al., 2002; Renné et al., 2003).

The first gene encoding a plastid transporter isolated from a plant mutant was *brittle-1* (*bt-1*). The maize (*Zea mays*) *bt-1* mutant was identified back in 1926 and is characterized by a severely decreased amount of starch in the endosperm. The *bt-1* locus in the mutant is disrupted by an integrated transposable element that was used to identify the *bt-1* locus and to clone the corresponding cDNA (Sullivan et al., 1991). Based on extensive biochemical evidence it has been proposed that the BT1 protein is involved in the transport of ADP-Glc, the substrate for starch biosyn-

Figure 1. Transport processes across the inner envelope membrane of plastids from higher plants. Only transporters that were characterized at the molecular level are shown. The names of the transport proteins are explained in Table I. All names refer to the proteins in Arabidopsis, except for the BT1 protein from maize. The origin of the transporters is indicated by a color code. Blue, Host cell; green, cyanobacterium; pink, horizontal gene transfer; black, unknown. GSH, Glutathione; Mal, malate; MTOP, 4-methylthio-2-oxobutanoate; OG, 2-oxoglutarate; P_i, inorganic phosphate; SAH, *S*-adenosylhomo-Cys; SAM, *S*-adenosyl-Met; Xyl5P, xylulose-5-P; γ-EC, γ-glutamyl-Cys.
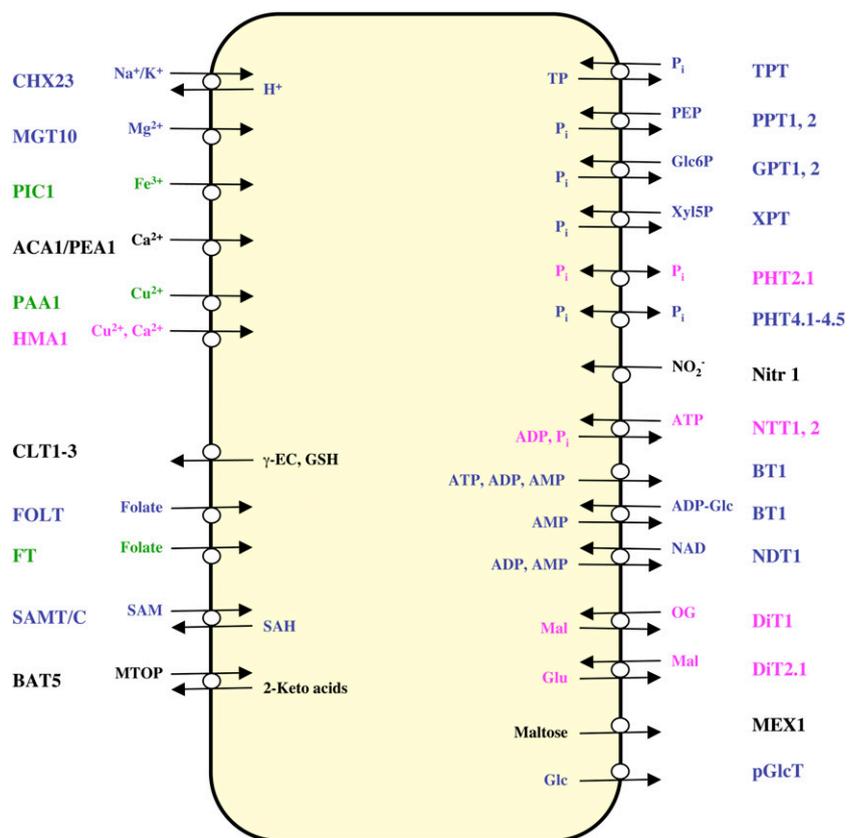
Table I. *Transporters of the inner envelope membrane of plastids from higher plants*

Only transporters with well-defined physiological functions are included. HGT, Horizontal gene transfer.

Name	Activity	Gene ID	Origin	Identification	Reference
TPT	TrioseP/phosphate translocator	At5g46110	Host cell	Protein labeling	Flügge et al. (1989)
PPT1	PEP/phosphate translocator 1	At5g33320	Host cell	Protein purification	Fischer et al. (1997)
PPT2	PEP/phosphate translocator 2	At3g01550	Host cell	Sequence homology	Knappe et al. (2003)
GPT1	Glc6P/phosphate translocator 1	At5g54800	Host cell	Protein purification	Kammerer et al. (1998)
GPT2	Glc6P/phosphate translocator 2	At1g61800	Host cell	Sequence homology	Knappe et al. (2003)
XPT	Xyl5P/phosphate translocator	At5g17630	Host cell	Sequence homology	Eicks et al. (2002)
PHT4.4	Phosphate transporter	At4g00370	Host cell	Sequence homology	Guo et al. (2008)
PHT4.2	Phosphate transporter	At2g38060	Host cell	Sequence homology	Guo et al. (2008)
PHT4.3	Phosphate transporter	At3g46980	Host cell	Sequence homology	Guo et al. (2008)
PHT4.5	Phosphate transporter	At5g20380	Host cell	Sequence homology	Guo et al. (2008)
PHT2.1	Phosphate transporter	At3g26570	HGT	Sequence homology	Versaw and Harrisson (2002)
Nitr1	Nitrite transporter	At1g68570	Unknown	cDNA Isolation	Sugiura et al. (2007)
NTT1	Nucleotide transporters	At1g80300	HGT	Sequence homology	Reiser et al. (2004)
NTT2	Nucleotide transporters	At1g15500	HGT	Sequence homology	Reiser et al. (2004)
BT1	Brittle 1 protein (maize)	BT016796	Host cell	Forward genetics	Sullivan et al. (1991)
BT1	Brittle 1 protein	At4g32400	Host cell	Sequence homology	Leroch et al. (2005)
NDT1	NAD transporter	At2g47490	Host cell	Sequence homology	Palmieri et al. (2009)
DiT1	Dicarboxylate transporter 1	At5g12860	HGT	Protein purification	Weber et al. (1995)
DiT2.1	Dicarboxylate transporter 2.1	At5g64290	HGT	Sequence homology	Taniguchi et al. (2002)
DiT2.2	Dicarboxylate transporter 2.2	At5g64280	HGT	Sequence homology	Renné et al. (2003)
pGlcT	Plastid Glc transporter	At5g16150	Host cell	Protein labeling	Weber et al. (2000)
Mex1	Maltose exporter 1	At5g17520	Unknown	Forward genetics	Niittylä et al. (2004)
CHX23	Cationic proton exchanger 23	At1g05580	Host cell	Sequence homology	Song et al. (2004)
MGT10	Magnesium transporter 10	At5g22830	Host cell	Sequence homology	Li et al. (2001)
PIC1	Permease in chloroplasts 1	At2g15290	Cyanobacterium	Sequence homology	Duy et al. (2007)
ACA1/PEA1	Arabidopsis calcium ATPase	At1g27770	Unknown	Antibody Screening	Huang et al. (1993)
PAA1	Copper P-type ATPase	At4g33520	Cyanobacterium	Forward genetics	Shikanai et al. (2003)
HMA1	Heavy metal ATPase 1	At4g37270	HGT	Proteomics	Seigneurin-Berny et al. (2006)
CLT1	CRT like transporter 1	At5g19380	Unknown	Forward genetics	Maughan et al. (2010)
CLT2	CRT like transporter 2	At4g24460	Unknown	Forward genetics	Maughan et al. (2010)
CLT3	CRT like transporter 3	At5g12160	Unknown	Forward genetics	Maughan et al. (2010)
FOLT1	Folate transporter 1	At5g66830	Host cell	Sequence homology	Bedhomme et al. (2005)
FT	Folate transporter	At2g32040	Cyanobacterium	Sequence homology	Klaus et al. (2005)
SAMT/C	SAM transporter/carrier	At4g39460	Host cell	Sequence homology	Bouvier et al. (2006)
BAT5	Bile acid transporter like 5	At5g12030	Unknown	Coexpression	Gigolashvili et al. (2009)

thesis. In cereals, this metabolite is synthesized in the cytosol and therefore has to be imported into amyloplasts. This theory was finally proved to be correct by direct measurement of the transport activity of the maize BT1 protein after heterologous expression in *Escherichia coli* (Kirchberger et al., 2007; but notice the different function of BT1 in plants other than cereals [Fig. 1; Table I]). A second transporter being involved in starch metabolism was identified in a screen for starch excess mutants. One mutant showed, in addition, elevated levels of maltose and was shown to be impaired in the export of maltose generated in the plastid during starch degradation at night. The mutated locus that was named *mex-1* (maltose excess) was shown to encode the plastid maltose transporter Mex-1 (Niittylä et al., 2004). A loss of Mex-1 leads to an accumulation of maltose in amyloplasts that in turn severely inhibits starch breakdown. Other transporters that were also identified by mutant analysis are listed in Table I. These examples clearly indicate that for-

ward genetics screens are valuable tools for plastid research.

Modern Times: Bioinformatics and Omics Revelations

The enormous amount of data emerging from the numerous genome sequencing projects offers unique opportunities for the identification of plastid transporters. Indeed, more than half of the transporters listed in Table I were identified based on their sequence homology to transporters of known function in other organisms, namely cyanobacteria (e.g. the folate transporter FT), parasitic bacteria (e.g. the ATP transporter NTT), yeast (e.g. the NAD transporter NDT or the cationic ion exchanger CHX23), plants (e.g. the XPT), or mammalian cells (the folate transporter FOLT). These homology searches can be combined with in silico analyses of their subcellular localization using several algorithms that have been developed to identify targeting signals within proteins. The wealth of data

garnered is publicly available in (plant) protein databases such as ARAMEMNON (http://aramemnon. botanik.uni-koeln.de/) or SUBA (http://suba.plantenergy. uwa.edu.au/).

Transcriptome projects offer a new analytic approach, the guilty-by-association approach, which is based on the concept that genes with similar function often display similar transcription profiles, meaning genes can be identified based on their coexpression with genes of known function (Armbruster et al., 2010). Recently, using this new in silico approach, a first plastid transporter was identified. BAT5 is a member of a small family of five proteins in Arabidopsis (*Arabidopsis thaliana*) having homology to bile acid carriers from mammalian cells. BAT5, but not the other members of the family, shows coexpression with several genes involved in the synthesis of aliphatic glucosinolates (Gigolashvili et al., 2009), a pathway that is divided between the cytosol and the stroma. This specific compartmentation implies the need for the transport of metabolites of this pathway across the envelope membranes. In a comprehensive analysis of *bat5* mutants it could be shown that BAT5 is catalyzing the import of 4-methylthio-2-oxobutanoate, which is produced from Met in the cytosol. In chloroplasts, 4-methylthio-2-oxobutanoate is then converted to homoketo acids of different chain length, followed by the export into the cytosol, most likely also by BAT5 (Gigolashvili et al., 2009). These first results demonstrate that transcriptome analysis is a powerful tool for the identification of genes in plants.

Another widely used high-throughput method is proteomics, i.e. the analysis of the total protein content of a particular type of cell or of a cellular compartment. Proteome studies of purified envelope membranes from chloroplasts of spinach (*Spinacia oleracea*), Arabidopsis, pea (*Pisum sativum*), and maize (from both mesophyll and bundle sheath cells) and also from proplastids of cauliflower (*Brassica oleracea*; e.g. Ferro et al., 2003; Fröhlich et al., 2003; Bräutigam et al., 2008; Majeran et al., 2008; Bräutigam and Weber, 2009) have been performed. These studies not only confirmed the number of 300 to 400 envelope membrane proteins identified by in silico analysis, but also caught a first glimpse of the dynamics of the envelope proteome, i.e. the similarities and differences between different plants species and between different types of plastids. Various proteins of unknown function have been identified in these studies. A first protein (HMA1) has been analyzed in more detail and shown to represent a copper transporter involved in the import of copper into plastids (Seigneurin-Berny et al., 2006; a second copper transporter [PAA1] has been identified by forward genetics [Table I]).

THE MEANING OF PLASTID TRANSPORTERS

Knowledge of the substrate specificities of a transporter is the prerequisite for understanding its phys-

iological function and its role in plant metabolism. Therefore, several techniques have been developed for the measurement of the transport activities of membrane proteins. These include the expression of cDNAs encoding a particular transporter in heterologous systems such as *E. coli* or yeast (*Saccharomyces cerevisiae*), followed by the measurement of the transport activity of the recombinant protein. The bacterial system has the advantage that the plant transport proteins are integrated into the bacterial plasma membrane. This enables the determination of the transport activity by measuring the import or export of radiolabeled substances into or out of intact *E. coli* cells. Several plastid transporters have been analyzed using this approach including the NTTs and BT1 from maize and Arabidopsis (Tjaden et al., 1998; Kirchberger et al., 2007). As the production of membrane proteins in *E. coli* is in some cases difficult to achieve, yeast cells have been widely used for this purpose as an alternative. As yeast cells do not possess plastids, plastid proteins are targeted to other internal membranes, i.e. to mitochondrial membranes and/or to the endoplasmic reticulum (ER; Loddenkötter et al., 1993). For further characterization, the recombinant protein is isolated from yeast membranes, in most cases by affinity chromatography, and reconstituted into artificial membranes to measure its transport activity (Loddenkötter et al., 1993). All proteins of the pPT family and several other plastid transporters have been characterized at the molecular level by this approach (Flügge et al., 2003).

Reverse Genetics: A Powerful Tool

The in vivo function of a transporter, however, cannot be deduced from its in vitro transport activity alone. Reducing gene expression in planta either by knockout (by insertion of a T-DNA or a transposon) or knockdown approaches (by RNA interference), or increasing gene expression by overexpression of an introduced second copy of the gene that is under the control of a specific promoter clarify the in vivo roles of transport proteins. I want to illustrate the enormous potential of these reverse genetics approaches by two examples, the analysis of the GPTs and the NTTs (for a broader overview covering many more proteins see, for example, Linka and Weber, 2010 and refs. therein).

Plastids of nongreen tissues from multicellular plants import carbon mainly in the form of Glc6P as a source for biosynthetic pathways, and ATP as source of energy. Several studies of carbon metabolism in plants indicate that Glc6P is used for three different purposes. It is the substrate for starch synthesis and fatty acid synthesis in nongreen plastids but is also fed into the oxidative pentose phosphate pathway for the production of reducing equivalents (NADPH). The import of Glc6P into heterotrophic plastids is mediated by the GPT (Kammerer et al., 1998). As Arabidopsis possesses two different functional GPTs, GPT1 and 2, the contribution of these two transporters to plant metabolism was originally not known.

Therefore, *gpt1* and *gpt2* mutants were analyzed. The deletion of the *gpt1* gene turned out to be lethal (Niewiadomski et al., 2005). A detailed analysis of heterozygous *GPT1*/*gpt1* plants revealed that the development of both the female and the male *gpt1* gametophyte are impaired. Two more recent investigations showed that GPT1 is also involved in embryo development and seed maturation (Rolletschek et al., 2007; Andriotis et al., 2010). A reduction of *GPT1* expression of about 50% in seeds of *Vicia narbonensis* by an antisense approach with a seed-specific promoter led to a shift in assimilate partitioning from starch/lipids into storage proteins, indicating that GPT1 exerts a significant control on seed filling and maturation (Rolletschek et al., 2007). A reduction of more than 80% of *GPT1* expression in Arabidopsis seeds is lethal because embryo development stops at the globular stage (Andriotis et al., 2010). The reason why a defect in Glc6P transport leads to such severe phenotypes is not clear so far. The existence of several starch-free mutants of Arabidopsis that retain full fertility demonstrates that starch accumulation per se is not a prerequisite for development. As the lipid content in Arabidopsis seeds with a reduced GPT1 activity is only slightly reduced, an interruption of fatty acid synthesis is not the cause of the lethality. Therefore, it has been suggested that a reduction in the production of NADPH via the oxidative pentose phosphate pathway impairs several NADPH-dependent processes necessary for plastid development and differentiation, loss of which inhibits gametophyte and embryo development (Niewiadomski et al., 2005; Andriotis et al., 2010). However, a direct proof of this hypothesis is still lacking.

In contrast, the *gpt2* knockout line shows no detectable phenotype. Only recently it could be shown that *GPT2* expression is up-regulated in mutants impaired in starch synthesis (Kunz et al., 2010). In an extensive analysis of *gpt2* plants and several double knockout lines, the contributions of both GPT1 and GPT2 to metabolism in vegetative tissues were dissected. Whereas the GPT1 is constitutively present in particular cells such as stomatal guard cells of leaves or cells of the root tip, GPT2 is induced when carbohydrate metabolism is impaired, e.g. at higher concentrations of soluble sugars (Kunz et al., 2010). Thus, the two GPT proteins, even though showing the same transport activity, have different physiological functions in Arabidopsis.

ATP represents the universal energy currency of all living cells. It is mainly synthesized in mitochondria and, in plant cells, also in chloroplasts. An ATP transport activity has been shown to be present in envelope membranes of chloroplasts and nongreen plastids (Heldt, 1969). Within the past decade, the physiological functions of the two ATP transporters (NTTs) in Arabidopsis have been characterized. Extensive analysis of *NTT* antisense, RNA interference, and knockout plants revealed distinctive physiological roles of the NTTs in different types of plastids (Reiser et al., 2004).

In nongreen plastids, the function of the NTTs, especially NTT2, is the supply of ATP for several anabolic pathways. These transporters exert significant control on starch synthesis in amyloplasts of potato (*Solanum tuberosum*) tubers and on lipid biosynthesis in developing Arabidopsis seeds (Geigenberger et al., 2001).

In contrast to nongreen plastids, the function of ATP transport in chloroplasts remained elusive. Chloroplasts show a high level of ATP synthesis by photophosphorylation but the ATP transport activity in these organelles is low. Therefore, it has been proposed that the function of ATP transport is to energize unknown processes in the stroma during the night, rather than to export ATP during photosynthesis (Heldt, 1969). Only very recently, this hypothesis could be validated through the analysis of Arabidopsis lines lacking both NTTs (Reinhold et al., 2007). These mutants showed a dwarf phenotype with necrotic lesions on the leaves under short-day conditions at low-light intensities, a phenotype that is caused by photooxidation due to the accumulation of protoporphyrin IX. The increased protoporphyrin IX levels are most likely due to a reduced activity of magnesium-chelatase, which requires ATP for catalysis and also for stabilization of the holoenzyme. Thus, one important ATP-dependent process during the night is magnesium-chelatase assembly (Reinhold et al., 2007). Whether the inhibition other energy-dependent processes in chloroplasts in the dark are also impaired in the mutant is not known so far.

TRANSPORT PROCESSES IN SECONDARY PLASTIDS: A NEW AND PROMISING FIELD

Compared to primary plastids, almost nothing is known about transport processes in secondary plastids that are derived from at least three different secondary endosymbiotic events, in which either a green or a red algae was taken up by a protist and subsequently reduced to a plastid. Although there is an ongoing debate about the complicated phylogeny of the different groups of algae and protists (with the possibility of tertiary endosymbiotic events; for more information see Archibald, 2009; Keeling, 2010) it can be clearly stated that these different endosymbiotic events gave rise to an enormous diversity of algae and nonphotosynthetic protists like the diatoms and the parasitic *Apicomplexa* (Gould et al., 2008). Secondary plastids are surrounded by three or four membranes representing the two envelope membranes of the primary plastid (the two innermost membranes), the plasma membrane of the endosymbiont and a fourth membrane that is continuous with the ER of the host cell. Thus, solute transport is much more complicated compared to primary plastids as solutes have to cross up to four membranes.

Recently, two transporters from the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* showing homology to plastid NTTs from higher plants

were identified and analyzed (Ast et al., 2009). By expression of these genes in *E. coli* cells it was shown that NTT1 transports different adenine nucleotides including dATP, while NTT2 transports a range of different nucleotides and deoxynucleotides. Thus, the substrate specificities of the algal transporters are much broader than those of their counterparts from higher plants (Fig. 1), indicating a different physiological function, namely the import of nucleotides into the plastids from the cytosol where their synthesis takes place. Thus, the permeability of the envelope membranes clearly reflects differences in the metabolism of diatoms and higher plants, here the distinct localization of nucleotide biosynthesis in the cytosol and in plastids.

Another important group of organisms bearing secondary plastids are the *Apicomplexa*, a phylum comprising several thousand obligate intracellular protozoan parasites. Several of these species are causing important human and animal diseases. Malaria caused by species of the genus *Plasmodium* remains one of the most devastating diseases worldwide, leading to more than 1 million deaths per year and imposing a massive economic burden on developing countries (Breman, 2001). *Toxoplasma gondii* causes severe toxoplasmosis in immunocompromised individuals while other species are important pathogens in wild and domestic animals (Wong and Remington, 1993). The discovery that *Apicomplexa* harbor a single secondary plastid, called apicoplast, more than a decade ago represented a major breakthrough in this field (McFadden et al., 1996). It is now widely believed that the apicoplast was derived from the uptake of a red algal cell and that the *Apicomplexa* are part of the eukaryotic supergroup *Chromalveolata* (Gould et al., 2008). The identification of a photosynthetic alga, *Chromera velia*, which belongs to the alveolates and is closely related to the *Apicomplexa* corroborates this phylogeny (Moore et al., 2008). Furthermore, *Apicomplexa* possess a number of plant-specific metabolic pathways, many of them associated with the apicoplast. Like plants, some apicomplexan species synthesize fatty acids via a bacterial type II fatty acid synthesis pathway located in apicoplasts (Mazumdar and Striepen, 2007). Second, isopentenyl diphosphate, the precursor of isoprenoids, is synthesized via the nonmevalonate or 1-deoxy-xylulose 5-phosphate pathway found only in plastids and bacteria. Several inhibitor and gene knockout studies have provided strong evidence that these plant-like metabolic pathways and the apicoplast where they are located in are essential for survival of the parasites and thus provide new possibilities for drug development (e.g. Jomaa et al., 1999).

A comprehensive in silico analysis of the genome of *Plasmodium falciparum* and other *Apicomplexa* revealed that their genomes encode less than 200 membrane transporters, which is about 10% of the transporters found in other, nonparasitic eukaryotes (Martin et al., 2005; R. Schwacke and K. Fischer, unpublished data). Obviously, these cells are minimalistic with regard to

transporters. Only a few transporters homologous to plastid transporters from higher plants have been identified, and only the apicoplast phosphate translocator (APT) has been characterized in detail (Brooks et al., 2010; Lim et al., 2010). While *Plasmodium* species possess two APTs, *T. gondii* has only one. The APTs show a significant similarity with the pPT family from higher plants and are located in the apicoplast envelope membranes, most likely in the innermost but also in the outermost membrane. A detailed analysis of the transport activities of the APTs revealed that they are able to transport TPs, 3-phosphoglycerate, PEP, and inorganic phosphate (Brooks et al., 2010; Lim et al., 2010). Thus, these transporters have the combined substrate specificities of a TPT and a PPT from higher plants. The physiological function of the APTs is the import of carbon in the form of TPs and PEP that are used as substrates for fatty acid synthesis and most likely also for the 1-deoxy-xylulose 5-phosphate pathway (Brooks et al., 2010). A disruption of the APT gene in *T. gondii* is lethal, clearly showing the importance of this transporter for parasitic metabolism.

The few examples of transporters from secondary plastids indicate that it is an important goal in the next decade to characterize their transport processes in more detail. Many of these organisms are of enormous medical importance while unicellular algae like the diatoms represent a major part of the phytoplankton and are responsible for a large part of the primary productivity in the ocean (Roberts et al., 2007).

WHERE DO THEY COME FROM? NEW INSIGHTS INTO THE EVOLUTION OF PLASTID TRANSPORTERS

After the uptake of a cyanobacterium by a host cell, an endosymbiotic relationship (or an enslavement of the bacterium, as suggested by some authors [Cavalier-Smith, 2000]) was established. A major factor in the process of bacterial integration into the host cell was the endosymbiotic gene transfer, i.e. the massive transfer of genes from the endosymbiont to the nucleus of the host cell. The endosymbiotic gene transfer had to be closely accompanied by the evolution of a plastid protein import machinery for the reimport of proteins that were now synthesized in the cytosol. Both processes are extensively discussed in the literature (e.g. Bhattacharya et al., 2007; Archibald, 2009). A second important process, the change in membrane permeability during the transformation of the bacterial membranes into the organellar envelope membranes, is, in contrast, rarely mentioned in the literature. This process was achieved by the loss of many bacterial transporters and by the insertion of new transporters with different transport characteristics. It has been proposed that such a system of new metabolite transporters was a critical and very early step in plastid evolution (Cavalier-Smith, 2000; Weber et al., 2006) based on the following arguments: The establishment

of an endosymbiotic relationship is driven mainly through complementation of the host cell's limited metabolic capabilities by the biochemical versatility of the endosymbiont (Nowack and Melkonian, 2010). Therefore, the integration of at least one or more transporters into the bacterial membranes to establish a connection between both cells would enable the export of bacterial metabolites and allow the host cell to benefit immediately from bacterial metabolism, i.e. photosynthesis and other processes, thereby stabilizing and sustaining endosymbiosis early on (Cavalier-Smith, 2000; Bhattacharya et al., 2007; Tyra et al., 2007).

In modern plants, carbon assimilated in photosynthesis is exported from chloroplasts via two different routes. During the day, carbon is exported in the form of TPs by the TPT (Flügge et al., 2003) while at night chloroplasts export mainly maltose and—to a lesser extent—Glc, both derived from starch breakdown (Weber et al., 2000; Niittylä et al., 2004). An extensive analysis of the phylogeny of the pPT family has clearly revealed that these transporters are related to a large superfamily of transporters, the nucleotide sugar transporters that are localized in the Golgi or ER membranes and transport nucleotide sugars like GDP-Man or UDP-Gal into the ER and Golgi lumen (Knappe et al., 2003; Weber et al., 2006). The pPTs are derived from a single nucleotide sugar transporter of the host cell that was rerouted from its location, the endomembranes, to the bacterial plasma membrane. This hypothesis is supported by recent evidence of membrane contact sites between the ER membranes and plastids that allow the exchange of lipids between both organellar membranes but that might also explain the rerouting of proteins from endomembranes to plastids during organellar evolution (Andersson et al., 2007). Thus, it is tempting to speculate that the first metabolites exported from the endosymbiont were nucleotide sugars like UDP-Gal or, as has been suggested very recently, ADP-Glc (Colleoni et al., 2010). During the evolution of plastids the substrate specificities of the pPTs later changed from nucleotide sugars to phosphorylated metabolites like TPs and, after the split into the different pPT subfamilies, to PEP and sugar phosphates.

To get more insight into the evolution of plastid envelope transporters, Bhattacharya and colleagues performed a detailed phylogenetic analysis of more than 80 plastid transporters commonly found in plants (Tyra et al., 2007). It turned out that about 60% of all transport proteins were derived from existing host membrane proteins. These include, besides the pPT family, phosphate transporters and transporters for S-adenosyl-Met and folate (Fig. 1; Table I; Tyra et al., 2007). In contrast, only 12% of the membrane proteins were contributed by the cyanobacterial endosymbiont, for example a second folate transporter (Klaus et al., 2005). For about 20% of the proteins their origin could not be conclusively determined. These include Mex1 that is found only in higher plants and a number of transporters of unknown function. Finally, a number

of proteins were identified having homologs only in obligate intracellular bacterial parasites like *Chlamydia* and *Rickettsia* (Tyra et al., 2007). These are the two NTTs, the metal ATPase HMA1, the phosphate transporter PHT2, and the dicarboxylate translocators (DiT1, 2). These surprising findings have recently been explained by a horizontal gene transfer between plants and bacteria. Although the direction of the transfer is a matter of discussion, phylogenetic analysis supports a *Chlamydia*-to-*Plantae* transfer (Tyra et al., 2007 and refs. therein).

The data discussed here clearly corroborate the assumption that the integration of plastid and host metabolism was mainly driven by the host cell and that their interrelationship is rather slavery than symbiosis.

CONCLUSION

In the last 20 years enormous progress has been achieved in understanding the transport processes across both the inner and outer envelope membranes of plastids and their role in connecting the metabolism of plastids with that of the other parts of a plant cell. However, we are far away from having completed the puzzle. The omics techniques (genomics, proteomics, transcriptomics, metabolomics, etc.) that have been developed in the last decade and that are now collectively called systems biology have had and will have an enormous impact on plant science. Several plastid transporters have been identified and characterized by these methods in combination with biochemistry and molecular biology. One goal ought to be the description and functional characterization of the proteome of a model plastid, namely the chloroplast of Arabidopsis, including the function of all transporters in both the inner and outer envelope membrane. A second goal would be to look at the differences of the proteomes of different types of plastids and at changes of the permeability of the plastid membranes during plastid differentiation. Studying the adaptation of the transport processes to different types of photosynthesis, namely C_3, C_4, and crassulacean acid metabolism and studying the transport into secondary plastids will lead to a better understanding of adaptations to changing environments and of the evolution of intracellular connections.

LITERATURE CITED

Andersson MX, Goksör M, Sandelius AS (2007) Optical manipulation reveals strong attracting forces at membrane contact sites between endoplasmic reticulum and chloroplasts. J Biol Chem **282:** 1170–1174

Andriotis VME, Pike MJ, Bunnewell S, Hills MJ, Smith AM (2010) The plastidial glucose-6-phosphate/phosphate antiporter GPT1 is essential for morphogenesis in Arabidopsis embryos. Plant J **64:** 128–139

Archibald JM (2009) The puzzle of plastid evolution. Curr Biol **19:** R81–R88

Armbruster U, Pesaresi P, Pribil M, Hertle A, Leister D (2010) Update on chloroplast research: new tools, new topics and new trends. Mol Plant **4:** 1–16

Ast M, Gruber A, Schmitz-Esser S, Neuhaus HE, Kroth PG, Horn M, Haferkamp I (2009) Diatom plastids depend on nucleotide import from the cytosol. Proc Natl Acad Sci USA **106:** 3621–3626

Bedhomme M, Hoffmann M, McCarthy EA, Gambonnet B, Moran RG, Rébeillé F, Ravanel S (2005) Folate metabolism in plants. An Arabidopsis homolog of the mammalian mitochondrial folate transporter mediates folate import into chloroplasts. J Biol Chem **280:** 34823–34831

Bhattacharya D, Archibald JM, Weber APM, Reyes-Prieto A (2007) How do endosymbionts become organelles? Understanding early events in plastid evolution. Bioessays **29:** 1239–1246

Bölter B, Soll J (2001) Ion channels in the outer membranes of chloroplasts and mitochondria: open doors or regulated gates? EMBO J **20:** 935–940

Bouvier F, Linka N, Isner JC, Mutterer J, Weber APM, Camara B (2006) Arabidopsis SAMT1 defines a plastid transporter regulating plastid biogenesis and plant development. Plant Cell **18:** 3088–3106

Bräutigam A, Hoffmann-Benning S, Weber APM (2008) Comparative proteomics of chloroplast envelopes from C₃ and C₄ plants reveals specific adaptations of the plastid envelope to C₄ photosynthesis and candidate proteins required for maintaining C₄ metabolite fluxes. Plant Physiol **148:** 568–579

Bräutigam A, Weber APM (2009) Proteomic analysis of the proplastid envelope membrane provides novel insights into small molecule and protein transport across proplastid membranes. Mol Plant **2:** 1247–1261

Breman JG (2001) The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. Am J Trop Med Hyg (1-2 Suppl) **64:** 1–11

Brooks CF, Johnsen H, van Dooren GG, Muthalagi M, Lin SS, Bohne W, Fischer K, Striepen B (2010) The toxoplasma apicoplast phosphate translocator links cytosolic and apicoplast metabolism and is essential for parasite survival. Cell Host Microbe **7:** 62–73

Cavalier-Smith T (2000) Membrane heredity and early chloroplast evolution. Trends Plant Sci **5:** 174–182

Colleoni C, Linka M, Deschamps P, Handford MG, Dupree P, Weber APM, Ball SG (2010) Phylogenetic and biochemical evidence supports the recruitment of an ADP-glucose translocator for the export of photosynthate during plastid endosymbiosis. Mol Biol Evol **27:** 2691–2701

Duy D, Wanner G, Meda AR, von Wirén N, Soll J, Philippar K (2007) PIC1, an ancient permease in Arabidopsis chloroplasts, mediates iron transport. Plant Cell **19:** 986–1006

Eicks M, Maurino V, Knappe S, Flügge UI, Fischer K (2002) The plastidic pentose phosphate translocator represents a link between the cytosolic and the plastidic pentose phosphate pathways in plants. Plant Physiol **128:** 512–522

Ferro M, Salvi D, Brugière S, Miras S, Kowalski S, Louwagie M, Garin J, Joyard J, Rolland N (2003) Proteomics of the chloroplast envelope membranes from *Arabidopsis thaliana.* Mol Cell Proteomics **2:** 325–345

Fischer K, Kammerer B, Gutensohn M, Arbinger B, Weber A, Häusler RE, Flügge UI (1997) A new class of plastidic phosphate translocators: a putative link between primary and secondary metabolism by the phosphoenolpyruvate/phosphate antiporter. Plant Cell **9:** 453–462

Flügge UI, Fischer K, Gross A, Sebald W, Lottspeich F, Eckerskorn C (1989) The triose phosphate-3-phosphoglycerate-phosphate translocator from spinach chloroplasts: nucleotide sequence of a full-length cDNA clone and import of the *in vitro* synthesized precursor protein into chloroplasts. EMBO J **8:** 39–46

Flügge UI, Häusler RE, Ludewig F, Fischer K (2003) Functional genomics of phosphate antiport systems of plants. Physiol Plant **118:** 475–482

Fröhlich JE, Wilkerson CG, Ray WK, McAndrew RS, Osteryoung KW, Gage DA, Phinney BS (2003) Proteomic study of the *Arabidopsis thaliana* chloroplastic envelope membrane utilizing alternatives to traditional two-dimensional electrophoresis. J Prot Res **2:** 413–425

Geigenberger P, Stamme C, Tjaden J, Schulz A, Quick PW, Betsche T, Kersting HJ, Neuhaus HE (2001) Tuber physiology and properties of starch from tubers of transgenic potato plants with altered plastidic adenylate transporter activity. Plant Physiol **125:** 1667–1678

Gigolashvili T, Yatusevich R, Rollwitz I, Humphry M, Gershenzon J, Flügge UI (2009) The plastidic bile acid transporter 5 is required for the biosynthesis of methionine-derived glucosinolates in *Arabidopsis thaliana.* Plant Cell **21:** 1813–1829

Gould SB, Waller RF, McFadden GI (2008) Plastid evolution. Annu Rev Plant Biol **59:** 491–517

Guo B, Jin Y, Wussler C, Blancaflor EB, Motes CM, Versaw WK (2008) Functional analysis of the Arabidopsis PHT4 family of intracellular phosphate transporters. New Phytol **177:** 889–898

Heldt HW (1969) Adenine nucleotide translocation in spinach chloroplasts. FEBS Lett **5:** 11–14

Heldt HW (2002) Three decades in transport business: studies of metabolite transport in chloroplasts—a personal perspective. Photosynth Res **73:** 265–272

Huang L, Berkelman T, Franklin AE, Hoffman NE (1993) Characterization of a gene encoding a Ca-ATPase-like protein in the plastid envelope. Proc Natl Acad Sci USA **90:** 10066–10070

Jomaa H, Wiesner J, Sanderbrand S, Altincicek B, Weidemeyer C, Hintz M, Türbachova I, Eberl M, Zeidler J, Lichtenthaler HK, et al (1999) Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs. Science **285:** 1573–1576

Kammerer B, Fischer K, Hilpert B, Schubert S, Gutensohn M, Weber A, Flügge UI (1998) Molecular characterization of a carbon transporter in plastids from heterotrophic tissues: the glucose 6-phosphate/phosphate antiporter. Plant Cell **10:** 105–117

Keeling PJ (2010) The endosymbiotic origin, diversification and fate of plastids. Philos Trans R Soc Lond B Biol Sci **365:** 729–748

Kirchberger S, Leroch M, Huynen MA, Wahl M, Neuhaus HE, Tjaden J (2007) Molecular and biochemical analysis of the plastidic ADP-glucose transporter (ZmBT1) from *Zea mays.* J Biol Chem **282:** 22481–22491

Klaus SM, Kunji ER, Bozzo GG, Noiriel A, de la Garza RD, Basset GJ, Ravanel S, Rébeillé F, Gregory III JF, Hanson AD (2005) Higher plant plastids and cyanobacteria have folate carriers related to those of trypanosomatids. J Biol Chem **280:** 38457–38463

Knappe S, Flügge UI, Fischer K (2003) Analysis of the plastidic phosphate translocator gene family in Arabidopsis and identification of new phosphate translocator-homologous transporters, classified by their putative substrate-binding site. Plant Physiol **131:** 1178–1190

Kunz HH, Häusler RE, Fettke J, Herbst K, Niewiadomski P, Gierth M, Bell K, Steup M, Flügge UI, Schneider A (2010) The role of plastidial glucose-6-phosphate/phosphate translocators in vegetative tissues of *Arabidopsis thaliana* mutants impaired in starch biosynthesis. Plant Biol (Stuttg) (Suppl 1) **12:** 115–128

Leroch M, Kirchberger S, Haferkamp I, Wahl M, Neuhaus HE, Tjaden J (2005) Identification and characterization of a novel plastidic adenine nucleotide uniporter from Solanum tuberosum. J Biol Chem **280:** 17992–18000

Li L, Tutone AF, Drummond RS, Gardner RC, Luan S (2001) A novel family of magnesium transport genes in Arabidopsis. Plant Cell **13:** 2761–2775

Lim L, Linka M, Mullin KA, Weber APM, McFadden GI (2010) The carbon and energy sources of the non-photosynthetic plastid in the malaria parasite. FEBS Lett **584:** 549–554

Linka N, Weber APM (2010) Intracellular metabolite transporters in plants. Mol Plant **3:** 21–53

Loddenkötter B, Kammerer B, Fischer K, Flügge UI (1993) Expression of the functional mature chloroplast triose phosphate translocator in yeast internal membranes and purification of the histidine-tagged protein by a single metal-affinity chromatography step. Proc Natl Acad Sci USA **90:** 2155–2159

Majeran W, Zybailov B, Ytterberg AJ, Dunsmore J, Sun Q, van Wijk KJ (2008) Consequences of C₄ differentiation for chloroplast membrane proteomes in maize mesophyll and bundle sheath cells. Mol Cell Proteomics **7:** 1609–1638

Martin RE, Henry RI, Abbey JL, Clements JD, Kirk K (2005) The 'permeome' of the malaria parasite: an overview of the membrane transport proteins of *Plasmodium falciparum.* Genome Biol **6:** R26

Maughan SC, Pasternak M, Cairns N, Kiddle G, Brach T, Jarvis R, Haas F, Nieuwland J, Lim B, Müller C, et al (2010) Plant homologs of the Plasmodium falciparum chloroquine-resistance transporter, PfCRT, are required for glutathione homeostasis and stress responses. Proc Natl Acad Sci USA **107:** 2331–2336

Mazumdar J, Striepen B (2007) Make it or take it: fatty acid metabolism of apicomplexan parasites. Eukaryot Cell **6:** 1727–1735

McFadden GI, Reith ME, Munholland J, Lang-Unnasch N (1996) Plastid in human parasites. Nature **381:** 482

Moore RB, Oborník M, Janouskovec J, Chrudimský T, Vancová M, Green DH, Wright SW, Davies NW, Bolch CJS, Heimann K, et al (2008) A photosynthetic alveolate closely related to apicomplexan parasites. Nature **451:** 959–963

Niewiadomski P, Knappe S, Geimer S, Fischer K, Schulz B, Unte US, Rosso MG, Ache P, Flügge UI, Schneider A (2005) The *Arabidopsis* plastidic glucose 6-phosphate/phosphate translocator GPT1 is essential for pollen maturation and embryo sac development. Plant Cell **17:** 760–775

Niittylä T, Messerli G, Trevisan M, Chen J, Smith AM, Zeeman SC (2004) A previously unknown maltose transporter essential for starch degradation in leaves. Science **303:** 87–89

Nowack ECM, Melkonian M (2010) Endosymbiotic associations within protists. Philos Trans R Soc Lond B Biol Sci **365:** 699–712

Palmieri F, Rieder B, Ventrella A, Blanco E, Thi Do P, Nunes-Nesi A, Trauth AU, Fiermonte G, Tjaden J, Agrimi G, et al (2009) Molecular identification and functional characterization of Arabidopsis thaliana mitochondrial and chloroplastic NAD carrier proteins. J Biol Chem **284:** 31249–31259

Reinhold T, Alawady A, Grimm B, Beran KC, Jahns P, Conrath U, Bauer J, Reiser J, Melzer M, Jeblick W, et al (2007) Limitation of nocturnal import of ATP into Arabidopsis chloroplasts leads to photooxidative damage. Plant J **50:** 293–304

Reiser J, Linka N, Lemke L, Jeblick W, Neuhaus HE (2004) Molecular physiological analysis of the two plastidic ATP/ADP transporters from Arabidopsis. Plant Physiol **136:** 3524–3536

Renné P, Dressen U, Hebbeker U, Hille D, Flügge UI, Westhoff P, Weber APM (2003) The Arabidopsis mutant *dct* is deficient in the plastidic glutamate/malate translocator DiT2. Plant J **35:** 316–331

Roberts K, Granum E, Leegood RC, Raven JA (2007) Carbon acquisition by diatoms. Photosynth Res **93:** 79–88

Rolletschek H, Nguyen TH, Häusler RE, Rutten T, Göbel C, Feussner I, Radchuk R, Tewes A, Claus B, Klukas C, et al (2007) Antisense inhibition of the plastidial glucose-6-phosphate/phosphate translocator in *Vicia* seeds shifts cellular differentiation and promotes protein storage. Plant J **51:** 468–484

Seigneurin-Berny D, Gravot A, Auroy P, Mazard C, Kraut A, Finazzi G, Grunwald D, Rappaport F, Vavasseur A, Joyard J, et al (2006) HMA1, a new Cu-ATPase of the chloroplast envelope, is essential for growth under adverse light conditions. J Biol Chem **281:** 2882–2892

Shikanai T, Müller-Moulé P, Munekage Y, Niyogi KK, Pilon M (2003) PAA1, a P-type ATPase of Arabidopsis, functions in copper transport in chloroplasts. Plant Cell **15:** 1333–1346

Somerville SC, Ogren WL (1983) An *Arabidopsis thaliana* mutant defective in chloroplast dicarboxylate transport. Proc Natl Acad Sci USA **80:** 1290–1294

Song CP, Guo Y, Qiu Q, Lambert G, Galbraith DW, Jagendorf A, Zhu JK (2004) A probable Na (K)/H exchanger on the chloroplast envelope functions in pH homeostasis and chloroplast development in Arabidopsis thaliana. Proc Natl Acad Sci USA **101:** 10211–10216

Sugiura M, Georgescu MN, Takahashi M (2007) A nitrite transporter associated with nitrite uptake by higher plant chloroplasts. Plant Cell Physiol **48:** 1022–1035

Sullivan TD, Strelow LI, Illingworth CA, Phillips RL, Nelson OE Jr (1991) Analysis of maize brittle-1 alleles and a defective *Suppressor-mutator*-induced mutable allele. Plant Cell **3:** 1337–1348

Taniguchi M, Taniguchi Y, Kawasaki M, Takeda S, Kato T, Sato S, Tabata S, Miyake H, Sugiyama T (2002) Identifying and characterizing plastidic 2-oxoglutarate/malate and dicarboxylate transporters in *Arabidopsis thaliana.* Plant Cell Physiol **43:** 706–717

Tjaden J, Schwöppe C, Möhlmann T, Quick PW, Neuhaus HE (1998) Expression of a plastidic ATP/ADP transporter gene in Escherichia coli leads to a functional adenine nucleotide transport system in the bacterial cytoplasmic membrane. J Biol Chem **273:** 9630–9636

Tyra HM, Linka M, Weber APM, Bhattacharya D (2007) Host origin of plastid solute transporters in the first photosynthetic eukaryotes. Genome Biol **8:** R212

Versaw WK, Harrison MJ (2002) A chloroplast phosphate transporter, PHT2;1, influences allocation of phosphate within the plant and phosphate-starvation responses. Plant Cell **14:** 1751–1766

Walker DA (2003) Chloroplasts in envelopes: CO_2 fixation by fully functional intact chloroplasts. Photosynth Res **76:** 319–327

Weber A, Menzlaff E, Arbinger B, Gutensohn M, Eckerskorn C, Flügge UI (1995) The 2-oxoglutarate/malate translocator of chloroplast envelope membranes: molecular cloning of a transporter containing a 12-helix motif and expression of the functional protein in yeast cells. Biochemistry **34:** 2621–2627

Weber A, Servaites JC, Geiger DR, Kofler H, Hille D, Gröner F, Hebbeker U, Flügge UI (2000) Identification, purification, and molecular cloning of a putative plastidic glucose translocator. Plant Cell **12:** 787–802

Weber APM, Fischer K (2007) Making the connections: the crucial role of metabolite transporters at the interface between chloroplast and cytosol. FEBS Lett **581:** 2215–2222

Weber APM, Linka M, Bhattacharya D (2006) Single, ancient origin of a plastid metabolite translocator family in Plantae from an endomembrane-derived ancestor. Eukaryot Cell **5:** 609–612

Weber APM, Schwacke R, Flügge UI (2005) Solute transporters of the plastid envelope membrane. Annu Rev Plant Biol **56:** 133–164

Wong SY, Remington JS (1993) Biology of *Toxoplasma gondii.* AIDS **7:** 299–316

Yoon HS, Hackett JD, Ciniglia C, Pinto G, Bhattacharya D (2004) A molecular timeline for the origin of photosynthetic eukaryotes. Mol Biol Evol **21:** 809–818