The chloroplast proteome: A survey from the *Chlamydomonas reinhardtii* perspective with a focus on distinctive features

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Abstract

The unicellular green alga *Chlamydomonas reinhardtii* has emerged to be an important model organism for the study of oxygenic eukaryotic photosynthesis as well as other processes occurring in the chloroplast. However, the chloroplast proteome in *C. reinhardtii* has only recently been comprehensively characterized, made possible by proteomics emerging as an accessible and powerful tool over the last decade. In this review, we introduce a compiled list of 996 experimentally chloroplast-localized proteins for *C. reinhardtii*, stemming largely from our previous proteomic dataset comparing chloroplasts and mitochondria samples to localize proteins. In order to get a taste of some cellular functions taking place in the *C. reinhardtii* chloroplast, we will focus this review particularly on metabolic differences between chloroplasts of *C. reinhardtii* and higher plants. Areas that will be covered are: Photosynthesis, chlorophyll biosynthesis, carbon metabolism, fermentative metabolism, ferredoxins and ferredoxin-interacting proteins.

Introduction

Chloroplasts are thought to have arisen from cyanobacteria through endosymbiosis (Goksoyr 1967) and are the site for important cellular processes such as photosynthesis, nitrogen assimilation, amino acid biosynthesis, sulfur metabolism and isoprenoid biosynthesis. Although contemporary chloroplasts still possess their own genome, it only encodes for a fraction genes as those of free-living cyanobacteria, indicating that many genes have been lost from plastids or transferred to the nucleus (Martin et al. 2002). Phylogenomic data comparing several photosynthetic eukaryotes with cyanobacteria reveal genes in the host nuclear genome that are of cyanobacterial origin and further analysis of these genes suggests a filamentous, nitrogen-fixing heterocyst cyanobacteria as a likely candidate for the chloroplast ancestor (Deusch et al. 2008). The unicellular green alga *Chlamydomonas reinhardtii* is an important model organism in many areas of research, including photosynthesis and other vital processes occurring in the chloroplast (Harris 2001). Despite a well-established history of research in *C. reinhardtii*, the proteomes of various cellular compartments are not yet fully characterized. In *C. reinhardtii*, the chloroplast itself retained 72 genes (Maul et al. 2002), but the chloroplast proteome is expected to consist of around 3,000 proteins (Abdallah et al. 2000). The majority of the proteins in the chloroplast are nucleus-encoded and imported to the chloroplast after translation in the cytosol. Unfortunately, prediction tools such as TargetP and ChloroP (Emanuelsson et al. 1999, 2007) are not optimized for Chlamydomonas proteins, leading to false localization. TargetP and ChloroP localized approximately 50% of the experimentally chloroplast-localized Chlamydomonas proteins correctly to the chloroplast (Terashima et al. 2010), showing large discrepancies between experimental and prediction tool localizations.

There have been numerous efforts within the last decade to characterize the chloroplast proteome of higher plants, which act as references for Chlamydomonas proteins as well. Kleffmann et al. identified 690 chloroplast proteins in *Arabidopsis thaliana* through MS/MS identifications (Kleffmann et al. 2004), which was followed by the publication of the Plant Proteomics Database at Cornell (PPDB) by Sun et al. that provides resources for experimentally identified proteins in *A. thaliana* and *Zea mays*. The PPDB contains 5,000+ accessions, 80+ published Arabidopsis proteome datasets from sub-cellular compartments and 1,500+ Arabidopsis proteins that were manually assigned to a sub-cellular location (Sun et al. 2009). Sub-cellular localization has also been compiled in the SUBA database as well (Heazlewood et al. 2007). Additionally, another chloroplast protein list was created for Arabidopsis for proteins localized to the chloroplast in two independent studies, resulting in a total of 1156 proteins (Yu et al. 2008; Baginsky and Gruissem 2009; Reiland et al. 2009). A table in a review by Baginsky compiles over 50 plant organelle proteomics studies performed largely in the first half of the last decade, showing that this is a well established field in plant biology (Baginsky 2009). A recent addition to the Arabidopsis chloroplast proteome is another database from Ferro et al., AT_CHLORO, which consists of 1,323 proteins from Arabidopsis leaves identified from LC-MS/MS-based analyses, localizing proteins to the stroma, thylakoids and the envelope membranes (Ferro et al. 2010). Overall proteomics in Arabidopsis is well established with numerous available resources (Wienkoop et al. 2010).

Experiment-based characterization of the chloroplast proteome is not nearly as established in *C. reinhardtii* as in *A. thaliana*. In the last five years, there have been milestones in the field of proteomics for Chlamydomonas, but very few in the realm of chloroplast as a whole. Allmer et al. identified 233 proteins from isolated thylakoid membranes (Allmer et al. 2006; Naumann et al. 2007), Stauber et al. characterized the LHCI and LHCII proteins (Stauber et al. 2003, 2009), and Yamaguchi et al. characterized the chloroplast ribosomes (Yamaguchi and Subramanian 2000; Yamaguchi et al. 2000, 2002, 2003). Concurrently, progress was being made in other organelle-based proteomics such as work from Schmidt et al. and Wagner et al. on the eyespot proteome and Atteia et al., characterizing the mitochondria proteome, to name a few (Schmidt et al. 2006; Wagner et al. 2008; Atteia et al. 2009). A recent review summarizes the proteomics-based research for *C. reinhardtii* (Rolland et al. 2009). A proteomics approach by Terashima et al. on isolated chloroplasts and mitochondria allowed for new insights on the chloroplast proteome (Terashima et al. 2010). This review stems from this previous work to further explore selected aspects of the Chlamydomonas chloroplast proteome.

From the data presented in Terashima et al., samples enriched in chloroplasts and mitochondria were measured using a mass spectrometer and the spectral counts for all the identified proteins were analyzed (Terashima et al. 2010). Chloroplasts isolated from *C. reinhardtii* usually contain about 10-15% mitochondria contamination, while mitochondria can be isolated to a high degree of purity from chloroplast proteins (Terashima et al. 2010). On the other hand cytosolic proteins co-migrated more abundantly in the mitochondria compared to the chloroplasts. For this reason, proteins identified from isolated chloroplasts were compared with proteins identified from mitochondria in order to identify the contaminants. From a total of 2,315 confidently identified proteins, an experimentally defined core chloroplast proteome of 606 proteins with an additional 289 proteins that are candidates for chloroplast localization were deduced. In order to create a more comprehensive list of Chlamydomonas chloroplast proteins, the list of 895 experimentally chloroplast-localized proteins was further extended to include proteins that were not identified in the proteomics data but are chloroplast-encoded as well as those proteins that are annotated in JGI Chlamydomonas gene model database (v3.1) and NCBI databases (BK000554.2 and NC_001638.1) as being chloroplast-localized. This amounted to a total of 996 chloroplast-localized proteins. These proteins were run through the NCBI Basic Local Alignment Search Tool algorithm (BLAST) (Altschul et al. 1990) against the NCBI non-redundant database (ftp://ftp.ncbi.nlm.nih.gov/blast/db) complemented with all *C. reinhardtii* proteins in order to create a simple distribution visualization of these proteins in context of BLAST hit results for higher plants and bacteria (Figure 1 and Supplementary Table 1). The purpose of the BLAST results are not to make any claims of specificity or characteristics in an evolutionary context for individual proteins, but to have a simple method of visualization of the 996 proteins in a larger scheme of bacteria, green algae, algae and higher plants. For this reason, no evolutionary conclusions should be made from these BLAST results. The blastp program was used with the default settings (word size: 3; scoring matrix: BLOSUM62; gap opening cost: 11; gap extension cost: 1; filter low complexity regions: Yes; E-value cutoff: 10). When all 996 chloroplast proteins were run through BLAST, there were 417,010 hits to over 10,600 organisms. As expected, the best hit for every protein was to *C. reinhardtii.* We evaluated the BLAST results according to bit scores. However, because the bit scores (as well as the E-values) depend on the length of the sequence, bit scores were normalized to the *C. reinhardtii* hit, resulting in a relative bit score in the range from zero to one. For every hit, we determined the corresponding genus by using the NCBI taxonomy database.

Fig. 1

In order to compile the BLAST results for the purpose of this review, we divided the hits for the 996 proteins into four groups, discarding hits that matched to none of these four groups: 1) Bacteria, 2) Green algae (Chlorophyta) 3) Algae (which consist of Chlorarachniophytes, Chlorophyta, Cryptomonads, Dinoflagellates, Euglenids, Glaucophyta, Haptophyta, Heterokonts and Rhodophyta) and 4) Vascular plants and mosses (Figure 1 and Supplementary Table 1). Green algae in addition to algae were investigated because the algae group includes a wide range of organisms. In addition, all hits to Chlamydomonas and Volvox genus were discarded because of the similarity of these organisms to *C. reinhardtii*. At this point, for each protein three lists are available (one for each taxonomic group). To determine a representative relative bit score for each of the groups, lists were shortened, discarding low scores, until all three lists had the same number of hits. Finally, the relative bit score for each group was determined by calculating the median of all relative bit scores in the list. A summary of the BLAST results can be found in Supplementary Table 1.

As expected, there are strong similarities between pathways found in higher plants and *C. reinhardtii*, as well as numerous conserved proteins among photosynthetic organisms, grouped as GreenCut proteins (Merchant et al. 2007) (Figure 1). GreenCut proteins are proteins conserved in Chlamydomonas, Ostreococcus, moss and Arabidopsis. There is a region of the photosynthetic proteins in Figure 1 that is not overlapping with the GreenCut proteins. This is because these consist of many chloroplast-encoded proteins, which were not considered during the generation of the GreenCut protein list (Merchant et al. 2007). There is also a pronounced variation between the chloroplast proteome of *C. reinhardtii* and vascular plants in terms of the different localization of conserved pathways, algae-specific metabolic pathways and bacterial pathways conserved in *C. reinhardtii* but not in vascular plants, which will be discussed in further detail in this review. Similarities of certain *C. reinhardtii* proteins to proteins from bacteria are seen in Figure 1A and 1B, especially for proteins involved in fermentation (visualized in red circles in Figure 1).

The aim of this review is not to characterize the entire *C. reinhardtii* chloroplast proteome, but rather to focus on describing some of the pathways and proteins represented in this 996 chloroplast- localized protein list that have been characterized to be different from the chloroplast proteome in higher plants. Topics that we will touch on are: photosynthesis, acclimation and adaptation of the photosynthetic electron transport chain towards metal deficiencies, chlorophyll biosynthesis, carbon metabolism, fermentative metabolism, ferredoxins and ferredoxin-interacting proteins.

Photosynthesis

Fig. 2

The chloroplast is perhaps best known as being the site for some of the most important reactions for photosynthetic organisms. Of the 996 chloroplast proteins, 118 proteins take part in photosynthesis, according to the functional "bins" from MapMan (http://mapman.gabipd.org/web/guest/mapmanstore) (Thimm et al. 2004) (Figure 2). *C. reinhardtii* has been a prime organism to study oxygenic eukaryotic photosynthesis (Hippler et al. 1998; Eberhard et al. 2008). Furthermore, powerful forward and reverse genetic approaches are available. In particular, due to an efficient homologous chloroplast recombination system, specific alterations of critical amino acid residues within the photosynthetic core complexes could be performed and functionally analyzed. Thus many mechanistic aspects known today of excitation energy and electron transfer within oxygenic eukaryotic photosynthetic complexes were discovered using the *C. reinhardtii* model system. In general, it can be stated that the overall architecture of the photosynthetic core complexes are very similar between *C. reinhardtii* and vascular plants (Nield et al. 2004). This is visualized by the clustering of proteins involved in photosynthesis when BLAST hits between algae and vascular plants and moss are compared in Figure 1C and 1D. In regard to the protein composition of the photosynthetic machinery, the light-harvesting system between green algae and vascular plants shows some major differences. *C. reinhardtii* and vascular plants both possess several trimer-forming *LHCB* gene products that functionally associate with PSII, yet the Chlamydomonas genes are no proper orthologs for the plant *LHCB1-3* genes. While the minor PSII antenna proteins Lhcb4 and 5 (CP29 and CP26) are present in green algae and vascular plants, *C. reinhardtii* lacks an ortholog to the minor antennae, CP24 (Elrad and Grossman 2004). In regard to the PSI antenna proteins, differences between *A. thaliana* and *C. reinhardtii* have also been described. While *A. thaliana* encodes for six (*LHCA1-6*), *C. reinhardtii* encodes for nine *LHCA* genes (designated *LHCA1* through *9*). In the latter case, all nine *LHCA* gene products were identified on the protein level (Stauber et al. 2003). The Lhca2 and Lhca9 subunits from *C. reinhardtii* appear to form an algae-specific

LHCI clade (Koziol et al. 2007). The crystal structure of the pea PSI-LHCI complex illustrated that four Lhca subunits form a half-moon shaped complex at the PsaF/PsaJ side of the PSI core (Ben-Shem et al. 2003; Amunts et al. 2007). Biochemical analysis of isolated LHCI complex indicated that the Chlamydomonas LHCI complex is significantly larger than its plant counterpart (Hippler et al. 2001; Stauber et al. 2003), an observation that has been further supported by results of electron microscopy studies (Germano et al. 2002; Kargul et al. 2003). From these studies it also became evident that the half-moon shaped LHCI complex at the PsaF/PsaJ side of the PSI core also exists in *C. reinhardtii*. Furthermore it was suggested that Lhca monomers may bind to the PsaH side of the PSI core (Nield et al. 2004). Using isotope dilution mass spectrometry, the number of LHCI polypeptides per PSI core from *C. reinhardtii* was estimated to be 7.5±1.4 (Stauber et al. 2009). Therefore it seems safe to conclude that the PSI-LHCI from *C. reinhardtii* is larger and probably more flexible in regard to Lhca polypeptide binding and composition as compared to the higher plant complement. Despite the low sequence identity between the Lhca proteins from *C. reinhardtii* and *A. thaliana*, the pigment binding and spectroscopic properties are very similar, with the highest resemblance to Lhca2 from *A. thaliana* (Mozzo et al. 2010). In *A. thaliana*, the minor LHCI-subunits Lhca5 and Lhca6, which are expressed at very low levels (Klimmek et al. 2006), have been implicated in NAD(P)H dehydrogenase-PSI complex formation and function (Peng et al. 2009). This multi-protein complex is involved in cyclic electron transfer in *A. thaliana,* but is lacking in *C. reinhardtii* (see below), pointing to a functional recruitment of Lhca subunits that is absent at the level of green algae.

Besides differences in Lhca and Lhcb polypeptides, *C. reinhardtii* codes for three light-harvesting genes that are named *LHCSR1*, *LHCSR3.1* and *LHCSR3.2*. These types of light-harvesting genes, encoding an ancient class of LHC proteins, are absent in all currently sequenced vascular plant genomes (Koziol et al. 2007). Gene and protein expression studies and microarray analyses demonstrated that the expression of *LHCSR* genes is induced under high light stress and under phosphorus, iron or sulfur deficiencies (Im et al. 2003; Zhang et al. 2004; Moseley et al. 2006; Naumann et al. 2007). The strong induction under stress implies a role of these proteins in acclimation to photo-oxidizing conditions. In such conditions, excess photons must be de-excited to avoid photodamage in high light. In plants the fastest response to excess light is provided by a mechanism called non-photochemical quenching (NPQ). The most important constituent of NPQ is qE, which regulates the thermal dissipation of excess absorbed light energy and operates at a time scale of seconds to minutes. In that way the qE mechanism provides efficient photo-protection. qE is also described as a feedback de-excitation mechanism since its occurrence is dependent on the formation of a pH-gradient across the thylakoid membrane due to active photosynthetic electron transfer. Most interestingly in *C. reinhardtii,* effective qE is indeed dependent on LHCSR3 (Peers et al. 2009). This has been demonstrated by LHCSR3 knock-down and knock-out mutant studies, where depletion and/or deletion of LHCSR3 had a severe impact on the development of qE (Peers et al. 2009). Contrarily in vascular plants, PSBS, a photosystem II (PSII) polypeptide, is essential for efficient qE (Li et al. 2000). There is currently no evidence that PSBS, in addition to LHCSR3, participates in the establishment of qE in *C. reinhardtii*, because no protein expression of PSBS in the alga has been reported. This is remarkable given the fact that two *PSBS* genes are encoded in the *C. reinhardtii* genome. Consequently, the function of PSBS in the green alga remains unclear. Interestingly, while *C. reinhardtii* uses LHCSR3 for driving qE, the moss *Physcomitrella patens,* which encodes for *PSBS* and *LHCSR* genes, utilizes both types of regulatory proteins to operate qE (Alboresi et al. 2010). This reveals an interesting aspect of the evolution of terrestrial plants, pointing to the fact that land plants evolved a novel PSBS-dependent qE mechanism before losing the ancestral LHCSR-specific type found in algae. It is also of note that qE is constitutive in vascular plants, whereas it is induced upon acclimation to high light in green algae (Peers et al. 2009).

Interestingly, the plant-specific CAS (Calcium Sensor) calcium binding protein and calcium appear to be involved in the regulation of the high light response and particularly in the regulation of expression of LHCSR3 in *C. reinhardtii* (Petroutsos et al., submitted). Depletion of CAS by ami-RNA or RNAi approaches resulted in a strong decrease in light-dependent induction of LHCSR3, a pronounced light-sensitivity, as well as a delay in PSII recovery after photoinhibition. Interestingly, the phenotypes can be rescued by the addition of a ten-fold higher Ca^{2+} concentration to the growth medium. CAS has initially been described as a plasma membrane-localized cell surface receptor that mediates extracellular Ca^{2+} sensing in guard cells (Han et al. 2003). In contrast, using functional proteomics, the CAS protein was found to be enriched in thylakoid membranes from *A. thaliana* (Peltier et al. 2004) and *C. reinhardtii* (Allmer et al. 2006). Independent data recently confirmed the chloroplast localization of the CAS protein (Nomura et al. 2008; Vainonen et al. 2008; Weinl et al. 2008). Experimental evidence indicated a role of CAS in the chloroplast-mediated control of external Ca^{2+}-induced cytosolic Ca^{2+} transients and stomatal closure (Nomura et al. 2008; Weinl et al. 2008). However, CAS does not play a role in the control of NPQ and particularly qE in *A. thaliana* (Vainonen et al. 2008). The finding that CAS-depletion resulted in a severe light-sensitivity is in contrast to the phenotype observed for A. thaliana knock-out mutant plants. Thus similar to PSBS, the CAS protein is another example that conserved orthologous proteins in *C. reinhardtii* and *A. thaliana* may differ in function and in their placement in the intrinsic cellular response and signal transduction pathways.

Besides qE, a process called state transition also contributes to NPQ (qT). State transitions are important to balance the excitation energy between PSI and PSII (Bonaventura and Myers 1969; Murata 1969). Under light conditions where PSII is preferentially excited, PSII core and LHCII proteins become phosphorylated. As a consequence, phosphorylated LHCII proteins detach from PSII and migrate to PSI (state 2). Because this process is reversible, under conditions where PSI is particularly excited, LHCII proteins are de-phosphorylated and migrate back to PSII (state 1). The extent of state transition between vascular plants such as *A. thaliana* and *C. reinhardtii* differs significantly. While the proportion of mobile LHCII antenna is about 80% in the alga, only 15-20% of LHCII in *A. thaliana* are transferred to PSI under state 2 conditions. From a functional point of view, the STT7 kinase in *C. reinhardtii* and its ortholog STN7 in *A. thaliana* are essential for LHCII phosphorylation and the corresponding initiation of state transitions (Depége et al. 2003; Bellafiore et al. 2005). For more in-depth details about the mechanism of state transitions, the reader is referred to (Lemeille and Rochaix 2010). As mentioned above, light-harvesting protein composition and function may differ between chloroplasts in algae and vascular plants, as well as the mechanistic properties of state transition. Recently, a state 2-specific PSI-LHCI supercomplex containing two minor monomeric LHCII proteins CP26 and CP29, as well as a major LHCII protein designated Lhcbm5 was isolated from *C. reinhardtii* (Takahashi et al. 2006). Intriguingly, CP29 RNAi lines, although having normal LHCII phosphorylation, have abolished pLHCII association to PSI (Tokutsu et al. 2009) underpinning the central role of CP29 in the LCHII-PSI attachment process in Chlamydomonas. Currently there is no evidence that CP29 participates in state transitions in vascular plants.

Chlorophyll biosynthesis

In contrast to vascular plants, chlorophyll biosynthetic genes in *C. reinhardtii* are controlled by copper-deficiency and oxygen depletion. CRD1 (Copper Response Defect) was first isolated in *C. reinhardtii* as a mutant with partial iron deficiency phenotype (development of chlorosis, reduced amounts of LHCI and PSI subunits) in absence of copper (Moseley et al. 2000). It is a di-iron containing enzyme, in which for the homolog in the purple bacterium *Rubrivivax gelatinosus,* the enzyme is involved in the aerobic oxidative cyclization of an intermediate of the bacteriochlorophyll a biosynthesis (Pinta et al. 2002). Indeed, a crd1-antisense line of *A. thaliana* displayed an overaccumulation of Mg-protoporphyrin IX (Tottey et al. 2003), supporting the notion that the protein function has been conserved from bacteria to land plants. *C. reinhardtii* possesses two isoforms of the enzyme, one common in copper sufficient conditions (CTH1) and one in copper deficient conditions (CRD1), both included in the list of 996 chloroplast proteins (Moseley et al. 2002). This is contrary to vascular plants, which only contain one homolog of *CRD1*, *CHL27* (Moseley et al. 2000; Tottey et al. 2003). Beside CRD1, also coproporphyrinogen III oxidase (CPX1) is induced under copper-deficiency (Quinn et al, 2000) Interestingly, *CRD1*, *CPX1* as well as other copper-deficiency induced genes are also induced by hypoxia. A recent study identified a transcription factor, CRR1 (Copper Response Regulator), with a plant-specific DNA-binding domain named SBP, ankyrin repeats, and a C-terminal Cys-rich region that is required for both activating and repressing target genes of a copper- and hypoxia-sensing pathway in *C. reinhardtii* (Kropat et al. 2005; Sommer et al. 2010).

Aside from CRD1, *C. reinhardtii* is unique in other aspects of chlorophyll biosynthesis because, in addition to the light-dependent chlorophyll biosynthesis seen in higher plants, it is able to synthesize chlorophyll in the dark (Fujita et al. 1996; Armstrong 1998). This pathway has been largely studied in anoxygenic photosynthetic bacteria (Zappa et al. 2010), but is also found in cyanobacteria, nonvascular plants, ferns and gymnosperms. *C. reinhardtii* has conserved both pathways, which enables adaptation to sudden onset of light and dark conditions because of the existence of light-dependent and light-independent protochlorophyllide reductases. The dataset of 996 chloroplast proteins contains the light-dependent protochlorophyllide reductase (POR) and subunits B, N, and L of the light-independent protochlorophyllide reductase.

In *C. reinhardtii*, the light-independent reduction of protochlorophyllide to chlorophyllide requires three chloroplast genes (*CHLL, CHLN, and CHLB*) along with at least seven nuclear loci (*y-1 to y-10*) (Li et al. 1993; Timko 1998). Mutants disrupted in these genes showed a "yellow-in-the-dark" phenotype, but were able to normally synthesize chlorophyll in the light (Li et al. 1993; Cahoon and Timko 2000). Cahoon and Timko showed that *CHLL* gene expression is negatively regulated by light in aerobic conditions. However, they observed that CHLL levels are high under anaerobic conditions in the light, similar to levels seen under dark conditions. In cyanobacteria, anaerobic induction of CHLL was also observed and recent studies have shown the light-independent protochlorophyllide reductase to be oxygen sensitive (Yamazaki et al. 2006; Yamamoto et al. 2009). Yamamoto et al. suggest that cyanobacteria have mechanisms to protect light-independent protochlorophyllide reductase from oxygen, enabling the protein to remain in oxygenic photosynthetic organisms (Yamamoto et al. 2009). Further investigation into these protective mechanisms would be interesting, especially for biotechnological applications such as the realization of an active hydrogenase under (semi-)aerobic conditions. If the protection mechanism for the light-independent protochlorophyllide reductase could be applied to protect the hydrogenase, one could perhaps alleviate the necessity to construct an oxygen-insensitive hydrogenase.

To return to the subject of chlorophyll biosynthesis, the ability to synthesize chlorophyll in the dark was obviously an advantage for *C. reinhardtii* living in environments that can switch between light and dark over short periods of time, allowing for immediate light energy utilization during light conditions without the need to induce chlorophyll synthesis. The light-independent reduction of protochlorophyllide to chlorophyllide is yet another example of characteristics shared between *C. reinhardtii* and certain prokaryotes.

Carbon metabolism

Fig. 3

The electron transport chain ultimately provides reducing power to fix CO_2 in the Calvin-Benson cycle, allowing for starch production and providing precursors for glycolysis, gluconeogenesis and the non-oxidative pentose phosphate pathway. Localization of the majority of these proteins to the chloroplast is conserved between *C. reinhardtii* and higher plants. In vascular plants, it has been shown that many proteins connecting the glycolytic pathway to the Calvin-Benson cycle are localized to the chloroplast. Joyard et al. recently confirmed the localization of these proteins in *A. thaliana* to the chloroplast (Joyard et al. 2010). Interestingly, the proteins involved in the second half of the glycolysis converting 3-phosphoglycerate ultimately to pyruvate appear not to be localized to the chloroplast in *C. reinhardtii* (Figure 3). Several studies have shown the phosphoglycerate mutase, enolase and the pyruvate kinase to be localized to the outside of the chloroplast through enzymatic activity assays, immunoblot analyses and mass spectrometric identifications (Klein 1987; Klock and Kreuzberg 1991; Mitchell et al. 2005; Terashima et al. 2010). *C. reinhardtii* possesses multiple isoforms of the phosphoglycerate mutase and the pyruvate kinase (PYK), which could suggest the localization of one of the isoforms to the chloroplast. However, from our previous proteomics study, we identified PYK 1-3 and 9, but they were not chloroplast-localized (although PYK3 abundance was too low to make any definite conclusions about the localization) (Terashima et al. 2010). On the other hand, pyruvate, phosphate dikinase (PPDK) was found in abundance in the chloroplast. PPDK catalyzes the interconversion between PEP and pyruvate, working in both directions unlike PYK, which can only work to synthesize pyruvate from phosphoenolpyruvate (Nevalainen et al. 1996; Slamovits and Keeling 2006). Therefore, perhaps PPDK catalyzes this reaction in the chloroplast instead of PYK. PPDK will be discussed further in the fermentation section of this review.

Considering that *C. reinhardtii* possesses only one isoform of enolase and the fact that it works in between phosphoglycerate mutase and PYK suggest that this second half of the glycolytic pathway is not present in the chloroplast (Figure 3), unlike in vascular plants. Contrarily, pathways leading to the production of 3-phosphoglycerate (which includes Calvin-Benson cycle, the first half of glycolysis, and also indirectly the non-oxidative pentose phosphate pathway, which relies on intermediates from glycolysis as well as sharing intermediates with the Calvin-

Benson cycle) have been localized to the chloroplast in *C. reinhardtii.* The majority of the proteins involved in these pathways have been identified in the 996 chloroplast proteins (Terashima et al. 2010). A reason for the split compartmentalization of glycolysis in *C. reinhardtii* could be for energy partitioning (Ginger et al. 2010). In *C. reinhardtii*, light-driven ATP production can easily support the ATP consumption phase of glycolysis in the chloroplast. Therefore, localizing the second "pay-back" phase of the pathway outside of the chloroplast provides additional ATP and NADH to fuel other metabolic functions. This type of separation of at least one of the ATP-producing steps to another cellular compartment is present in other organisms. For further insights on cross-compartmentalization of metabolism in protist, please refer to the recent review by Ginger et al. (Ginger et al. 2010).

Fermentative metabolism

Fig. 4

Overall, there is a large similarity of chloroplastic proteome between the green alga *C. reinhardtii* and higher plants, which is visualized by the up-shift of the distribution of the proteins when comparing relative BLAST hit scores between algae and plants and mosses (Figure 1D) from to the distribution pattern between algae and bacteria (Figure 1B). However, the fermentative proteins, represented in red in Figure 1, show a different trend because they shift down towards the algae axis in Figure 1D. This is because in *C. reinhardtii*, the fermentative pyruvate metabolism, along with photosynthetically-driven hydrogen production (Melis and Happe 2001), are absent in higher plants (Hemschemeier and Happe 2005) (Figure 4). When the hit scores are compared only to green algae, some fermentative proteins show a different trend. Two proteins, corresponding to ACK1 and PAT2, localize to the y-axis (Figure 1C, Supplementary Table 1). These proteins appear to have not been conserved in other green algae species (aside from *C. reinhardtii* and *Volvox*) and have hits only to bryophyte Physcomitrella and lycophyte Selaginella (E-values for ACK1 and PAT2 hits to Physcomitrella were e^{-95} and e^{-126} and for Selaginella e^{-88} and e^{-81}, respectively).

Both hydrogen production and the fermentative pyruvate metabolism are induced under anaerobic conditions. Hydrogenases in general are found in a number of organisms from bacteria to green algae and are utilized in both directions: to provide an electron source under nutrient-deprived conditions by the oxidation of H_2 and, in the reverse direction, to act as an electron outlet to prevent over-reduction of the electron transport chain (Appel and Schulz 1998; Esper et al. 2006). Many organisms, usually found in anaerobic environments, can grow by using H_2 as an electron source (Weaver et al. 1980). Similarities between prokaryotic hydrogenases usually operating in the opposite direction to the algal hydrogenase are demonstrated by the fact that heterologous expression of Chlamydomonas hydrogenase (*HYD1*) in *Clostridium acetobutylicum* and *Scenedesmus obliquus,* without co-transformation of the hydrogenase assembly factors, HydEF and HydG, results in a functionally active hydrogenase (Girbal et al. 2005). This feat is also possible in *Escherichia coli*, but only if the assembly factors are co-expressed (Posewitz et al. 2004).

Green algae and cyanobacteria are unique in that they are capable of both oxygenic photosynthesis and hydrogen production (Schutz et al. 2004). *C. reinhardtii* possesses two isoforms of the FeFe-hydrogenase, HydA1 and HydA2 (Florin et al. 2001; Happe and Kaminski 2002; Forestier et al. 2003), although HydA1 appears to be more prominently expressed in *C. reinhardtii* (Happe and Naber 1993; Kamp et al. 2008). In *C. reinhardtii*, the HydA1 works in association to the photosynthetic electron transport chain, accepting electrons from ferredoxin, encoded by *PETF,* under anaerobic conditions (Happe and Naber 1993; Happe and Kaminski 2002; Happe et al. 2002; Winkler et al. 2010). FeFe-hydrogenase is oxygen sensitive and as a result expressed under dark anaerobic conditions, transiently under anaerobic conditions in the light and under sulfur deprivation in the light where oxygen consumption exceeds production (Gaffron and Rubin 1942; Melis et al. 2000).

Recent data have indicated that in *C. reinhardtii* mutant strains where the expression of PGRL1 is depleted, hydrogen production is significantly induced after the onset of of sulfur-deficiency (Petroutsos, Tolstygina, Hippler, unpublished results)). In *A. thaliana* PGRL1 was described to be important for cyclic photosynthetic electron transfer (CEF) (DalCorso et al. 2008). In *C. reinhardtii* the expression of PGRL1 is induced under low iron conditions (Naumann et al. 2007) and required for efficient CEF under iron deprivation (Petroutsos et al. 2009). Anaerobic conditions also induce significant CEF in *C. reinhardtii*, which is impaired in PGRL1-depleted strains (Petroutsos, Tolstygina, Hippler, unpublished results). Aside from hydrogen production, the range of pyruvate metabolism demonstrates the numerous pathways that *C. reinhardtii* can utilize under anaerobic conditions, where the standard respiratory electron transport chain is inhibited and the $NAD(P)^+/NAD(P)H$ level needs to be re-balanced. A large majority of the fermentative metabolism appears to be present in the chloroplast as well as some parallel pathways existing in mitochondria. Previous studies by Atteia et al. have localized pyruvate formate lyase (PFL), acetate kinase 2 (ACK2) and phosphate acetyltransferase 1 (PAT1) to be mitochondrial (Atteia et al. 2006). This was further confirmed by the presence of these proteins in mitochondria-enriched samples through mass spectrometric analyses (Terashima et al. 2010). PFL appears to be present also in the chloroplast (Atteia et al. 2006) as well as isoforms ACK1 and PAT2 (Terashima et al. 2010). Additionally, the pyruvate ferredoxin oxidoreductase 1 (PFR1) and the alcohol dehydrogenase 1 (ADH1) are also localized to the chloroplast. ACK1, PAT2, PFR1 and ADH1 are included in the 996 chloroplast protein list, making up a small portion of the chloroplast proteome as a whole (Figure 2, Supplementary Table 1).

Especially under anaerobic conditions, the chloroplast increasingly becomes a reducing environment. ADH1 oxidizes two molecules of NADH per acetyl-CoA, helping to replenish the chloroplast with NAD^+ (Figure 4). *C. reinhardtii* possesses a bifunctional aldehyde/alcohol dehydrogenase, which was described for the non-photosynthetic chlorophyte *Polytomella sp*. as likely being localized to the mitochondrion (Atteia et al. 2003). Very little studies have been done specifically on *C. reinhardtii* ADH1 in terms of the origin of the enzyme. However, studies comparing ADH1 in the facultative anaerobic protozoa *Entamoeba histolytica* and *Giardia lamblia* show the enzyme to be bacterial origin for both species, but appear not necessarily to have the same origin, indicating independent horizontal transfer of this gene for each of the species (Rosenthal et al. 1997). Although ADH1 has been associated with anaerobic metabolism (Mus et al. 2007), recent findings indicate *ADH1* transcript level to be regulated by circadian cycles instead of oxygen availability (Whitney et al. 2010). Interestingly, ADH1 showed induction during the day under photosynthetic conditions instead of during the night (Whitney et al. 2010). A possible explanation for *ADH1* transcript levels to increase during the day and decrease at night could be that ADH1 would act in competition with the PAT2-ACK1 pathway at night, which yields one ATP per acetyl Co-A (Grossman et al. 2010).

Under dark conditions when light-driven ATP production is not possible, it would make sense that the PAT2-ACK1 ATP-producing pathway is utilized. ADH1 is then induced again during the day when photosynthesis can drive enough ATP production so that there is no dependence on the PAT2-ACK1 pathway and the remaining reducing equivalents can be utilized by ADH1 to rebalance the $NAD(P)^+/NAD(P)H$ ratio.

It is of note that ADH1 is significantly induced under iron-deficiency especially under photo-heterotrophic conditions (Höhner and Hippler, unpublished results). Interestingly the iron deficiency response is dependent on the metabolic status of the cells (Naumann et al. 2007; Busch et al. 2008; Terauchi et al. 2010). Under photoheterotrophic conditions, PSI is rapidly degraded to a content of 20-30 % as compared to the iron-sufficient situation, the iron storage protein ferritin is up-regulated and cells maintain high growth rates by increasing respiration. Photoautotrophic cells are less impacted by iron deficiency. They maintain both photosynthetic and respiratory function and their associated Fe-containing proteins in conditions where heterotrophic cells lose photosynthetic capacity. Such balanced adaptation strategies that are dependent on the trophic status of the organism are absent in vascular plants, which are obligate photoautotrophs. In this scenario ADH1 could be involved in rebalancing the $NAD(P)^+/NAD(P)H$ ratio under conditions where consumption of photosynthetically produced NADPH by carbon fixation is compromised.

Working upstream of ADH1, PFR1 allows for pyruvate decarboxylation in anaerobic organisms, a step usually catalyzed by the pyruvate dehydrogenase under oxygenic conditions. PFR1 enables the production of acetyl CoA without the requirement of NAD^+ as electron acceptors by reducing ferredoxin instead (Figure 4) (Charon et al. 1999; Ragsdale 2003). Unlike the pyruvate dehydrogenase, PFR1 can also catalyze the reverse reaction, producing pyruvate from CO_2 and acetyl CoA (Evans et al. 1966). PFR1 is utilized by microorganisms that inhabit absolute or partially anaerobic environments and is present in numerous prokaryotic (such as sulfate-reducing bacteria and clostridia) (Hatchikian and Le Gall 1970; Wahl and Orme-Johnson 1987) and a few eukaryotic organisms (such as protozoa and green algae) (Chen and Gibbs 1992a; Rosenthal et al. 1997; Terashima et al. 2010). The exact origin of the gene in Chlamydomonas is unknown. Research on anaerobic protozoa seems to reveal that the origin of the PFR gene in eukaryotes is more complicated than emerging simply from a single gene through vertical inheritance (Horner et al. 1999; Rotte et al. 2001; Embley et al. 2003). Clustering of various eukaryotic PFR sequences suggests a common origin of the gene (Embley 2006). However, the exact identity of this ancestor still remains a mystery because the PFR eukaryotic sequence cluster is not closest to α-proteobacteria, suggesting that this gene did not originate from the mitochondrial ancestor (Embley 2006). Where exactly *C. reinhardtii* fits into this story still remains to be discovered; a more recent phylogenetic study that included *C. reinhardtii* by Hug et al. discussed the likelihood that PFR derived from a few early lateral gene transfers from anaerobic bacteria to the eukaryotic cell, followed by a loss of the gene in the aerobic lineages (Hug et al. 2010). They also considered the possibility that the gene was incorporated and maintained only in anaerobic eukaryotes at a later state of evolutionary history through multiple independent lateral gene transfers, but conclude that the phylogenetic trees support the earlier acquisition of the gene for PFR. Nevertheless, the study by Hug et al. clearly indicates that PFR did not originate from the single α-proteobacterial mitochondrial ancestor, which is also in line with the results from Embley et al. as well as the chloroplast localization of this protein (Embley et al. 2003; Embley 2006).

The exact role of PFR1 in *C. reinhardtii* is unclear. It has been speculated that PFR1 induced under anaerobic conditions could reduce ferredoxin, which could subsequently donate electrons to the hydrogenase, explaining the low amounts of hydrogen observed in the dark when the photosynthetic electron transport chain could not account for the hydrogen production (Gfeller and Gibbs 1984; Kreuzberg 1984; Ohta et al. 1987; Atteia et al. 2006; Mus et al. 2007). However, recent findings have shown that electron sources do come predominantly from the electron transport chain under sulfur starvation-induced hydrogen production, suggesting that PFR1 is by no means the major contributor to hydrogen production, at least in the light (Hemschemeier et al. 2008).

Ragsdale suggested that PFR1 must have an anabolic role because organisms containing PFR1 cannot grow on substrates more complex than acetate (Ragsdale 2003). Interestingly, it has been postulated that PFR1 could work in the opposite direction to synthesize pyruvate in *C. reinhardtii* (gray arrow in Figure 4) (Chen and Gibbs 1992a; Melis 2007). Using a strain that lacked a complete reductive pentose-P pathway due to the absence of phosphoribulokinase, Chen and Gibbs demonstrated the probable presence of the reductive carboxylic acid cycle due to detection of PFR1 and α-ketoglutarate synthase activity in cell extracts (Chen and Gibbs 1992a). Many anaerobic environments naturally inhabited by *C. reinhardtii* are rich in acetate (Harris 2008) and Chen and Gibbs showed that *C. reinhardtii* does take up CO_2 in the dark coupled to the oxidation of H_2 through the reverse reaction of hydrogenase in minimal aerobic conditions (1% O_2) (Chen and Gibbs 1992b). They proposed that the reductive carboxylic acid cycle could be a significant pathway for CO_2 assimilation when the Calvin-Benson cycle is compromised (Chen and Gibbs 1992a). This would suggest that the hydrogenase or other reductants could reduce ferredoxin in dark anaerobic conditions, which could work with PFR1 to synthesize pyruvate, in turn leading to the production of the reductive carboxylic acid intermediates. Oxaloacetate can be synthesized directly from pyruvate by means of pyruvate carboxylase (PYC) or through a two-step process of first synthesizing phosphoenolpyruvate (PEP) by the PPDK, followed by oxaloacetate production through PEP carboxylase (PEPC) (Figure 4). Although PPDK catalyzes the same reaction as pyruvate kinase, it is able to work bidirectionally, while pyruvate kinase works irreversibly to synthesize pyruvate (Hatch and Slack 1968; Reeves 1968; Nevalainen et al. 1996; Slamovits and Keeling 2006). Another possibility for pyruvate metabolism is the direct production of malate through the malic enzyme (MME). Dubini et al. have suggested the synthesis of oxaloacetate or malate from a pyruvate precursor to explain the succinate accumulation under anaerobic conditions in a strain incapable of hydrogen production, which consequently induced *MME4* (Dubini et al. 2009). They presented a model for the accumulation of these metabolites in *C. reinhardtii*, which fits with the possibility that PFR1 is acting in the direction of pyruvate synthesis, connecting to the reductive carboxylic acid, resulting in the production of succinate using acetate (a phenomenon shown by Yoon et al. in the green sulfur bacteria *Chlorobium tepidum* (Yoon et al. 1999)). In *C. reinhardtii*, PYC, MME5, and PPDK have been localized to the chloroplast and are among the 996 chloroplast proteins (Figure 4, Supplementary Table 1) (Terashima et al. 2010). MME4 was not present in the mass spectrometric dataset, but MME isoforms 1, 2 and 6 were identified, with MME1 and 6 likely to be localized outside of the chloroplast and MME2 localization is inconclusive (Terashima et al. 2010). Currently, there is no evidence for PEPC localization to the chloroplast. The enzyme has been localized to the cytosol in higher plants (Chollet et al. 1996) and appears to also be cytosolic in *C. reinhardtii* (Giordano et al. 2003). There are two classes of PEPC isoforms in *C. reinhardtii* (Rivoal et al. 1998). The second class is more abundant in *C. reinhardtii* and is an unusual hetero-oligomeric, high-molecular mass complexes, which has to date only been found in green microalgae and in developing castor oilseed endosperms (Rivoal et al. 2001; Blonde and Plaxton 2003). Both the less abundant class 1 PEPC and the dominant class 2 PEPC are induced at the transcript and protein level at lower concentrations of NH_4^+, likely having a non-photosynthetic role (Mamedov et al. 2005; Moellering et al. 2007). Because the export of PEP from the chloroplast to the cytoplasm is feasible, as it is commonly seen in higher plants (Rumpho and Edwards 1984; Flugge 1999), it is conceivable that if pyruvate is converted to PEP, it can be exported out of the chloroplast in *C. reinhardtii*.

Although this phenomenon of pyruvate feeding into a reductive carboxylic acid cycle has since not been revisited in detail in *C. reinhardtii*, this phenomenon has been characterized and linked to the reductive carboxylic acid in the green alga *Selenastrum minutum* under anaerobiosis induced by nitrogen starvation (Vanlerberghe et al. 1989, 1990). By tracing the incorporation of radio-labeled carbon as well as measuring key metabolic intermediates, Vanlerberghe et al. showed that under anaerobiosis, *S. minutum* partially relies on a reductive carboxylic acid cycle converting PEP to oxaloacetate, malate and finally to succinate as an accumulating product of anaerobic metabolism (Vanlerberghe et al. 1989). Accumulation of succinate described in the dark by Dubini et al. as well as CO_2 fixation in the dark observed by Chen et al., all point to the fact that *C. reinhardtii* could possibly utilize PFR1 in the opposite direction to produce pyruvate and allow for increased NAD(P)H oxidation (Figure 4) (Chen and Gibbs 1992b; Dubini et al. 2009). Replenishing the cell with $NAD(P)^+$ allows for partial oxidative TCA cycle to continue, because the classic fermentative products (such as ethanol and lactate) can only accommodate for reoxidizing NADH generated through glycolysis and additional reductive pathways are necessary to provide NAD^+ for partial continuation of the TCA cycle in anaerobic conditions, as proposed by Vanlerberghe et al. (Vanlerberghe et al. 1989).

The possibility of PFR working to synthesize pyruvate is also seen in many eukaryotic organisms experiencing anaerobic conditions that possess hydrogenosomes, an alternative organelle to mitochondria. Lindmark and Müller first characterized the hydrogenosomes in the anaerobic flagellate *Tritrichomonas foetus* (Lindmark and Müller 1973), where key enzymes in this organelle were described to be PFR and the hydrogenase, likely to be working in both directions. Furthermore, it has been shown that in the unicellular microaerophilic eukaryote *Trichomonas vaginalis*, MME enzymes are central in addition to PFR in the hydrogenosomal carbohydrate metabolism (Müller 1993; Xu et al. 2004). Linking PFR and MME with pyruvate metabolism has been shown widely in hydrogenosome-containing anaerobic eukaryotes, suggesting that this pathway would be conceivable to exist in the *C. reinhardtii* chloroplast, because it contains a highly similar set of proteins that are anoxic-induced. Under anaerobic conditions, where reducing equivalents are known to accumulate in the chloroplast (Klein and Betz 1978), it is likely that PFR activity in the direction of pyruvate synthesis followed by MME reductive carboxylation of pyruvate, resulting in malate production, is favored, because this would result in the consumption of NAD(P)H. Additionally, PFR activity has also been linked to MME activity and malate production in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1, which could further support the presence of the reductive carboxylic acid cycle (Fukuda et al. 2005). Regardless of the directionality of PFR function, the fact that green algae have maintained these bacterial pathways unlike higher plants reflects the plasticity required for a unicellular organism that faces a wide range of environmental conditions.

Ferredoxins and ferredoxin-interacting proteins

C. reinhardtii has at least six plant-type ferredoxins (FD) (Merchant et al. 2006). FD (also known as PETF), FDX2, FDX3, FDX5 and FDX6 have recently been localized to the chloroplast (Jacobs et al. 2009; Terauchi et al. 2009) and the proteomics data from Terashima et al. (Terashima et al. 2010) indicate FDX4 localization also to the chloroplast. The 996 chloroplast protein list contains all of these six proteins. The phylogenetic tree presented in Terauchi et al. shows FD and FDX2-6 to be closely related to those in other plant and algae species (Terauchi et al. 2009). The many isoforms of Ferredoxins existing in *C. reinhardtii* imply specialized roles for each ferredoxin, whether it is substrate or condition-specific (Terauchi et al. 2009). FD preferentially accepts electrons from PSI and FDX2 has a primary role in nitrogen assimilation, as the nitrite reductase favored electrons from FDX2. FDX5 has been shown to be induced under anaerobic conditions and under copper deficiency (Jacobs et al. 2009; Terauchi et al. 2009). However, Jacobs et al. showed FDX5 not to be the primary donor of electrons to hydrogenase, suggesting a different role of FDX5 as a reductant under these conditions (Jacobs et al. 2009). Several postulations have been made for the role of FDX5, including the possibility of reducing the aerobic oxidative cyclase in chlorophyll biosynthesis or possibly reducing proteins such as PFL activase, HydEF and HydG (Jacobs et al. 2009; Terauchi et al. 2009). Along the same lines, it is also plausible that FDX5 is responsible for interacting with PFR1. In addition, Terauchi et al. mentioned the possibility that FDX5 could supplement FD in accepting electrons from PSI. It is also feasible that FDX5 directs electrons to Cytochrome b_6f complex for cyclic electron transfer. A recent review by Winkler et al. summarizes the currently known roles of the ferredoxin isoforms in various conditions (Winkler et al. 2010).

Aside from the photosynthetic electron transport chain, there are many other ferredoxin-interacting proteins localized to the chloroplast. These include proteins such as Fd-Sulfite reductase (SIR) 1 and 2, Fd-Thioredoxin reductase (FTRC), Fd-dependent Glutamate synthase (FGS) and Phycocyanobilin-FDoxidoreductase-related protein (PCYA), HydA1 as well as PFR1 introduced earlier. PCYA is particularly interesting because it catalyzes the production of phycocyanobilin, a precursor for the pigment phycobiliproteins for the light harvesting antennae (Frankenberg et al. 2001) not known to be found in *C. reinhardtii*. Phycobiliproteins are synthesized from heme precursors in which the conversion to biliverdin IXα is the first committed step (Falkenberg et al. 2001). It is peculiar that a PCYA-like protein was detected among the chloroplast proteins, because phycobilisomes are not known to be naturally present in *C. reinhardtii* and are usually found in cyanobacteria, red and cryptophyte algae (Gantt et al. 1971; Grossman et al. 1993; MacColl 1998; Adir 2005). Interestingly, there is a species of cyanobacteria, *Prochlorococcus marinus*, which also possesses PCYA despite lacking phycobilisome antennae, having in place chlorophyll antennae (Dammeyer et al. 2008). This PCYA protein is similar to that of *C. reinhardtii* (E-value: 10^{-21}). It has been suggested that, despite the lack of phycobilisomes in *Prochlorococcus sp.*, these genes must be conserved for a reason (Hess et al. 1999; Steglich et al. 2001, 2003, 2005). Demmeyer demonstrated that some phycobilisome pigment biosynthesis genes are also incorporated in cyanophages that infect *Prochlorococcus sp*., suggesting that these genes contribute to the fitness of the cell and the conservation of PCYA-similar genes in non-phycobilisome-containing organisms such as Chlamydomonas and Prochlorococcus has a beneficial effect (Dammeyer et al. 2008; Zhaxybayeva et al. 2009). However, the exact role of PCYA remains to be discovered. The existence of phycocyanobilin in non-phycobiliprotein-containing green algae is not a novel idea, as Wu et al. demonstrated the existence of both phycocyanobilin and phytochromobilin in *Mesotaenium caldariorum* (Wu et al. 1997). Additionally, Kirilovsky also showed specific binding and energy transfer between *C. reinhardtii* PSII particles and phycobilisomes from cyanobacteria *Fremyella diplosiphon* (Kirilovsky and Ohad 1986), another demonstration that the structure of PSII is largely conserved from cyanobacteria through higher plants.

Conclusions

There are many cellular processes occurring in the chloroplast, as demonstrated from the diversity of metabolic pathways that the 996 experimentally chloroplast-localized proteins are a part of (Figure 2). The BLAST visualizes the similarity of these 996 chloroplast proteins to other algae species, bacteria and to vascular plants and mosses (Figure 1). This is demonstrated by the overlap between the clustering of the photosynthetic proteins and GreenCut proteins when algae hits were compared to vascular plants and mosses (Figure 1C and 1D) and to bacteria (Figure 1A and 1B) because many chloroplast-localized GreenCut proteins, which by definition are proteins conserved in photosynthetic organisms, are involved in photosynthesis (Merchant et al. 2007). The non-overlapping region contains mostly chloroplast-encoded photosynthetic proteins, which were not considered during the generation o the GreenCut list. Components of the photosynthetic machinery and the carbon metabolism demonstrate the similarities between *C. reinhardtii* and higher plants. However, certain differences are evident in the proteins and/or pathways present in *C. reinhardtii* chloroplast, as demonstrated the light-harvesting proteins and key proteins involved in important photosynthetic processes such as NPQ. The cross-compartmentalization of the glycolytic enzymes to within and outside of the chloroplast (Figure 3 and 4) and the unique fermentation metabolism (Figure 4) not present in higher plants also are interesting aspects of *C. reinhardtii* chloroplasts. There are several topics that are of particular interest to investigate in the future. To examine which pathways are responsible for the metabolite export from the chloroplast and the accumulation of succinate described by Dubini et al. (Dubini et al. 2009), and whether *C. reinhardtii* synthesizes pyruvate through the PFR1, as suggested by Chen and Gibbs (Chen and Gibbs 1992a) (Figure 4), will provide further insights into the metabolic network in *C. reinhardtii*. Widening our understanding of the anaerobic metabolism will, for example, provide new strategies for altering the destinations of photosynthetically-derived reducing equivalents.

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Figure legends

Figure 1. Distribution of the 996 *C. reinhardtii* chloroplast proteins based on normalized BLAST bit scores for green algae/algae vs. bacteria (A, B) and green algae/algae vs. plants and mosses (C, D). The 996 chloroplast-localized proteins were run through the NCBI BLAST, the bit scores of the hits were normalized to the score for *C. reinhardtii*, resulting in a relative bit score from zero to one. Hits to proteins stemming from bacteria, green algae, algae (both excluding genus Chlamydomonas and Volvox) and vascular plants and mosses were extracted. The bit score for each protein represented in the figure is the median relative bit score for the same number of hits for each group (Baceria, algae and plants and mosses). The number of hits used to derive the median relative bit score for each protein was determined by taking the maximum number of hits possible, starting from the high scores, while requiring the same number of hits for each group. Photosynthetic and fermentative proteins are determined according to functional "bins" from MapMan (Thimm et al. 2004) and are represented in blue and red, respectively. GreenCut proteins are conserved proteins in the green lineage of the Plantae, determined for nuclear encoded genes (Merchant et al. 2007) and are represented in green. Axes represent relative bit scores (bit score divided by highest bit score). Areas have been added by calculating contour lines around individual points to denote the spatial distribution of each group.

Figure 2. Distribution of the 996 chloroplast proteins in terms of function and functional subsets. Proteins were grouped according to functional "bins" from MapMan (Thimm et al. 2004). For those proteins with known function that were not classified into bins from MapMan, the sorting was performed manually. Selected protein bins are shown here.

Figure 3. The carbon metabolism in the *Chlamydomonas reinhardtii* chloroplast. Unlike in vascular plants (reviewed in (Joyard et al. 2010)) where the entirety of this pathway is localized to the chloroplast, the second half of glycolysis appears to occur outside of the chloroplast in *C. reinhardtii*. This figure is adapted from the figure presented by Joyard et al. (Joyard et al. 2010). Abbreviations: G6P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; 1,3BPG, 1,3-diphosphoglycerate; 3PGA, 3-phosphoglycerate; 2PGA, 2-phosphoglycerate; F6P, fructose-6-phosphate;

F1,6P, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; E4P, erythrose-4-phosphate; S7P, sedoheptulose-7-phosphate; Xu5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; Ru1,5BP, ribulose-1,5-bisphosphate; PEP, Phosphoenolpyruvate.

Figure 4. The pyruvate metabolism in the context of the photosynthetic electron transport chain and carbon metabolism in *Chlamydomonas reinhardtii*. Fermentative metabolism (shown in purple) involves pyruvate catalyzed by PFR1 and PFL1 (Hemschemeier and Happe 2005; Grossman et al. 2007). The PFL1 pathway of the fermentative metabolism occurs in parallel between the chloroplast and mitochondria (Atteia et al. 2006, 2009; Terashima et al. 2010). Pathways involving MME5, PYC, PPDK have been localized to the chloroplast (Terashima et al. 2010), but their role in fermentative metabolism as well as the notion that PFR1 is working to synthesize pyruvate (shown in gray arrows), as discussed in the text, have been speculated and not fully described biochemically (Chen and Gibbs 1992a; Melis et al. 2007; Dubini et al. 2009). Fermentation pathways are linked to the electron transport chain through HYDA1, which is reduced by FDX. Linear electron flow (depicted in green) provides reducing power for the carbon fixation pathways. Linear electron flow consists of electron transfer from PSII, PQ, Cytb_6f, PC, PSI, FDX and to FNR. Cyclic electron flow (depicted in blue) is modulated by PGRL1 (Petroutsos et al. 2009; Iwai et al. 2010) or alternatively through NDA2 (Mus et al. 2005; Jans et al. 2008; Desplats et al. 2009) and possibly NDA3 ((Terashima et al. 2010)). Figure for electron transport chain inspired by Peltier et al. (Peltier et al. 2010). As depicted in Figure 3, the energy consuming initial steps are localized to the chloroplast along with steps converting GAP to 3PGA. Steps converting 3PGA to pyruvate is localized outside of the chloroplast (multiple reaction steps are depicted with dashed arrows). Abbreviations: 3PGA, 3-phospho-glycerate; ACK, acetate kinase; ADH, Alcohol dehydrogenase; Cytb_6f, cytochrome b_6f complex; FDX, ferredoxin; FMR, fumarate reductase; FNR, ferredoxin $NADP^+$ reductase; FUM, fumarase; GAP, glyceraldehyde-3-phosphate; HydA1, Hydrogenase; MDH, malate dehydrogenase; MME, Malic enzyme; NDA2 and 3, a type II NAD(P)H dehydrogenase; PAT, Phosphate acetyltransferase; PC, plastocyanin; PEP, Phosphoenolpyruvate; PEPC, Phosphoenolpyruvate carboxylase; PFL, Pyruvate formate lyase; PFR, Pyruvate ferredoxin oxidoreductase; PPDK,

Pyruvate, phosphate dikinase; PQ, plastoquinones; PSI and PSII, photosystem 1 and 2; PYC, pyruvate carboxylase.

Supplementary Table header

Supplementary Table 1. A summary of the BLAST results for 996 *Chlamydomonas reinhardtii* chloroplast proteins. The 996 chloroplast proteins were run through the NCBI BLAST and the bit scores of the hits were normalized to the score for *C. reinhardtii*, resulting in a relative bit score from zero to one. Hits to proteins stemming from bacteria, green algae (Chlorophyta excluding genus Chlamydomonas and Volvox), algae (which consist of Chlorarachniophytes, Chlorophyta, Cryptomonads, Dinoflagellates, Euglenids, Glaucophyta, Haptophyta, Heterokonts and Rhodophyta, but excluding genus Chlamydomonas and Volvox) and vascular plants and mosses were extracted. The bit score for each protein is the median bit score for the same number of hits for each set of comparison groups (baceria, algae and plants and mosses or bacteria, green algae and plants and mosses). The number of hits used to derive the median bit score for each protein was determined by taking the maximum number of hits possible, starting from the high scores, while requiring the same number of hits for each group. Photosynthetic and fermentative proteins are determined according to functional "bins" from MapMan (Thimm et al. 2004). GreenCut proteins are conserved proteins in the green lineage of the Plantae, determined for nuclear encoded genes (Merchant et al. 2007). The proteins are matched to the JGI v4.0 protein numbers. For those proteins without exact matches (a total of 17 proteins), the significant best BLAST hit (E-value less than e^{-05}) is listed for reference preceded by "similar to". Unmatched proteins with no significant hits are listed as "no significant hits". Abbreviations in column headers: CP encoded, chloroplast-encoded proteins; PS bin, proteins grouped in the photosynthetic functional bin from MapMan; Fern bin, proteins grouped in the fermentation functional bin from MapMan; Count, the number of hits used to determine the median relative bit score.

Figures 1-4

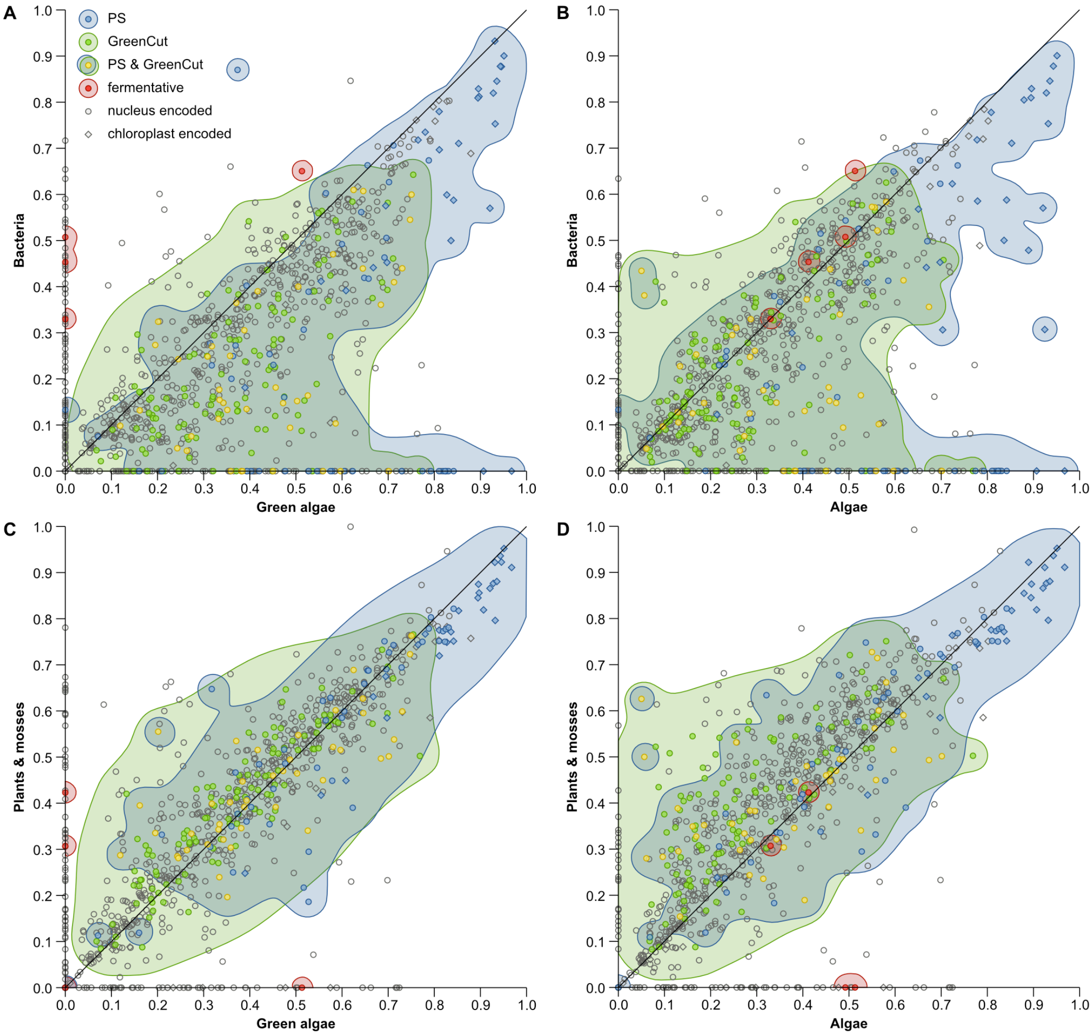
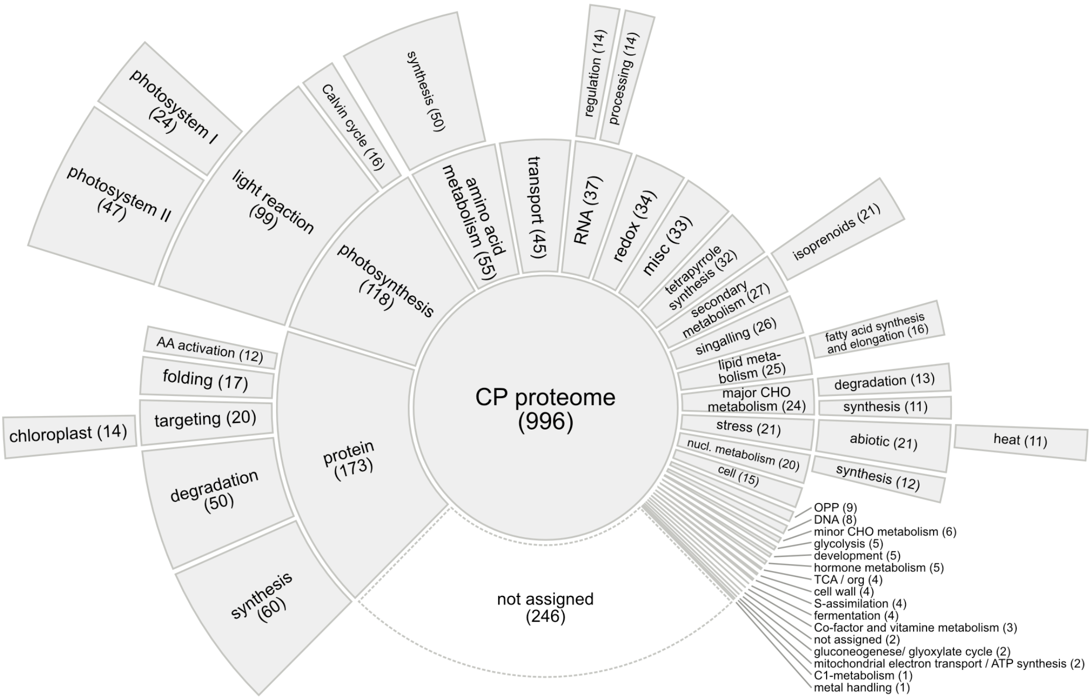


Figure 1


CP proteome
(996)
not assigned
(246)
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photosynthesis
(118)
light reaction
(99)
photosystem II
(47)
photosystem I
(24)
Calvin cycle (16)
synthesis (50)
amino acid
metabolism (55)
transport (45)
RNA (37)
regulation (14)
processing (14)
redox (34)
misc (33)
tetrapyrrole
synthesis (32)
secondary
metabolism (27)
isoprenoids (21)
singalling (26)
lipid meta-
bolism (25)
fatty acid synthesis
and elongation (16)
major CHO
metabolism (24)
degradation (13)
synthesis (11)
stress (21)
abiotic (21)
heat (11)
nucl. metabolism (20)
synthesis (12)
cell (15)
AA activation (12)
folding (17)
targeting (20)
chloroplast (14)
degradation
(50)
synthesis
(60)
OPP (9)
DNA (8)
minor CHO metabolism (6)
glycolysis (5)
development (5)
hormone metabolism (5)
TCA / org (4)
cell wall (4)
S-assimilation (4)
fermentation (4)
Co-factor and vitamine metabolism (3)
not assigned (2)
gluconeogenese/ glyoxylate cycle (2)
mitochondrial electron transport / ATP synthesis (2)
C1-metabolism (1)
metal handling (1)

Figure 2

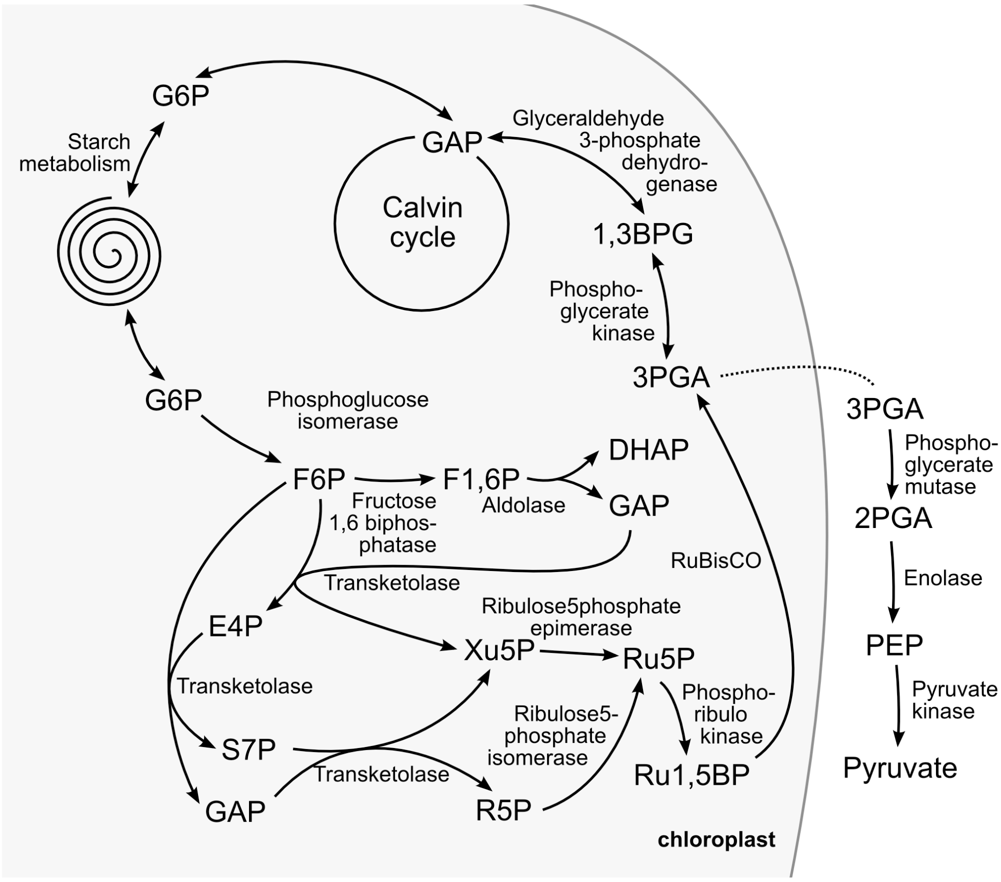
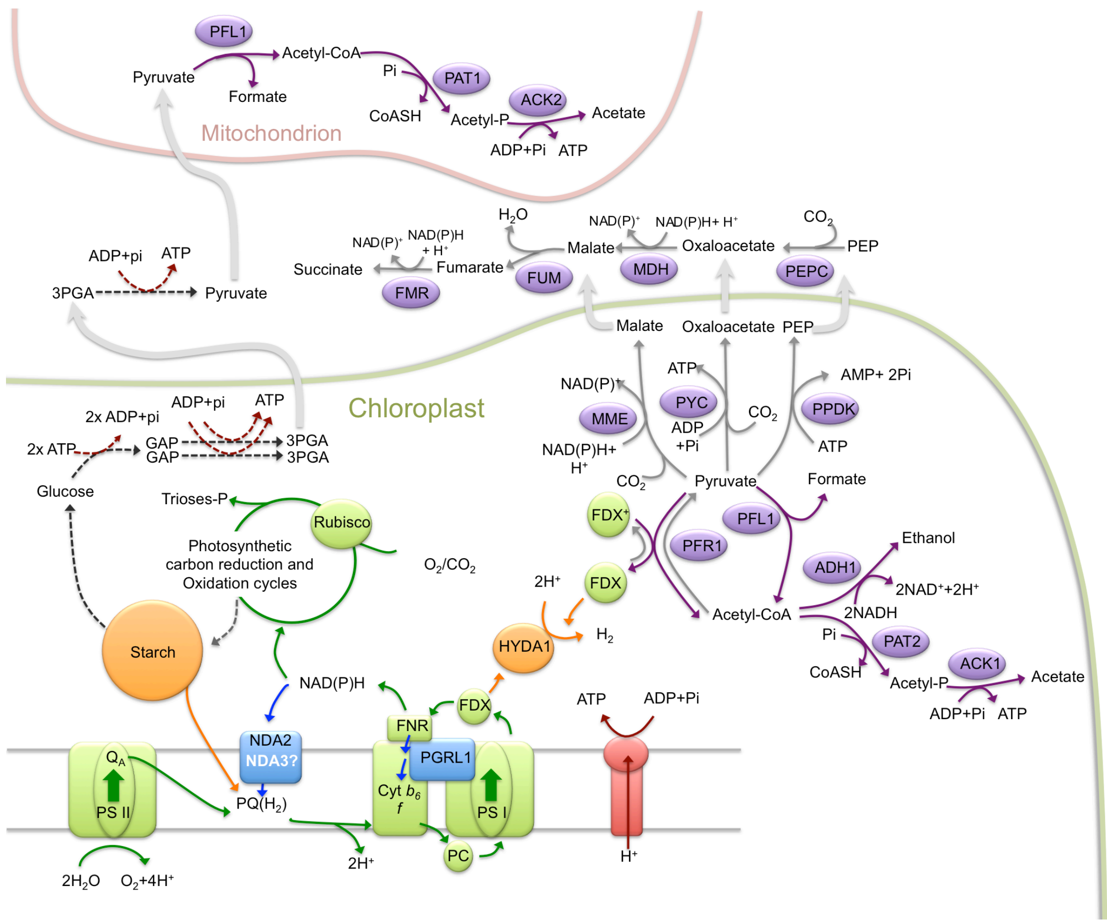

G6P
Starch metabolism
GAP
Calvin cycle
Glyceraldehyde 3-phosphate dehydro-genase
1,3BPG
Phospho-glycerate kinase
3PGA
G6P
Phosphoglucose isomerase
F6P
F1,6P
DHAP
GAP
Fructose 1,6 biphos-phatase
Aldolase
Transketolase
RuBisCO
E4P
Ribulose5phosphate epimerase
Xu5P
Ru5P
Transketolase
Phospho-ribulo kinase
Ribulose5-phosphate isomerase
S7P
Transketolase
Ru1,5BP
GAP
R5P
chloroplast
3PGA
Phospho-glycerate mutase
2PGA
Enolase
PEP
Pyruvate kinase
Pyruvate

Figure 3



PFL1
Pyruvate
Acetyl-CoA
Formate
Pi
PAT1
CoASH
ACK2
Acetyl-P
Acetate
ADP+Pi
ATP
Mitochondrion
H_2O
$NAD(P)^+$
NAD(P)H+ H^+
CO_2
NAD(P)H + H^+
Malate
Oxaloacetate
PEP
Succinate
Fumarate
FUM
MDH
PEPC
FMR
ADP+pi
3PGA
Malate
Oxaloacetate
PEP
$NAD(P)^+$
ATP
PYC
AMP+ 2Pi
MME
PPDK
ADP +Pi
NAD(P)H+ H^+
Chloroplast
2x ADP+pi
2x ATP
GAP
GAP
3PGA
3PGA
Glucose
Trioses-P
Rubisco
Photosynthetic carbon reduction and Oxidation cycles
O_2/CO_2
FDX
PFR1
$2H^+$
H_2
HYDA1
Ethanol
ADH1
$2NAD^++2H^+$
2NADH
PAT2
ACK1
Starch
NAD(P)H
NDA2
NDA3?
FNR
PGRL1
Q_A
PS II
$PQ(H_2)$
Cyt b_6f
PS I
PC
$2H_2O$
O_2+4H^+
$2H^+$
H^+

Figure 4