Research review

When the lights go out: the evolutionary fate of free-living colorless green algae

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Summary

The endosymbiotic origin of plastids was a launching point for eukaryotic evolution. The autotrophic abilities bestowed by plastids are responsible for much of the eukaryotic diversity we observe today. But despite its many advantages, photosynthesis has been lost numerous times and in disparate lineages throughout eukaryote evolution. For example, among green algae, several groups have lost photosynthesis independently and in response to different selective pressures; these include the parasitic/pathogenic trebouxiophyte genera Helicosporidium and Prototheca, and the free-living chlamydomonadalean genera Polytomella and Polytoma. Here, we examine the published data on colorless green algae and argue that investigations into the different evolutionary routes leading to their current nonphotosynthetic lifestyles provide exceptional opportunities to understand the ecological and genomic factors involved in the loss of photosynthesis.

Introduction

The rise, spread, and loss of eukaryotic photosynthesis

Approximately 1.5 Gyr ago (Yoon et al., 2004), eukaryotes acquired photosynthetic capabilities by establishing an endosymbiotic relationship with a cyanobacterium – an event that ultimately gave rise to the plastids of all photosynthetic eukaryotic lineages (Keeling, 2010). It is widely accepted that the plastids of glaucophytes, red algae, green algae, and land plants – which together form the Archaeplastida supergroup – evolved directly from a common cyanobacterial ancestor (i.e. ‘primary’ plastids; Fig. 1). The plastids of other eukaryotic lineages, however, were acquired through more recent eukaryote–eukaryote endosymbioses, and are known as ‘secondary’ or ‘tertiary’ plastids (Keeling, 2010). For instance, the plastids of stramenopile algae (e.g. diatom, brown, and golden algae), various alveolates (e.g. dinoflagellates and chromerids), haptophytes, and cryptophytes arose from secondary endosymbioses with red algae. Conversely, the plastids of chlorarachniophytes (Rhizaria) and photosynthetic euglenids (euglenophytes; Excavata) evolved from independent secondary endosymbioses with green algae (Fig. 1a) (Keeling, 2010).

Understanding plastid evolution becomes even more complex when considering that many algal lineages have lost photosynthesis independently (Fig. 1a) (Keeling, 2010). Photosynthesis was also lost in land plants, as exemplified by the parasitic, nonphotosynthetic land plants Epifagus virginiana, Orobanche minor, Rafflesia lagascae, and different Cuscuta isolates (Wicke et al., 2013), which obtain water and other nutrients directly from the vascular system of the parasitized host. Most eukaryote lineages that have lost photosynthesis still retain colorless (lacking photosynthetic pigments) plastids, which continue to perform crucial nonphotosynthetic metabolic functions (Wicke et al., 2013).

Unraveling the events that resulted in the astonishing diversity of photosynthetic eukaryotes and their nonphotosynthetic close relatives requires a deep understanding of the adaptive and nonadaptive forces and genomic consequences associated with the
gain and loss of photosynthesis. Here, we examine recently published data on colorless chlorophyte green algae and argue that investigations into the different evolutionary routes leading to their nonphotosynthetic lifestyles provide exceptional opportunities to understand the ecological and genomic factors involved in the loss of photosynthesis.
Mixotrophism: from heterotrophism to photoautotrophism and back

Heterotrophism and autotrophism take advantage of different carbon sources (organic vs inorganic), and organisms evolved various ways to utilize one or the other. However, ‘mixotrophic’ organisms can make use of both inorganic (via photoautotrophism) and organic (via chemoheterotrophism) carbon sources; the latter involves prey consumption through phagocytosis (phago-mixotrophism), endocytosis, or the intake of small organic compounds via osmosis (osmo-mixotrophism). Whether or not photoautotrophy or chemoheterotrophy is the main form of nutrient assimilation varies among mixotrophic organisms and depends on the availability of light and organic compounds in the environment (Troost et al., 2005). Consequently, mixotrophs should outcompete obligate photoautotrophs in environments where light or low inorganic supplies limit the photosynthetic activity, and have an advantage over strict heterotrophs when prey or dissolved organic compounds are scarce (Tittel et al., 2003). Despite its apparent benefits, mixotrophy has a major drawback: it is costly to maintain the molecular machinery needed for both trophic strategies. It is estimated that mixotrophic protists spend five times more energy and nutrient allocation on maintaining the photosynthetic apparatus than on heterotrophic abilities (Raven, 1997). This implies that under certain conditions, such as when the energy costs of maintaining the photosynthetic apparatus outweigh the benefits of its products, the selective pressures on preserving photoautotrophic machinery can be relaxed and the loss of photosynthesis – even under favorable light conditions – can be an ecological advantage (De Castro et al., 2009).

Indeed, the presence of numerous plastid-harboring nonphotosynthetic groups demonstrates that photosynthesis is dispensable under certain conditions, and that the loss of photosynthesis is not uncommon among mixotrophic algae (Stoecker, 1998). Extant colorless algal lineages have either phagotrophic or osmotrophic lifestyles, and this is generally a reflection of the heterotrophic strategy employed by their mixotrophic relatives. For example, phagotrophic colorless algae can be found among dinoflagellates, stramenopiles and cryptophytes; this lifestyle is consistent with the presence of phagotrophism in their close mixotrophic relatives. Other colorless algae, such as the chlorophyte green algae Helicosporidium, Prototheca, Polytoma, and Polytomella, are closely related to omo-mixotrophic chlorophytes and adopted an osmotrophic strategy, where the source of dissolved organic matter can be either a host (in the case of pathogenic/parasitic species) or the environment (in free-living species). Interestingly, although there are no reported cases of phagotrophic colorless green algae, a few examples of phago-mixotrophic prasinophytes are known (Maruyama & Kim, 2013).

Chlorophyte green algae as models in which to study the loss of photosynthesis

The Chlorophyta comprises a diverse assemblage of green algae traditionally classified into Chlorophyceae, Trebouxiophyceae, Ulvophyceae and Prasinophyceae. The loss of photosynthesis has occurred several independent times among chlorophytes: at least twice in the order Chlorellales (Trebouxiophyceae) and at least three times in the order Chlamydomonadales (Chlorophyceae) (Fig. 1b). These unicellular nonphotosynthetic algae are particularly interesting because they each have distinct and disparate ecological and evolutionary histories leading to their obligate heterotrophic lifestyles: colorless species from the order Chlorellales evolved as opportunistic parasites/pathogens, whereas the colorless Chlamydomonadales lost photosynthesis as free-living organisms.

Parasitism and the loss of photosynthesis in Chlorellales (Trebouxiophyceae)

The genera Prototheca and Helicosporidium (Trebouxiophyceae, Chlorellales) include unicellular nonflagellated parasites/pathogens that still retain vestigial plastids. Members of the genus Prototheca are ubiquitous opportunistic animal pathogens that can be found in diverse habitats, such as soil detritus, fresh and brackish water, and plant- and animal-derived foods for human consumption. Prototheca is the causative agent of protothecosis, a disease that develops after Prototheca comes in contact with skin wounds, causing cutaneous lesions, bursitis, and major systemic alterations in immunosuppressed hosts (Lass-Flörl & Mayr, 2007). Protothecosis is rare in humans with only 160 cases documented in the medical literature between 1964 and 2011 (Todd et al., 2012). Helicosporidium infections are common in insects, mites, trematodes and cladocerans (Tartar, 2013). Recent reports indicate that Helicosporidium infections can affect between 10% and 70% of coleopteran populations (Yaman, 2008).

The loss of photosynthesis probably occurred in the ancestors of Prototheca and Helicosporidium during their shift from mixotrophy to parasitism (Pombert et al., 2014). It is unclear, however, if these two closely related genera lost their photosynthetic abilities independently. Recent phylogenetic analyses of nuclear 18S rRNA and β-tubulin data have shown that some Prototheca wickerhamii isolates are more closely related to photosynthetic taxa (e.g. Chlorella spp.) than to other Prototheca species (Mancera et al., 2012). Our maximum likelihood (ML) analyses of nuclear 18S rRNA (Fig. 2a) and plastid 16S rRNA (Fig. 2b) sequences from various trebouxiophytes depict P. wickerhamii SAG 263-11 as a nonsister lineage to the other Prototheca and Helicosporidium species. These data suggest that the loss of photosynthesis has occurred at least twice in the evolution of parasitic/pathogenic Chlorellales (Fig. 2). Moreover, the mixotrophic capabilities of various Chlorella species (Lee et al., 1996), which are able to use different organic compounds (e.g. glucose, glycerol, ethanol, acetate, and butyrate) as carbon sources, imply that nonphotosynthetic Chlorellales probably evolved from commensals (e.g. saprophytes; similar to Prototheca species living in animal integumentary tissues) that ultimately harnessed their heterotrophic abilities to invade novel ecological niches.
Multiples cases of photosynthesis loss in free-living colorless Chlamydomonadales (Chlorophyceae)

*Polytoma* and *Polytomella* are two nonphotosynthetic genera that belong to the Chlamydomonadales (Chlorophyceae) (Fig. 3) (Nakada et al., 2008). Both lineages consist of free-living, flagellated heterotrophs that live in fresh water and have vestigial plastids with notable morphological similarities to the colorless plastids of certain *Chlamydomonas reinhardtii* nonphotosynthetic mutants (Inwood et al., 2008). *Polytomella* and *Polytoma* species can use various compounds as carbon sources, including organic acids (pyruvate, acetate, succinate and butyrate), alcohols (ethanol and butanol) and monosaccharides (glucose and glyceraldehyde) (Links et al., 1961).

Phylogenetic analyses using different molecular markers indicate that *Polytomella* is a monophyletic group, whereas the *Polytoma* genus is polyphyletic and comprises at least two independent lineages (Nedelcu, 2001; Nakada et al., 2008). Our ML phylogenetic analysis of nuclear 18S rRNA sequences (Fig. 3a) indicates that certain *Polytoma* species (*Polytoma uvella*, *Polytoma mirum*, *Polytoma obtusum*, and others) are closely related to the osmomixotrophic *Chlamydomonas leiostraca* and *Chlamydomonas humicola* as well as to other photosynthetic species, including *Chlamydomonas applanata*, *Chlamydomonas pulsatilla*, and *Chlamydomonas pumilia*. The colorless *Polytoma osiforme* (SAG 62-27), however, branches independently of other *Polytoma* species and forms a sister lineage to the photosynthetic *Chlamydomonas chlamydogama* and *Chlamydomonas* sp. Itas 9/21 T-4w (Fig. 3a). Although the position of *Polytomella* species is not well resolved in our 18S rRNA analysis, they do form a monophyletic group closely related to the photosynthetic species *C. reinhardtii* and *Volvox carteri* (Smith & Lee, 2014). The ML analysis of the plastid 16S rRNA (Fig 3b) is consistent with the 18S rRNA ML trees, placing (1) *P. uvella*, *P. obtusum*, and *P. mirum* close to the photosynthetic *C. leiostraca*, *C. humicola* and *C. applanata*, and (2) *P. osiforme* as sister to *C. chlamydogama* (100% BS; note that *Polytomella* species were not included in the plastid tree because they have lost their plastid genomes (Smith & Lee, 2014)). Taken together, these
phylogenetic analyses suggest that the loss of photosynthesis has occurred at least three times independently within the Chlamydomonadales.

Loss of photosynthesis in Chlamydomonadales: insights from Chlamydomonas reinhardtii colorless mutants

Chlamydomonas reinhardtii and other species, such as C. humicola and Chlamydomonas acidophila, can grow in total darkness, heterotrophically, with acetate as the only carbon source. When C. reinhardtii is grown under mixotrophic and saturating light conditions, the use of acetate as a carbon source significantly inhibits photosynthetic metabolism without affecting the rate of cell growth. Even under optimal light conditions for photosynthesis, the number of carbon compounds derived from acetate consumption can replace up to 50% of the photosynthetically fixed carbon (Heifetz et al., 2000). Thus, it is reasonable to hypothesize that, under particular environmental conditions (e.g. low inorganic nutrients and low light intensity), the photosynthetic machinery can become expendable in mixotrophic algae.

Studies of certain nonphotosynthetic C. reinhardtii mutants suggest that the loss of photosynthesis can emerge from single-nucleotide mutations of genes involved in photopigment biosynthesis (e.g. phytoene or chlorophylls). For instance, the C. reinhardtii 'white' mutant las1-204, defective in phytoene synthase (an enzyme catalyzing the first step of carotenoid biosynthesis), and 'yellow' mutants, defective in Mg-protoporphyrin IX methyltransferase (an enzyme involved in tetrapyrrole biosynthesis), and 'yellow' mutants, defective in Mg-protoporphyrin IX methyltransferase are unable to survive even under low light conditions. The lack of carotenoids in C. reinhardtii colorless mutants can grow in the dark using acetate as a carbon source, but are unable to survive even under low light conditions.
nearly neutral in environments where photosynthesis is not critical for carbon assimilation, and offers an ecological scenario and a plausible explanation for the origin of free-living heterotrophic colorless algae. Furthermore, the numerous *Chlamydomonas* ‘white’ and ‘yellow’ mutants are promising models for studying the physiological and genetic mechanism underlying this major trophic shift.

**Genomic consequences of the loss of photosynthesis: different routes, different endpoints?**

The different selective pressures and lifestyles (e.g. parasitism vs free-living) associated with the evolution of the colorless green algae discussed in the previous subsection are likely to be reflected in distinct genomic evolutionary processes in these lineages. For instance, parasitism is expected to result in genome reduction (e.g. large-scale loss of genes) or compaction (e.g. shorter genes and intergenic regions; loss of introns). However, the recently sequenced nuclear genome of the parasite *Helicosporidium* sp. ATCC 50920 does not exhibit the large levels of reduction generally observed in nuclear genomes of other unicellular parasitic species (e.g. apicomplexans, microsporidians and ciliates (Corradi et al., 2010; Coyne et al., 2011; Heitlinger et al., 2014). Although the *Helicosporidium* genome shows some evidence of compaction, most of the cellular functions found in free-living, photosynthetic Chlorellales are still present in *Helicosporidium*, with the exception of those involved in photosynthesis (Pombert et al., 2014). The lack of nuclear genome reduction in *Helicosporidium* possibly reflects a recent transition to a parasitic lifestyle and might also be associated with the ability of *Helicosporidium* to grow independently of its host in experimental cultures (Pombert et al., 2014).

**Plastid genomics of nonphotosynthetic chlorophytes**

The plastid genomes (ptDNA) of chlorophytes have revealed interesting evolutionary patterns. Comparative analyses of diverse chlamydomonal lineage species have shown that ptDNA size and amount of noncoding regions correlate positively with the level of cellular organization, whereby multicellular taxa or taxa with large cells have larger ptDNAs than unicellular species or those with small cells (Smith et al., 2013). Furthermore, the ptDNAs of photosynthetic Chlamydomonadales exhibit remarkable architectural diversity (Smith et al., 2013). For example, the 525-kb ptDNA of *V. carteri*, which is c. 80% noncoding and contains c. 96 genes (protein-coding genes, rRNAs and tRNAs), is among the largest plastid chromosomes sequenced thus far (Smith & Lee, 2010); the ptDNAs of the chlamydomonal clades *Dunalellales* and *Pleodorina starrii* are also very large (both c. 270 kb) and contain 104 and 103 genes, respectively. The ptDNAs of Chlorellales also show a range of architectures: *Chlorella variabilis* (124 kb and c. 102 genes; HQ914635), *Chlorella sorokiniana* (109 kb and c. 100 genes; GenBank: KJ742376), *Chlorella vulgaris* (150 kb and c. 101 genes; AB001684), *Parachlorella kessleri* (c. 124 kb and c. 105 genes; FJ968741), *Oocystis solitaria* (incomplete ptDNA; 96 kb and 108 genes; FJ968739) and *Auxenochlorella protothecoides* (incomplete ptDNA; 84 kb and 99 genes; KC631634).

In contrast to the number of data available for photosynthetic chlorophyte lineages, little is known about the evolutionary patterns characterizing the plastid genomes of their nonphotosynthetic relatives. The loss of photosynthesis is typically associated with ptDNA reduction and the erosion of photosynthesis-related genes (De Koning & Keeling, 2006). The ptDNAs of *Helicosporidium* and *Prototheca* species have shorter intergenic regions, fewer introns, and reduced coding capacities as compared with photosynthetic Chlorellales (De Koning & Keeling, 2006). For instance, the *Helicosporidium* sp. ATCC 50920 ptDNA is 37.5 kb with only 29 protein-coding genes, none of which are associated with photosynthesis. Similarly, the available c. 55-kb ptDNA sequence for *P. wickerhamii* encodes 18 proteins unrelated to photosynthesis (Tartar & Boucias, 2004). Although additional data are still needed to clarify whether or not *Prototheca* and *Helicosporidium* evolved independently or share a common non-photosynthetic ancestor, it appears that evolution towards parasitism has produced similar plastid gene content in colorless parasitic green algae (De Koning & Keeling, 2006). In contrast, ptDNA data from free-living, colorless chlamydomonal lineages are rather limited. Interestingly, a recent investigation of *Polytomella* species has demonstrated that, although the nonphotosynthetic plastids of these colorless algae are metabolically active, they have completely lost their genome, taking the process of genome reduction to the ultimate extreme (Smith & Lee, 2014). *Polytomella* is one of only two known examples of ptDNA loss (the other is the parasitic angiosperm *Rafflesia lagascae*, Molina et al., 2014). By contrast, *Polytoma* species do contain a ptDNA, but available information is restricted to 16S rRNA and few protein-coding sequences.

**Comparative ‘omics’ and the plastid functions in colorless chlorophytes**

As discussed above, phylogenetic, genomic, and physiological data all indicate that the widespread mixotrophic capabilities of unicellular green algae underlie the multiple ‘successful losses’ of photosynthesis. Central to understanding the evolution of colorless green algae is the identification of physiological (e.g. particular pathways) and genetic (e.g. key mutations) mechanisms involved in the ‘no-return’ transition from a mixotrophic lifestyle to an obligate heterotrophic one, and the role of nonphotosynthetic plastids in colorless algae. Further comprehensive genomic and functional investigations are critical to a better understanding of how these major trophic shifts occurred several times independently in chlorophytes.

Nuclear genome sequencing of various photosynthetic chlamydomonal and trebouxiophyte algae has revealed a surprising amount of genomic architectural diversity. The nuclear genomes of the chlamydomonal clade *C. reinhardtii* (121 Mbp) (Merchant et al., 2007) and *V. carteri* (138 Mbp) (Prochnik et al., 2010) are more than twice the size and have one-third more protein-coding genes (c. 14 800 vs c. 9800; see Table 1 for details) compared with those of the trebouxiophyte *C. variabilis* (46 Mb) (Blanc et al., 2010) and *Coccomyxa subellipsoidea* (48.8 Mb) (Blanc et al., 2012)
family expansions are also observed in *Helicosporidium*, including 14 genes encoding glycosyl hydrolases of the GH18 chitinase family; these expansions are probably associated with the evolution towards parasitism (Pombert et al., 2014).

Previous transcriptomic investigations of *Helicosporidium* sp. and *Prototheca wickerhamii* have suggested that the plastids of these colorless Chlorellales house critical biosynthetic pathways (Borza et al., 2005; Pombert et al., 2014). The *Helicosporidium* genome sequence has confirmed that most of the plastid enzymes participating in biosynthesis of aromatic and hydrophobic side-chain amino acids, fatty acids, tetrapyrrole, and terpenoids are encoded in the nucleus. Most of the genes involved in the Calvin–Benson cycle, starch biosynthesis, the TIC/TOC (translocons at the inner and outer envelope membranes, respectively, of plastids) machinery (plastid protein import), and the general secretory (SEC) pathway (protein export) are also nuclear encoded. Although proteins for many plastid-localized pathways are still encoded in the nuclear genome, nearly 44% of the plastid-targeted proteins present in other viridiplants (green algae and land plants) have probably been lost from the *Helicosporidium* metabolic repertoire (Pombert et al., 2014). The presence of conserved plastid pathways in *Helicosporidium* and *Prototheca* indicates that these biochemical routes are indispensable for the algal cell, even for nonphotosynthetic pathogens. Remarkably, some of the Chlorellales-shared plastid pathways (e.g. fatty acid, isoprenoid, and tetrapyrrole biosynthesis) are also retained in the colorless plastid of apicomplexans, which possess a secondary, red-algal-derived plastid (Fig. 1a) (De Koning & Keeling, 2006).

There are no complete nuclear genomes available yet for free-living colorless chlorophytes, but such data will be important to investigate the evolutionary patterns following the loss of photosynthesis under an ecological scenario that is different from the parasitic/pathogenic lifestyle underlying the evolution of *Helicosporidium* and *Prototheca*. The analysis of transcriptomic data of diverse *Polytoma* species (Smith & Lee, 2014) and *Polytoma uvella* nuclear genome data (our unpublished data) has revealed numerous putative nuclear-encoded, plastid-targeted enzymes (Table 2) shared with *Helicosporidium* and *Prototheca* (Borza et al., 2005), suggesting that key nonphotosynthetic functions are maintained in both free-living and parasitic/pathogenic nonphotosynthetic green algae.

Overall, we argue that genomic and transcriptomic studies of colorless green algae have the potential to greatly improve our understanding of photosynthesis and its evolutionary loss. The available data are largely skewed towards pathogenic/parasitic species and, thus, are impacted by the gene-repertoire reduction associated with both the loss of photosynthesis and parasitism. Current transcriptomic data suggest that there is a certain degree of ‘convergence’ in the plastid protein repertoire among diverse colorless algae and land plants; however, the data available for colorless Chlamydomonadales are limited.

### Conclusions

Mixotrophy has been an important driving force in the loss of photosynthesis across diverse green algal lineages. The genera

### Table 1 Comparison of nuclear genome data from several Chlamydomonadales and Chlorellales green algae

|        | Chlre | Volca | Chlva | Cocsu | Helic |
|--------|-------|-------|-------|-------|-------|
| Genome size (Mb) | 121.0 | 138.0 | 46.0  | 48.8  | 17    |
| Chromosome number | 17    | 14    | 12    | 20    | 10    |
| Number of predicted genes | 15 143 | 14 520 | 9791  | 9851  | 6035  |
| Exons per gene (%) | 8.3   | 7.0   | 7.3   | 7.0   | 2.3   |
| Coding sequences (%) | 16.7  | 18.0  | 29.0  | n/a   | n/a   |
| Repeated sequences (%) | 16.7  | 23.8  | 8.9   | 7.2   | n/a   |

*Chlre*, *Chlamydomonas reinhardtii*; *Volca*, *Volvox carteri f. nagariensis*; *Chlva*, *Chlorella variabilis* NC64A; *Cocsu*, *Coccomyxa subellipsoidea* C-169; *Helic*, *Helicosporidium* sp. ATCC 50920; n/a, data not available.
| Phenylalanine tyrosine and tryptophan biosynthesis | Helic | Prowi | Poluv | Polpa |
|-------------------------------------------------|-------|-------|-------|-------|
| Anthranilate phosphoribosyltransferase           | ●     | ●     |       | ●     |
| 3-Phosphoshikimate 1-carboxyvinyltransferase     | ●     |       |       | ●     |
| Aspartate aminotransferase, chloroplastic        | ●     | ●     | ●     | ●     |
| Histidinol-phosphate aminotransferase            | ●     |       |       |       |
| Shikimate kinase                                 |       |       |       |       |
| 3-Deoxy-7-phosphoheptulonate synthase (aroF)     | ●     | ●     | ●     | ●     |
| Anthranilate synthase component I                | ●     |       |       |       |
| Anthranilate synthase component II               | ●     | ●     | ●     | ●     |
| Tryptophan synthase alpha chain (trpA)           | ●     |       |       |       |
| Tryptophan synthase beta chain (trpB)            | ●     | ●     |       |       |
| 3-Dehydroquinase synthase (aroB)                 |       | ●     |       | ●     |
| Chorismate synthase                              | ●     |       |       |       |
| Chorismate mutase                                |       |       |       |       |
| Arogenate/prephenate dehydratase (pheA)          | ●     | ●     | ●     | ●     |
| 3-Dehydroquinase dehydratase/shikimate dehydrogenase | ●     | ●   | ●     | ●     |
| Arogenate dehydrogenase (NADP+), plant          | ●     |       |       |       |
| Aspartate aminotransferase and glutamate/aspartate-prephenylalanine aminotransferase | ●     | ●     | ●     | ●     |
| Terpenoid backbone biosynthesis                  |       |       |       |       |
| 1-Deoxy-D-xylulose-5-phosphate reductoisomerase  | ●     |       |       |       |
| Protein-S-isoprenylcysteine O-methyltransferase  |       |       |       |       |
| Acetyl-CoA C-acetyltransferase                   | ●     |       |       |       |
| Farnesyl diphosphate synthase (fps1)             | ●     | ●     | ●     | ●     |
| 4-Diphosphocytidyl-2-C-methyl-D-erythritol kinase| ●     | ●     | ●     | ●     |
| 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase | ●     |       |       |       |
| Hydroxymethylglutaryl-CoA synthase               | ●     |       |       |       |
| 1-Deoxy-D-xylulose-5-phosphate synthase          | ●     |       |       |       |
| 2-C-methyl-D-erythritol 2,4-cyclophosphate synthase | ●     |       |       |       |
| Isopentenyl-diphosphate delta-isomerase          | ●     |       |       |       |
| (E)-4-hydroxy-3-methylbut-2-ethyl-diphosphate synthase (gcpE)| ●     | ● |       |       |
| 4-Hydroxy-3-methylbut-2-ethyl diphosphate reductase | ●     |       |       |       |
| All-trans-nonaprenyl-diphosphate synthase        | ●     |       |       |       |
| Protein farnesyltransferase subunit beta         | ●     |       |       |       |
| Protein farnesyltransferase/geranylgeranyltransferase type-1 subunit alpha | ●     |       |       |       |
| Ditrans, polyisoprenyl diphosphate synthase      | ●     |       |       |       |
| Geranyl diphosphate synthase                     | ●     |       |       |       |
| Prenylcysteine alpha-carboxyl methyltransferase  | ●     |       |       |       |
| Terpenoid backbone biosynthesis                  |       |       |       |       |
| Valine, leucine and isoleucine biosynthesis      |       |       |       |       |
| 3-Isopropylmalate dehydrogenase (leuB)           | ●     |       |       |       |
| Ketol-acid reductoisomerase (ilvC)               | ●     | ●     | ●     | ●     |
| Branched-chain amino acid aminotransferase       | ●     | ●     | ●     | ●     |
| 2-isopropylmalate synthase                       | ●     | ●     | ●     | ●     |
| Aceetoacetate synthase I/II/III large subunit (ilvI)| ●     |       |       |       |
| Aceetoacetate synthase I/III small subunit (ilvH)| ●     |       |       |       |
| Dihydroxy-acid dehydratase                       | ●     |       |       |       |
| 3-Isopropylmalate/(R)-2-methylmalate dehydratase large subunit | ●     | ● |       |       |
| 3-Isopropylmalate/(R)-2-methylmalate dehydratase small subunit | ●     | ● |       |       |
| Threonine dehydratase                            | ●     |       |       |       |
| Biosynthesis of unsaturated fatty acids          |       |       |       |       |
| 3-Oxoacyl-[acyl-carrier protein] reductase (fabG) | ●     | ●     | ●     | ●     |
| Acyl-CoA oxidase                                 | ●     |       |       |       |
| Stearoyl-CoA desaturase (delta-9 desaturase)     | ●     |       |       |       |
| Acyl-[acyl-carrier protein] desaturase           | ●     | ●     | ●     | ●     |
| Acetyl-CoA acyltransferase 1                     | ●     |       |       |       |
| Omega-6 fatty acid desaturase (delta-12 desaturase) | ●     |       |       |       |
| Very-long-chain enoyl-CoA reductase              | ●     |       |       |       |
| Very-long-chain (3R)-3-hydroxyacyl-[acyl-carrier protein] dehydratase | ●     | ●     | ●     | ●     |
| Porphyrin and chlorophyll metabolism             |       |       |       |       |
| Protochlorophyllide reductase                    | ●     |       |       |       |
| Coproporphyrinogen III oxidase                  | ●     |       |       |       |
| Oxygen-dependent protoporphyrinogen oxidase     | ●     |       |       |       |
Helicosporidium and Prototheca are examples of loss of photosynthesis associated with the transition to parasitic/pathogenic lifestyles and sequencing additional nuclear genomes from Helicosporidium and Prototheca species will be important to understand the evolution of this trophic transition. For example, is the relative lack of genomic reduction in Helicosporidium sp. ATCC 50920 a common trend among pathogenic/parasitic trebouxiophytes? Unlike Helicosporidium and Prototheca, Polytomella and Polytoma are not believed to have gone through pathogenic/parasitic stages en route to losing photosynthesis. Thus, they should lack the genomic consequences typically associated with parasitism, such as high nucleotide substitution rates, gene loss, and reduced rates of recombination, and can provide a different perspective on our understanding of the loss of photosynthesis. Important insights could come from comparative genomic studies of Polytomella and Polytoma species and their close photosynthetic Chlamydomonas relatives.

For example, the colorless-photosynthetic ‘sister taxa’ pairs P. uvella–C. leiostraca and P. oviforme–C. chlamydogama represent exceptional duos for investigating the consequences of the loss of photosynthesis in free-living algae without the confounding effects associated with adopting a parasitic/pathogenic lifestyle. Our preliminary analyses of Polytoma uvella ptDNA sequence data reveal similar patterns of gene loss between the genomes of free-living and parasitic chlorophytes. These similarities are notable given the different ecological scenarios that presumably drove the independent evolution toward heterotrophism, and suggest that the convergence in ptDNA gene content after the loss of photosynthesis has been shaped by similar constraints.

Other questions to be addressed concern the nuclear genomic complements of colorless green algae. For example, how do the gene collections of different Polytoma lineages and Polytomella species compare to the repertoire of their closely related photosynthetic taxa? Has the loss of photosynthesis caused expansions or
contractions of particular gene families in colorless algae? Are there ‘unique’ genes, or even complete pathways, encoded in the nuclear genomes of the colorless species not present in those of their photosynthetic relatives? Has horizontal gene transfer had any role in the evolution of the colorless Chlamydomonadales? How did the organelles of cyanobacterial origin recruited > 1 billion yr ago become essential for other cellular roles beyond the photosynthesis? Are there other biochemical and molecular functions, other than photosynthesis, critical for the establishment of primary plastids?

Finally, the study of the Polytomella and Polytoma nuclear genomes and plastid proteomes will be key to understand in detail the physiological roles of their colorless organelles. The very distinct genomes and plastid proteomes will be key to understand in detail the physiological roles of their colorless organelles. The very distinct

organisms even after ‘the lights went out’.

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