

REVIEW PAPER

Chloroplast-mitochondria cross-talk in diatoms

Judit Prihoda¹, Atsuko Tanaka¹, Wilson B. M. de Paula², John F. Allen², Leïla Tirichine¹ and Chris Bowler^{1,*}

¹ Environmental and Evolutionary Genomics Section, Institut de Biologie de l'Ecole Normale Supérieure (IBENS), CNRS UMR 8197 INSERM U1024, Ecole Normale Supérieure, Paris, 46 rue d'Ulm, 75230 Paris Cedex 05, France

² School of Biological and Chemical Sciences, Queen Mary, University of London, Mile End Road, London E1 4NS, UK

* To whom correspondence should be addressed. E-mail: cbowler@biologie.ens.fr

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Abstract

Diatoms are unicellular, mainly photosynthetic, eukaryotes living within elaborate silicified cell walls and believed to be responsible for around 40% of global primary productivity in the oceans. Their abundance in aquatic ecosystems is such that they have on different occasions been described as the insects, the weeds, or the cancer cells of the ocean. In contrast to higher plants and green algae which derive from a primary endosymbiosis, diatoms are now believed to originate from a serial secondary endosymbiosis involving both green and red algae and a heterotrophic exosymbiont host. As a consequence of their dynamic evolutionary history, they appear to have red algal-derived chloroplasts empowered largely by green algal proteins, working alongside mitochondria derived from the non-photosynthetic exosymbiont. This review will discuss the evidence for such an unusual assemblage of organelles in diatoms, and will present the evidence implying that it has enabled them with unorthodox metabolisms that may have contributed to their profound ecological success.

Key words: Alternative oxidase, chloroplast, diatom, mitochondria, secondary endosymbiosis, urea cycle.

Introduction

Diatoms are eukaryotic unicellular organisms inhabiting aquatic and other humid ecosystems. Most diatom species are photosynthetic, and because they represent a significant component of both planktonic and benthic ecosystems in the contemporary ocean they are believed to be responsible for about 20% of primary productivity on Earth (Falkowski *et al.*, 1998; Field *et al.*, 1998) and to produce around 40% of marine organic carbon (Nelson *et al.*, 1995). Another well-known characteristic of diatoms is their ornate cell wall constructed of amorphous silica, which makes the cells heavy and susceptible to sinking when they die or when they are grazed. By generating oxygen through photosynthesis and sequestering atmospheric carbon into the ocean interior, they are therefore likely to have influenced significantly the Earth's atmosphere and climate (Brzezinski *et al.*, 2002).

The reasons underlying the evolutionary and ecological success of diatoms are largely unknown, but the availability of whole genome sequences from two divergent diatom species (Armbrust *et al.*, 2004; Bowler *et al.*, 2008) has

provided a genetic context for exploring their evolutionary trajectory and for predicting their biochemical potential. Studies that have exploited these genome sequences and the enabling resources that allow their dissection suggest unorthodox interactions between diatom plastids and mitochondria. The results have implications for understanding the basics of diatom cell physiology and their response to environmental change, as well as for exploiting these organisms in applied applications such as nanotechnology and for biofuels (Dismukes *et al.*, 2008; Kröger and Poulsen, 2008). These recent results have been reviewed here and their potential evolutionary and metabolic significance is discussed.

Evolutionary aspects

Based on evidence from the fossil record and from molecular clocks, diatoms are believed to have appeared after the Permian–Triassic mass extinctions (250 million years ago), and the first reliable fossils date to around

180 million years ago (Sims *et al.*, 2006; Kooistra *et al.*, 2007; Armbrust, 2009). The two major diatom divisions that are now recognized based on their symmetry, the centrics (radially symmetrical) and pennates (bilaterally symmetrical), diverged during the Cretaceous around 90 million years ago and their populations expanded and diversified enormously around 30 million years ago at the Eocene–Oligocene boundary, from where we can trace back the appearance of many modern diatoms (Bowler *et al.*, 2010). It is now believed that there may be as many as 100 000 extant species, comprising two groups each within the centric (radial and bi/multipolar) and pennate (raphid and araphid) lineages. But from where did diatoms arise and what do we know about their origins?

The secondary endosymbiont hypothesis of diatom origins

Plants and algae are believed to have originated through a process where a non-photosynthetic eukaryote engulfed (or was invaded by) a cyanobacterium, thereby acquiring a photosynthetic apparatus that became housed within an organelle surrounded by two membranes (Fig. 1). This event, known as a primary endosymbiosis, is believed to have occurred around 1.8 billion years ago, and it conveniently explains the monophyletic origins of all plastids within eukaryotic cells (Gould *et al.*, 2008; Keeling, 2010). The host cell can be termed the exosymbiont (Hamm and Smetacek, 2007), whereas the cyanobacterium is known as an endosymbiont. This initial endosymbiotic event gave rise to the

green and red algal lineages, as well as to the glaucophytes (Fig. 1). Land plants arose following the evolution of multicellularity within the green algal lineage.

The endosymbiotic process also involved the transfer of thousands of genes from the cyanobacterial genome to the host eukaryotic nucleus (Martin *et al.*, 2002), whereas the genome within the chloroplast (a photosynthetic plastid) became reduced to only a few hundred genes. In contrast to the small genome of the chloroplast, this organelle contains a large number of plastid proteins, probably between 2000 and 5000 (van Wijk and Baginsky, 2011), the majority of which are nuclear encoded, synthesized on cytosolic ribosomes, and targeted to the plastid using amino-terminal targeting peptides (Soll and Schleiff, 2004). Interestingly, dual protein targeting to mitochondria and plastids is also quite a common phenomenon and, in some cases, these plastid proteins do not even have targeting sequences (Jarvis, 2004; Jarvis and Robinson, 2004; Millar *et al.*, 2006; Radhamony and Theg, 2006).

By contrast with the evolution of land plants and green algae, the evolutionary history of diatoms is believed to have followed a rather different path (Fig. 1). Specifically, diatoms and their relatives are thought to have originated when a second non-photosynthetic eukaryote engulfed a photosynthetic eukaryote about 1.4 billion years ago (Yoon *et al.*, 2002). This is known as a secondary endosymbiosis, and a single event is currently believed to be at the origin of the whole Chromalveolata supergroup, which comprises heterokonts (also known as stramenopiles, and to which the diatoms belong), alveolates, haptophytes, cryptophytes, and perhaps also rhizaria (Bhattacharya

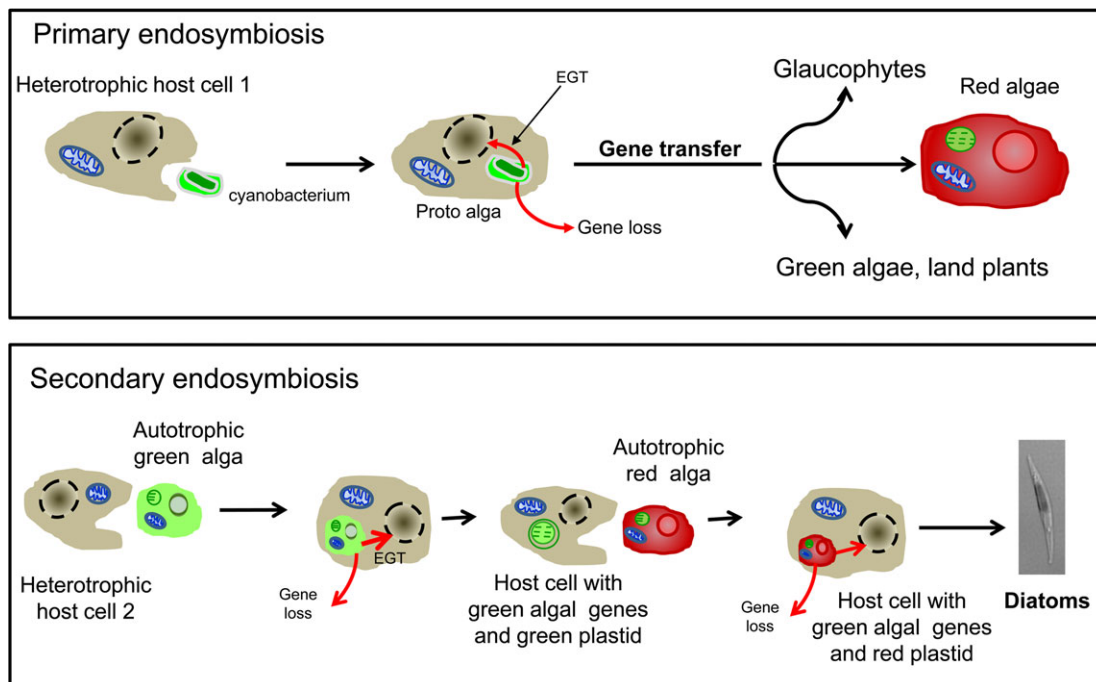


Fig. 1. Schematic representation of the secondary endosymbiont hypothesis of diatom evolution. The upper panel shows the conventional primary endosymbiosis at the origin of green and red algae and Glaucophytes. By contrast, diatoms are now believed to be derived from a serial secondary endosymbiosis (lower panel) in which a heterotrophic host cell (exosymbiont) combined first with a green alga and subsequently with a red alga (Moustafa *et al.*, 2009). EGT, endosymbiotic gene transfer.

et al., 2007; Cavalier-Smith, 1999; Keeling, 2010). Important evidence for the secondary endosymbiosis is that the plastids in many of these organisms are surrounded by four rather than two membranes, corresponding to (from outside to inside) the exosymbiont endomembrane, the plasma membrane of the engulfed alga, and the two membranes of the primary plastid. These nested cellular compartments provide clues about the evolutionary history of these organisms. For example, a vestige of the nuclear genome of the endosymbiont (known as a nucleomorph) can sometimes be found between the inner two and the outer two membranes surrounding the plastid (Ben Ali *et al.*, 2001), albeit not in diatoms. The nucleomorphs of cryptophytes still encode 18S rRNA, and it has been shown that the endosymbiont was most probably related to red algae (Van de Peer *et al.*, 1996). By extension, it would therefore appear that the chromalveolate secondary endosymbiosis involved a red alga (Yoon *et al.*, 2002). This further supports earlier molecular phylogenetic studies, which place the plastids of heterokonts as close relatives to those of red algae and cryptophytes (as reviewed in Keeling, 2010; Green, 2011).

Diatom chloroplasts are typical secondary plastids surrounded by four membranes (Fig. 2). The outer envelope is termed the chloroplast endoplasmic reticulum (CER) and is continuous with the nuclear envelope. Diatom plastids are also characterized by a 'girdle lamella' that runs in parallel with the four membranes that surround them (Fig. 2D). The girdle lamella and the thylakoids are always found in bundles of three but are never partitioned between stacked and unstacked grana, as in some green algae and higher plants. A further contrast with green algae is that all the algae within the chromalveolate grouping have chlorophyll *a* and *c*, whereas green algae contain chlorophyll *a* and *b* (Delwiche, 1999; Green, 2011). Furthermore, diatoms do not use state 1/state 2 transitions to balance absorbed excitation energy distribution between Photosystem I (PSI) and Photosystem II (PSII) (Owens, 1986), most likely because the photosystems are not distributed between stacked and unstacked grana (Pfannschmidt *et al.*, 2009). The ultrastructure of red algal thylakoids is the simplest among eukaryotes (<http://science.jrank.org/pages/48292/Algae.html>">Algae-Prokaryotic Algae), with single thylakoids that are not stacked. They use chlorophyll *a* and phycobilins, and the phycobilisomes that house them are attached to the stromal surface of the thylakoids (<http://science.jrank.org/pages/48292/Algae.html>">Algae-Prokaryotic Algae). Phycobiliproteins have been lost in diatoms but they are still present in cryptophytes (Delwiche *et al.*, 1995; Gibbs, 1981). Evidence for the derivation of diatoms and other chlorophyll *c*-containing algae from the red lineage is therefore somewhat anecdotal, but molecular phylogenetic analyses are consistent with this hypothesis (Van de Peer *et al.*, 1996; Delwiche *et al.*, 1995).

The light-harvesting peripheral antennae of the diatom photosynthetic machinery are composed of fucoxanthin chlorophyll *a/lc* binding proteins (FCPs) structured into oligomeric complexes (Büchel, 2003; Beer *et al.*, 2006). These complexes possess a large number of carotenoids only found within photosynthetic heterokonts and haptophytes, such as

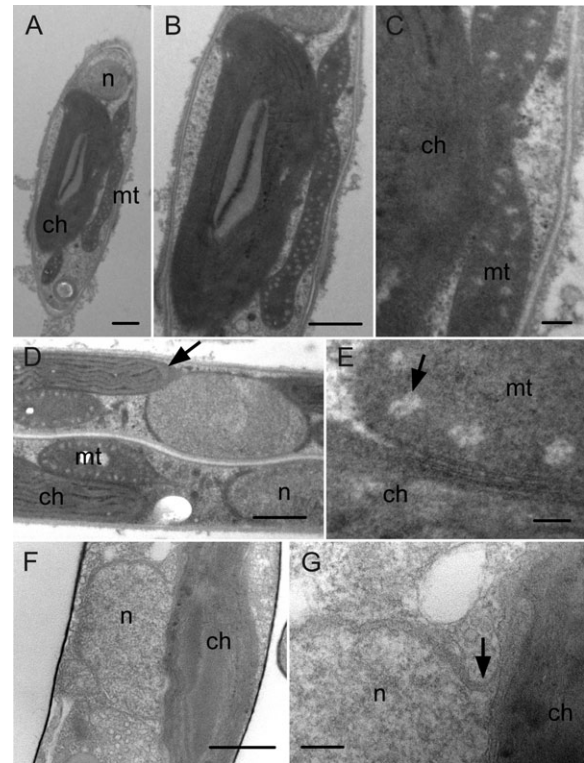


Fig. 2. Chloroplasts and mitochondria are closely juxtaposed inside diatom cells. The images show the spatial relationship between mitochondria and chloroplasts in *P. tricornutum*. (A–C) Longitudinal sections and (D–G) vertical sections of two daughter cells just after cell division. (A) Mitochondria (mt), chloroplast (ch), and nucleus (n) are closely located; (B) the mitochondrion lies alongside the chloroplast and (C) partly attaches to the chloroplast. (D, E) The girdle lamella is continuous around the end of chloroplast just under the chloroplast envelope (D, arrow). Both daughter cells have mitochondria with clear tubular cristae (E, arrow). (E) Mitochondrial membrane and chloroplast outer membrane (chloroplast endoplasmic reticulum; CER) are not completely attached. (F, G) Longitudinal sections with nucleus and partial chloroplast. The CER continues to the nuclear membrane (G, arrow). Scale bars: 0.5 μ m (A, B, D, F) and 0.1 μ m (C, E, G).

fucoxanthin (Fx), as well as diadinoxanthin (Ddx) and diatoxanthin (Dtx) pigments that are involved in their unique xanthophyll cycle (Lepetit *et al.*, 2011). Besides the FCP proteins, three other antenna protein families have been found: Lhcf (the classical light-harvesting proteins), Lhcr (red algal-related proteins), and the less abundant (Westermann and Rhiel, 2005) Lhcx proteins (previously known as LHCSR and Li818) (Eppard *et al.*, 2000; Green, 2007; Koziol *et al.*, 2007; Lepetit *et al.*, 2011). Diatoms also have a PSI-specific antenna (Veith and Büchel, 2007; Veith *et al.*, 2009; Lepetit *et al.*, 2011) that has a higher amount of Ddx and Fx than the main FCP proteins (Lepetit *et al.*, 2011).

Diatom thylakoid membranes contain the same classes of lipids as higher plants and green algae (Goss and Wilhelm, 2009; Lepetit *et al.*, 2011), although in contrast to plants and green algae which have mostly neutral galactolipids, diatom thylakoid galactolipids are mainly negatively

charged (Vieler et al., 2007a, b; Goss et al., 2009; Lepetit et al., 2011). In higher plants the most abundant galactolipids are MGDG (monogalactosyldiacylglycerol), while in diatoms the main lipid is the anionic SQDG (sulphoquinovosyldiacylglycerol) (Goss et al., 2009). Another difference is that diatom thylakoid membranes contain the phospholipid phosphatidylcholine (PC), while in plants this lipid is not present in thylakoids (Lepetit et al., 2011).

As mentioned above, only about 2% of the original plastid genome is retained in the chloroplast, while most of the other genes have either been lost or have become incorporated into the nucleus of the host. This was presumably advantageous for avoiding Muller's ratchet, the accumulation of deleterious mutations when population sizes are small (Muller, 1932). Furthermore, the plastid genome may be subject to higher mutation rates due to increased oxidative stress as a result of the high levels of reduced oxygen species (ROS) that can be generated from the photosynthetic apparatus (Allen and Raven, 1996). It has been argued that the large number of plastid genomes per cell favours net gene transfer from plastid to the nucleus, simply by a 'diffusion gradient' argument, compared with the less probable transfer from a single copy nuclear genome to multiple copies of the plastid genome (Martin, 2003). These factors perhaps combined to provide a selective advantage to moving genes from the plastid to the nucleus. Although only a small percentage of the original plastid genome remained, the fact that it did remain signifies that it has an important role. According to Puthiyaveetil et al. (2010) plastid-encoded genes are required for redox regulation within the organelle, while the genes encoding ribosomal proteins and RNAs support the primary redox regulatory control of photosynthesis (Allen, 2003; Puthiyaveetil et al., 2010).

Like other eukaryotes, diatom cells contain mitochondria evolved from a single primary endosymbiotic event involving an α -proteobacterium. No trace of mitochondria remain in the secondary endosymbiont, and so it is believed that diatom mitochondria are derived from the exosymbiont. In support of this, heterokont, haptophyte, and dinoflagellate mitochondria are all characterized by tubular cristae, as in animals, whereas green and red algae (as well as cryptophytes) all contain flattened cristae (<http://science.jrank.org/pages/48292/Algae.html>)>Algae-Prokaryotic Algae). The functional significance of this difference is unclear (Frey and Mannella, 2000), but given that the cristae contain and organize the respiratory electron transport chain and the proton-motive ATP synthase of oxidative phosphorylation, the issue warrants further investigation.

Following secondary endosymbiosis, the different genomes of the exosymbiont and endosymbiont are predicted to have combined to form a novel and unique set of genes (Falkowski et al., 2004) dispersed within the nuclear, mitochondrial, and chloroplast genomes (Fig. 1). Considering that such a fusion is thought to involve two nuclear genomes, two mitochondrial genomes, and one plastid genome, the process must have been highly complex and, because it happened so long ago, reconstructing its history

has proven extremely difficult. The following section will address how the availability of completed diatom genome sequences has been able to contribute to addressing this fundamental question.

Genome sequences and the secondary endosymbiosis theory

The first diatom species whose genome was fully sequenced, *Thalassiosira pseudonana*, belongs to the bi/multipolar centric family of diatoms known as Mediophyceae. Armbrust et al. (2004) found that this diatom has a 34 Mb nuclear genome, a 129 kb plastid genome, and a 44 kb mitochondrial genome. In total, 11 242 protein-coding genes are predicted in the nuclear genome, 127 in the plastid, and 34 genes in the mitochondria.

Following the centric diatom *T. pseudonana*, a raphid pennate diatom species (Bacillariophyceae) *Phaeodactylum tricornutum* was sequenced, revealing more information about diatom adaptation to the varied aquatic environments which they inhabit. *P. tricornutum* has a smaller nuclear genome, of about 27.4 Mb, a chloroplast genome of 117.4 kb, and a mitochondrial genome of 77.4 kb, predicted to have 10 402, 130, and 34 genes, respectively (Oudot-Le Secq et al., 2007; Bowler et al., 2008; Oudot-Le Secq and Green, 2011).

The two aforementioned groups of diatoms diverged about 90 million years ago (Sims et al., 2006; Kooistra et al., 2007), and so significant differences are expected between their genomes. It is interesting to note that the divergence is so large between these two types of diatoms that it can be compared with the difference between *Homo sapiens* and *Takifugu rubripes* (puffer fish), which diverged around 550 million years ago. This comparison indicates a high rate of diatom gene modification, losses and gains of genes and introns, and gene exchanges with other organisms, all of which probably contributed to high diatom diversification rates. Notwithstanding, about 57% of genes are shared between *T. pseudonana* and *P. tricornutum*.

The secondary endosymbiont hypothesis posits that diatoms acquired genes from both non-photosynthetic and photosynthetic ancestors, therefore gaining a chimeric genome from a varied origin. Further studies of the *T. pseudonana* genome provided strong support for this hypothesis. Of particular interest, in a comparison with animals (*Mus musculus*) and green plants (*Arabidopsis thaliana*), 806 proteins homologous to sequences found only in the animal were discovered, whereas 865 proteins matched sequences found in the plant but not in the mouse. It was therefore proposed that these two sets of sequences were derived from the exo- and endosymbionts at the origin of diatom evolution (and by extrapolation all chromalveolates) (Armbrust et al., 2004). In spite of the large number of sequences that can be traced with confidence to *Arabidopsis*, it was noted with surprise that the *T. pseudonana* genome contained relatively few genes of red algal origin. At the time this was attributed to the poor taxon sampling of the red lineage, because the only whole genome

sequence available was that of *Cyanidioschyzon merolae* (Matsuzaki *et al.*, 2004), which is not particularly representative of the red lineage because it is known to be highly derived due to its extremophile lifestyle. In a subsequent comparison of the *P. tricornutum* genome with the red algal genome, Bowler *et al.* found just 171 genes of red algal origin, 108 of which were also found in *T. pseudonana* (Bowler *et al.*, 2008). A high number of these, specifically 74 genes (43%), encode plastid-related functions, and 11 genes are also shared with oomycetes, non-photosynthetic heterokonts which are also believed to be derived from the same secondary endosymbiotic event with a red alga, but which subsequently lost photosynthesis. By contrast, when the diatom sequences are compared with those in a cyanobacterium (*Nostoc* sp. PCC 7120), numerous proteins are found to be shared with photosynthetic clusters found in both green and red lineages.

As mentioned earlier, diatom plastids are surrounded by four membranes, and the outer one is contiguous with the endoplasmic reticulum (ER) that surrounds the nucleus (Fig. 2G). This suggests that diatoms must have developed a special way of transferring proteins from the ER into the chloroplast. Analysis of nuclear-encoded chloroplast targeted proteins in diatoms indeed shows the presence of ER signal sequences in addition to the more conventional chloroplast targeting transit peptides found in plants (Kilian and Kroth, 2005). The functionality of these dual targeting sequences has also been verified experimentally to the extent that it is now possible to predict whether a nuclear-encoded protein in diatoms is likely to be plastid targeted (Kroth, 2002).

The incorrectly folded proteins in the ER that cannot be repaired must be relocated into the cytosol for degradation. This process occurs via the ER-associated degradation of luminal proteins (ERAD-L) system (Nakatsukasa and Brodsky, 2008). Sommer *et al.* (2007) suggested that all chromalveolates with four membranes, including diatoms, have an ERAD-derived system (called SELMA; symbiont-specific ERAD-like machinery) that is located in the plastid/periplastid thylakoid of the endosymbiont-derived plastid, and that it coexists with the host ERAD system of the same cell. They also proposed that this machinery serves as a transport channel for plastid/periplastid compartment (PPC) preproteins that are encoded in the nucleus to be transported from the ER into the periplastid space. The Der1 protein is considered to be the central component of the ERAD-L system (Knop *et al.*, 1996). In chromalveolates with four membrane-bound plastids, including *P. tricornutum*, two host-specific Der1 (hDer1-1 and hDer1-2), and two symbiont-specific Der1 (sDer1-1 and sDer1-2) proteins were found (Sommer *et al.*, 2007). Hempel *et al.* (2009) confirmed that the symbiont-specific Der1 proteins are indeed located within the second outermost membrane of the plastid. This protein complex interacts with the periplastid-targeted proteins, but not with the stromal-targeted ones, indicating that the Der1 system could have an important role in discriminating between the preproteins that are transported into the PPC across the two outermost membranes.

Components of the ERAD-specific ubiquitination machinery in diatoms include Der1-1, Der1-2, Cdc48, Ufd1, E1 (Uba1), E2 (Uba2), ubiquitin (Sommer *et al.*, 2007; Hempel *et al.*, 2009), as well as ptE3P (the likely ubiquitin ligase) and ptDUP (the de-ubiquitinase) (Hempel *et al.*, 2010). While Felsner *et al.* (2010) confirmed that all SELMA components can be traced back to their red algal origins, their data indicate that the SELMA system has apparently not arisen in a single endosymbiotic event, but rather through serial secondary endosymbioses with rhodophytes, thus contradicting the chromalveolate hypothesis. On the other hand, based on sequence analysis of a newly discovered protein related to Toc75 from the red alga *C. merolae*, and the discovery that all Omp85-like proteins from chromalveolates form an individual clade related to Toc75 from *C. merolae*, Bullmann *et al.* (2010) found consistency with the hypothesis that the plastids of chromalveolates share a common evolutionary origin.

In summary, the collected data suggests multiple gene transfers from the endo- and exosymbiont, from one genome to another, such as genes transferred from the algal plastid to the algal nucleus, and then incorporated into the host, in this case diatom, nucleus. While the genome sequences therefore provided support for the secondary endosymbiosis theory, it has been difficult to establish using phylogeny whether the photosynthetic endosymbiont was of green or red algal origin. However, a re-examination of the gene content of diatom plastid genomes does indicate more similarity with red plastids than with green plastids (Fig. 3) (Allen JF *et al.*, 2011). Furthermore, Chloroplast Sensor Kinase (CSK), a nuclear-encoded bacterial-type sensor kinase that plays a major role in transcriptional control of plastid genes, is of clear red algal origin in diatoms (Puthiyaveetil *et al.*, 2010).

Diatom mitochondria are considered to have been derived from the exosymbiont. The mitochondrial genomes from both diatoms (as well as a third species *Synedra acus*) were found to display almost the same gene composition as in the mitochondrial genomes of haptophytes and cryptophytes (Oudot-Le Secq and Green, 2011). While there is evidence of significant gene transfer from the plastid and mitochondrial genomes to the nucleus in higher plants (Martin, 2003) and dinoflagellates (Hackett *et al.*, 2004), in the diatoms no proof of such gene transfers from the mitochondria to the nucleus were found. There are no red algal mitochondrial genes found in the *T. pseudonana* or *P. tricornutum* nuclear genomes, which strengthens the conclusion that the red algal mitochondrion was lost and that only the exosymbiont mitochondria was retained during the evolutionary process. The general properties of diatom mitochondrial genomes are indeed more similar to animal than to plant mitochondrial genomes (Oudot-Le Secq and Green, 2011). Furthermore, recent studies have highlighted the novel metabolisms within diatom mitochondria, that are quite unlike those of green algae and higher plants, such as a urea cycle (see later).

Besides providing evidence for the ancient secondary endosymbiotic event at the origin of diatoms (and perhaps

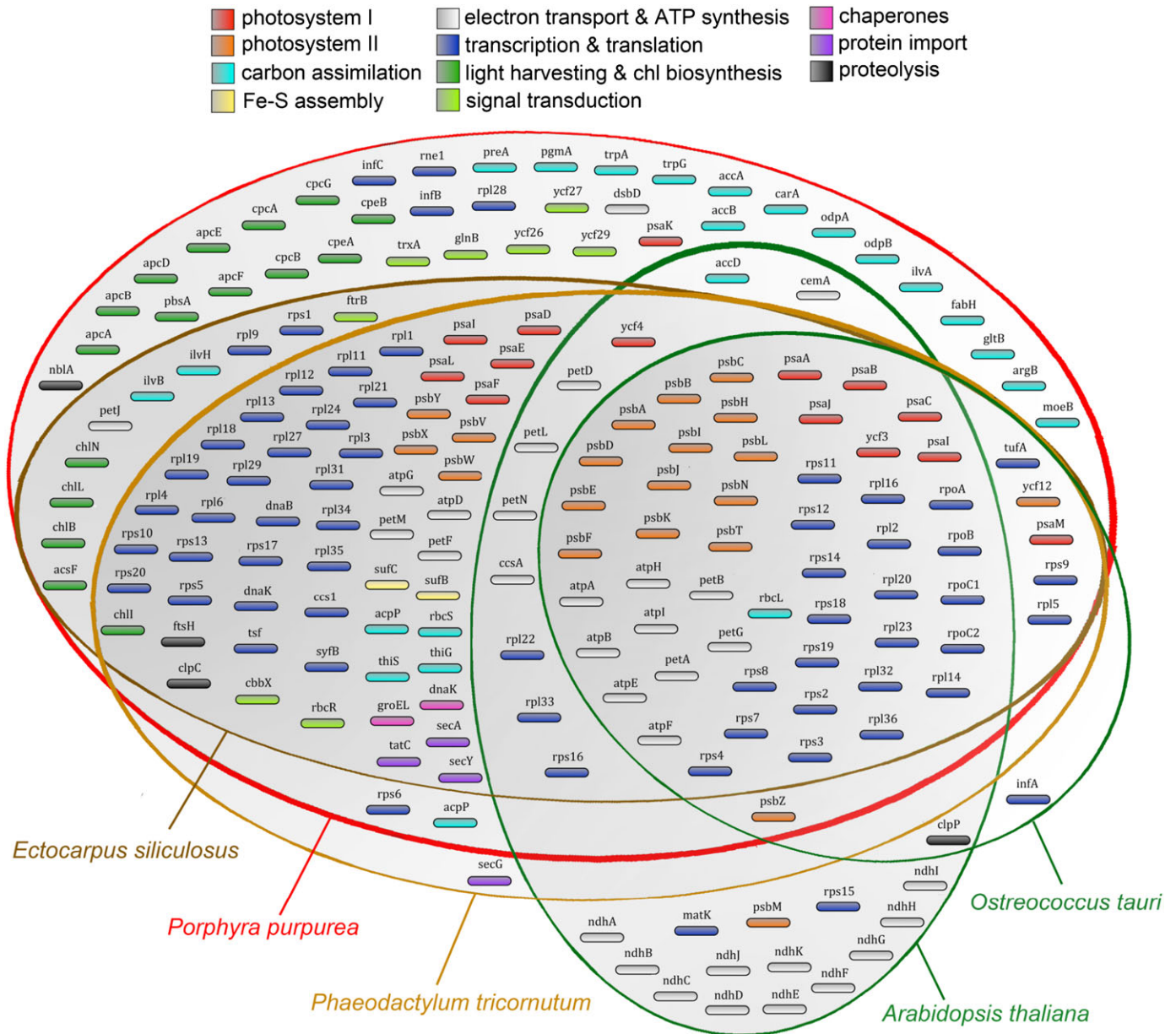


Fig. 3. Venn diagram of gene content of the plastid genomes of *Arabidopsis thaliana*, the green alga *Ostreococcus tauri*, the red alga *Porphyra purpurea*, the brown macroalga *Ectocarpus siliculosus*, and *P. tricornutum*. Larger overlap between the diatom and *Arabidopsis* than between the diatom and *Ostreococcus* is likely to be an effect of genome reduction in *Ostreococcus*.

all chromalveolates), it was found that diatom genomes also contain a large number of genes (587 in *P. tricornutum*) proposed to be derived from bacteria by horizontal gene transfer (Bowler et al., 2008). These genes were not found in any other photosynthetic eukaryotes (other than other heterokonts), and it is believed that they have provided diatoms with increased flexibility in their regulatory and metabolic networks that may have contributed to their evolutionary success. For example, diatoms probably acquired genes enabling novel metabolic offshoots from the urea cycle through such processes (see later). Equally striking are a bacterial glutamine synthetase targeted to the mitochondria, and a bacterial NAD(P)H nitrite reductase (Allen et al., 2006). Such observations hint that nitrogen metabolism has been configured in a unique manner in diatoms.

Evidence for a third partner in the secondary endosymbiosis

As mentioned above, although multiple evidence indicates that diatoms and other chromalveolates gained a plastid of red algal origin through secondary endosymbiosis, the initial analysis of whole genome sequences did not provide incontrovertible evidence about the origin of diatom plastids (Armbrust et al., 2004; Bowler et al., 2008). The subject was reinvestigated by Moustafa et al. (2009), who used a phylogenomic analysis pipeline based on the wholesale generation of phylogenetic trees from each diatom protein sequence encoded by the two diatom nuclear genomes. Using this approach, in *T. pseudonana* 2423 genes were identified as being of red or green algal origin, while in

P. tricornutum 2533 such genes were found. Subsequent analysis revealed that more than 70% of these sequences were more similar to green algal proteins than to red algal proteins, contrasting with the prediction of the chromalveolate hypothesis (Cavalier-Smith, 1999). The >1700 identified 'green' genes comprise around 16% of the diatom genome, in contrast to the 'red' genes which only represented around 3% of the nuclear genome. Some green proteins known to be encoded in the nucleus of green algae and higher plants, such as naphthoate synthase, haem oxygenase, pyruvate dehydrogenase, and GUN4, can similarly be found in the diatom nucleus even though these genes are encoded in the plastids of red algae. Rather than being of red algal origin and present in the plastid genome, they are therefore absent in the red algal-derived plastid of diatoms and are instead of green algal origin and encoded in the nucleus. Another impressive example of green gene acquisition is phytoene desaturase, which catalyses one of the early steps in carotenoid biosynthesis, as well as many other genes encoding enzymes of this pathway. This is of particular significance because diatoms are known to contain a diatom-specific xanthophyll cycle involved in photoprotection (Lohr and Wilhelm, 1999; Coesel *et al.*, 2008). It would therefore appear that this innovation was derived from genes originally encoded in green algae.

The results from Frommolt *et al.* (2008) indicated for the first time that the cryptic green alga might have been a Prasinophyte, a primitive group of green algae. The conclusion from the phylogenomic studies of Moustafa *et al.* (2009) was, therefore, that a cryptic green alga was involved in the secondary endosymbiotic event, in addition to the red alga (Fig. 1). The fact that diatom plastids have clear biochemical and genetic affiliations with red algal plastids was interpreted as meaning that the green algal endosymbiont was acquired first, and that the red alga followed in a serial secondary endosymbiotic event. In such a scenario, the data would imply that the genes derived from the green algal nucleus were largely retained and that the majority of red algal nuclear genes were lost because they were not required due to the availability of green algal nucleus-derived orthologues already present in the host nucleus. By contrast, the data in Fig. 3 indicate that the genes already present within the plastid of the red algal endosymbiont were largely retained. The same analytical pipeline, when used to interrogate the genome sequences available from other heterokonts and alveolates (as well as the haptophyte *Emiliania huxleyi*, whose phylogeny is uncertain with respect to the heterokonts and alveolates), indicated similar overall patterns, suggesting that these same serial secondary endosymbiotic events were at the origin of all the chromalveolates (Moustafa *et al.*, 2009). Such data therefore confirm the phylogenetic affiliation of the chromalveolates (Steinkötter *et al.*, 1994; Petersen *et al.*, 2006; Frommolt *et al.*, 2008; Reyes-Prieto *et al.*, 2008), although the implication of a third partner in the secondary endosymbiotic event was a major surprise.

A further question that was addressed by Moustafa *et al.* (2009) was what kind of green alga was party to the serial

secondary endosymbiosis. Among the green lineages of the Viridiplantae, several unicellular members have been studied at the whole genome level, in particular within the Chlorophyta and Streptophyta clades (Tirichine and Bowler, 2011). The green genes from the two diatoms were found to share most similarity with those present in prasinophytes, which lie within the Streptophyta and constitute a primitive group of green algae most commonly found in marine environments (Derelle *et al.*, 2006). It was therefore proposed that a related alga was most likely to have been the source of the green genes in diatoms.

In summary then, the evolutionary trajectory of diatoms has brought together a highly unorthodox combination of genes derived by endosymbiotic gene transfer from two secondary endosymbionts to the exosymbiont nucleus, as well as by horizontal gene transfer that permitted numerous additional acquisitions from bacteria and Archaea. Conversely, exosymbiont-derived mitochondria gained the opportunity to work alongside a red-algal-derived plastid that was powered largely by green algal genes. It can therefore be predicted that diatoms display novel biochemical pathways that drive physiological processes that are unknown in plants, animals, and fungi. The following section reveals that this is indeed the case.

Biochemical aspects

Interorganellar co-ordination of metabolism has been studied in green algae and higher plants, for example, regarding the exchange of metabolites between chloroplasts, peroxisomes, and mitochondria during photorespiration (Raghavendra and Padmasree, 2003; Noctor *et al.*, 2007; Parker *et al.*, 2008). However, the preceding discussion has presented evidence that diatom chloroplasts and mitochondria both have unusual characteristics with respect to those in better studied experimental organisms. An additional curiosity is that these two organelles are often found in very close proximity within the diatom cell (Fig. 2A–E). This juxtaposition suggests some important metabolic interactions that perhaps go beyond those in the green lineage. Some possibilities are discussed below.

Mitochondrial alternative oxidase as a valve for electrons from the chloroplast

Photosynthesis occurs by the light-dependent generation of energy in the chloroplast through a process known as linear electron flow (LEF), where electrons are transferred from water to NADP via three major complexes: Photosystem II (PS II), the cytochrome *b₆f* complex (cyt *b₆f*) and Photosystem I (PS I). The produced NADPH is used in the Calvin–Benson–Bassham cycle, where carbon dioxide is fixed to produce organic compounds. Optimized functioning of photosynthesis requires fine-tuning between conversion of light into chemical energy as NADPH and ATP, and its use by metabolic reactions such as carbon fixation. The metabolic demand for ATP and NADPH varies under

different physiological and environmental conditions, so their relative abundances have to be finely controlled. In green algae and higher plants, the water–water cycle (WWC; also known as chlororespiration or pseudo-cyclic electron flow) and cyclic electron flow around PS I (CEF) play physiologically important roles in both the regulation of photosynthesis and the alleviation of photoinhibition by consuming excess electrons and maintaining an appropriate NADPH/ATP ratio (Grossman et al., 2010; Wilhelm and Selmar, 2011). In *Arabidopsis* and *Chlamydomonas*, CEF appears to be particularly important for balancing light absorption with carbon fixation, whereas in cyanobacteria and the prasinophyte *Ostreococcus* WWC appears to dominate, mediated by the plastoquinol terminal oxidase (PTOX) (Finazzi et al., 2010).

In diatoms, xanthophyll-dependent non-photochemical quenching appears to have a prominent role in managing the flow of photosynthetic electrons, whereas alternative pathways involved in the diversion of electrons to other reactions such as CEF and WWC do not appear to be particularly active (Wilhelm et al., 2006; Lepetit et al., 2011). Furthermore, oceanic diatoms contain constitutively low levels of iron-rich PS I complexes and some diatoms appear to replace the iron-rich cytochrome c_6 with the copper-containing plastocyanin in the photosynthetic electron transport chain during conditions of chronic iron limitation (Strzepek and Harrison, 2004; Peers and Price, 2006). Some diatoms are therefore well adapted to growing at the low iron concentrations that persist in offshore surface waters. Their rates of carbon dioxide fixation per unit of iron used are much higher than in their coastal counterparts, thus reducing their demand for iron relative to the supply (Strzepek and Harrison, 2004).

In line with these studies that indicate physiological adjustments of diatom photosynthesis in response to environmental conditions, it was proposed recently that in iron-limiting conditions diatoms employ an additional mechanism that reroutes photosynthetic electrons towards the respiratory chain within their mitochondria (Allen et al., 2008). It was further shown in the same study that mitochondrial alternative oxidase (AOX) activity and mRNA levels were significantly higher under iron-depleted conditions, implying that the mitochondria are a major sink for reducing equivalents in diatoms (Allen et al., 2008). AOX has been well studied in plants, where it acts as an alternative to the cytochrome pathway to transfer electrons from the respiratory chain to molecular oxygen (Van Aken et al., 2009a). Although it cannot generate ATP like the conventional respiratory terminal oxidase, AOX plays an important role as an electron sink for excess reductants diverted from photosynthesis during conditions of stress. It therefore appears to have an equivalent function in diatoms during iron limitation. However, its role as an electron sink appears to be more important because other mechanisms that are operative in plants and green algae, such as CEF and WWC, appear to be insignificant in diatoms (Wilhelm et al., 2006; Lepetit et al., 2011).

The rerouting of electrons during iron-limiting conditions suggests cross-talk between mitochondria and chloroplasts

in diatoms in order to regulate the redox balance of the cell and an efficient and appropriate allocation of essential resources that adjusts cellular metabolism. The flow of electrons might be directed into mitochondria through the malate–aspartate shuttle or by as yet unidentified transporters (see later). By analogy with higher plants (Yoshida et al., 2007), through this mechanism the high levels of AOX would be expected to act as an electron sink for excess electrons from photosynthetic LEF, thus also decreasing reactive oxygen species (ROS) accumulation. In conditions such as iron limitation where photosynthesis is dramatically reduced, besides the rerouting of excess reductant to AOX which evacuates ROS, *P. tricornutum* appears able to cope with sustained illumination through the action of specialized light-harvesting complex (LHC) proteins known as LHCX (Bailleul et al., 2010). Interestingly, one of the isoforms of LHCX, known as LHCX1, appears to operate in normal conditions to optimize photosynthesis further (Bailleul et al., 2010).

AOX is widely represented among most kingdoms of life, including animals (McDonald, 2009) (Fig. 4). The *P. tricornutum* genome encodes one AOX protein whereas *T. pseudonana* has two copies. The proteins from both diatoms contain the redox-sensitive residues that are the targets of thioredoxin regulation, as in AOX proteins from all other organisms examined to date (Van Aken et al., 2009b). In addition, the diatom AOX sequences contain calmodulin-like calcium-binding motifs known as EF hands at their amino termini, suggesting a calcium-mediated regulation of AOX activity. This is further supported by the up-regulation of calcium-responsive genes during conditions in which AOX activity is increased, such as genes encoding a heat shock protein (HSP), and proteins such as myosin, gelsolin, copine, and scramblase involved in cytoskeleton and cell membrane regulation and modification (Allen et al., 2008). EF hand domains appear to be specific to the diatom AOX proteins, and so it will be well worth addressing whether they impart some diatom-specific roles to AOX function.

The diatom urea cycle

One of the first surprises to emerge from the sequencing of diatom genomes was the presence of an apparently fully functional urea cycle (Fig. 5) (Armbrust et al., 2004). Diatoms were, in fact, the first photosynthetic organisms to contain all the genes for such a cycle, and so it was of great interest to understand its role in the context of a photosynthetic cell. Prior to its discovery in diatoms, it was believed to have been a metazoan innovation, invented to cope with the high amino acid diets that accompanied their adaptation to life on land by removing excess nitrogen from the body in the form of urea, a relatively innocuous organic nitrogenous compound. Its discovery in diatoms overturned this theory and pushed back the origins of the urea cycle by more than a billion years (Armbrust et al., 2004; Bowler et al., 2008; Allen et al., 2011).

The first committed step of the urea cycle is driven by a mitochondrially-localized carbamoyl phosphate synthase,

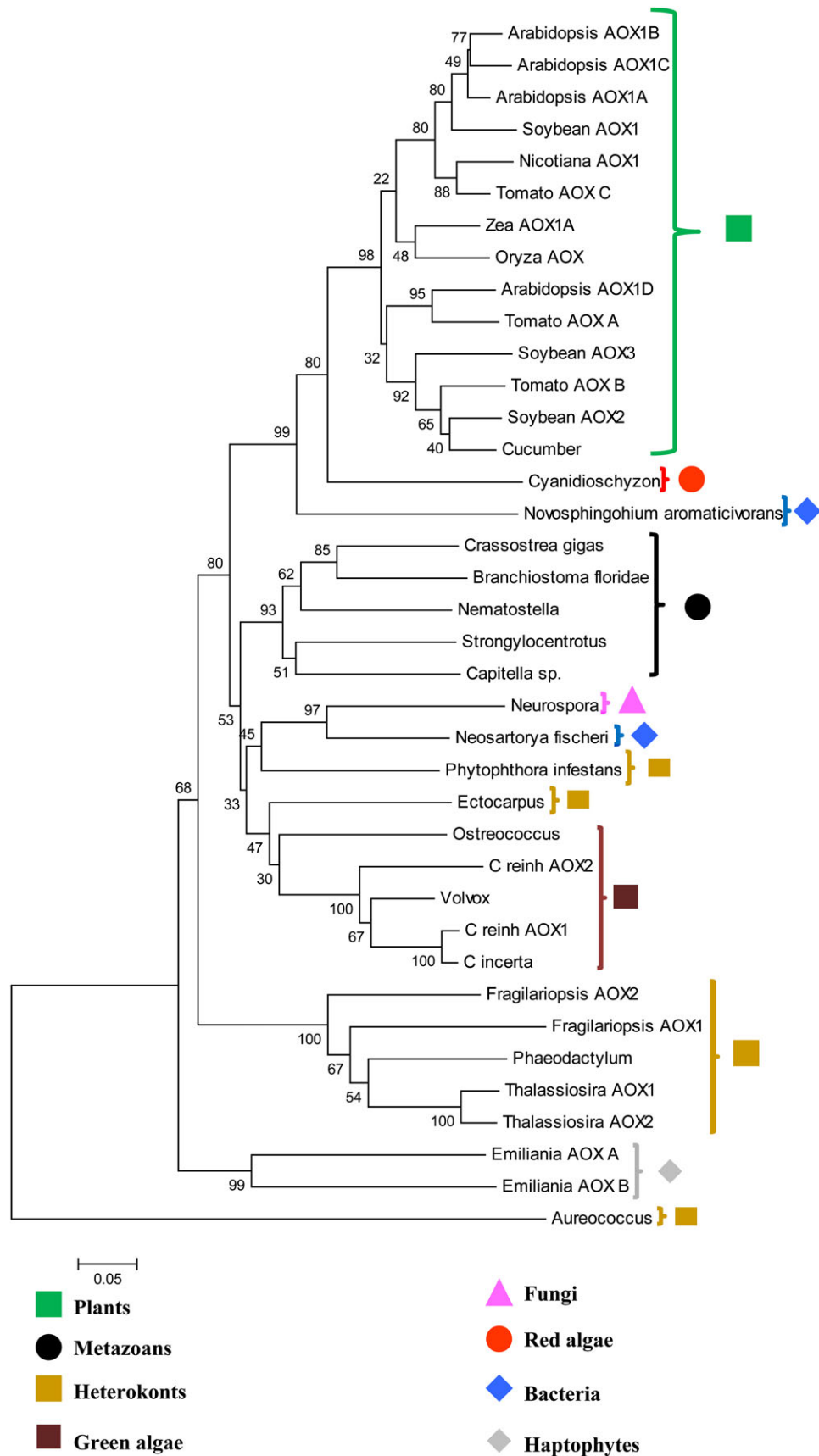


Fig. 4. Phylogenetic tree of AOX proteins. The phylogenetic tree was constructed with the full-length protein using the Neighbor-Joining method with the Mega 4 software (Tamura et al., 2007). C. Reinh is *Chlamydomonas reinhardtii* and C. incerta is *Chlamydomonas incerta*.

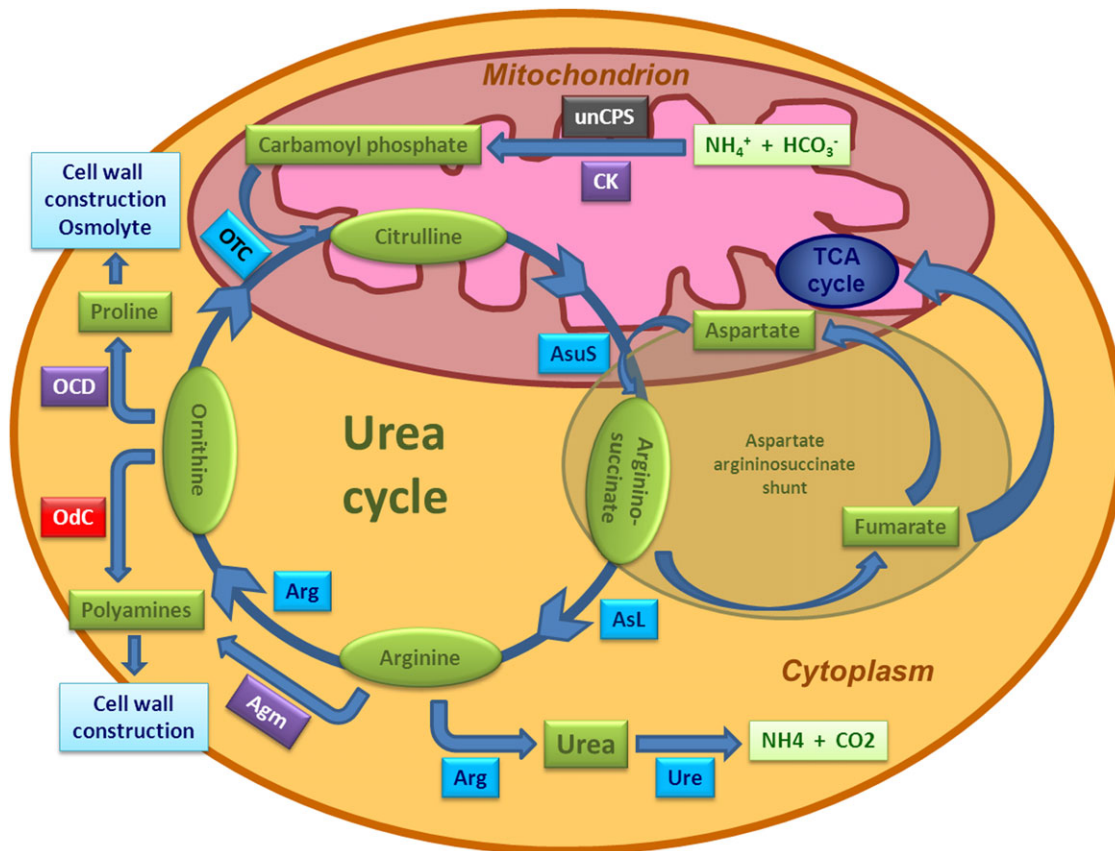


Fig. 5. The urea cycle in diatoms. Scheme showing the principal components of the diatom urea cycle. Genes encoding enzymes of bacterial origin are indicated in purple, metazoan origin in black, and red algal origin in red. Those with uncertain affiliation are shaded in blue. Abbreviations: Agm, agmatinase; Arg, arginase; ASL, argininosuccinate lyase; AsuS, argininosuccinate synthase; CK, carbamate kinase; OCD, ornithine cyclodeaminase; OdC, ornithine decarboxylase; OTC, ornithine transcarbamylase; unCPS, mitochondrial carbamoyl phosphate synthase III; Ure, urease.

now known as unCPS (Allen *et al.*, 2011), which provides the substrate carbamoyl phosphate for the cycle (Fig. 5). Although absent in plants, in metazoans it plays an important role in vertebrate metabolic adaptations (Anderson, 1980; Guppy, 1986; Mommsen and Walsh, 1989; Hong *et al.*, 1994; Lawson *et al.*, 1996; Holden *et al.*, 1999). Phylogenetic analysis of the unCPS sequences from *T. pseudonana* and *P. tricornutum* revealed their similarity with the metazoan enzymes and confirmed their absence in red and green algae, but indicated that other heterokont genomes, as well as the haptophyte *E. huxleyi*, all contained a similar sequence (Allen *et al.*, 2011). It was therefore proposed that the enzyme was most likely to be of exosymbiont origin, but that the gene had undergone several duplications during its evolutionary history that can explain its neo-functionalization.

Other components of the diatom urea cycle closely resemble those found in the metazoan cycle from a functional point of view, although the genes do not all appear to be of exosymbiont origin (Fig. 5). Furthermore, diatom genomes appear to encode several enzymes acquired by horizontal gene transfer from bacteria that potentially can greatly expand the functionality of the cycle with respect to its animal counterpart. Of particular interest, both diatom nuclear genomes encode an ornithine cyclodeaminase (OCD), predicted to convert ornithine into proline, as well

as agmatinase (Agm), which has been predicted to link the urea cycle to polyamine biosynthesis (Allen *et al.*, 2006).

Hints about the role of the urea cycle in diatoms have been derived from analysis of the expression of integral and peripheral urea cycle genes (Allen AE *et al.*, 2011). On the one hand, studies of their expression patterns in cultures grown in different nitrogen sources indicated that their expression is co-ordinately regulated. On the other hand, examination of their expression over an even broader range of growth conditions (Maheswari *et al.*, 2010) indicated that they were particularly expressed during conditions that favour anabolic rather than catabolic metabolism. These findings were interpreted as suggesting that the urea cycle may be principally involved in the biosynthesis of organic nitrogenous compounds rather than their breakdown, as is the case in animals (Lee *et al.*, 2000; Esteban-Pretel *et al.*, 2010).

To determine the role of the urea cycle in diatoms better, Allen *et al.* generated unCPS knockdown mutants in *P. tricornutum* (Allen AE *et al.*, 2011). It was found that with respect to wild-type cells these lines displayed a delayed recovery following conditions of nitrogen limitation, mirrored also in the delayed accumulation of metabolites derived from the urea cycle. Further analysis of metabolite profiles in these lines provided clues as to the metabolic roles of the cycle in diatoms, and it has been proposed to be of particular

importance as a recycling hub for anaplerotic carbon fixation into organic nitrogen compounds. Particularly important offshoots of the pathway appear to go to proline and polyamines. Proline is the major osmolyte in diatoms (Krell *et al.*, 2007), and proline-rich proteins are common cell wall structural components. By contrast, arginine and ornithine are precursors for polyamines such as spermidine and putrescine (Kröger and Poulsen, 2008), which are, in turn, converted into highly modified long-chain polyamines that are used for building the diatom-specific siliceous cell walls (Kröger and Poulsen, 2008). Both these offshoots of the urea cycle are probably enabled by bacterial genes (Fig. 5). Hence, the diatom urea cycle appears to play an important role in building organic nitrogen compounds that are particularly critical for building the unique features of diatom cells. In addition, it was proposed that the urea cycle is connected to glutamine synthetase/glutamate synthase (GS/GOGAT) and to the tricarboxylic acid (TCA) cycle through the aspartate–argininosuccinate shunt (Fig. 5). Such interactions are not unique to diatoms, but it is of interest that the shunt provides an additional point of integration for the urea cycle into central metabolism. In summary, the urea cycle appears to function in the recycling and biosynthesis of organic nitrogen compounds, rather than for their breakdown as in animals. Due to its positioning between the mitochondria and cytoplasm, it is likely to be of special importance for the exchange of nutrients between these two cellular compartments. It remains to be determined whether it also exchanges nutrients with plastids, but given their close juxtaposition with diatom mitochondria such a possibility certainly warrants investigation. One hypothesis that has been proposed is that the urea cycle may interact with photorespiratory pathways, another is that C₄-type photosynthesis may operate in some diatoms by metabolite exchange between chloroplasts and mitochondria (Kroth *et al.*, 2008; Parker *et al.*, 2008).

Diatom nutrient transporters

For endosymbiosis to be successful, it was of the utmost importance to the endosymbiotic organelles (both mitochondria and chloroplasts) to control the exchange of metabolites between the host and themselves. Connections between the host and the endosymbiont metabolism were shown by Linka and Weber (2010), Weber and Linka (2011), and Palmieri *et al.* (2011), who found that the diatom plastid triose-phosphate antiporter likely evolved from an existing ER/Golgi metabolite translocator protein family. This suggests that the plastid endosymbiont had already developed an endomembrane system before it was incorporated into the host cell (Bhattacharya *et al.*, 2007).

Furthermore, the proposed involvement in diatoms of the mitochondrial AOX as an electron sink for excess reductant generated by the photosynthetic electron transport chain (Allen *et al.*, 2008; Finazzi *et al.*, 2010) implies that electrons generated in the chloroplasts must be exported to the mitochondria using metabolic carriers. Such co-operation between chloroplasts and mitochondria implies translocators

such as the *Arabidopsis* oxoglutarate malate transporter (OMT/Dit1) and dicarboxylate transporter (DCT/Dit2) (Linka and Weber, 2010; Weber and Linka, 2011; Palmieri *et al.*, 2011). Examination of the *P. tricornutum* genome revealed the presence of two Dit1 proteins (Kroth *et al.*, 2008, Prihoda *et al.*, unpublished results), and the Dit1 gene was also found in other diatoms such as *T. pseudonana* and *Fragilariopsis cylindrus*. However, neither Dit2 nor DCT could be detected in any of the three diatom genomes (Prihoda *et al.*, unpublished results). This suggests that diatom Dit1 may either replace Dit2 and DCT or that another gene product fulfils their function. Obvious candidates are the numerous triose-phosphate transporters identified in *P. tricornutum* (Weber and Linka, 2011). However, the fact that diatom chloroplasts are surrounded by four rather than two membranes means that analogies with plant and green algal systems are only of limited value, and that fundamental studies of putative diatom transporters are required (Hempel *et al.*, 2010). For each candidate transporter, its localization needs to be determined to the level of a specific membrane, and its substrate specificity needs to be defined.

Mitochondrial transporters may be easier to define by analogy with transporters in mitochondria of other eukaryotes because of their likely orthodoxy. But although studies in organisms from the green lineage are quite advanced (reviewed in Palmieri *et al.*, 2011), almost no studies have been performed in diatoms. These transporters are expected to work together with the mitochondrial inner membrane uncoupling protein (UCP) to maintain redox balance (Noctor *et al.*, 2007; Linka and Weber, 2010). Another component to consider is thioredoxin. Sequence analysis and localization studies in *P. tricornutum* have identified eight thioredoxins, three of which are localized to the chloroplast and one to the mitochondria (Kroth *et al.*, 2008; Weber *et al.*, 2009). By analogy with higher plants, the mitochondria-localized thioredoxin is likely to be important for the redox regulation of the diatom AOX.

Conclusions

Diatoms have a remarkably dynamic genome derived from multiple sources, the exosymbiont, a green and red alga, as well as multiple bacterial genes acquired through HGT. Although a major challenge to unravel, it is clear that this unique combination of genes has endowed them with novel metabolisms that may have contributed to their ecological success in contemporary oceans. Although our knowledge of diatom biology has expanded enormously following the availability of two sequenced genomes, it is sobering to recall that more than half of their genes have no known function nor clear phylogenetic affiliation. The availability of a wide range of tools to interrogate gene function is a major asset to anyone interested to explore their unique properties further, and it is truly an exciting time to open up these organisms to molecular examination. Regarding chloroplast–mitochondria interactions, key questions that

need to be addressed relate to how interactions between the photosynthetic and respiratory electron transport chains are mediated and how they are regulated. An important component of such studies will be the analysis of nutrient transporters on the chloroplast and mitochondrial membranes. Investigation of such fundamental processes is likely to lead to major new insights that will alter our rather green-centric view of how photosynthetic metabolism is integrated into a eukaryotic cell.

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