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PII: S0981-9428(24)00329-2

DOI: https://doi.org/10.1016/j.plaphy.2024.108661

Reference: PLAPHY 108661

To appear in: *Plant Physiology and Biochemistry*

Received Date: 17 December 2023

Revised Date: 14 April 2024

Accepted Date: 23 April 2024

Please cite this article as: N. Foresi, M.A. De Marco, F. Del Castello, L. Ramirez, A. Nejamkin, G. Calo, N. Grimsley, N. Correa-Aragunde, G. Martinez-Noel, "The Tiny Giant of the Sea, Ostreococcus's Unique Adaptations", *Plant Physiology et Biochemistry*, https://doi.org/10.1016/j.plaphy.2024.108661.

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"The Tiny Giant of the Sea, Ostreococcus's Unique Adaptations"

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Ostreococcus spp. are unicellular organisms with one of the simplest cellular organizations. The sequencing of the genomes of different *Ostreococcus* species has reinforced this status since *Ostreococcus tauri* has one most compact nuclear genomes among eukaryotic organisms. Despite this, it has retained a number of genes, setting it apart from other organisms with similar small genomes. *Ostreococcus spp.* feature a substantial number of selenocysteine-containing proteins, which, due to their higher catalytic activity compared to their selenium-lacking counterparts, may require a reduced quantity of proteins. Notably, *O. tauri* encodes several ammonium transporter genes, that may provide it with a competitive edge for acquiring nitrogen (N). This characteristic makes it an intriguing model for studying the efficient use of N in eukaryotes. Under conditions of low N availability, *O. tauri* utilizes N from abundant proteins or amino acids, such as L-arginine, similar to higher plants. However, the presence of a nitric oxide synthase (L-arg substrate) sheds light on a new metabolic pathway for L-arg in algae. The metabolic adaptations of *O. tauri* to day and night cycles offer valuable insights into carbon and iron metabolic configuration. *O. tauri* has evolved novel strategies to optimize iron uptake, lacking the classic components of the iron absorption mechanism. Overall, the cellular and genetic characteristics of *Ostreococcus* contribute to its evolutionary success, making it an excellent model for studying the physiological and genetic aspects of how green algae have adapted to the marine environment. Furthermore, given its potential for lipid accumulation and its marine habitat, it may represent a promising avenue for third-generation biofuels.

Keywords

Alga, Iron, Lipids-Starch, Mamiellales, Mamiellophyceae, Nitrogen, Picoeukaryote, Picophytoplankton

Introduction

Ostreococcus, a member of the picophytoplankton, plays a vital role as a primary producer, contributing significantly to oxygen production, carbon sequestration, and serving in the foundation of the marine food web. Its sensitivity to environmental changes makes it an important biological stress sensor, essential for the conservation and management of marine ecosystems (Derelle et al., 2006; Worden et al., 2004).

Ostreococcus spp. have become the subject of diverse scientific investigations since the initial discovery of *O. tauri* in 1994 (Courties et al., 1994). In the *Ostreococcus* genus, only a limited number of species have undergone in-depth study (Box 1), and most of them are available in the Roscoff-Culture-Collection (http://www.roscoff-culture-collection.org/). *O. tauri* is almost imperceptible under optical or fluorescence microscopy during field studies. However, its presence was first detected by flow cytometry and it was found to be a major component of phytoplankton populations (Derelle et al., 2006). Different *Ostreococcus* species prevail in oceans worldwide, including tropical and temperate environments (Box 1). This review aims to highlight *Ostreococcus* as an algal model or as a plant cell, because its simplicity should help understanding complex metabolic pathways in photosynthetic organisms (Table 1).

In this review, we compare the available knowledge about the *Ostreococcus* genus with other algae, in different metabolic pathways associated with their ability and flexibility to overcome hostile environments. We emphasize important physiological aspects without attempting to provide a complete overview, presenting little about research on virus-host interactions, marine ecology and mating-type evolution (see for example Weynberg et al., 2017; Leconte et al., 2020; Yau et al., 2020; Benites et al., 2021; Listmann et al., 2023 for further information on these topics).

Ostreococcus cells, nuclear and organellar genomes

Each cell contains a nucleus, a chloroplast, one mitochondrion, one Golgi body, and a remarkably reduced cytoplasmic compartment (Henderson et al., 2012). The presence of a starch granule and chlorophyll a and b indicates that it is a member of the phylum Chlorophyta (green algae), while the presence of a Chl c-like pigment led to its classification (Chretiennot-Dinet et al., 1995) in the now outdated group of prasinophyte algae. In Figure 1, TEM images of three strains of *Ostreococcus* are shown, highlighting their cellular simplicity. The small size and DNA content of *Ostreococcus* cells make them the smallest eukaryotes known to date (Chretiennot-Dinet et al., 1995).

Advances in green algal biology are being driven by genome sequencing of numerous unicellular green algae (Vandepoele et al., 2013), including *Chlamydomonas* and seven species in the order Mamiellales, four being *Ostreococcus spp*. Although green algae and plants share many central metabolic pathways, the genome analysis of *C. reinhardtii* reveals the retention of genes from its heterotrophic eukaryotic ancestor, which have been lost in other members of the green eukaryote clade, such as the moss *Physcomitrella patens* and the angiosperm *Arabidopsis thaliana* (Peers and Niyogi, 2008).

The genomes of different species of the *Ostreococcus* genus display strong interspecies synteny, with some surprising regions of heterogeneity within their genomes (Peers and Niyogi, 2008). The availability of genomic sequences for several related *Ostreococcus* spp. enables the identification of conserved non-coding sequences, and facilitates gene prediction and annotation (especially for small open reading

frames). Furthermore, the completed genome of *O. lucimarinus* shed light on the unique metal metabolism of these organisms. *Ostreococcus* species encode a substantial number of selenocysteine-containing proteins, which are probably more catalytically active than their selenium-lacking counterparts, potentially requiring fewer of these proteins. Additionally, selenoenzymes, novel fusion proteins, and the loss of some major protein families associated with chromatin are considered crucial adaptations for *Ostreococcus* to achieve its small cell size (Palenik et al., 2007).

The mitochondrial and chloroplast genomes of *O. tauri* were also fully sequenced. The mitochondrial genome appears as a circular molecule containing 65 genes. Notably, the intergenic regions have an average length of only 42 base pairs, making it the most gene-dense mitochondrial genome among all Chlorophyta. Moreover, it exhibits a unique segmental duplication, encompassing 22 genes and covering 44% of the genome. This duplication is a previously unobserved phenomenon in green algae, although it is also found in the mitochondrial genomes of higher plants (Robbens et al., 2007). In terms of genome size and gene content, the chloroplast genome of *Ostreococcus* is also the smallest among all known green algae. Strikingly, numerous lytic viruses such as the *O. tauri* viruses (OtV5 and OtV2), have been identified from the Phycodnaviridae family, genus Prasinovirus (Weynberg et al., 2011). Host-virus coevolution can be rapid in natural systems enhancing propagation strategies and selective advantages in their specific niches (Listmann et al., 2023; Weynberg et al., 2011). Prasinoviruses are abundant, accompanying their hosts worldwide, and their ~200 kb long DNA genomes encode many proteins that intervene in cellular metabolism, such as phosphate transporters, ammonium transporters and amino acid synthesis (Derelle et al., 2015; Giaccardi et al., 2022; Hingamp et al., 2013; Leconte et al., 2020; Monier et al., 2017, 2012; Moreau et al., 2010; Weynberg et al., 2011). Host resistance to prasinoviruses develops rapidly and involves genes on specialized chromosomes whose gene content can be reshuffled (Yau et al., 2020, 2018, 2016), but the exact mechanism of resistance remains to be elucidated. Unlike higher plants, whose viruses are mostly rather small with few genes, viruses of *Ostreococcus* are very large and carry about 250 genes, some of which came from their host. These viruses are found everywhere in the oceans where *Ostreococcus* is found, and the physiology of their genes is being studied.

The integrity and organization of the *O. tauri* genome is partly assured by sexual recombination, which has been inferred (Grimsley et al., 2010) but not yet observed experimentally. *O. tauri* encodes the most ancient mating type locus known in the plant kingdom, spanning a region of suppressed recombination about 450-650 kb long (Benites et al., 2021).

Ostreococcus is becoming a promising model organism for molecular and physiological processes. It can be easily cultured in the laboratory, and cellular transformation gives about 10^4 transformants per microgram of DNA using a selectable marker gene (Sanchez et al 2019). While the more complex eukaryote, *Chlamydomonas*, has been the primary focus so far, the simplicity of *Ostreococcus* offers a new alternative for research in the research field. *Ostreococcus* spp. often have one copy of genes that are multigene families in other photosynthetic organisms, so the modification of a gene by directed mutagenesis is a simplified strategy to assign genes the biological role in this species. The available *Ostreococcus* molecular toolkits are described in Box 2 and the different ways of culture growth are explained in Table 2.

In summary, *Ostreococcus* is a fascinating group of unicellular organisms with unique characteristics, and their genomes provide valuable insights into the evolution and biology of green algae. Through continued research, *Ostreococcus* holds the potential to unravel further secrets of eukaryotic cellular organization

and function. In the following sections, we will describe the main metabolic pathways studied so far in *Ostreococcus* focusing on the outstanding and unique adaptations and its differences with the other model green alga *Chlamydomonas.* The taxonomically distant marine pelagic unicellular diatom *Phaeodactylum tricornutum* is also being developed as a model for basic and biotechnological studies, but its biology differs in many respects to that observed in the green lineage. *P; tricornutum* has a predominantly diploid life-cycle, with a nuclear genome length about twice as long as *Ostreococcus* spp.; its plastid and mitochondrial genomes have also been sequenced (Bowler et al., 2008, Giguere et al., 2022).

Simplified circadian rhythm of *Ostreococcus*

Circadian rhythms coordinate biological processes with the daily light period, ensuring that physiological processes are optimal for an organism to develop, grow, survive, and proliferate. In *Chlamydomonas*, forward genetic studies allowed large-scale identification of circadian genes (Matsuo et al., 2008). In short, numerous genes controlling rhythm are similar to those in *A. thaliana,* and few genes are specific to *Chlamydomonas* (Waltenberger et al. 2001; Schmidt et al., 2006).

Interestingly, *O. tauri* possesses an extremely simplified circadian clock mechanism compared to *Chlamydomonas* and higher plants, making it an attractive model organism for studying the fundamental principles of circadian regulation. Corellou et al. (2009) identified two master clock genes in *O. tauri*: Timing of Cab Expression 1 (TOC1) and CCA1. The mRNA level of TOC1 peaks around dusk, coincident with the decay of the CCA1 transcript. CCA1 expression reaches a plateau in the middle of the night, decreasing before dawn, followed by TOC1 increase. TOC1 activates CCA1 transcription, which in turn represses TOC1 expression by binding to a conserved evening element (EE) sequence found in the TOC1 promoter, thus, the level of TOC1 falls as CCA1 rises, similar to that of the *A. thaliana* homologue (Alabadí et al., 2002; Corellou et al., 2009). The expression time profiles of CCA1 and TOC1 were accurately modeled as a two-gene negative feedback loop independent of the light parameter (Thommen et al., 2010). In addition, experimental and modeling studies indicate that coupling circadian rhythm to light quality/intensity is mediated by photoreceptors (Pfeuty et al., 2012). *Ostreococcus* blue light-sensitive light, oxygen, or voltage sensing histidine kinase (LOV-HK) has a rhythmic pattern of expression in light/dark cycles peaking at dawn, indicating that it is under circadian control (Djouani-Tahri et al., 2011). Both overexpression and downregulation of LOV-HK alter the cyclic expression pattern of CCA1, revealing that LOV-HK is required for the circadian rhythm in *Ostreococcus* integrating light qualities (Djouani-Tahri et al., 2011). Moreover, the histidine kinase rhodopsin (HRK) is a photoreceptor whose transcript peaks at dusk and is involved in *Ostreococcus* circadian rhythm (Luck et al., 2019; Pfeuty et al., 2012). Both LOV-HK and HRK might perceive the light spectrum at different day times and depths in the water column entraining the master clock regulator TOC1 by protein phosphorylation (Luck et al., 2019; Pfeuty et al., 2012). In contrast, the regulation of diurnal rhythm in *P. tricornutum* and other non-green algae is more closely related to that in animals, as bHLH-PAS nuclear proteins are involved (Annunziata et al., 2019).

Understanding the simplified circadian rhythm of *Ostreococcus* offers relevant information about the development and operation of circadian clocks in photosynthetic species. It also opens new lines for chronobiological study and the formulation of strategies to boost photosynthetic output and efficiency in crop plants and algae.

Photobiology of *Ostreococcus*

Numerous authors have investigated the responses of marine algae to light, reflecting intense interest in this field (Jaubert et al, 2017 for a review). Recently, Sands et al. (2023) demonstrated that *Ostreococcus* ecotypes originating from different light quality environments exhibit divergent responses. Specifically, *Ostreococcus* ecotype RCC809 (Fig; 1) showed remarkable responses to light quality. Transcriptomics analysis revealed variations in gene expression between cells acclimated to monochromatic red, green, and blue light (Sand et al., 2023). The gene expression pattern under blue light differed markedly from that under red light, with intermediate phenotypes being observed under green light. In particular, responses to light quality in *Ostreococcus* differed from those observed in terrestrial plants, where the effects of green light counteract those of red and blue wavelengths (Wang and Folta, 2013). This distinctive pattern may be attributed to the presence of a distinct set of photoreceptors. Whereas cryptochrome and phototropin photoreceptors are universally found in plants and algae (Kianianmomeni and Hallmann, 2014), *Ostreococcus* has the LOV-HK and HKR (histidine kinase rhodopsin) involved in responses to blue and green light respectively (Djouani-Tahri et al., 2011; Luck et al., 2019), both being absent in land plants. However, *Ostreococcus* lacks phytochromes and the identity of a photoreceptor responsible for perceiving red light is as yet unknown. These findings suggest that the differential ability to respond to light quality may contribute to the specialization of the phytoplankton ecotypes in different environments (Sands et al., 2023). In Diatoms, specialized receptors called Aureochromes have evolved to respond to blue light, and an extended family of light harvesting complex proteins provide protection to absorbed light.

The main difference between RCC809 and other *Ostreococcus* species lies in their exposure to light. While most *Ostreococcus* species are exposed to moderate to high light levels and may experience photoinactivation due to oxidative stress from the photosynthetic chain, RCC809, as a deep-sea organism, is exposed to low light intensity. Unlike other *Ostreococcus* strains that require a photosystem inhibitor to regulate photosystem 2 activity and inhibit electron transfer chains (as nitric oxide commonly does in mitochondrial respiratory chains, Brown et al., 1999), RCC809 does not need such inhibition. Instead, it needs to maximize light absorption for photosynthesis. The presence of Nitric Oxide Synthase (NOS) in *O. tauri* and *O. lucimarinus* and other green algal phyla argues for a common ancestor of this phylum that has not been conserved in land plants (Weisslocker-Schaetzelet et al., 2017). In fact, NOS are present in all *Bathycoccaceae* genomes except that of *Ostreococcus* RCC809 (http://genome.jgi.doe.gov/). This could explain a possible function of NOS in photosynthetic organisms (Foresi et al., 2010; Weisslocker-Schaetzelet et al., 2017). Understanding these photobiological divergences in *Ostreococcus* is crucial for unraveling the evolutionary adaptations of these microorganisms to diverse light environments and their ecological roles in marine ecosystems. Ongoing research continues to shed light on the intricate mechanisms governing *Ostreococcus* photobiology and its implications for global carbon cycling and aquatic ecosystems.

Fundamental differences in iron metabolism in *Ostreococcus*

Iron concentration is low in well oxygenated water. Most (~99%) of the dissolved iron in the ocean is

bound to organic compounds with a very high affinity for iron, and its availability is often a crucial factor limiting phytoplankton growth. Whereas prokaryotes uptake mainly siderophore-complexed iron, eukaryotic phytoplankton uptake mainly porphyrin-complexed iron (Hutchins et al., 1999). Iron availability has a strong impact on phytoplankton growth in some ocean regions where primary production is low despite high concentrations of major nutrients such as nitrate, phosphate and silicate. Iron is a vital element for all living organisms since as a cofactor it is involved in DNA synthesis, photosynthesis, N fixation, mitochondrial respiration, and detoxification of reactive oxygen species. Despite its great abundance in the earth's crust, several environmental factors such as pH, carbonate concentration, and temperature affect the bioavailability of iron. Consequently, specific and high affinity mechanisms have evolved in organisms to acquire this element (Martín-Barranco et al., 2021). Additionally, in the presence of oxygen produced by photosynthesis, iron can react with water to produce free radicals (the Fenton reaction), which are highly toxic. The mechanisms of iron absorption and intracellular storage must therefore be strictly regulated. In *Chlamydomonas*, multiple iron acquisition systems have been identified (Blaby-Haas and Merchant, 2017). In the reductive strategy for iron acquisition, a ferric reductase (FRE) reduces Fe^{3+} to the soluble iron form Fe^{2+} in close vicinity to the cell membrane. The accumulated Fe^{2+} pool is re-oxidized to Fe^{3+} via a multicopper oxidase (FOX), which has been suggested to confer a high metal specificity to this high-affinity iron uptake system (Herbik et al., 2002). FOX is presumed to form a complex with the permease (FTR), which internalizes the oxidized Fe^{3+} into the cytosol.

Ostreococcus appears to lack the classical components of the reductive iron uptake strategy (Lelandais et al., 2016; Palenik et al., 2007). Jancek et al. (2008) found two *FRE* genes and a *FOX* gene in *O. tauri* which are absent in *O. lucimarinus*. However, no clear evidence of ferrireductase activity was found even under iron limiting conditions in *O. tauri* (Lelandais et al., 2016; Sutak et al., 2012).

Interestingly, both ferric and ferrous iron uptake were induced in *O. tauri* after the cells were shifted to iron deficient conditions (Sutak et al., 2012). This observation, without the parallel induction of a FRE activity, suggests that this organism can take up iron without prior reduction, as shown for the marine diatom *Phaeodactylum tricornutum* (Morrissey et al., 2015). In *P. tricornutum* the protein ISIP2a, belonging to the transferrin family, has been shown to concentrate Fe^{3+} at the cell surface and facilitates its uptake. ISIP2a is transcriptionally induced in response to iron limitation, presents a C-terminal Fea1 domain, and has been experimentally confirmed to directly bind Fe^{3+}, thereby increasing iron uptake (McQuaid et al., 2018; Morrissey et al., 2015). On one hand, as in *P. tricornutum*, an iron binding protein named Ot-Fea1, was identified under iron-limited and copper sufficient conditions in *O. tauri* (Lelandais et al., 2016; Scheiber et al., 2019). As a copper-regulated protein, Ot-Fea1 establishes a new iron-copper connection in *O. tauri*, which remains poorly understood to date. Ot-Fea1 contains duplicated Fea1 domains and is structurally different from Cr-Fea1/Fea2, the major proteins secreted during iron-deficiency in *C. reinhardtii* (Allen et al., 2007).

On the other hand, six genes encoding ZIP family transporters involved in iron acquisition and intracellular trafficking have been found in *O. tauri,* only one of which, *OsIrt1*, is likely to be the ZIP protein playing a significant role in iron metabolism (Lelandais et al., 2016). This protein is predicted to be localized at the plasma membrane and might have high iron-binding capacity as it contains the iron-binding motif found in *A. thaliana* Iron Regulated Transporter 1 (IRT1) (Lelandais et al., 2016).

In comparison with the iron uptake mechanisms, less is known about how phytoplanktonic species adapt

to iron scarcity. Different responses have been observed among *Ostreococcus* strains to growth in iron-limiting conditions, such as the modulation of the chlorophyll and protein cell content, the photosynthetic activity, and the iron cell content (Botebol et al., 2017). In nano and microphytoplankton, the reduction of cell surface/volume ratio has been reported as the main strategy to cope with low iron availability. However, in an *Ostreococcus* strain that requires low iron levels (RCC802), acclimatization to this condition consists of a decrease in iron demand by reducing cell biomass through a reduction in the photosynthetic machinery and protein content (Botebol *et al.*, 2017). *Ostreococcus* spp., like numerous other marine phytoplankton, can use flavodoxins instead of ferredoxins (which contain iron) in the photosynthetic electron transfer chain if the iron reservoir is scarce (La Roche et al 1996; Erdner et al 1999; Palenik et al., 2007). Additionally, genes for Cu/Zn- and Mn-containing superoxide dismutases, but not a Fe-SOD have been identified in the *O. tauri* genome, which may contribute to reducing iron quotas.

One of the best-known iron storage mechanisms in algae, ferritin, is diurnally regulated in *O. tauri.* Botebol *et al.* (2017) have suggested that the main function of ferritin in *O. tauri* would not be primarily long-term storage of iron but rather temporary storage during the day/night cycle. In the night, ferritin might keep the intracellular iron stock intact from damaged/oxidized iron-containing proteins. At dawn, iron is released from ferritin and becomes available for assembly into new iron-containing complexes synthesized *de novo* during the next day.

The mechanisms of iron uptake and utilization by *Ostreococcus* differ fundamentally from those described in *Chlamydomonas* or in diatoms (Sutak et al., 2020). We propose the *Ostreococcus* species as a new model for the investigation of iron metabolism since, due to its particular characteristics; it can contribute to the knowledge of new mechanisms related to iron homeostasis in marine microalgae.

Nitrogen transport, signaling and metabolism

Nitrogen (N) is often considered a regulatory factor for the growth of phytoplankton populations. The genetic ability to use different N sources conditions survival and provides evidence of the evolutionary history of microorganisms (Moschonas et al., 2017). Most algae, like terrestrial plants, primarily use nitrate as a source of N. The main nitrate and nitrite transport system includes, the high- and low-affinity nitrate transporters, NTR1 and NTR2 (and their accessory protein NAR2) respectively, and the NAR1 protein (transports nitrite to the chloroplast) (Sanz-Luque et al., 2015). In the *O. tauri* genome, the NTR1, NTR2, NAR2 and NAR1 genes were identified (McDonald et al., 2010; Sanz-Luque et al., 2015), while *Chlamydomonas* has one gene for NTR1 and NAR2, and six genes for the NTR2 (Sanz-Luque et al., 2015) and NAR1 (NAR1.1-6, Mariscal et al., 2006). Additionally, genes involved in nitrate assimilation were identified in *O. tauri* genome, including the enzymes nitrate reductase (NR), proteins for the synthesis and transporter of the molybdate cofactor, nitrite reductase (NiR), glutamine synthetase (GLN) and glutamate synthase (GSN/GSF) (McDonald et al., 2010; Sanz-Luque et al., 2015). The *Chlamydomonas* genome also contains these genes involved in N assimilation, but with four GLN genes and two genes for GSN/GSF and MOT2 (molybdate transporter) (Sanz-Luque et al., 2015). The arrangements of these genes, located in clusters, are reminiscent of prokaryotes (Rocap et al., 2003). The physical proximity of genes likely facilitates the coordination of their regulation and inheritance, increasing selection pressure for resource optimization (Mugford et al., 2013; Nützmann and Osbourn, 2015; Osbourn et al., 2012; Trowsdale, 2002).

Eukaryotic algae are generally considered ineffective competitors for ammonium (Worden et al., 2004). However, in the *O. tauri* genome, four genes encoding ammonium transporters (AMT) were identified, two related to the green lineage (AMT1) and the other two similar to prokaryotes (AMT2) (Derelle et al., 2006; McDonald et al., 2010). The discovery of a N transporter originating from a host organism, found within an *Ostreococcus* virus named OtV6, is significant. The algal virus OtV6 carries a host-derived AMT that can modify N uptake in its host marine phytoplankton. This finding establishes a connection between viral and host functions in the context of N acquisition in marine ecosystems (Monier et al., 2017).

The large number of AMT genes in *Ostreococcus*, as opposed to, for example, *Chlamydomonas*, suggests that there may be strong competition for this N source in marine ecosystems.

Furthermore, different AMT genes within single genomes can have varying functions, localizations, substrate affinities, and transport efficiencies, providing a favorable adaptation to fluctuating environmental conditions (Fernandez and Galvan 2007). However, the activity and regulation of these transporters have been little investigated (Derelle et al., 2006; McDonald et al., 2010). *Ostreococcus tauri* is also able to grow with urea as the only source of N (Worden et al., 2004). A recent study showed that *O. tauri* growing with L-arg as the only N source, promotes a sustained growth rate and an increase in chlorophyll levels (Foresi et al., 2022). The addition of L-arg also reduces the levels of transcripts of genes involved in N absorption and metabolism, which are increased under N deficiency (Foresi et al., 2022). In this sense, *C. reinhardtii* is capable of efficiently metabolizing L-arg, although there is an activation of N catabolic genes and of the responses induced by the lack of N (Muñoz-Blanco et al., 1990; Munz et al., 2020). *O. tauri* lacks the enzymes described in photosynthetic organisms for L-arg degradation (such as arginase, arginine deimidase, arginine decarboxylase and L-amino oxidase). However, a genuine NOS is present (Foresi et al., 2010; Weisslocker-Schaetzel et al., 2017). Foresi et al. (2022) propose a new pathway for the metabolism of L-arg by the enzyme NOS in *O. tauri* (OtNOS), where L-arg is broken down to NO and L-citrulline, then the NO could be oxidized in a non-enzymatic way to nitrite and nitrate and reincorporated into the N metabolism (Figure 2). In addition, Sanz-Luque et al. (2015) reported that NO acts in the signaling of nitrate transport and assimilation in *Chlamydomonas*. Despite the crucial role of nitrate as a nutrient and growth promoter, few studies regarding nitrate signaling pathways were published in algae (Gao et al., 2023; Yang et al., 2021). The discovery of a peak of calcium (Ca^{2+}) and the activation of Ca^{2+} dependent protein kinases (CDPKs) after nitrate sensing were already described in land plants (Liu et al., 2017; Riveras et al., 2015). In this context, Calo et al. (2017) have shown the existence of functional CDPKs in *O. tauri.* Moreover, the transcriptional regulation of these kinases was subject to N status and N stress treatments, suggesting their ancient conserved role as N response proteins.

Unique Carbon Metabolism in *Ostreococcus*

The main products from photosynthesis and carbon fixation in the Calvin cycle are carbohydrates (Moreira et al., 2022). Polysaccharides obtained from microalgae are safe, stable, biocompatible, and biodegradable. Thus, much effort is being invested to unveil the mechanism behind their metabolism and regulation. Carbohydrate synthesis in microalgal cells occurs in the chloroplast, and when superfluous sugars are not used as an energy source, they are converted to starch in this organelle.

As a distinctive feature, O. tauri accumulates starch in a unique granule inside the chloroplast. The molecular composition is amylose/amylopectin with α-1,4 glycosyl bonds and α-1,6 branches, such as other chlorophytes and higher plants, showing high complexity despite being a picoalga. Starch

biosynthesis in *O. tauri* uses ADPG (adenosine diphosphate glucose) as glycosyl donor and the enzymes ADPG pyrophosphorylase (ADPGPPase), granule-bound Starch Synthase I (GBSSI), soluble starch synthase (SS I-VI), starch branching enzyme (SB I and II) and isoamylase (ISA I and II). Thus, *O. tauri* has all the synthesis pathway steps found in green algae and plants. Furthermore, *O. tauri* presents three different isoforms of SS in contrast to most plants, showing a high complexity despite its compact genome (Barchiesi et al., 2018). *O. tauri* also has "bacterial like '' enzymes in relation to starch metabolism such as one isoform of SS (SIII-C) which is active but lacks the N-terminal starch binding domain (Barchiesi et al., 2018). As in *Chlamydomonas*, OtGBSS is part of the synthesis pathway of amylose and also amylopectin. One particular difference in *O. tauri* is that the OtADPGPPase is not regulated by redox state, which leads to hypothesizing that the enzyme regulation by redox conditions has evolved after the separation of the picoalga from land plants (Figueroa et al., 2018). Instead, the regulation of ADPGPPase in *O. tauri* through 3-phosphoglycerate and orthophospshate is conserved with plants, being activated or inhibited respectively. Recently, a new plastidial protein without catalytic activity (Carbohydrate Binding Domain 20 Containing Protein, CBM20CP) that could increase starch synthesis through the interaction with OtSSIII-B was discovered (Hedin et al., 2022). *O. tauri* cannot synthesize glycogen due to the lack of the enzymes related to that metabolic pathway. The picoalga has an interesting mechanism of starch granule partition in which prior to the cell division, the granule grows in size in order to segregate one for each divided chloroplast. Therefore, *O. tauri* modifies the starch granule size according to the environmental conditions but does not degrade this carbon reservoir completely even in the dark (Ral et al., 2004). With this particular mechanism *O. tauri* does not need to prime starch granules or regulate their number.

Unexpectedly, in metabolic profiling experiments sucrose was not detected in *O. tauri* (Hirth et al., 2017). Instead, trehalose was found to be the more abundant sugar, showing its highest content during the night (1.6 mM) (Hirth *et al*., 2017). Trehalose concentration is subject to a great variation dictated by the diel cycle, and its high content at the end of the night may be a consequence of starch degradation. The switch from anabolic to catabolic metabolism that occurs in chloroplast in the dark is driven by the inactivation of the reductive Calvin cycle and the activation of the oxidative pentose phosphate pathway (OPPP). In relation to this, another interesting and specific feature of *Ostreococcus* is that the Calvin cycle is inactivated by the formation of GapAB (glyceraldehyde-3-phosphate dehydrogenase) complexes (Robbens et al., 2007). In land plants, two main actors, the glyceraldehyde-3-phosphate dehydrogenase (GapAB) and CP12, regulate the Calvin cycle at night. While GapA and CP12 are found in all plants including green algae, GapB is exclusive to Streptophytes. Interestingly, *O. tauri* and *O. lucimarinus* genomes contain the GapB gene, while CP12 is absent, being the first exception.

O. tauri also activates accumulation of C reserves when the N/C ratio is altered (Caló et al., 2022; Foresi et al., 2022). The protein that senses the N/C balance in cyanobacteria and algae is the PII protein, which perceives cellular glutamine and 2-oxoglutarate levels and the ATP/ADP ratio. The PII protein regulates not only important steps of N and C metabolism but rather controls a wide range of transporters, besides being involved in the production of signaling molecules (Forchhammer et al., 2022). A distinctive characteristic of *O. tauri* is that, unlike green algae and plants (Chlorophyta and Streptophyta), it lacks a typical PII homologue (Selim et al., 2020). Then, how does this picoalga perform this regulatory function?. The absence of a typical PII is striking since it must be present in the core of Chlorophyta (Leliaert et al., 2011). Thus, there are open interesting avenues to further explore for the N and C metabolism signaling pathways in *O. tauri*, especially in a fluctuating environment.

Lipid regulation in *Ostreococcus*

Microalgae are considered a promising source of renewable energy because of their high photosynthetic activity and faster growth than higher plants, as well as their ability to accumulate carbon reserves. Microalgae are known to accumulate starch and lipids under suboptimal growth conditions through a complex spatio-temporal mechanism involving the endoplasmic reticulum and chloroplasts (Li-Beisson and Peltier, 2013). Typically, the accumulation of these crucial compounds coincides with growth arrest (Collyer and Fogg, 1955). To date, several environmental stressors have been studied as possible triggers of lipid accumulation in microalgae, including light exposure, oxidative stress, salt concentrations, and nutrient deficiencies (Chen *et al*., 2017, Yonghua Li-Beisson and Gilles Peltier, 2013). Among these stressors, phosphorus and nitrogen deprivation have been the most extensively studied, especially in the unicellular microalgae *C. reinhardtii* (Boyle et al., 2012; Kamalanathan et al., 2016; Siaut et al., 2011). Like *Chlamydomonas*, under nutrient starvation, *O. tauri* also tends to accumulate triacyl glycerides (TAGs) to a greater extent (Degraeve-Guilbault et al., 2017). As described in *Chlamydomonas*, in *O. tauri* the addition of L-arg or NO_2^- as sole N sources showed a similar increase in lipid content to N deprivation, though culture growth is not severely affected (Foresi et al., 2022; Munz et al., 2020).

Nutrient deprivation is a crucial factor determining the ultrastructure and lipid composition of *O. tauri*. When subjected to N or P deprivation, *O. tauri* cells increase in size and tend to accumulate TAGs by storing them in oil bodies. Moreover, N-deprived *O. tauri* cells seem to accumulate plastidicoil bodies, which may play a significant role in TAG synthesis. An analysis of the lipid profile of *O. tauri* revealed that those in the plastid were enriched in C18-PUFAs and that ω-3 docosahexaenoic acid was exclusively extraplastidial, but that highly unsaturated TAGs originate from both endoplasmic reticulum and plastid precursors (Degraeve-Guilbault et al., 2017).

The Diacylglycerol acyltransferases (DGATs) are key enzymes for producing lipids in animals, plants, and yeast. DGAT enzymes catalyze TAG formation in the Acyl-CoA-dependent pathway. Additionally, the phospholipid: diacylglycerol acyltransferase (PDAT) enzyme operates in an alternative pathway, known as the Kennedy pathway, for TAG formation, which is Acyl-CoA-independent (Turchetto-Zolet et al., 2011; Yoon et al., 2012). In *Chlamydomonas*, several DGAT and PDAT enzymes, such as DGAT1, DGAT2 (DGTT1-5), DGAT 3 and PDAT1 (Bagnato et al., 2017; Iwai et al., 2014; Miller et al., 2010; Nguyen et al., 2011), have been identified and are essential for stress-induced TAG biosynthesis (Boyle et al., 2012; Carro et al., 2022; Iwai et al., 2014; Liu et al., 2016; Msanne et al., 2012). Three orthologous genes of type 2 DGAT (DGTTA-C) have been identified in the genome of *O. tauri*. In contrast, no orthologue sequences of DGAT1 have been found in this picoalga (Wagner et al., 2010). Bagnato *et al*. (2017) identified a putative DGAT3 protein but its prediction was difficult as there are undetermined amino acids in its sequence. Also, they identified a putative sequence of the PDAT protein but did not discuss it. A putative *Micromonas* PDAT that belongs to the same phylogenetic clade as CrPDAT has been described so far (Pan et al., 2015). Despite these findings, the role of these enzymes under different abiotic stress conditions in *O. tauri* remains unknown. It would be interesting to investigate how DGAT and PDAT are regulated under various stressors and assess their contributions to TAG accumulation in *O. tauri*.

Another important aspect of lipid accumulation in microalgae is its connection to the Target of Rapamycin (TOR) protein, a conserved protein kinase crucial for cell growth and development (Kennedy and Lamming, 2016). In plants and green algae, only the TOR1 complex has been described so far (Brunkard

et al., 2020; Pacheco et al., 2021; Rodríguez-Ruiz et al., 2019). TORC1 is integrate by TOR kinase, the Regulatory-associated protein of TOR (RAPTOR), and the Lethal with Sec13 protein 8 (LST8) protein. Most studies on the TOR signaling pathway in green microalgae have been limited to *C-reinhardtii.* In the last decade, TOR activity was shown to be regulated by nutrient availability (Couso et al., 2020; Upadhyaya and Rao, 2019) and its inhibition by TOR inhibitors (Rapamycin, AZD8055 or PP242) induces lipid accumulation in *C. reinhardtii* (Imamura et al., 2016; Jüppner et al., 2018). *O. tauri* has the components of TORC1 and recently OsTOR inhibition by PP242 was found to arrest cell growth and increase carbon reserves. TOR is also involved in nutritional stress response because after 24 h of N starvation, TOR transcripts are downregulated and lipid levels increase. A surprising observation in *O. tauri* is that the cells excrete lipids into the extracellular medium when TOR is inhibited or under N starvation conditions (Caló et al., 2022). Pinto *et al*. (2021) have shown that *Marinobacter algicola* takes advantage of *O. tauri* exudates. Understanding the regulatory mechanisms of lipid excretion and its metabolic significance in *O. tauri* demands further study.

Phosphate deprivation is known to increase TAG content in microalgae. In *Chlamydomonas*, P metabolism is regulated by the transcription factor PSR1 (phosphate starvation response) which is an effector upstream of TOR (Bajhaiya et al., 2016; Couso et al., 2020). Fiore *et al*. (2021) described a putative PSR1 protein in *O. tauri* which has conserved domains. Since P deprivation increases TAG content in *O. tauri* cells, the role of the putative OsPSR1 is an important issue for future research. In *Chlamydomonas*, another important transcription factor involved in the stress response is the NITROGEN RESPONSE REGULATOR 1 (NRR1). NRR1 transcript is induced under N deprivation conditions and plays a key role in the accumulation of TAGs in cells (Boyle et al., 2012). So far, this kind of transcriptional control has not been investigated in *O. tauri*.

O. tauri provides a valuable model for the study of TAG accumulation pathways and nutritional stress responses. Furthermore, given its potential to accumulate lipids and its marine environment, it may represent a promising avenue for third generation biofuels.

Conclusion

In this review, we aimed to highlight the main advantages of *Ostreococcus* spp. as good models for unveiling signaling pathways of microalgae and plants (Table 1). Thus, *Ostreococcus* could be proposed as a model, for example, of iron deficiency signaling and for efficient use of N in marine phytoplankton. The apparent absence of a canonical PII protein is particularly interesting, as is the presence of the NOS enzyme and the poorly characterized pathway of lipid excretion. Moreover, the sequenced *Ostreococcus* genome may yet yield unexpected discoveries, along with surprising genes that could provide the basis for exciting biochemical studies in the near future. Research on *Ostreococcus* is not only important for the understanding of biochemical and molecular processes but also for marine ecology and in the evolution and diversification of the green lineage (van Baren et al., 2016).

Despite all the advances in the study of the *Ostreococcus* genus in the last few years, the generation of feasible tools to mutate *Ostreococcus* genes remains a great challenge that is awaiting resolution. Although vectors and different methodologies to transform *Ostreococcus* cells have been developed, only one article so far reports *Ostreococcus* insertion mutant lines (Thomy et al., 2021). Modern genetic

techniques such as CRISPR-CAS9 on *Ostreococcus* cells remain to be developed and should facilitate and accelerate the genetic manipulation of this picoalga. This will open the possibility of using *Ostreococcus* for biotechnological tools, such as environmental biosensors.

There is no doubt that the possibilities of *Ostreococcus* as a tool and study model are just beginning and that it has great potential that will expand as genetic methodologies advance.

Figure Legends

Figure 1. Transmisión Electronic Microscopy of *Ostreococcus* cells. A) *Ostreococcus tauri* (RCC4221). B) Ostreococcus RCC809. C) *Ostreococcus lucimarinus.* The bar represents 500 nm. Chl–chloroplast; Cyt–cytoplasm, n–nucleus, m–mitochondrion, sg–starch grain.

Figure 2. *Ostreococcus* exhibits distinctive and optimized features for N metabolism. This model was generated principally from *O. tauri* genome information. The NTR1, NTR2, NAR2 and NAR1 genes were identified for NO_3 transport (McDonald et al., 2010; Sanz-Luque et al., 2015). Furthermore, the genes involved in nitrate assimilation, including the enzymes nitrate reductase (NR), proteins for the synthesis and transport of the molybdate cofactor, nitrite reductase (NiR), glutamine synthetase (GLN) and glutamate synthase (GSN/GSF) (McDonald et al., 2010; Sanz-Luque et al., 2015). Cationic amino acid transporters (APC) carry out arginine uptake from the medium. Intracellular Arginine is metabolized by nitric oxide synthases (NOS) to produce citrulline and NO. NO can be rapidly oxidized non-enzymatically in the presence of O_2 to produce N oxides (such as NO_2) which can be assimilated and reduced to NH_4 by NiR. Furthermore, NO may also be reduced to nitrous oxide (N_2O gas) by flavodiiron proteins (FLVs) or cytochrome P450 (CYP55), under light or dark conditions respectively (Foresi et al., 2022). Strikingly, four genes encoding ammonium transporters (AMT) were identified, two related to the green lineage (AMT1) and the other two similar to prokaryotes (AMT2) (Derelle et al., 2006; McDonald et al., 2010). In addition, *O. tauri* is capable of growing with urea as the only source of N and has a gene encoding for an urea transporter (Dur3) and three genes encoding for the multi-subunit urease (UreGD, UreABC, UreF) in its genome (Worden et al., 2004; Derelle et al., 2006).

Acknowledgements

We thank Marie-Line Escande for the TEM photos of Ostreococcus. We apologize to those authors whose papers are not discussed or cited due to space limitation.

Conflict of interest

The authors have declared no competing interest.

Funding

This research was funded by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT, PICT2019-2118), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET PIP-11220200101701CO/11220210100268CO) and Universidad Nacional de Mar del Plata (UNMdP, EXA1036/21, EXA1142/23).

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919 **Table 1. Peculiarities of the *Ostreococcus* cell and metabolism**

Cell organization and peculiarity features of metabolism of *Ostreococcus*	
Findings	***Reference***
Cells	
Ostreococcus has a nucleus, a single chloroplast, mitochondrion, and Golgi body. It lacks a visible cell wall under transmission electron microscopy and has a notably reduced cytoplasmic compartment.	*Henderson et al.,2007* *Ral et al., 2004*
This picoalga utilizes an intriguing mechanism for starch granule partition, where the granule grows in size before cell division and then divides, resulting in one granule per daughter cell allocated to each divided chloroplast.	
Nuclear and organell genoma	
These algae have the smallest genome size and gene content among all known green algae. Only 19% of *O. tauri* genes have introns. However, EST analysis of *O. lucimarinus,* which has the most compact eukaryotic genome, reveals an excess of introns in highly expressed genes.	*Robbens et al., 2006* *Palenik et al., 2007* *Derelle et al., 2006*
Ostreococcus encodes a high proportion of selenoproteins with greater activity.	
Circadian rhythm	
O. tauri possesses an extremely simplified circadian clock.	*Correleau et al, 2009*
Only two master clock genes. Timing of Cab Expression 1 (TOC1) and Circadian Clock-Associated 1	
Iron Metabolism	
Lacks the classic components of the iron absorption reduction strategy.	*Palenik et al., 2007* *Lelandais et al., 2016*
The iron-binding proteins Irt1 and Fea1 might be involved primarily in iron uptake	
Nitrogen Metabolism	*Rocap et al., 2003*
Genes associated with nitrate, ammonium, and urea metabolism in the genome are clustered.	*Foresi et al., 2010* *Foresi et al., 2022*
O. tauri demonstrates the ability to grow using L-arg as the sole source of N, potentially through the activity of OtNOS.	*Weisslocker-et al., 2017* *Sanz-Luque et al., 2015* *Rocap et al., 2003*
Carbon Metabolism	
Accumulates trehalose, does not synthetizes sucrose.	*Hirth et al., 2017*
Accumulates starch in a unique granule inside the chloroplast.	*Barchiesi et al., 2017*
N/C balance	
Prasinophytes does not contains PII protein (except *Micromonas pusilla* and *M. commoda*)	*Selim et al., 2020*
Lipid metabolism	
Nutrient deprivation results in growth arrest, increased cell volume, and elevated TAG content in *O. tauri*.	

Additionally, it exhibits a tendency to secrete lipids into the extracellular medium through an unknown mechanism.	*Liu et al., 2016*
	Wagner et al., 2010
C18-PUFAs accumulate inside the plastid, while ω-3 docosahexaenoic acid is exclusively found extraplastidially. Unsaturated TAGs are derived from both ER and plastidial precursors.	*Calo et al., 2022*
	Fiore et al., 2021
Three orthologous Diacylglycerol O-Acyltransferase 2 genes have been identified.	*Couso et al., 2020*
Components of TORC1, a master regulator of the nutritional stress response, are present.	*Degraeve-Guilbault et al., 2017*
A putative sequence of the PSR1 protein, which regulates phosphorus metabolism, has been identified.	

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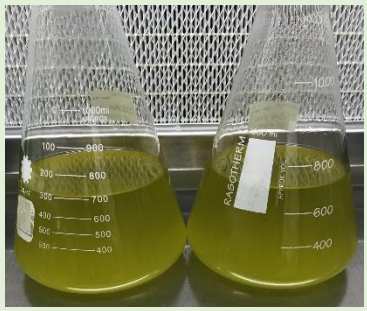
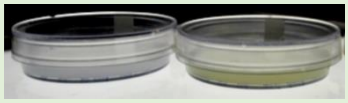
935 **Table 2. *Ostreococcus* growth culture conditions in laboratory**

Different ways to grow *Ostreococcus tauri* OTTH0595 (RCC745)		
O. tauri cells are cultured in Keller (K) medium (Keller et al. 1987) supplemented with a vitamin f/2 solution (Guillard and Ryther 1962, Guillard 1975) in the presence of antibiotic mixture (penicillin 25 µg/ml, neomycin 20 µg/ml and kanamycin 25 µg/ml). *O. tauri* cultures can be grown in seawater or artificial seawater (ASW), in order to be able to modify nutrient concentrations specifically. In general, cultures are grown in liquid medium at different volumes, but depending on the type of experiments, they can also be grown in semi-solid medium to qualitatively evaluate the effects on growth. *O. tauri* cultures are maintained under a light:dark photoperiod regime of 12:12h, 60 µE light intensity and a temperature of 20 ± 1°C. The cultures must be shaken periodically every 6h for 5 min each time.		
Liquid cultures	***K medium based in seawater*** The seawater comes from surface samples collected from the see. In our case the samples were taken from Permanent Station of Environmental Studies, EPEA) in the Argentine Sea (38º28' S, 57º41'W; 27 nautical miles south of Mar del Plata, Argentina). The seawater is pre-filtered through a 0.45 µm pore size filter and filtered through a 0.2 µm pore size membrane (Durapore). Then, the components of the K medium (macro and micronutrients) are added and the medium is autoclaved for 20 min at 121°C. Finally, the medium is fractionated based on the necessary volume and supplemented with the vitamin solution and the antibiotic mixture.	
	K medium based in artificial seawater *O. tauri* cells are cultured in K medium based on ASW. Sea salts (420 mM NaCl, 10 mM KCl, 20 mM $MgCl_2$, 10 mM $CaCl_2$, 25 mM $MgSO_4.7H_20$, 2,5 mM $NaHCO_3$) are dissolved in deionised water. Then, K medium components (macro and micronutrients) are added. The final media is then sterilised, fractionated and supplemented with vitamins and antibiotic mixture.	
	K medium based in artificial seawater (nutrient-defficient) *Ostreococcus* cells are cultured in K medium based in ASW. Cultures are centrifuged and resuspended in K medium deficient in one or more nutrients. For N-deficient experiments, cells were cultured in K medium based in ASW lacking $NaNO_3$ and NH_4Cl, which were replaced by equal concentration of NaCl.	
Semi-solid cultures	***Semi-solid K medium based in seawater or artificial seawater*** For qualitative growth assays, cultures can be grown on semi-solid medium. Exponential phase cultures are concentrated by centrifugation and resuspended in ASW-based K medium. For growth experiments in vials, 1 ml of *O. tauri* culture is mixed with molten agarose (0.15% final concentration). The mixture could be placed in 2 ml vials, Petri dishes or multi-well plates and incubated under normal growth conditions. The cells are kept in suspension in the semi-solid culture medium matrix.	  

BOX 1

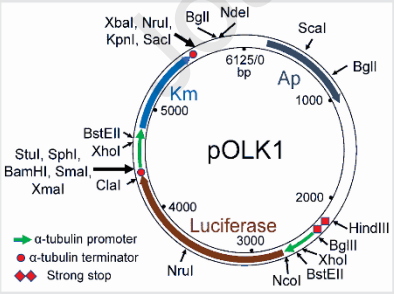
***Ostreococcus* strain**

***O. tauri* strain OTTH 0595**
Isloated from the Thau lagoon on the Mediterranean coast of France.
Adapted to 800 µmol photons $m^{-2}s^{-1}$ (Chrétiennot-Dinet et al. 1995).
***Ostreococcus* sp. strain CCE9901 also known as *O. lucimarinus* (nomen nudum).**
Isolated from the Pacific. Adapted to the ocean surface (Worden et al., 2009).
***Ostreococcus* sp. RCC809**
Isolated from the deep euphotic zone of the tropical Atlantic Ocean at 105 m depth
Acclimated only up to 400 µmol photons $m^{-2}s^{-1}$ (Rodriguez et al., 2005).
***Ostreococcus* sp. RCC802**
Isolated at 65 m depth from the Sicilian channel, Italy (Botebol et al., 2017).
***Ostreococcus mediterraneus* (RCC789)**
Isolated from the surface water of Barcelona harbor, Spain (Guillau et al 2004., Subirana et al., 2013).
***O. bengalensis* (nomen nudum)**
Isolated from the Bay of Bengal, predominantly in mesotrophic, potentially moderate salinity, continental shelf regions of the Atlantic Ocean (Strauss et al., 2023)

BOX 2

Ostreococcus molecular toolkits

1. The pOLK vectors, incorporating synthetic sequences for visible (luciferase) and algal selectable markers (kanamycin) into the cloning vector pUC57 (Corellou et al., 2009, Sanchez *et al.,* 2009).
2. A simplfied Ostreococcus transformation protocol has recently been described. (Sanchez *et al.*, 2019). A treatment with polyethylene glycol (PEG) was more efficient (short incubation time and higher number of transformants per µg of DNA) than the electroporation protocol described by van Ooijen *et al*., 2013.
3. The construction pHAT:Luc involves the utilization of the phosphate high-affinity phosphate transporter (HAPT) promoter of *O. tauri.* This promoter responds to variation in phosphate concentration, has been used for anti-sense constructions (Corellou *et al*., 2009) and is characterized as non-toxic (Djouani-Tahri *et al*., 2011).



4. Figure 1. pOLK vectors used for transformation. Km- eukaryotic G418 (or Kanamycin) resistance. Synthetic sequences for the visible (Luciferase) and algal selectable markers flank the BamHI site of the polylinker. Commonly used restriction endonucleases cutting once or twice are shown, and thicker arrows indicate multiple-enzyme cloning sites. In the vectors pOLK2, pOLK3, pOLK4, pOLK5 (not shown), the BgIII- NcoI fragment was replaced by promoter sequences from Histone 2A, Histone 3, Ubiquitin and thioredoxin, respectively (Sanchez *et al.,* 2019).

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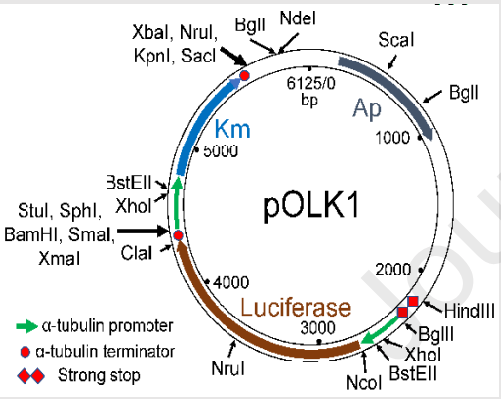

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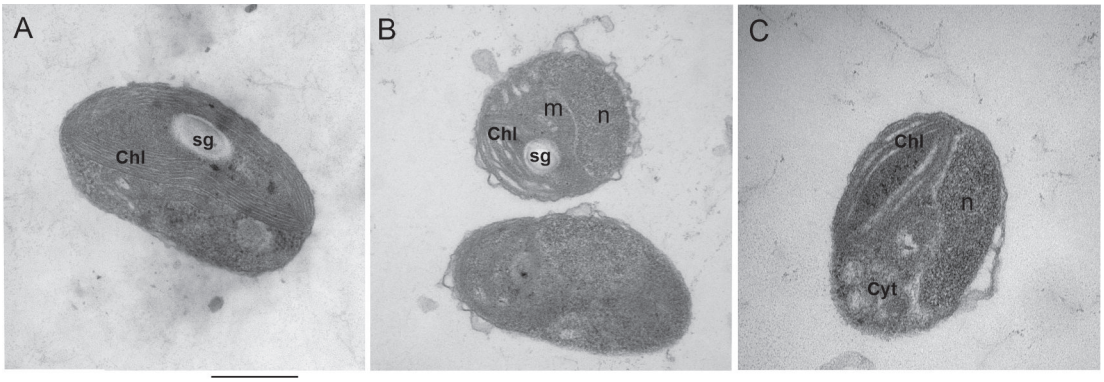

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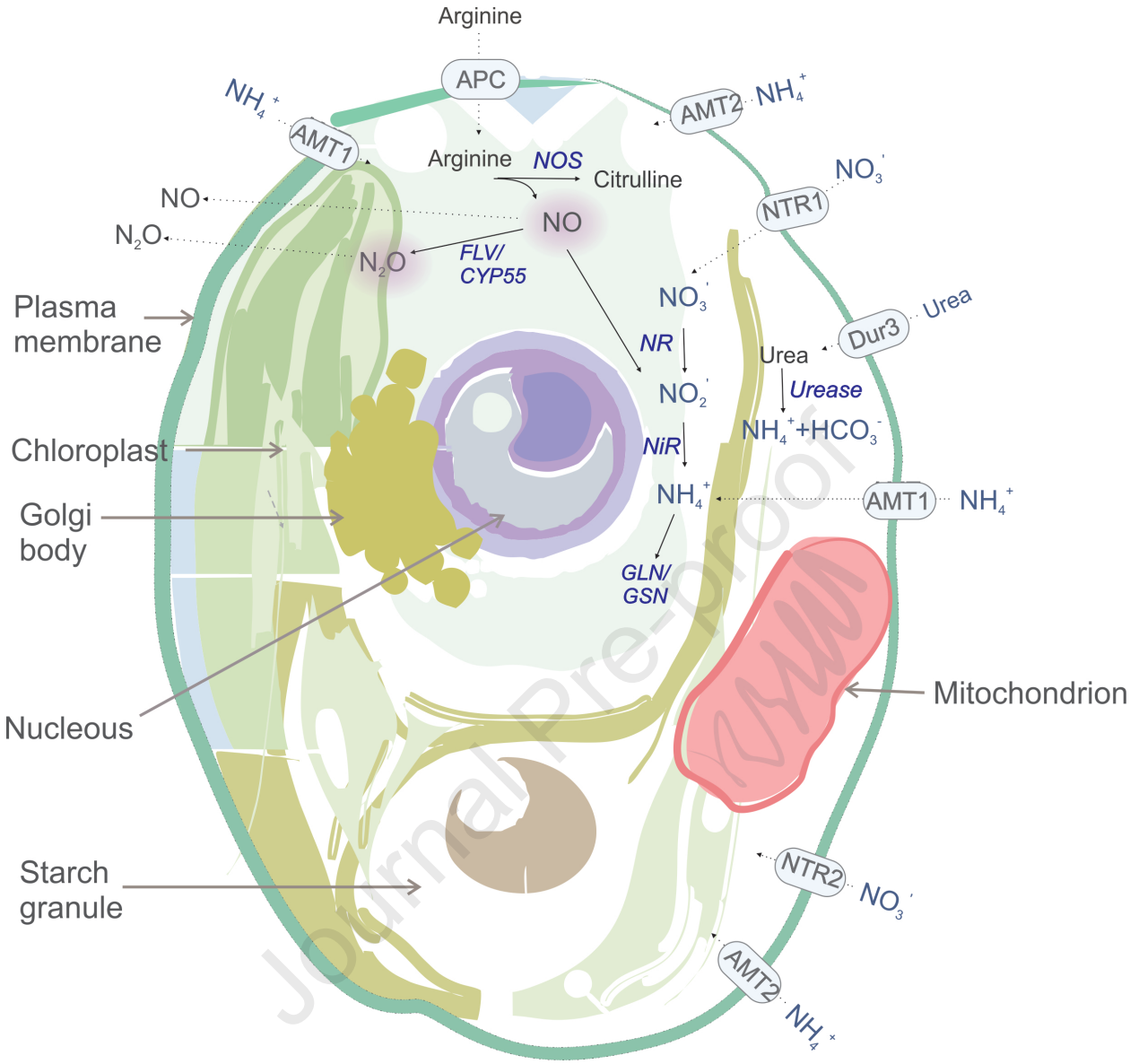

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The manuscript explores the compact genomes, metabolic pathways, and unique strategies of Ostreococcus spp., shedding light on their physiological and genetic adaptations. This contributes valuable insights into the broader understanding of green algae biology and their role in marine ecosystems.

Studying Ostreococcus is timely as it addresses pressing environmental concerns. Insights into its unique features may pave the way for sustainable solutions, including its potential for third-generation biofuels, aligning with the global need for alternative, environmentally friendly energy sources.

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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