



Doctoral Thesis

Multiomics Characterization of the Responses to Diurnal and Seasonal Cycles in the Marine Picoeukaryote *Ostreococcus tauri*

Dissertation presented by Ana Belén Romero Losada to obtain the PhD Degree by Universidad de Sevilla.

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Abstract

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Introduction

Chronobiology

Simple direct observations of our surroundings easily unveil many cyclic processes. We are so familiar with them that we are mainly unconscious about their drastic influences over our lives and bodies, in particular, and life on Earth, in general.

Four environmental cycles are the most apparent ones on Earth: tides (which repeat every 12 and half hours), lunar cycles (lasting 28.5 days), days (every 24 hours) and years (every 365.25 days). These cycles are also called circatidal, circalunar, circadian, or circannual (Numata et al., 2015) . They arise from gravitational forces exerted by the Sun and the Moon over the Earth. Their key characteristic is full predictability. For example, it is possible to determine the exact time of a high tide months in advance. We constantly see how full moons are always followed by new moons, and everybody goes to bed certain that the sun will rise the next morning. The exact date and even time for the next solar equinox, the moment when Earth's rotational axis is perpendicular to the line linking the Sun and the Earth, is known and written in all calendars all over the world. Therefore, sequential and periodic seasonal changes are never a surprise.

Since these four cycles affect earth with an overwhelming precision, earthlings have evolved endogenous molecular systems, referred to as molecular clocks, that allow them to anticipate those cyclic changes. This has motivated the emergence of *chronobiology*, a young area of research that studies how these rhythmic environmental changes affect organisms responses and adaptions (Edmunds, 1983; Kuhlman et al., 2018).

Molecular clocks can be seen as central oscillators susceptible to be entrained by external environmental cyclic inputs (rhythmic environmental changes) that, in turn, generate rhythmic outputs controlling a myriad of biological processes. Chronobiologists have found biological rhythms regulated by the clock in a wide range of spatial scales, ranging from the molecular level (transcription, translation, protein degradation, metabolites biosynthesis, etc), to the cellular and tissue level (cell division, synaptic connections, apoptosis, etc.) or even at the level of whole organisms, populations and ecosystems (Edmunds, 1983; Merrow et al., 2005; Sharma et al., 2022).

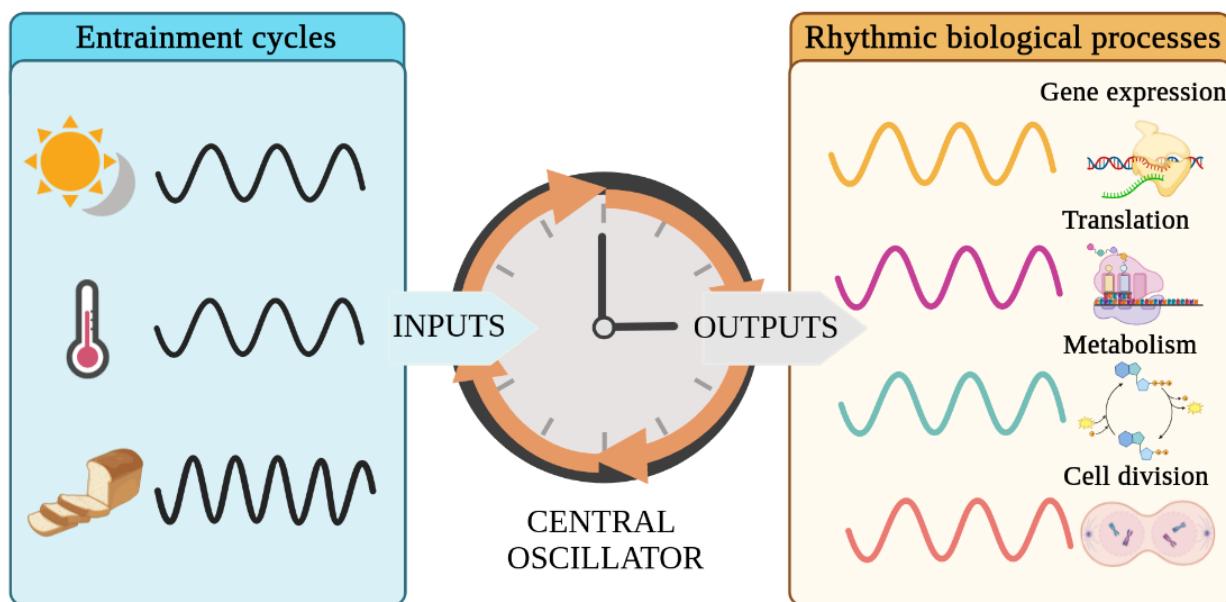


Figure 1: Diagram of the central oscillator known as clock. The clock acts as a central oscillator that is entrained by external cyclic inputs, such as as light/dark cycles or photoperiod, temperature changes and nutrients availability, generated by diurnal, seasonal, lunar or tidal cycles. As a response, the clock produce rhythmic outputs of biological processes that allow organisms to anticipate those environmental changes. Figure created using Biorender.

A distinctive and defining characteristic of clock-regulated biological rhythms is self-sustainability under constant non cycling environmental signals (Pittendrigh, 1960; Roenneberg & Merrow, 2005). Typically, when the response of an organism to rhythmic environmental changes (light/dark cycles, food availability, temperature changes, etc.) is studied (Fig. 2-A), multiple biological processes are found showing similar rhythmic profiles to the corresponding environmental signals (Fig. 2-B,C). However, on the one hand, some of these processes would not maintain their rhythmicity under constant conditions indicating that their rhythmic profiles are direct responses to changes in the environment rather than being generated by endogenous oscillating systems or molecular clocks. Therefore, when environmental signals are constant, the profiles of such biological processes stop oscillating and remain constant as well (Fig. 2-B). On the other hand, a subset of biological processes would maintain a rhythmic profile under constant environmental conditions, revealing that their rhythmicity is self-sustained and, thus, regulated by endogenous oscillating systems acting as molecular clocks (Fig. 2-C).

Accordingly, chronobiology experiments are commonly designed as follows (Fig. 2-A). A temporal series consisting of consecutive days when the organism is exposed to a rhyth-

mic environmental condition (called *zeitgeber*, which is used as a synchronizer, literally a *time giver*) are followed by several consecutive days when the organism is exposed to constant non cycling conditions termed as *free-running* conditions (Kuhlman et al., 2018). Data are collected with a specific time interval, every few hours, minutes or seconds depending on the complexity of the data. Specifically, in the case of circadian experiments, as the ones performed in this thesis, the *zeitgeber* is the alternation of light-dark cycles and the free-running conditions consist of constant light and constant dark periods. Under this experimental design, circadian processes can be identified and distinguished from light/dark responding processes as those that maintain their oscillating profile under free running conditions.

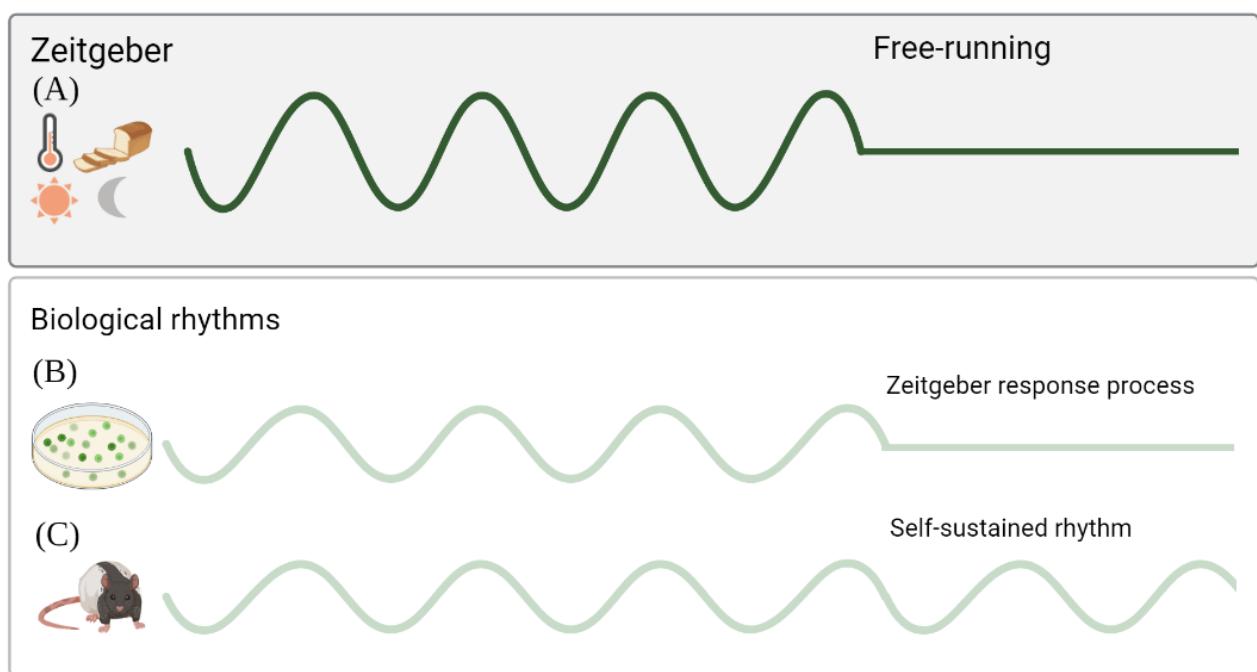


Figure 2: Different patterns of response to rhythmic environmental changes. A) Under a *zeitgeber* (rhythmic environmental input such as light/dark, temperature and food availability) two kind of biological processes show rhythmic patterns: (B) the ones only responding to the *zeitgeber* that stop cycling under constant free running conditions; (C) the ones with self-sustained oscillations under free running conditions for which there is evidence of a regulation exerted by an endogenous clock that is still functional when the environmental condition stops cycling. Figure created using Biorender.

Following this experimental design, chronobiologists have described self-sustained biological rhythms reacting to the four different previously described environmental cycles acting as *zeitgebers*. In particular, some marine organisms has been shown to produce self-sustained circatidal rhythms when they are kept in laboratory tanks without its *zeitgeber*, in

this case, tidal rhythms (Rock et al., 2022). As an example, the marine diatom *Hantzschia amphioxys* descends to the sand at high tides and rises to the surface at low tides (Fauré-Fremiet, 1951). In contrast, self-sustained circalunar processes are yet more unknown (Andreatta & Tessmar-Raible, 2020). An eminent example is the larvae of the insect called Ant Lion, which builds small holes in the sand as traps for insects. Scientists found that the size of the traps changes showing a circalunar profile that is maintained under constant conditions (Youthed & Moran, 1969).

However, scientific studies about circadian or circannual rhythms are much more numerous, and they have been found in a wide range of organisms (Merrow et al., 2005; Pfeuty et al., 2012; Roenneberg & Merrow, 2005). In animals, circadian rhythms studies focus mainly on activity-rest cycles (Roenneberg et al., 2022; Zee & Abbott, 2020). Nevertheless, thousands of other parameters from ethology to gene expression also show circadian profiles. For example, the olfactory discrimination in mice is higher at night time, even at subjective nights during free running (Granados-Fuentes et al., 2006). Also fungi show circadian rhythmic phenomena, for instance, *Neurospora crassa* generates asexual spores according to a rhythm of 24 h that is maintained under constant darkness (Correa & Bell-Pedersen, 2002). Circadian rhythmic profiles in plants are found in leaf movement, growth rate, stomatal opening, as well as the expression of a wide range of genes (Merrow et al., 2005). In general, organisms in the green lineage react in many different ways to circadian cycles (Noordally & Millar, 2015), one of the most known ones is the 24 h cyclic movement in the water column adjusted to their metabolism requirements (Lebert et al., 1999).

Day length or photoperiod is a crucial signal for the circannual timing system. Seasonal changes of photoperiod have been strongly connected to reproduction. In fact, all animal reproductive systems are related to seasons, from gene expression profiles to anatomical structures. Hamsters kept in short day condition have 10-fold smaller testes than the ones kept in long day condition (Klante & Steinlechner, 1994; Nishiwaki-Ohkawa & Yoshimura, 2016). But also in plants, flowering and seed production are photoperiod-regulated (Brandoli et al., 2020; Serrano-Bueno et al., 2017).

Since light is the main source of energy for photosynthetic organisms, they are highly synchronized with cyclic environmental changes involving light such as circadian rhythms and photoperiods. Nevertheless, the chronobiology of microalgae is yet barely studied com-

pared with other photosynthetic organisms despite representing one of the largest polyphyletic groups in the eukaryotic domain (Fig. 3). The genetics and molecular techniques used to identify clock components in other taxa have not been widely applied to microalgae yet (Noordally & Millar, 2015). This thesis aims to contribute to the chronobiology community by describing, for the first time, circadian rhythms emerging under different seasonal variations in diurnal cycles in the model marine planktonic microalga *Ostreococcus tauri*, considered the evolutionary eldest sister in the green lineage.

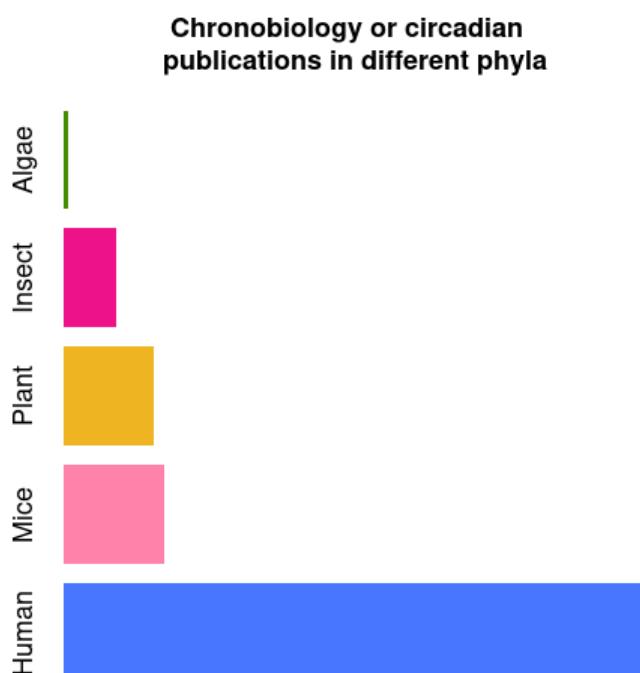


Figure 3: Number of publications found in PubMed using "chronobiology" or "circadian" keywords in their abstracts in July of 2022. For the algae group (using the generic term "algae", "microalgae" and "*Ostreococcus tauri*", "*Chlamydomonas reinhardtii*" and "*Polyedra*" as main model organisms of this group), only 493 publications were found. For the insect group (including the generic term "insect" and "*Drosophila melanogaster*" as main model organism), 5717 publications were found. For plants (including the generic term "plant" and "*Arabidopsis thaliana*" as main model organism), 9734 publications were found. For mice and human, 10771 and 62305 publications were found, respectively.

Circadian research

Our society is worldwide structured in a 24 h / 7 days system. People from different countries and cultures are experiencing jet-lags, shift work, exposure to artificial light and lack of outdoor activities. Circadian clock research is gaining relevance since this phenomena

has a crucial impact on human health, behavior and quality of life (Mermet et al., 2017; Roenneberg et al., 2019, 2022; Roenneberg & Merrow, 2016). Understanding the physiology, genetics and epigenetics (Ripperger & Merrow, 2011) at a laboratory level using different model organisms besides mice such as plants, fungi and microalgae will greatly contribute to advance our comprehension of the mechanisms and functioning of circadian clocks in living organisms on Earth beyond humans.

The synchrony that exists between sunrise/twilight and organisms functioning have been so obvious for scientists that the underlying molecular mechanisms remained ignored and unexplored for centuries. The first observation indicating that daily rhythms were programmed took place in the 18th century. Jean Jacques d'Ortous De Mairan, a French astronomer, described in less than 350 words (nowadays, less than two tweets) how a mimosa plant inside a closet maintained its daily leaf movement (De Mairan, 1729). For some scientists, it was a clear proof that leaf movement was not controlled by the alternation of light and dark cycles indicating the existence of an endogenous oscillating system. De Mairan invited botanists to investigate his discovery to confirm that leaf movement was also maintained when temperature changes were absent (which inside its closet was difficult to ensure). However, it took 30 years to confirm his observations taking into account temperature for the first time (Kuhlman et al., 2018; Roenneberg & Merrow, 2005).

The history of circadian research was sparse in results with large gaps between discoveries during its early stages. For instance, the physiology of the endogenous nature of the clock in plants was not studied until 1832, despite De Mairan's observations being reported in 1729. Similar phenomena in animals took another century to be identified, and 50 years more were needed to find them in humans (Kuhlman et al., 2018; McClung, 2006; Roenneberg & Merrow, 2005). Although circadian research and chronobiology were born in the 18th century, they only became relevant and coherent in the second half of 20th century. One of the breaking points in circadian research history was the international conference in Cold Spring Harbor in 1960, where 157 pioneering scientist in the field such as Colin Pittendrigh, Patricia Decourse and Franz Halberg met together for the fist time (Evans, 1961). From all the data shared, Pittendrigh summarized the qualities of circadian clocks in 16 generic empirical features that he predicted to be true in all organisms (Pittendrigh, 1960). Nowadays, 62 years later, these empirical features are still useful in spite of the huge development in the field. Recent technological developments have enabled a

more controlled experimentation and data analysis in chronobiological studies. Chronobiologists have already identified circadian rhythms in organisms spanning almost the entire tree of life, even non-photosynthetic prokaryotes (Eelderink-Chen et al., 2021). Also the physiology and genetics behind the circadian clock have been broadly studied in a wide range of phyla, since the first clock gene was described in 1971 by Seymour Benzer and Ronald Konopka using mutant screening in *Drosophila melanogaster* (Konopka & Benzer, 1971; Takahashi, 2021) (Fig. 4).

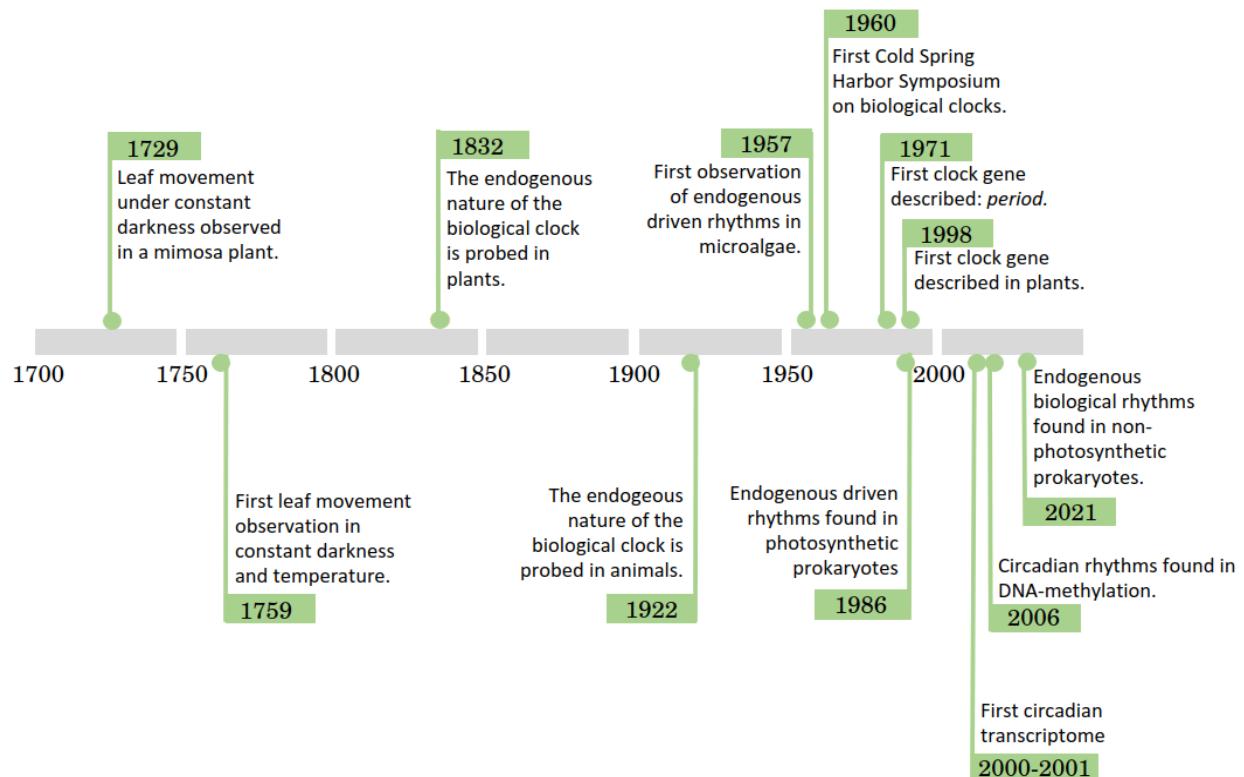


Figure 3: Timeline of circadian research. The main circadian discoveries have been listed in chronological order. (Akhtar et al., 2002; De Candolle, 1832; De Mairan, 1729; Duhamel du Monceau, 1759; Eelderink-Chen et al., 2021; Eissenberg & Elgin, 2006; Evans, 1961; HASTINGS & SWEENEY, 1957; Konopka & Benzer, 1971; Kuhlman et al., 2018; Mitsui et al., 1986; Richter, 1922; Roenneberg & Merrow, 2005; Somers et al., 1998)

As it can be observed in the timeline, circadian rhythms discoveries are very diverse. Chronobiologists usually have strong roots in other fields such as anatomy, physiology, molecular biology, genetics, ecology, mathematics and/or computer science. The knowledge obtained from each field have been shared and integrated in order to obtain a precise characterization of circadian rhythms (Klante & Steinlechner, 1994; Merrow et al.,

2005; Nishiwaki-Ohkawa & Yoshimura, 2016). In recent years, mathematics and computer science have strongly influenced the entire field of research by proposing a paradigm in which biological rhythms are studied as waves. Wave patterns repeat themselves periodically, maintaining several features that define their profiles. These features can be modelled as wave parameters and used to quantitatively compare different waves. Cosinusoïdal parametrizations of the following form are commonly applied in circadian research to model rhythmic phenomena:

$$Y = m + \alpha \cos\left(\frac{2\pi}{\tau}(t - \varphi)\right)$$

where Y represents the measurements of the phenomena under study such as gene expression or protein abundance in this thesis; m is the mesor or wave mean value around which oscillations take place; α is the wave amplitude or range between the maximum and minimum wave values; τ is the wave period or the time length between successive repetition of the same wave pattern which is fixed to 24 h in circadian research and φ is the wave phase or time point when the maximum wave value is reached (Fig. 4)

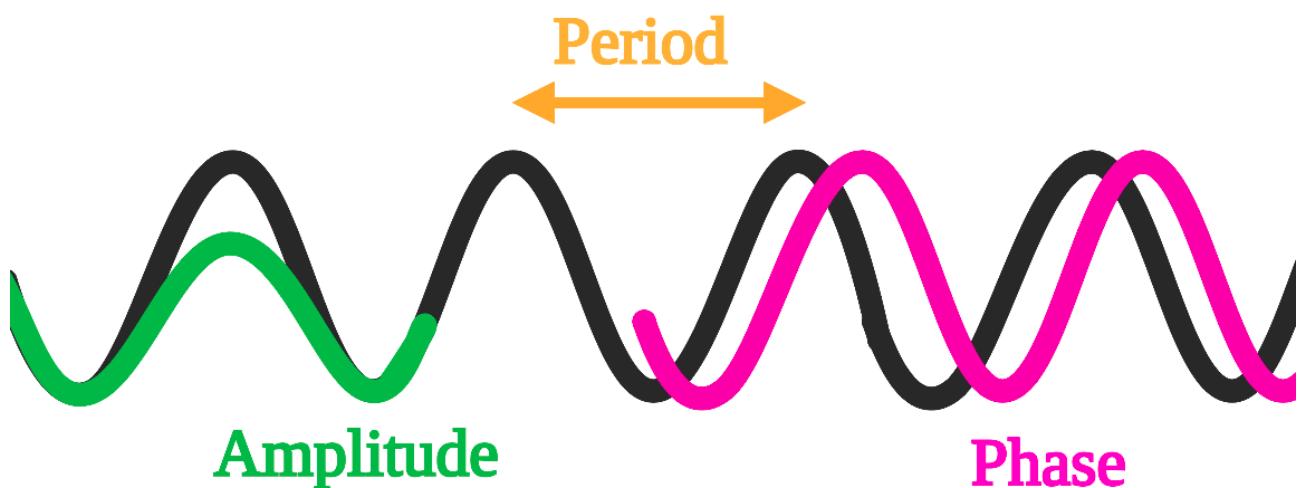


Figure 4: Graphical representation of cosinusoidal wave model parameters. Period is represented in orange and it corresponds to the time interval between consecutive repetitions of the wave pattern; amplitude is represented in green and it corresponds to the difference between the highest and lowest points the wave reaches; and phase is represented in pink and it corresponds to the time point where the wave reaches its maximum value

These parameters can be estimated from experimental data applying methods such as non-linear least squares for different rhythmic phenomena and the statistical significance of the differences between them can be assessed (McClung, 2006; Parsons et al., 2020). A major drawback of the previous parametric models consists in their ability to detect only symmetric waves failing to identify more complex rhythmic patterns. Therefore, non-parametric models have been proposed to complement these limitations detecting rhythmic patterns of arbitrary form. Robust non-parametric models have been developed based on rank tests for umbrella alternatives (Mack & Wolfe, 1981; Thaben & Westermark, 2014). Specifically, in this thesis these two complementary approaches, parametric and non-parametric, have been applied to obtain a deep characterization of circadian rhythms at three different biological levels (transcriptome, proteome and physiology) in *Ostreococcus tauri* subjected to different seasonal variations of diurnal cycles.

Ostreococcus tauri

The green lineage (*Viridiplantae*), comprehends two of the most important groups of oxygen photosynthetic eukaryotes: green microalgae and their descendants, terrestrial plants.

Microalgae are a very diverse group of photosynthetic microorganisms of special interest due to their plastic physiology and their biotechnological applications. They can be found in different water-based habitats, from freshwater to oceans, as well and land ecosystems, including deserts. Microalgae grow under a broad range of temperature, salinity, pH and light intensity values. More than 5000 species of microalgae have been identified in the oceans accounting for the production of 50% of the oxygen necessary to sustain life on Earth (El Gamal, 2010). Microalgae play a central ecological role as primary producers of biomass establishing the base of aquatic trophic chains. They have also been of great interest for the scientific community due to the large and yet increasing number of their biotechnological applications. Specifically, microalgae have been described as a high yield source of carbon compounds and good candidates to mitigate CO₂ emissions. The fixation of CO₂ is coupled with growth and biosynthesis of compounds of biotechnological interests such as polysaccharides, lipids, vitamins and antioxidants. They are currently large-scale cultured to produce biostimulants in agriculture, health supplements, pharmaceuticals and cosmetics, as well as, successfully applied in wastewater treatment coupled with the fixation of atmospheric CO₂ (Abinandan et al., 2018; Borowitzka, 2013; H. Chen et al., 2019; García-Cubero et al., 2018).

Recently, the development of high-throughput sequencing is contributing to the clarification of the evolutionary history of the green lineage (Bachy et al., 2022; Becker & Marin, 2009; Benites et al., 2021; Leliaert et al., 2012; Merchant et al., 2007). These two main clades are, as previously mentioned, *Streptophyta*, including *Embryophyta* or land plants and *Charophyta* (land plant's closest algal ancestors or evolutionary eldest algal sisters); and *Chlorophyta*, comprising the core chlorophytes (*Chlorophyceae* and *Trebouxiophyceae*) and the evolutionary eldest sisters in this clade and the entire green lineage: *Prasinophyta*, which include *Mamiellales* (Bachy et al., 2022; Leliaert et al., 2012; Tragin & Vaultot, 2019) (Fig. 5).

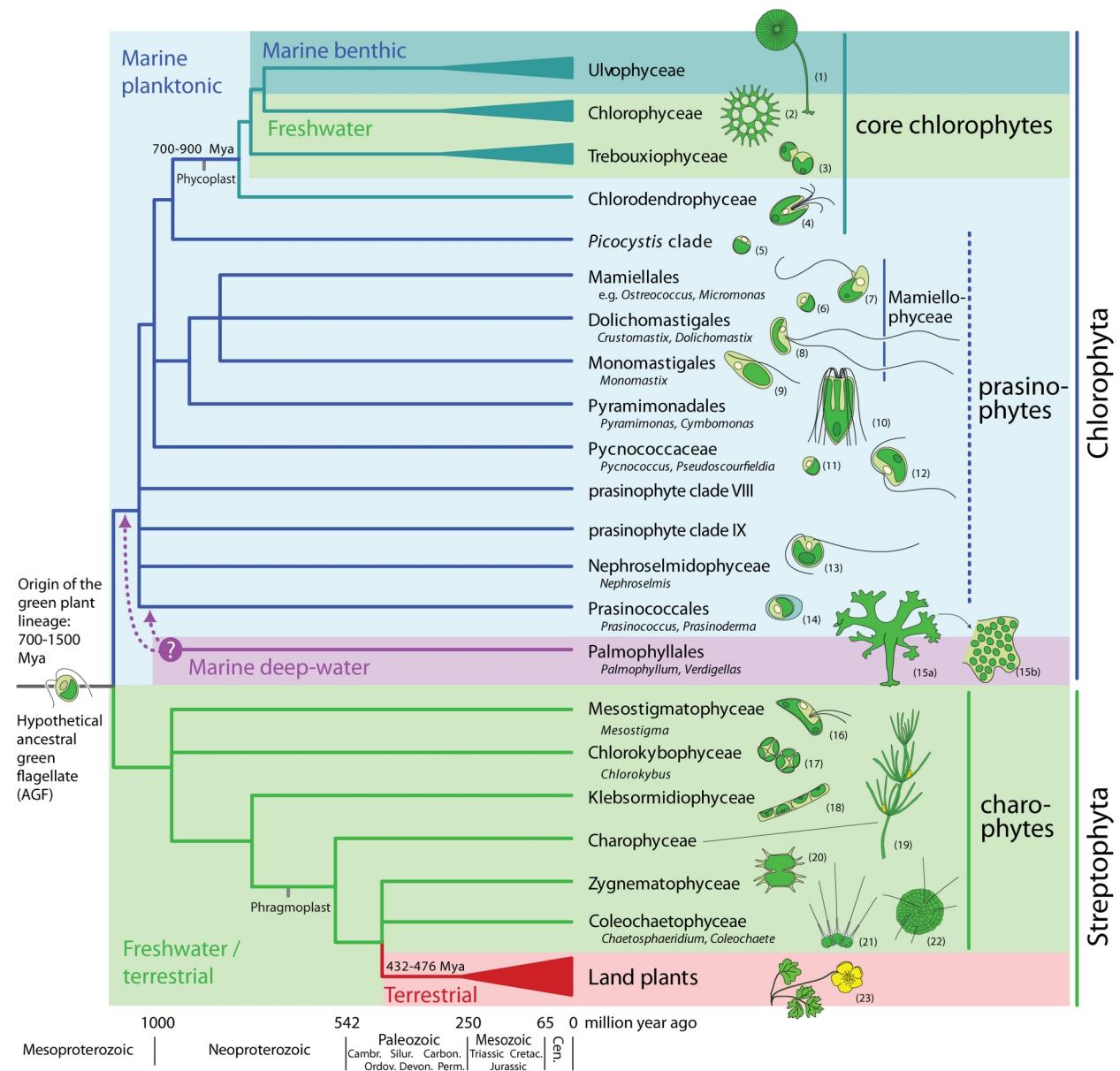


Figure 5: Phylogenetic relationships among the orders in the green lineage or Viridiplante. Figure obtained from (Leliaert et al., 2011). The tree topology is a composite on accepted relationships based on molecular phylogenetic evidence. Uncertain phylogenetic relationships are indicated by polytomies. The divergence times are rough approximations based on the fossil record and molecular clock estimates. Drawings illustrate representatives of each lineage: (1) Acetabularia, (2) Pedialstrum, (3) Chlorella, (4) Tetraselmis, (5) Picocystis, (6) Ostreococcus, (7) Micromonas, (8) Crustomastix, (9) Monomastix, (10) Pyramimonas, (11) Pycnococcus, (12) Pseudoscourfieldia, (13) Nephroselmid, (14) Prasinococcus, (15) Verdigellas (a: general habit, b: individual cells in a gelatinous matrix), (16) Mesostigma, (17) Chlorokybus, (18) Klebsormidium, (19) Chara, (20) Xanthidium, (21) Coleochaete, (22) Chaetosphaeridium, (23) Ranunculus.

Initial biodiversity studies on marine phytoplankton posited the domination of oceans by the so-called red lineage conformed by diatoms and dinoflagellates while the green lineage was thought to have less importance in marine ecosystems, being confined to fresh waters and terrestrial environments (Worden et al., 2004). These two lineages are early divergent from the endosymbiotic events that gave rise to chloroplasts. Whereas a single endosymbiotic event took place in *Viridiplantae*; two or even more endosymbiotic events are described in the red lineage (Leliaert et al., 2012).

However, in the last decade, metabarcoding studies have completed previous studies based solely on microscopic and traditional molecular techniques. These studies have unveiled the relevance of the green lineage in marine waters. Also, the cosmopolitan distribution of *Prasinophyta* has been described, especially for the order *Mamiellales* (Collado-Fabbri et al., 2011; Demir-Hilton et al., 2011; Leconte et al., 2020; Tragin & Vaulot, 2019; Worden et al., 2004).

In that taxonomic context, *Ostreococcus tauri* is classified as a green mamiellale microalgae (Fig 5-6). Its contribution to the marine phytoplankton is crucial in a wide range of oceans and seas all over the world (Benites et al., 2021; Collado-Fabbri et al., 2011; Demir-Hilton et al., 2011). As examples of *Ostreococcus* cosmopolitan character, it is worth mentioning that different strains have caused blooms in the Atlantic and Pacific Oceans (O'Kelly et al., 2003; Worden et al., 2004) and that a specific *Ostreococcus* strain has been found to be the most prevalent *Mamiellale* in the Mediterranean Sea (Tragin & Vaulot, 2019). Beyond its ecologically important role (Chapman, 2013; Worden et al., 2004) and cosmopolitan presence in marine environments, *Ostreococcus tauri* key feature consists in being considered the world's smallest free-living eukaryote known to date (around 1 µm). Due to its small size, *Ostreococcus tauri* have been "invisible" to field researchers for a long time. It was first detected by flow cytometry as a rounded microalgae in a bloom that took place on the Mediterranean French lagoon, Étang de Thau, known for its semi-intensive farming of oysters. Hence, it was referred to as an "oysters coccus form the Thau lagoon" or *Ostreococcus tauri*. Its subcellular structure was resolved consisting of a nucleus, a single mitochondrion, a single chloroplast containing one starch granule and a very reduced or almost non-existent cytoplasmic compartment (Fig 6) (Moreau, H, Grimsley, N. Derelle, E, Ferraz, C, Escande, ML, Eychenié, S , Cooke, R, Piganeau, G, Desdevises, Y, Bellec, 1995). (CITA MAL)

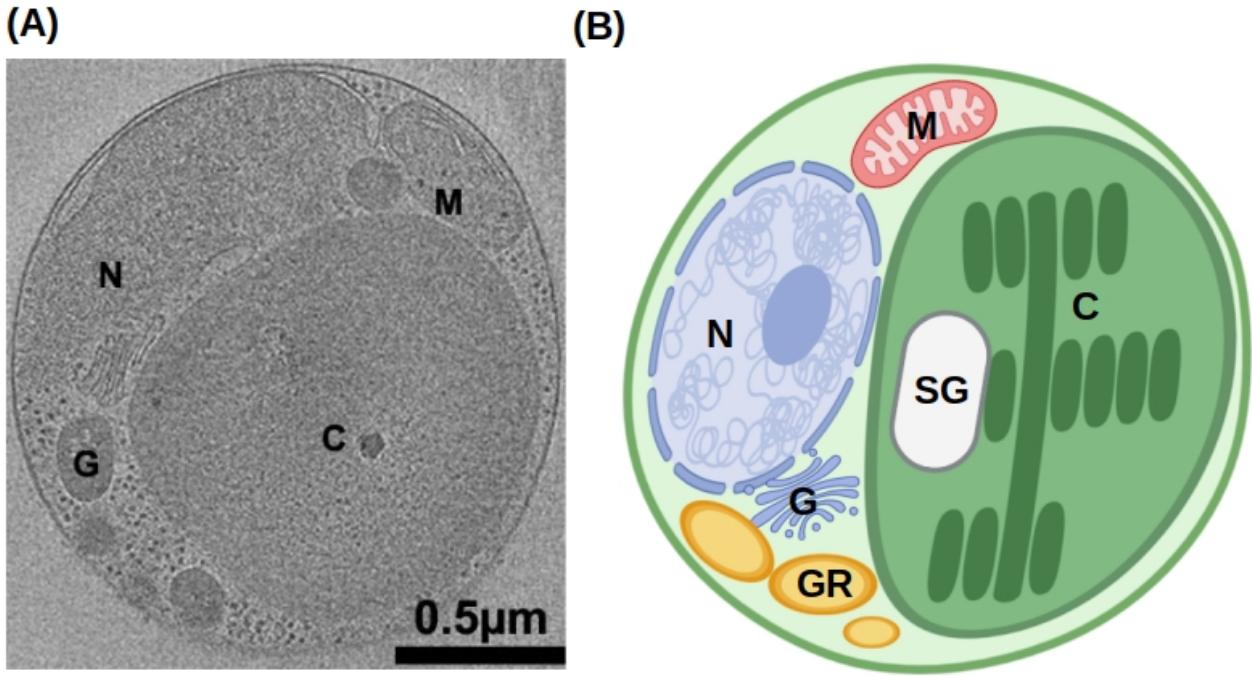


Figure 6: *Ostreococcus tauri*. (A) Tomographic 2D section from an *Ostreococcus tauri* cell from (Henderson et al., 2012). (B) An illustrated diagram of an *Ostreococcus tauri* cell (created using BioRender) based on the previous 2D segmentation. Labels: nucleus N, chloroplast C, Golgi body G, mitochondrion M, starch granule SG and granules GR.

At that time, genome sequencing studies to understand marine phytoplankton were mainly focused on prokaryotes (Berube et al., 2018; Palenik et al., 2003). *Ostreococcus tauri* genome was not sequenced for the first time until 2006 (Derelle et al., 2006). From that point on, an increasing number of picoeukaryotes genomes were sequenced, including a second version of *Ostreococcus tauri* genome (Blanc-Mathieu et al., 2014). These contributions allowed the identification of unique genomic features, showing that its simplicity goes beyond its reduced size and cell structure. Initially, no remarkable characteristic was identified in its 12.56 Mb genome arranged into 20 chromosomes when compared, for example, to *Saccharomyces*. Nevertheless, a unique feature is uncovered when the fascinating fact comes when the number of genes of these two organisms are compared. With a similar genome size, *Saccharomyces cerevisiae* has around 6275 protein coding genes, while *Ostreococcus tauri* has 8166 (Blanc-Mathieu et al., 2014; Derelle et al., 2006; Engel et al., 2014), making it the most gene dense free-living eukaryote known to date. This simplicity is also clear when genome size, number of genes and transcription factors in *Ostre-*

Ostreococcus tauri is compared to other photosynthetic species such as *Arabidopsis thaliana* (*Embryophyta*), *Klebsormidium nitens* (*Charophyta*), *Chlamydomonas reinhardtii* (*Chlorophyta*) and *Phaeodactylum tricornutum* (*Heterokontophyta*), Table 1.

Other key genomic characteristics in *Ostreococcus tauri* are its short intergenic regions and reduced gene family sizes comprising only a single gene copy or even merging different genes into a single one. These features contribute to its intense degree of genome compaction. With the only exception of a long internal duplication on chromosome 19, hypothesized to be of recent origin due to its lack of divergence. There are more special characteristics in chromosomes 19 and 2. They contain 77% of the transposable elements of the genome, have lower G+C content and a different codon usage (Blanc-Mathieu et al., 2014; Derelle et al., 2006). The first hypotheses about these chromosomes were that they had a different origin than the rest of the genome being the product of horizontal gene transfer events. Currently, only chromosome 19 is considered an “alien” chromosome since most of its protein coding genes are not related to the green lineage. However, chromosome 2 protein coding genes are essential housekeeping genes not duplicated and related to the green lineage (Fig 7). Alternatively, this chromosome has recently been considered as a sex-related or mating-type chromosome (Benites et al., 2021; Blanc-Mathieu et al., 2014) .

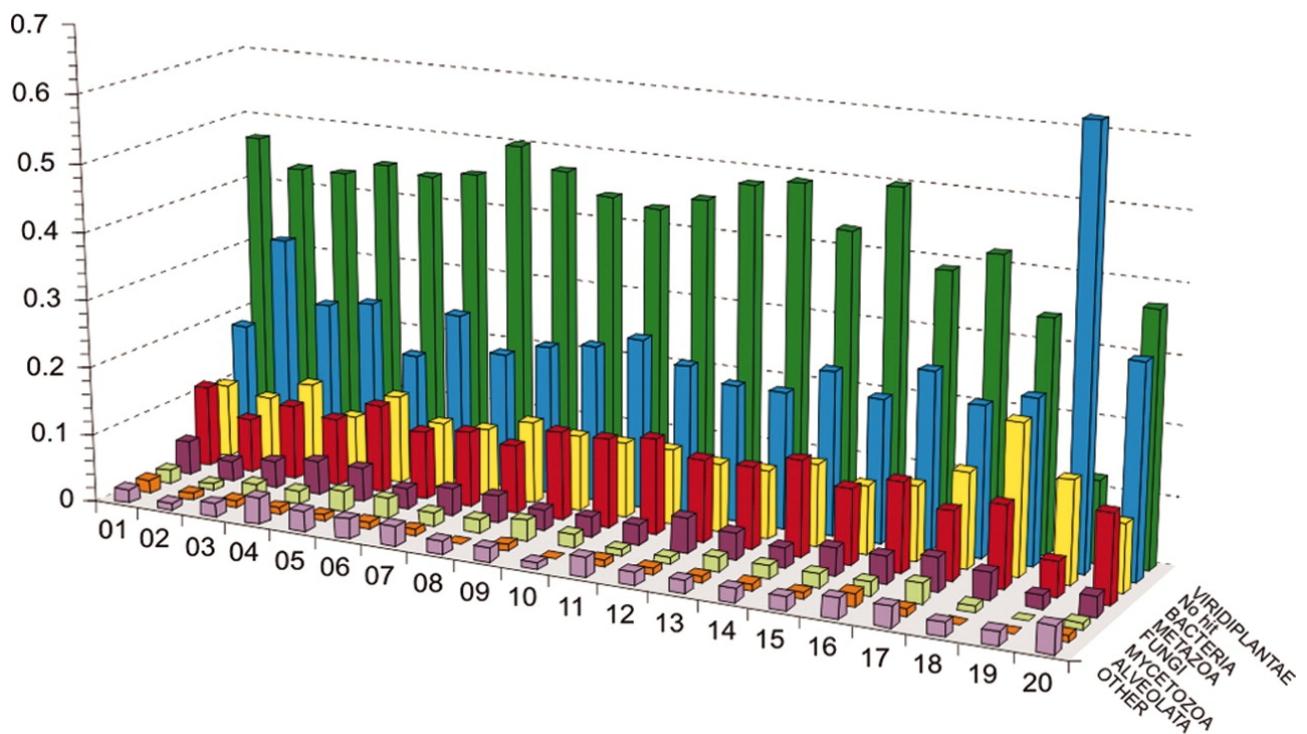


Figure 7: Taxon distribution of best hits for genes from each *Ostreococcus tauri* chromosome.

Figure from (Derelle et al., 2006). The number corresponding to each chromosome is represented in the x axis, the percentage of best hits is represented in y axis. Each color stands for: viridiplantae, green; no hit, blue; bacteria, yellow; metazoa, red; fungi, purple; mycetozoa, light yellow; alveolates, orange; others, light purple.

Although sex is now accepted as a ubiquitous and ancestral feature of eukaryotes (Sekimoto, 2017; Swanson et al., 2011), direct observation of sex is still missing in most unicellular eukaryotic lineages. The genomic regions, so-called mating-type, involved in sex have been characterized in other Chlorophyta (Sekimoto, 2017) and, recently, in *Ostreococcus tauri*, which appears to encode two highly divergent haplotypes. These *Mamiellales* mating-type candidate regions are likely to be the oldest mating-type loci described to date (Benites et al., 2021; Leconte et al., 2020).

Overall, *Ostreococcus tauri* is proposed as a novel model organism due to its structural and genomic features. In addition, inside the green lineage there is a lot of diversity and actually it is difficult to find a model organism that can represent the hole lineage (Cock & Coelho, 2011). *Ostreococcus tauri* taxonomical classification as the eldest sister in *Prasinophyta* makes it an ideal such organism potentially representing the green lineage eldest ancestor (Derelle et al., 2006; Leliaert et al., 2012). Therefore, *Ostreococcus tauri* molecular systems could be considered as representative of the fundamental systems in

the green lineage and the knowledge gained from them could be somehow extrapolated to a wide range of photosynthetic organisms.

Also very relevant to this thesis, research in Molecular Systems Biology often faces challenges produced by the complexity of the model organisms used in the corresponding field and the high economical and computational costs associated to the generation and analysis of the necessary massive amounts of omics data (De Keersmaecker et al., 2006; Jamers et al., 2009; Joyce & Palsson, 2006; Weckwerth, 2011). In this respect, due its genomic simple features *Ostreococcus tauri* is an ideal model organism in Molecular Systems Biology when compared with other models in the green lineage such as *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*, Table 1 (de los Reyes, Romero-Campero, Ruiz, Romero, & Valverde, 2017; Derelle et al., 2006; Krumholz et al., 2012; Le Bihan et al., 2011a; Lelandais et al., 2016). Indeed, in this thesis, *Ostreococcus tauri* has allowed us to generate, analyze and interpret massive multiomic and physiological data to characterize responses to seasonal variations of diurnal cycles in potentially fundamental systems in the green lineage.

Table 1: Genomic features of different green lineage model organisms. Their genome size and protein coding genes (Blaby et al., 2014; Blanc-Mathieu et al., 2014; Craig et al., 2021; Derelle et al., 2006; Hori et al., 2014; Lamesch et al., 2012; Swarbreck et al., 2008; M. Yang et al., 2018) are compared with their number of predicted transcription factors. (Rayko et al., 2010; Zheng et al., 2016)

	Genome size (Mb)	Number of protein coding genes.	Number of transcription factors.
<i>Arabidopsis thaliana</i>	135	27474	1779
<i>Klebsormidium nitens</i>	104	17055	286
<i>Chlamydononas reinhardtii</i>	110	14000	279
<i>Phaeodactylum tricornutum</i>	27.4	10567	212
<i>Ostreococcus tauri</i>	12.56	6275	102

Ostreococcus tauri allowed us to generate, analyze and interpret massive data from the complete system with less computational and experimental costs, so a holistic understanding of the chronobiology in this mammelleale was finally achieved.

Molecular Systems Biology

There is an ancient Indian fable that illustrate how a holistic view and ontological reasoning contributes to knowledge, it is called “Blind men and an elephant”. A poem of John Godfrey Saxe is one of the most famous written versions of it:

“ It was six men of Indostan To learning much inclined, Who went to see the Elephant (Though all of them were blind), That each by observation Might satisfy his mind.	Is very like a snake!" The Fourth reached out an eager hand, And felt about the knee. "What most this wondrous beast is like Is mighty plain," quoth he; "This clear enough the Elephant Is very like a tree!"
The First approached the Elephant, And happening to fall Against his broad and sturdy side, At once began to bawl: "God bless me! but the Elephant Is very like a wall!"	The Fifth, who chanced to touch the ear, Said: "E'en the blindest man Can tell what this resembles most; Deny the fact who can This marvel of an Elephant Is very like a fan!"
The Second, feeling of the tusk, Cried, "Ho! what have we here So very round and smooth and sharp? To me 'tis mighty clear This wonder of an Elephant Is very like a spear!"	The Sixth no sooner had begun About the beast to grope, Than, seizing on the swinging tailgeneraliza- tions That fell within his scope, "I see," quoth he, "the Elephant Is very like a rope!"
The Third approached the animal, And happening to take The squirming trunk within his hands, Thus boldly up and spake: "I see," quoth he, "the Elephant	And so these men of Indostan Disputed loud and long,

Each in his own opinion
Exceeding stiff and strong,
Though each was partly in the right,
And all were in the wrong! (...)"

After touching different parts of the animal, one of the blind wise men concluded that the elephant was like a snake, he was touching only the trunk of the elephant. Another one concluded that it was like a fan since he was only touching the ear of the animal, and so on with the other blind wise men. Each one of them was confident about his individual findings and reaching an agreement between them was impossible. The subject of study was so massive that they individually were only able to collect information from very specific regions that were treated as independent and contradictory truths instead of considering them as complementary parts of a whole.

Until recently, studies producing holistic views of living organisms were impossible to carry out due to the lack of suitable technologies. The development of omics technologies together with advances in computational science and artificial intelligence have made possible such systemic approaches in biology (Ideker et al., 2001; Karahalil, 2016; Veenstra, 2021; Weckwerth, 2011).

In the last 50 years, sequencing methods have exponentially improved. First generation DNA sequencing methods were developed around 45 years ago shortly anticipating the development of one of the biggest international research projects in History, the Human Genome Project, which started about 20 years ago (Ideker et al., 2001; Veenstra, 2021). In this project an extremely ambitious goal was pursued, the sequencing of the whole human genome. However ambitious, its impact in Science was beyond expected transcending the initial purpose. Different groups cooperated all over the world to sequence the complete human genome which promoted the development of new sequencing methods in order to make the process easier and cheaper. These methods are now applicable to study the molecular systems codified in the genome of living organism spanning the entire tree of Life (Abascal et al., 2020).

During the Human Genome Project, 13 years were needed to sequence a single complete human genome and the overall costs were 2.7 billion dollars. Currently, with the emergence of Next Generation Sequencing (NGS) methods, around 100000 human genomes have been sequenced in the last 6 years. The Illumina HiSeq System generates around 500 gigabase sequences per second, dropping the cost of sequencing a complete human

genome to \$1500 in only 10 years (Prendergast et al., 2020; Veenstra, 2021). Ultimately, these methods have changed how scientific projects are approached in biology.

These new approaches have been applied to sequence, identify and quantify complete transcriptomes, total mRNA transcripts from samples. This is generating unprecedented massive amounts of biological information. Although, transcriptomic studies are extremely informative they only focus on one layer of the complexity in biological systems leaving out the actual molecular components that perform most cellular functions, the proteins. This has promoted the development of methodologies based on High Performance Liquid Chromatography (HPLC) coupled to Mass Spectrometry (MS) aiming to the identification and quantification of the complete proteome, total set of proteins from biological samples. Moreover, in order to obtain a more complete picture of biomolecular systems, methods identifying and quantifying specific metabolites need to be explored developing targeted metabolomics. Finally, phenomics or the systematic acquisition of phenotypic data, using for example cell cytometry for the case of unicellular microorganisms, is necessary to link the most relevant molecular levels of complexity in living organisms with their physiology. Although, these molecular components are identified and quantified independently by different methods, genes, transcripts, proteins and metabolites highly interact with each other establishing different positive and negative regulatory loops underpinning cellular physiology. Based on these technologies and this view, researchers in Life Sciences are beginning to approach the study of living organisms and processes as systems with interacting modules comprising genes, transcripts, proteins and metabolites that constitute large biomolecular networks organized into cellular structures establishing the field of Molecular Systems Biology (Joyce & Palsson, 2006; Veenstra, 2021; Weckwerth, 2011).

While this holistic view of biological systems is gaining strength growing steadily during the first decades of the XXI century (Fig. 8), traditional reductionist methods focusing on individual molecular components, single genes, proteins and/or metabolites, are still the most popular ones in the study of specific organisms such as microalgae. Scientific projects in these research areas were limited by the time, economic resources and software tools needed to analyze and integrate massive amounts of biological data (Karahalil, 2016; Mazzocchi, 2012; Veenstra, 2021).

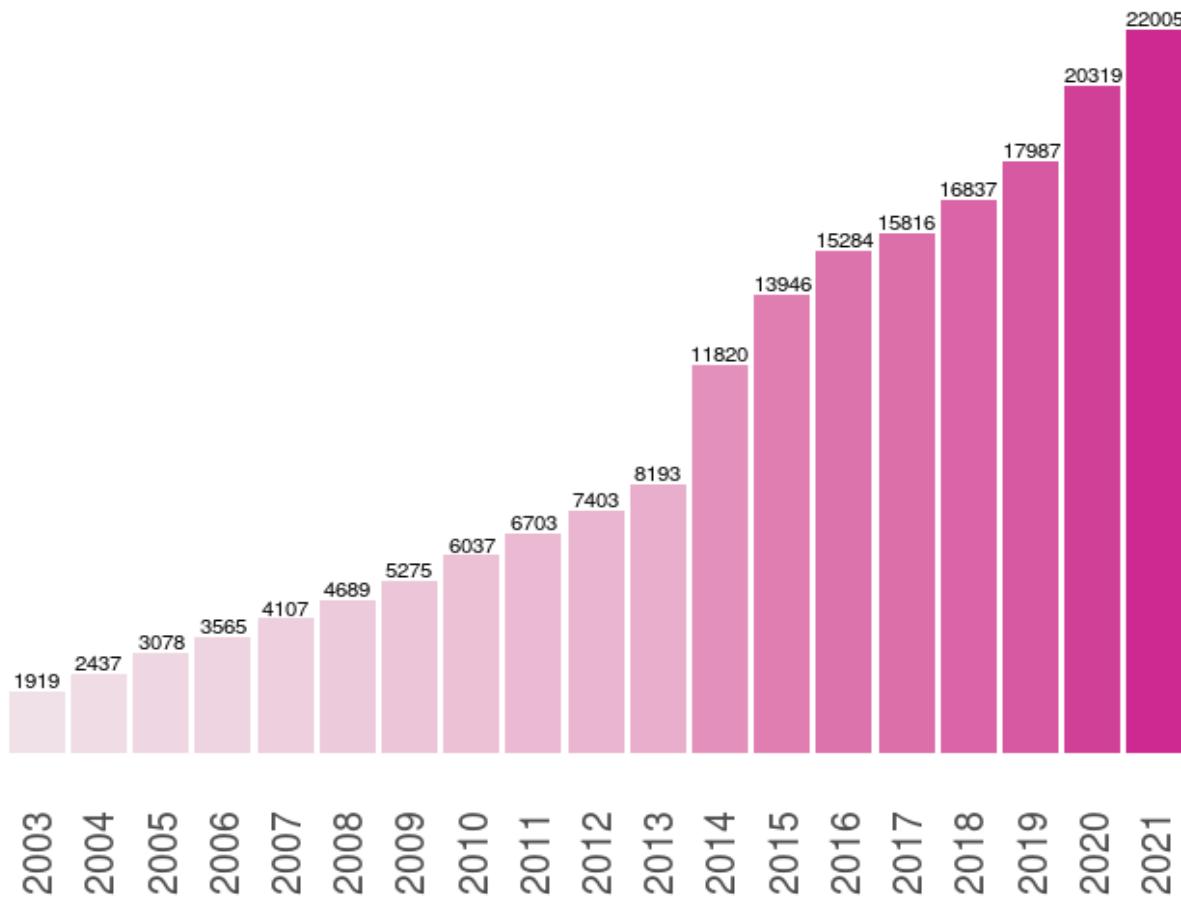


Figure 8: Publications on systems biology. Exponential increase in the number of publication using the term "systems biology" in PubMed since the year when the Genome Human Project was completed (2003). The number on the top of each bar corresponds to the exact number of publications of that year.

Nevertheless, on the one hand, the sequencing of an increasing number of microalgae genomes (Blanc et al., 2012; Bowler et al., 2008; Cheng et al., 2019; Derelle et al., 2006; Hori et al., 2014; Merchant et al., 2007; Morimoto et al., 2020; Prochnik et al., 2010) has allowed the application of omics technologies transcriptomics (de los Reyes, Romero-Campero, Ruiz, Romero, Valverde, et al., 2017; Ditz et al., 2021; Hoys et al., 2021; Serrano-Pérez et al., 2022), proteomics (Le Bihan et al., 2011b; Weckwerth, 2011) and metabolomics (Hoys et al., 2021; Serrano-Pérez et al., 2022; Weckwerth, 2011) in microalgae research. On the other hand, there was a lack of specific software tools for microalgae to analyze and interpret omics data. In order to contribute to resolve this deficiency in the microalgae research community, in the first steps of my doctoral thesis I developed the web-app ALGAEFUN with MARACAS, microALGAE FUNctional enrichment tool for Mi-

croAlgae RnA-seq and Chip-seq AnalysiS (Romero-Losada et al., 2022). These tools allow researchers without expertise in computational data analysis to process omic raw data to obtain list of relevant genes or genomic loci in their research and to perform functional enrichment analysis over them. This is contributing to the establishment of Microalgae Molecular Systems Biology. Following the main theme in Systems Biology the goal consists in improving our understanding of microalgae through the integration of partial characterizations obtained for their different biological components. Here the typical methodology in Molecular Systems Biology is adopted starting with the generation of massive amounts of omic data and its subsequent integrative analysis. The results obtained from computational analysis based on statistical and Artificial Intelligence methods must be validated by independent wet lab experiments revising these results if necessary. The final expected result is the development of predictive models enabling researchers in microalgae biotechnology to anticipate the physiological response of these organisms under specific conditions (Jamers et al., 2009; Veenstra, 2021; Weckwerth, 2011; Zurbriggen et al., 2012).

In this sense, this thesis aims at providing a molecular systems biology characterization of the responses in the prasinophyceae microalgae *Ostreococcus tauri* to seasonal variations in diurnal cycles. Accordingly, generation and integrative analysis of massive amounts of data from two different omics techniques, transcriptomics and proteomics, have been performed. Moreover, physiological measurements for cell cycle phases, photosynthetic activity, carotenoid and starch content, as a first approach towards phenomics, has been integrated into our analysis. Specifically, the transcriptomic method used in this thesis was RNA sequencing, RNA-seq, the up to date method used for this omic. The application of next-generation sequencing methods to study transcriptomes has produced the development of RNA-Seq analyses which has enabled researchers to identify and quantify a wide variety of different classes of RNAs without requiring transcript-specific probes which has almost driven microarrays to become obsolete previous method used in transcriptomic analyses), and not only to identify but also to quantify abundance of transcripts (Ditz et al., 2021; Veenstra, 2021; Wang et al., 2009). Respectively, the proteomic technology applied in this thesis was SWATH-MS, Sequential Window Acquisition of all Theoretical fragment ion spectra Mass Spectrometry.

The field of proteomics have been directly connected with the development of mass spectrometry (MS) technology. During the first part of this century, there were two lines of de-

velopment working separately for what would be known as proteomics today: identification (2-D electrophoresis gel) and quantification (using isotope tags) of proteins. Nowadays, proteomic technologies have enable the development of proteins identification and quantification methods so the number of proteins that can be quantified/identified today is around several thousands (Shen et al., 2022; Veenstra, 2021). Precisely, SWATH-MS is a data independent acquisition (DIA) method in which liquid chromatography y coupled with two round of MS for protein identification and characterization, as well as label free relative quantification (M. X. Chen et al., 2021; Ludwig et al., 2018).

Molecular systems biology with its increasing amount of data generated and the biological complexity it has unveiled is demanding a new generation of qualified researchers proficient in computer science, mathematics/statistics and molecular biology. My main goal when developing this thesis is to join this generation of young researchers who are working to develop software applications, efficient data analysis algorithms and user-friendly app-tools to enable the progress of systems biology studies making it more accessible to the scientific community (Coletto-Alcudia & Vega-Rodríguez, 2020; Romero-Campero et al., 2016; Romero-Losada et al., 2022).

Materials and Methods

Organism and culture growth conditions.

Organism and growth medium.

The sequenced strain of *Ostreococcus tauri*, RCC4221, was used in this study. Cells were grown photoautotrophically on sterilized artificial sea water (ASW) (Kester et al., 1967) supplemented with nitrates, phosphates, trace metal and vitamins. Components and concentrations of the medium are described in Table 2.

Table 2: Components supplementing ASW as *Ostreococcus tauri* culture medium.

	Concentration in solution (g L ⁻¹)	Concentration in medium (mg L ⁻¹)
Solución I	400 NaNO ₃	222.2 NaNO ₃
Solución II	2.8 Na ₂ HPO ₄ 10 K ₂ HPO ₄	1.6 Na ₂ HPO ₄ 5.6 K ₂ HPO ₄
Solución III	5.4 NH ₄ Cl 10.4 Fe-EDTA 74.4 Na ₂ -EDTA 4.6·10 ⁻² ZnSO ₄ 2.8·10 ⁻² CoSO ₄ 1.6·10 ⁻² Na ₂ MoO ₄ · 2H ₂ O 5.0·10 ⁻³ CuSO ₄ 3.4·10 ⁻² H ₂ SeO ₃ 3.6·10 ⁻² MnCl ₂ · 4H ₂ O	3.0 NH ₄ Cl 5.8 Fe-EDTA 41.3 Na ₂ -EDTA 2.6·10 ⁻² ZnSO ₄ 1.6·10 ⁻² CoSO ₄ 8.9·10 ⁻² Na ₂ MoO ₄ · 2H ₂ O 2.8·10 ⁻³ CuSO ₄ 1.9·10 ⁻² H ₂ SeO ₃ 2.0·10 ⁻² MnCl ₂ · 4H ₂ O
Solución IV	0.2 Thiamin-HCl 1.5·10 ⁻³ Biotin 1.5·10 ⁻³ Cyanocobalamin	0.2 Thiamin-HCl 1.7·10 ⁻³ Biotin 1.7·10 ⁻³ Cyanocobalamin

Continuous culture conditions in photochemostats.

Continuous culture was performed in 2.0 L capacity (0.07m diameter, 0.50 m height) jacketed sterilized photochemostat (bubble columns) containing 1.8 L of cells suspension, continuously sparged with air (1L (L culture⁻¹) h⁻¹) (Fig. 9). Culture conditions (pH, dilution rate and illumination regime) were constantly measured and computationally controlled by

a LabJack. Temperature was maintained at 20°C, and pH at 7.5 by on demand injection of CO₂ into the air stream entering the culture.

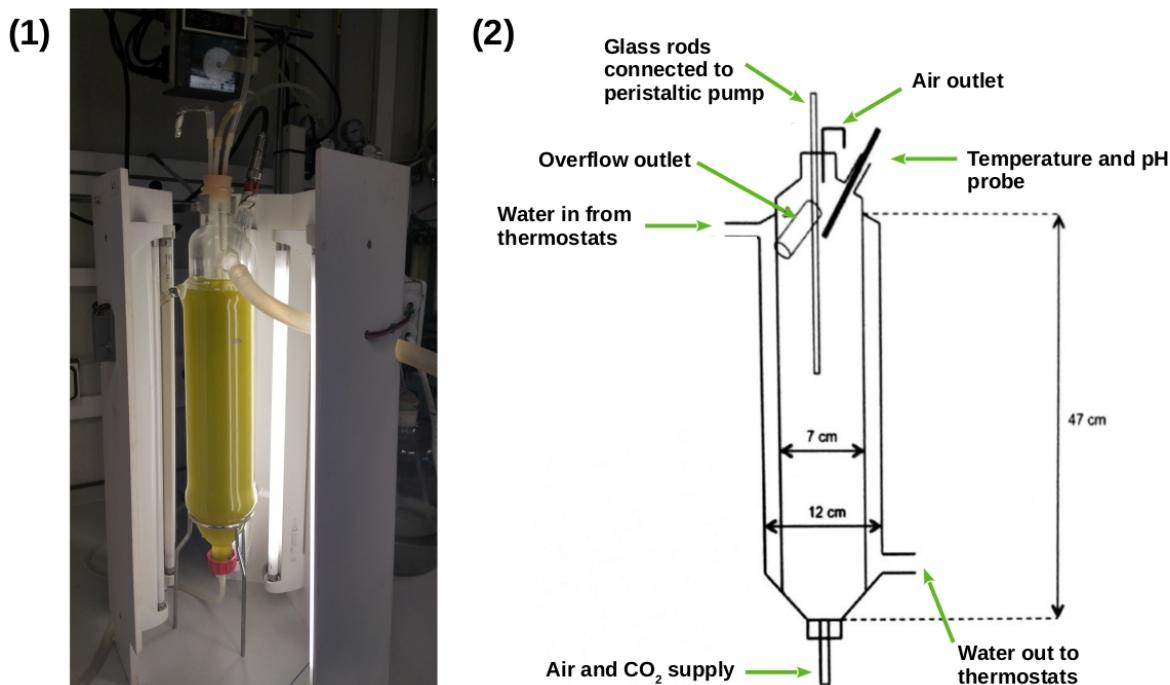


Figure 9: Growth system used for continuous cultures. (1) Picture of one of the photochemostats used. (2) Schematic detailed design of photochemostats.

Six Phillips PL-32W/840/4p white-light lamps, which provided a maximal incident irradiance of 1500 E m⁻² s⁻¹ on the reactor surface, were used as light inputs. Initially, the reactors were inoculated with batch-grown cells and operated on batch mode for about 3–4 d, with incident irradiance being progressively increased, until reaching stationary phase. Then, they were switched to operate on continuous mode, fresh medium being continuously fed during the light period at a flow rate of 45 mL h⁻¹ (dilution rate (D), 0.3 d⁻¹ under long day conditions and 0.15 d⁻¹ under short day conditions), with withdrawal of culture at the same rate. Once steady state conditions were achieved, samples were collected and analytical determinations were performed.

In addition, instead of a sudden transition from dark to light and from light to dark, our LabJack controlled system gradually increased light until an irradiance of 1500 µE m⁻² s⁻¹ is reached simulating the natural photoperiod (Fig. 10). Furthermore, they are surrounded by a wooden box and a completely opaque fabric to avoid any external light input.

Experimental design.

Our experiments are focused on two extreme photoperiods: summer long day conditions (LD, 16h light : 8h dark) and winter short day conditions (SD, 8h light : 16h dark). The experimental design (Fig. 10) consisted of three days under the specific photoperiod, LD or SD, followed by three days under free running conditions (constant light or constant dark). Cells were harvested at specific times during diurnal cycles, represented as zeitgeber time (ZT), where ZT0 corresponds to dawn, ZT4 to 4h after dawn and so on. Samples were taken every 4h (from ZT0 to ZT20) during the three days of alternating light/dark cycles. Therefore, a total of 6 samples for each day of sampling were generated. No samples were collected during the first day of free running condition to allow culture acclimation. Then, samples were collected every four hours starting at subjective dawn, during two days.

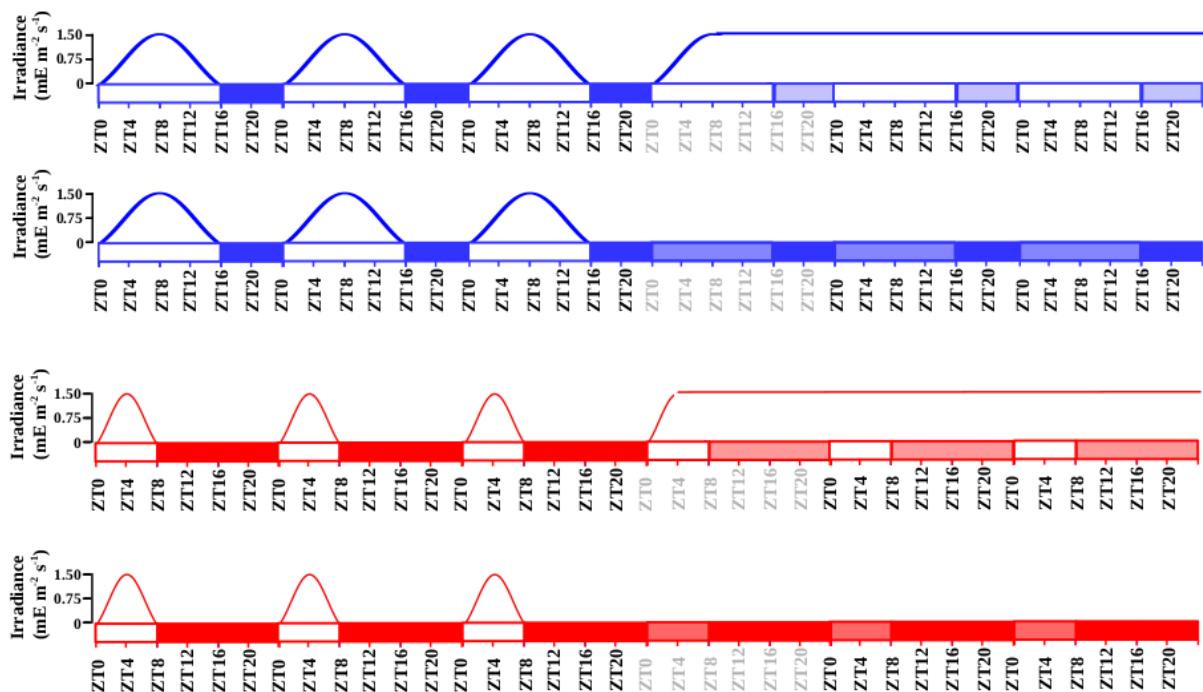


Figure 10: Schematic description of the experimental design. Gradually increased and decreased irradiance is represented during each light phase. Long day conditions are represented in blue and short day conditions in red. Photoperiods (light periods) correspond to white rectangles and skotoperiods (dark periods) to blue/red filled rectangles. Light blue and red filled rectangles are used to represent subjective photoperiods and skotoperiods under free running conditions.

Transcriptomic analysis

Sample Collection

From each chemostat, a volume of 50mL of cell suspension were harvested (4 min centrifugation at 5000 x g and 4°C) for each time point. Pellets were washed using Phosphate-buffered saline solution (PBS) and flash frozen with liquid Nitrogen before stored at -80°C.

Cell disruption

Frozen pellets were resuspended in 400 µL of disruption buffer (García-Domínguez & Florencio, 1997) and directly added to a 1,5 mL Eppendorf tube (RNase free and phenol-proof) containing 400 µL of phenol:chloroform 1:1 and 100 µL of acid washed glass beads (0.25–0.3 mm diameter; Braun, Melsungen, Germany). Mechanical disruption was performed by 30 min of repeated cycles of 60 s of vortexing and 60 s of incubating on ice.

RNA extraction

Extracts were centrifuged (4 °C) for 15 min at 13000 x g, producing three differentiated phases: an upper aqueous phase containing RNA, a white interphase containing DNA and a lower organic phase containing proteins, lipids and glass beads. The upper aqueous phase was collected, mixed with 400 µL of phenol:chloroform 1:1 and centrifuged for 5 min (4 °C at 13000 x g). This process was repeated three more times. In the last wash, only chloroform was used to avoid phenol contamination of the RNA samples. The supernatant was incubated overnight at -20 °C in a solution of 80 µL 10 M LiCl and 550 µL 100% EtOH for RNA precipitation. Finally, samples were centrifuged 10 min at 13000 x g 4 °C. Pellets were dried to avoid EtOH contamination.

RNA purification

RNA purification was performed using the Isolate II RNA Plant Kit (Bioline). Washing, DNase treatment and elution were carried out following the manufacturer instructions. The final RNA concentration and integrity were measured using a bioanalyzer 2100 (Agilent RNA 6000 Nano Kit).

RNA sequencing and processing

Library was prepared in accordance with the manufacturer's instructions and the sequencing was carried out on the Illumina NextSeq500 sequencer. Approximately, 10 million 75nt long single end reads were generated for each sample. The *Ostreococcus tauri* genome sequence and annotation v3.0 were used as reference genome (Blanc-Mathieu et al., 2014). Further computational analysis were carried out using the software tools developed in this thesis ALGAEFUN with MARACAS, microalgae FUNctional enrichment tool for MicroAlgae RnA-seq and Chip-seq AnalysiS (Romero-Losada et al., 2022).

Proteomic analysis

Sample collection

Sample collection for proteomic analysis followed the same procedure as the one previously described for RNA analysis.

Cell disruption

Directly onto frozen pellets, 1 mL of Trizol, 100 μ L of acid washed glass beads (0.25–0.3 mm diameter; Braun, Melsungen, Germany) and 40 μ L of Protein Inhibitor Cocktail PIC (25x) were applied, followed by 3 disruption cycles (60s agitation-60s incubation on ice) using a Mini-Beadbeater (BioSpec Products).

Proteins extraction

Proteins were extracted using TRIsure™ Reagent (Sigma-Aldrich), according to the manufacturer's instructions. The resulting proteins pellets were resuspended with 2 mL of 0.3 M guanidine solution in 95 % EtOH using 10 sonication cycles (30 s sonication - 30 s of incubating at 4 °C) in a Diagenode Bioruptor Pico Sonicator and then centrifuged at 4 °C during 5 min at 8000 x g. This washing process was repeated twice, followed by two additional washing using 90 % EtOH. The final pellets were resuspended in NH₄HCO₃ 50 mM / 0.2 % Rapigest (Waters) and total proteins were quantified using Qubit system.

Proteins digestion.

First, 50 µg of proteins were incubated with dithiothreitol (DTT 4.5 mM) for 30 min at 60 °C. Then, iodoacetamide was added to a final concentration of 10 mM and incubated for 30 min, under total darkness at room temperature. Treatment with trypsin was done overnight at 37 °C in a 1:40 trypsin:protein. After that, formic acid (**concentración?**) was added and incubated at 37°C for 1h. Finally, 2% acetonitrile (v/v) were added to reach a concentration of the digested sample around 0.5 µg of protein/µl of solution.

SWATH acquisition

Equipment and data acquisition method.

Proteomic data acquisition was performed on a time-of-flight TOF triple quadrupole hybrid mass spectrometer MS (5600 plus, Sciex) equipped with a nano electrospray source coupled to an nanoHPLC Eksigent model 425. The Sciex software Analyst TF 1.7 was used for equipment control and data acquisition. Peptides were first loaded onto a trap column (Acclaim PepMap 100 C18, 5 µm, 100 Å, 100 µm id × 20 mm, Thermo Fisher Scientific) under isocratical order in 0.1 % formic acid/2% acetonitrile (v/v) at a flow rate of 3 µL/min for 10 min. Subsequently, they were eluted on a reversed-phase analytical column, with the built-in emitter (New Objective PicoFrit column, 75 µm id × 250 mm, packed with ReproSil-PUR 3 µm). In the case of the samples corresponding to the short day conditions, the analytical column was Acclaim PepMap 100 C18, 3 µm, 100 Å, 75 µm id × 250 mm, Thermo Fisher Scientific, coupled to a PicoTip emitter (F360-20-10-N-20_C12 from New Objective). Formic acid 0.1 % (v/v) was used as solvent A and 2% acetonitrile with formic acid 0.1 % (v/v) were used as solvent B. Peptides were eluted with a linear gradient of 5-35 % (v/v) of solvent B in 120 min at a flow rate of 300 nL/min. The source voltage was selected at 2600 V and the temperature was maintained at 100 °C. Gas 1 was selected at 20 PSI, gas 2 at zero, and curtain gas at 25 PSI.

For proteins identification, Data Independent Acquisition DIA method was used. It consisted of a TOF-MS with a scan window of 400-1250 m/z (accumulation time of 250 ms) followed by 50 MS/MS with a scan window of 230-1500 m/z (accumulation time of 65 ms) and with a cycle time of 2574 s.

Library construction

The spectral library was constructed by making one run with a mixture of the biological replicates corresponding to each time point (ZT0, ZT4, ZT8, ZT12, ZT16, ZT20) with the DIA method described. ProteinPilot v5.0.1 software (Sciex) was used to identify the proteins in the library. A pooled search of all runs was performed. The parameters of the Paragon method were: trypsin as enzyme and iodoacetamide as cysteine alkylating agent.

The *Ostreococcus tauri* annotated proteome file from ORCAE (Sterck et al., 2012) linked to a Sciex Contaminants database were used in library construction. A false positive analysis (FDR) was performed and those with FDR ≤ 0.01 were considered.

SWATH runs

For each sample, the equivalent of 1 μg of digested protein was injected into each run. Before that, a standard (MS synthetic peptide calibration kit from Sciex) was injected to self-calibrate the equipment, control the sensitivity and chromatographic conditions. The described DDA method was used for SWATH runs with 60 ms of accumulation time and 3.7 s of cycle time. (preguntar a rocio si cambia el metodo o no)

Data processing

The library generated by DIA (1% FDR) was used in the analysis using the Sciex software PeakView 2.2 with the microapp SWATH 2.0, together with the data obtained from the SWATH runs. Using this program, the chromatographic traces of the ions were extracted and dumped into the Marker view 1.2.1.1 program where the list of identified proteins with their corresponding areas were generated. The parameters for extraction of ions and obtaining the areas were: 10 peptides per protein, 7 transitions of each peptide, threshold of confidence of the peptides set at 90 and FDR 1%. The software NormalizerDE 1.6.0 (Willforss et al., 2019) was used to test several normalization methods in order to probe which one achieved the minimum replicate variation relative to Log2. Quantile normalization was selected as normalization method based on this comparison. Data were imputed with mean (mean imputation method), which means that the missing value on a certain variable is replaced by the mean of the available cases.

Cell cycle analysis

Sample collection and cell fixation method

A volume of 1.5 mL of cell suspension was harvested for each time point. These samples were diluted 1:10 in PBS to ensure a suitable cell concentration for the assay. Two mL of these dilutions were centrifuged (**condiciones??preguntar a Meriyou**) and cells in the pellets were fixed with 10 mL of 100% EtOH before stored at -20°C for, at least, 24h.

Cell staining method

After fixation, cell suspensions were centrifuged for 5 min at 3500 x g (room temperature) and resuspended in 1 mL of PBS, washed once with PBS and sonicated for 3 minutes in an Ultrasonic Cleaner (JSP, US21, ultrasonic power 50W), in order to eliminate cell clumps and aggregates before staining.

In the staining process, 2 μ L of the Vibrant Dye Cycle Green (V35004, Thermo Fisher) (10 μ M final stain concentration) were added to each sample and incubated 30 min (37°C) for selective DNA labeling. After incubation, cells were washed (**con qué??? preguntar a meriyou**) and transferred to flow cytometry tubes for cell cycle analysis.

Data acquisition and processing

Flow Cytometry acquisition were performed with a BD FACS Canto II (BD Biosciences) where stained DNA were excited by a 488nm laser and emission was collected in a 530/30 nm PMT. Flow rate was low and linear amplification were established for the acquisition.

Data were analyzed using FlowJo v.10.6.1 (Becton Dickinson & Company BD). Analysis was performed using one of the univariate cell cycle methods provided by FlowJo, specifically the Watson pragmatic algorithm (Watson et al., 1987) to adjust the data to the model.

Analysis of photosynthetic activity

Sample collection

Fresh culture was harvested at the different specific times of the day. The samples were diluted 1:1 with growing medium and incubated at 20 °C in total darkness during 10 min.

Data acquisition

In order to analyze photosynthetic parameters, Pulse-Amplitude-Modulation PAM fluorometry measurements were performed using a Waltz DUAL-PAM-100. After darkness incubation, the non-actinic modulated light (450 nm, 2.8 $\mu\text{E m}^{-2} \text{ s}^{-1}$) was turned on, in order to measure F_o (fluorescence basal level). Then, to determine F_M (the maximum fluorescence level), a saturating red light pulse of 655nm and 5000 $\mu\text{E m}^{-2} \text{ s}^{-1}$ was applied to the sample during 400 ms. The F_v/F_M , that corresponds to the maximum potential quantum efficiency of Photosystem II when all reaction centers were open, was calculated as:

$$\frac{F_v}{F_M} = \frac{(F_M - F_o)}{F_M}$$

Analytical determinations

Sample collection

At each time point, 50 mL of fresh culture was harvested and centrifuged at 7000 x g 10 min. Then pellets were washed with 1% ammonium formate (p/v) to eliminate salts from the growing medium and lyophilized.

Starch Content

Cell disruption

Approximately 2-3 mg of lyophilized biomass were added to hermetic tubes containing 1 mL of glass beads (0.25–0.3 mm diameter) and 2 mL of chloroform:methanol (2:1). Three disrupting cycles (60 s agitation - 60 s incubation on ice) were applied using Mini-Bead-beater (BioSpec Products). Then cellular extracts were separated from the beads and saved in new tubes. Cellular extracts were centrifuged for 4 min at 13000 x g and the supernatant was discarded. The addition of chloroform:methanol (2:1) and centrifugation steps were repeated until the pellets were white in order to ensure the elimination of pigments and lipids that could disturb the determination process. Finally, pigment free pellets were dried.

Starch solubilization and digestion

The proposed protocol for plants in (Rufty & Huber, 1983) was adapted to *Ostreococcus tauri*.

Starch granules in dry pellets were alkaline solubilized with 1mL of 0.2 M KOH and heated at 100 °C. After 30 min, samples were gradually cooled and pH was adjusted to 5.0 adding 300 µL of 1 M acetic acid.

To starch digestion, 7.4 U of α-amylase were added and incubated 30 min at 37 °C, breaking down starch in small linear and branched oligosaccharides. After that, 5 U of amyloglucosidase were added and incubated 1-2 h at 55 °C releasing glucose residues. Finally, in order to stop enzymatic reaction, the samples were incubated at 100 °C 2 min and centrifuged at 13000 x g for 10 min discarding pellet. Enzymes were prepared in 0.1 M of sodium acetate pH 4.5.

Spectrophotometric quantification

The quantification of released glucose residues from starch was achieved following (Rufty & Huber, 1983) protocol. The method combined two enzymatic activities: hexokinase, that phosphorylated glucose residues, and glucose-6-phosphate dehydrogenase (G6PDH) that reduced NAD⁺ oxidizing the phosphorylated glucose. NADH generated could be measured at 340 nm and correlated to glucose concentration in a ratio 1:1 (Fig. 11).

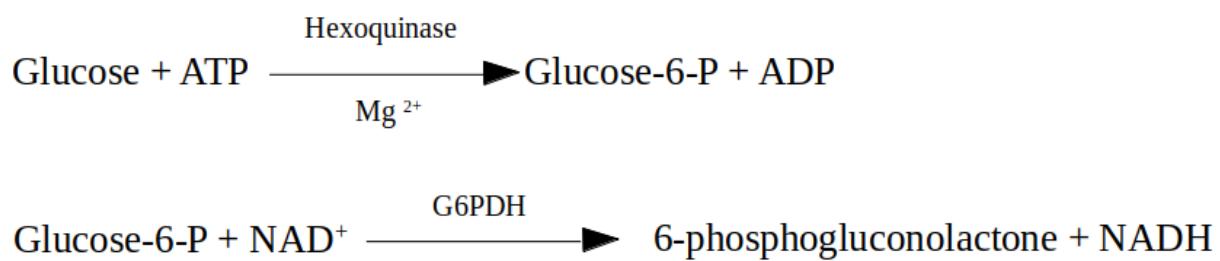


Figure 11: Enzymatic activities that link NADH production to glucose residues released from starch.

To achieve that measurement, quartz spectrophotometer cuvettes were used containing:

- 100 µL of the sample

- 500 µL of hexokinase buffer (100mM HEPES pH 7.7, 10 mM MgCl₂, 0.04% BSA p/v, 1mM DTT)
- 100 µL of ATP mix (containing 10mM ATP diluted in Hepes 100 mM ph 7.7)
- 100 µL of NAD⁺ mix (containing 4 mM NAD⁺ diluted in Hepes 100 mM ph 7.7)
- 2 µL of 2.5 U µL⁻¹ glucose-6-phosphate dehydrogenase
- 200 µL of destile water.

The absorbance of that mixture was measured at 340 nm followed by the addition of 5 µL of 1 U µL⁻¹ hexokinase enzyme and the incubation at 27 °C for 20 min. After that, a second measurement allowed to determine the amount of NADH produced during the reaction. NADH absorbance was related to the amount of glucose residues and consequently to the initial amount of starch in each the sample from calibration curves with commercial starch.

Carotenoid Content

Cell disruption

Four milligrams of lyophilized biomass were added to a hermetic tube containing 1 mL of glass beads (0.25–0.3 mm diameter) and 1 mL of pure acetone. Three disrupting cycles (60 s agitation – 60 s incubation on ice) were applied using Mini-Beadbeater (BioSpec Products).

Carotenoids extraction

Carotenoids extraction was achieved following the method proposed by (Del Campo et al., 2004). Darkness was maintain during the entire process to avoid pigments degradation.

After centrifugation for 4 min at 13000 × g, cellular extracts were collected and saved in new tubes. Again, 1 mL of pure acetone was added to wash glass beads, centrifuged and the supernatant was collected. This process was repeated until the supernatant turned colorless. Supernatants were pooled together in the same tube and acetone was evaporated using a stream of nitrogen gas. Finally, 350 µL of acetone were added for HPLC analysis.

Carotenoids determination and quantification

A Hitachi HPLC (Elite LaChrom), equipped with a photodiode-array detector (Hitachi L-2455) was used. Separation was performed on a Waters NovaPak C-18 (3.9×150 mm, 4 µm particle size, 60 Å pore size) column. Following the method proposed by (Böhme et al., 2002), the eluents used to create a gradient through the mobile phase were: eluent A (0.1 M ammonium acetate and 15:85 v/v H₂O-methanol) and eluent B (44:43:13 v/v methanol-acetonitrile-acetone). Temperature was maintained constant (20 °C) during the whole process and eluents flowed at 800 µL min⁻¹.

Different carotenoids were identified based on the retention times and absorption profiles of carotenoids patterns previously analyzed.

Rhythmic patterns analysis

Rhythmic patterns detection

The non parametric methods implemented in the R package RAIN (Thaben & Westermark, 2014) from Bioconductor were used to statistically identify rhythmic patterns in the different collected data. These rhythmic patterns were also independently identified using the cosinusoidal parametrizations developed in the R package CircaCompare (Parsons et al., 2020).

Three complete diurnal cycles from both photoperiods were used to detect rhythmic patterns in the different data of this study: expression levels of genes, abundance of proteins, maximum potential quantum efficiency of Photosystem II, cells in the different cell cycle phases and carotenoids and starch contents.

Rhythmic patterns with a single maximum point over a complete diurnal cycle were detected by setting the period parameter from RAIN to 24 hours. Similarly, more complex rhythmic patterns (the ones with two or even three maximum points per day) were detected changing the period parameter from RAIN to 12 and 8 hours respectively. A 0.05 p-value threshold was used in all scenarios.

In addition, the last two diurnal cycles and two consecutive days of continuous light or darkness were considered for the RAIN and CircaCompare analysis described above. In that way, RAIN and CircaCompare could statistically test if a similar pattern was main-

tained after changing the cycling light regime to free running conditions consisting in constant light or darkness preventing a bias towards any of the two conditions.

Rhythmic patterns comparison

The rhythmic patterns were fitted to a co-sinusoidal wave in order to be characterized and to enable comparison between them. The statistical significance of the differences in these waves parameters between different of rhythmic patterns was performed using the R package CircaCompare (Parsons et al., 2020) with a p-value threshold of 0.05. The significance of the global differences in the different rhythmic parameters was performed using the Mann-Whitney-Wilcoxon non parametric test implemented in the R function *wilcox.test*.

Data and code availability.

The RNA-seq transcriptomic data and the SWATH proteomic data generated and analysed in this thesis have been deposited in the Gene Expression Omnibus (NCBI) database and respectively and are freely available to the research community identified with the accession numbers GSE155535.

The codes for MARACAS (MicroAlgae RnA-seq and Chip-seq AnalysisS) pipeline and AlgaeFUN (microALGAE FUNctional enrichment tool) are freely available from the following Github repositories:

- <https://github.com/fran-romero-campero/MARACAS>
- <https://github.com/fran-romero-campero/AlgaeFUN>.

The actual R code developed in this thesis to perform all the analysis is available as an appendix generated from a Rmarkdown report, SANDAL (SeAsonal aNd DiurnAL cycles in Ostreoccocus), whose code is available from the Github repository:

<https://github.com/fran-romero-campero/SANDAL>

The code of the shiny app MINOTAUR, , developed to facilitate the exploration of the results presented in this thesis is available from the Github repository:

<https://github.com/ABRomeroLosada/MINOTAUR>

Hypothesis and Objectives

Seasonal variations in diurnal cycles play a central role in the physiology and development of living organisms on Earth in general. Therefore, it is expected that seasonality highly affects microalgae physiology in particular. For example, seasonal oceanic massive microalgae blooms have been observed from outer space. Nevertheless, there is a lack of omics analysis in microalgae for these responses. Specifically, these studies were limited in *Ostreococcus tauri* to a single transcriptomic analysis based on microarrays and an independent single proteomic study. Both studies were performed over neutral day conditions (12 h of light : 12 h of dark). Most of the *Ostreococcus* transcriptome has been reported as rhythmic, but *bona fide* circadian genes maintaining rhythmicity under different seasonal conditions and free-running conditions are not identified yet. Moreover, comparative analysis between rhythmic patterns at the transcriptomic, proteomic and physiological levels have not been performed and are expected to shed light on the responses of living organisms to seasonal variations in diurnal cycles.

The general objective in this thesis consist in carrying out a *multiomic characterization of the responses to photoperiodic seasonal variations in diurnal cycles in Ostreococcus tauri*. This is achieved pursuing the following specific objectives:

- 1 Development of software tools, computational pipelines and models to promote systems biology studies in microalgae in general and the exploration of the results in this thesis in particular.
- 2 Characterization of the transcriptomic responses to seasonal variations in diurnal cycles and free running conditions.
- 3 Characterization of the proteomic responses to seasonal variations in diurnal cycles and integration with the transcriptomic responses.
- 4 Characterization of responses of central physiological processes to seasonal variations in diurnal cycles and multiomics integration with transcriptomic and proteomic data.

Results

Chapter 1. ALGAEFUN with MARACAS, microALGAE FUNCtional enrichment tool for MicroAlgae RnA-seq and Chip-seq Analysis.

In order to characterize the molecular systems regulating microalgae physiology, high throughput sequencing technologies have been recently applied obtaining the genome sequences of a wide range of microalgae (Blanc et al., 2012; Bowler et al., 2008; Cheng et al., 2019; Corteggiani Carpinelli et al., 2014; Hori et al., 2014; Merchant et al., 2007; Moreau et al., 2012; Morimoto et al., 2020; Ottesen et al., 2013; Palenik et al., 2007; Polle et al., 2017; Roth et al., 2017; Worden et al., 2009). This has promoted the emergence of molecular systems biology studies and the use of different omics such as transcriptomics, based on RNA-seq data (Hoys et al., 2021; Serrano-Pérez et al., 2022), and cistromics, based on ChIP-seq data (Ngan et al., 2015; X. Zhao et al., 2021) in microalgae. Nonetheless, the progress of this type of studies on microalgae is limited by the lack of freely available and easy-to-use online tools to analyze and integrate omics data. Processing of the massive amount of high-throughput sequencing data and analysis of the resulting sets of genes and genomic loci obtained from molecular systems biology studies requires computational power, time, effort and expertise that some research groups on microalgae may lack. In addition, researchers must explore different data-bases separately, which makes the integration of the results and the generation of biological meaningful information difficult. Therefore, it is imperative the development of frameworks integrating microalgae genome sequences and annotations with tools for high-throughput sequencing data analysis and functional enrichment of gene and genomic loci sets.

In order to cover these needs for the microalgae research community and to promote studies in molecular systems biology we have developed the web portal ALGAEFUN with MARACAS (<https://greennetwork.us.es/AlgaeFUN/>) using the R package Shiny (<https://shiny.rstudio.com/>) other Bioconductor packages such as clusterProfiler (Wu et al., 2021; Yu et al., 2012) as well as annotation packages for microalgae developed in this thesis.

Our web portal consists of two different tools. First, MARACAS (MicroAlgae RnA-seq and Chip-seq Analysis) implements a fully automatic computational pipeline receiving as input

RNA-seq or ChIP-seq raw data from microalgae studies and producing set of differentially expressed genes or lists of genomic loci, respectively. These results can be further analyzed using our second tool, ALGAEFUN (microAlgae FUNctional enrichment tool). When receiving the results from an RNA-seq analysis, sets of genes are functionally annotated by performing Gene Ontology (GO) (Ashburner et al., 2000; Carbon et al., 2019) and metabolic pathways enrichment analysis (Kanehisa et al., 2016; Moriya et al., 2007; Ogata et al., 1999). When genomic loci from a ChIP-seq analysis are inputted, a set of potential target genes is generated together with the analysis of the distribution of the loci over gene features, as well as metagene plots representing the average mapping signal. This set of potential target genes can be further studied using the features for functional enrichment analysis in ALGAEFUN, as described above. The code for ALGAEFUN with MARACAS is publicly available at their respective GitHub repositories from the following links: <https://github.com/fran-romero-campero/ALGAEFUN> and <https://github.com/fran-romero-campero/MARACAS>.

Implementation

Integration of different microalgae databases.

ALGAEFUN with MARACAS supports 14 different microalgae species that cover an ample spectrum of their phylogeny (Fig.12): *Chlamydomonas reinhardtii* (Blaby et al., 2014; Merchant et al., 2007), *Volvox carteri* (Prochnik et al., 2010), *Chromochloris zofingiensis* (Roth et al., 2017), *Dunaliella salina* (Polle et al., 2017), *Haematococcus lacustris* (Morimoto et al., 2020) (Chlorophyceae), *Coccomyxa subellipsoidea* (Blanc et al., 2012) (Trebouxiophyceae), *Ostreococcus tauri* (Blanc-Mathieu et al., 2014; Palenik et al., 2007), *Bathycoccus prasinos* (Moreau et al., 2012), *Micromonas pusilla* CCMP1545 (Worden et al., 2009) (Mamiellophyceae), *Phaeodactylum tricornutum* (Bowler et al., 2008; M. Yang et al., 2018), *Nannochloropsis gaditana* (Corteggiani Carpinelli et al., 2014; Radakovits et al., 2012) (Stramenopiles), *Klebsormidium nitens* (Hori et al., 2014), *Mesotaenium endlicherianum* (Cheng et al., 2019) and *Spirogloea muscicola* (Cheng et al., 2019) (Charophyceae). Supported species include microalgae used in basic scientific research, as well as those used in biotechnology industry like *H. lacustris* (Hoys et al., 2021), *N. gaditana* (Ajjawi et al., 2017) or *P. tricornutum* (Cui et al., 2019; Pereira et al., 2021).

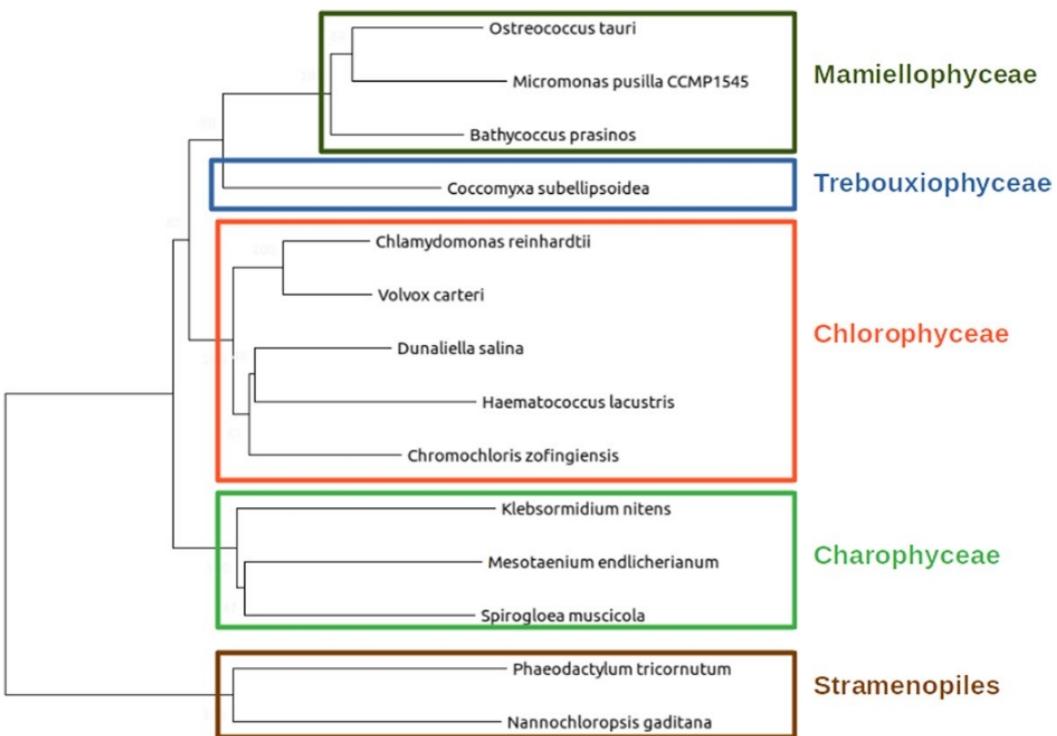


Figure 12: Phylogenetic relationship between the different microalgae species supported in AL-GAEFUN with MARACAS.

One of the goals of our tool is to integrate available genome sequences, functional annotations and genomic feature annotation files (Gene transfer files, GTF) for the already sequenced microalgae species in order to generate easily accessible resources. These data have been systematically collected from different freely available data bases, depending on the microalgae, Table 3. Specifically, for *N. gaditana* and *P. tricornutum*, we accessed Ensembl protist (Howe et al., 2021), a web based unicellular species genome browser storing gene annotation; *B. prasinus* is included in Orcae (Sterck et al., 2012) an online genome annotation resource built on the wiki philosophy; for *C. reinhardtii*, *V. carteri*, *C. zofingiensis*, *D. salina* and *C. subellipsoidea* the JointGenome Institute (JGI) / Phytozome (Goodstein et al., 2012), a web portal integrating omics for photosynthetic organisms was queried; for *M. endlicherianum* and *S. muscicola* a figshare associated to the corresponding publication was accessed; *M. pusilla*, *O. tauri*, *B. prasinus* and *K. nitens* genome sequence and annotation was downloaded from the JGI / PhycoCosm (Grigoriev et al., 2021), a comparative algal genomic resource; finally the genome of *H. lacustris* was available at NCBI genome database (https://www.ncbi.nlm.nih.gov/genome/67110?genome_assembly_id=839686).

Table 3: Resources used to collect genome sequences, functional and gene feature annotations for each supported microalgae.

Ensembl protists	PhycoCosm	Phytozome	Genomes NCBI datasets	Figshare associated to publication
<i>N. gaditana</i>	<i>B. prasinos</i>	<i>C. reinhardtii</i>	<i>H. lacustris</i>	<i>M. endlicherianum</i>
<i>P. tricornutum</i>	<i>K. nitens</i>	<i>V. carteri</i>		<i>S. muscicola</i>
	<i>M. pusilla</i>	<i>C. zofingiensis</i>		
	<i>O. tauri</i>	<i>D. salinas</i>		
		<i>C. subellipsoidea</i>		

Genome sequence and gene feature annotation files were downloaded in fasta and GTF format, respectively. When necessary, different chromosome and/or scaffold files were colligated programmatically to produce a single genome file. The GTF format in the gene feature annotation files consists of a data frame with nine columns. Each line corresponds to a specific gene feature. The first eight columns must contain information related to the gene feature (3' UTR, 5' UTR, gene, CDS or mRNA), chromosome start and end positions, the strand where it is positioned, and some other attributes in a well-defined format. The ninth column is not restricted to a specific format and can contain any type of information. Nonetheless, the mappers used in MARACAS assume that this last column follows the format taken by GTF files in the data-base Ensembl. In order to be able to use GTF files from other databases besides Ensembl, we developed an R script to translate any GTF file into the format followed by Ensembl and required by the read mapper HISAT2 (Kim et al., 2015).

Systematic functional annotation files consisting of Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genome) Orthology (KO) terms were also downloaded for each microalga from the previously mentioned databases. Gene Ontology terms seeks the development of a human-readable and machine-readable hierarchical vocabulary to relate genes with their molecular functions, biological processes in which they are involved and

the cellular components where they perform their function (Ashburner et al., 2000; Carbon et al., 2019). Complementary, KEGG Orthology terms associate genes to metabolic pathways and modules based on their orthologous relationships in sequenced genomes (Kanehisa et al., 2016; Moriya et al., 2007; Ogata et al., 1999). However, for microalgae species lacking these annotation systems, HMMER (biological sequence analysis using profile hidden Markov models) (Potter et al., 2018) was used to identify protein domains according to the PFAM (Protein Family) nomenclature (Mistry et al., 2021). PFAM terms were subsequently converted into GO terms using pfam2go (<http://current.geneontology.org/ontology/external2go/pfam2go>). KO terms were associated to genes applying KAAS (KEGG Automatic Annotation Server) (Moriya et al., 2007). Whenever possible, other systematic functional annotation format were also included: Protein Analysis Through Evolutionary Relationships (PANTHER) terms (Mi et al., 2021), that classifies, based of evolutionary families and subfamilies, gene products into classes capturing molecular function, biological process and metabolic pathways; Enzyme Commission numbers (EC numbers), that consists of a numerical classification identifier for enzymes, related to the biochemical reactions they perform; and Eukaryotic Orthologous Groups (KOG) terms, used to identify orthologue and parologue groups of proteins (Galperin et al., 2021).

Development of functional annotation and genomic packages.

In order to use all these annotation systems in ALGAEFUN two different types of R annotation packages were developed and were made freely available on our Github repository (<https://github.com/fran-romero-campero/AlgaeFUN/tree/master/packages>).

The systematic sources of functional annotation mentioned previously were gather together using the function makeOrgPackage from the Bioconductor R package AnnotationForge (Carlson & Pagès, 2019) which generated annotation packages for each microalga. These packages are instrumental when performing functional enrichment analysis over gene sets obtained from RNA-seq data analysis.

Gene featuring annotation of each microalgae from the previously downloaded and processed GTF files is stored in different packages applying the function makeTxDbFromGFF from the Bioconductor R package GenomicFeatures (Lawrence et al., 2013). These packages are central to carry out analysis over genomic loci obtained in ChIP-seq analysis.

Both functional annotation and gene feature packages of each microalga are freely available on our Github repository, in order to enable the research community in microalgae to use them and perform omics analysis independently from the tools available from ALGAE-FUN with MARACAS.

MARACAS implementation: high-throughput sequencing data processing.

The computational core of this tool consists of a parallel fully automatic computational pipeline or workflow synchronized through blackboards. This workflow is managed by the job scheduling system SLURM (Simple Linux Utility for Resource Management) and bash scripting. The inputs to our pipeline comprise the pre-computed index of the corresponding microalga reference genome, the previously processed gene feature annotation file in GTF format (both already included in MARACAS) and the raw sequencing data in fastq format from RNA-seq and ChIP-seq microalgae studies provided by the user. In turn, this workflow produces, as outputs, two lists of differentially expressed genes or DEGs (activated and repressed genes) when the fastq files correspond to a RNA-seq study; or a list of genomic loci or regions significantly occupied by the transcription factor or histone modification of interest in the case of a ChIP-seq study.

MARACAS requires the user to input specifications such as the microalga of interest, the names for control and experimental conditions, number of replicates and location of raw high-throughput sequencing files. In case of analyzing already published omic data, its accession number can be specified instead. Additionally, the user can set the statistical parameters to perform the corresponding analysis. Specifically, the fold-change and significance level cutoff thresholds for the identification of differentially expressed genes can be selected. In addition, user can choose as read mapper software either the short read mapper HISAT2 (Hierarchical Indexing for Spliced Alignment of Transcripts 2) or the pseudoalignment method implemented in kallisto. Whereas HISAT2 is an exact method requiring several hours for processing a typical sample (Kim et al., 2015; Pertea et al., 2016), kallisto produces near-optimal gene expression quantification in only a few minutes (Bray et al., 2016). Meanwhile, in the MARACAS ChIP-seq data analysis, pipeline is used the ultra-fast and memory-efficient short read mapper bowtie2 (Langmead & Salzberg, 2012). All this information is collected into a parameters file (Table 4 and Table 5), which is the main input received by the pipeline.

Table 4: Description of parameters file used as input in MARACAS for RNA-seq analyses.

Parameters file for RNA-seq analyses	
data_source	This parameter indicates the source of the data to be analyzed. It can take the value <i>FILES</i> when the fastq files are already located in a folder in the computer where MARACAS is installed or the value <i>DB</i> when the data has been already deposited in the GEO database.
cluster	Parameter specifying the execution mode. <i>SERVER</i> mode executes MARACAS with a sequential analysis of the different samples. Whereas <i>SLURM</i> mode executes MARACAS in a parallel manner, processing samples simultaneously in different computational nodes. In this last case, SLURM needs to be installed in user's computer cluster.
number_processors	Number of processors that can be used by MARACAS.
paired_end	It can take the values <i>FALSE</i> , when your data is single end, and <i>TRUE</i> , when your data is paired end.
working_directory	It indicates the location where the analysis folder will be generated.
microalgae	Name of the microalgae of interest.
read_mapper	This parameter specifies the software tool to perform read mapping. Two different options are provided: <i>HISAT2</i> and <i>Kallisto</i> .
main_folder	Name of the folder that will be created at the working directory to contain the outputs from the analysis.
number_of_samples	Total number of samples to be analyzed.
control_condition_name	Name of control condition.
experimental_condition_name	Name of experimental condition.
loc_sampleN	When <i>paired_end</i> : <i>FALSE</i> and <i>data_source</i> : <i>FILES</i> , this parameter indicates the path and file name of sampleN. N will take values from 1 to <i>number_of_samples</i> .
acc_sampleN	When <i>data_source</i> : <i>DB</i> , this parameter specifies accession number of fastq files in GEO.
loc_sample_leftN loc_sample_rightN	When <i>paired_end</i> : <i>TRUE</i> and <i>data_source</i> : <i>FILES</i> , this parameters indicates the path and file name of the fastq samples containing the left and right reads, respectively.
condition_sampleN	This parameters specifies which condition name of the ones chosen in <i>control_condition_name</i> or <i>experimental_condition_name</i> correspond to each sample.
fold_change q_value	These parameters specify the fold-change and q-value used to determine differential expressed genes in the experimental condition when compared to the control condition.

Table 5: Description of parameters file used as input in MARACAS for ChIP-seq analyses.

Parameters file for ChIP-seq analyses	
data_source	<i>FILES</i> or <i>DB</i> as it is explained in Table 4.
cluster	<i>SERVER</i> or <i>SLURM</i> as it is explained in Table 4.
number_processors	Number of processors that can be used by MARACAS.
paired_end	<i>FALSE</i> or <i>TRUE</i> as it is explained in Table 4.
working_directory	It indicates the location where the analysis folder will be generated.
microalgae	Name of the microalgae of interest.
main_folder	Name of the folder that will be created at the working directory to contain the outputs from the analysis.
number_of_replicates	Total number of samples to be analyzed.
included_control	It can receive <i>yes</i> if your experimental design includes a control condition such as input, mock or similar. Use <i>no</i> in negative cases.
mode	Use the values <i>transcription_factor</i> or <i>histone_modification</i> to specify if your ChIP-seq data was generated for a transcription factor or a histone modification study.
transcription_factor	When <i>mode: transcription_factor</i> this parameter specifies the name of the chosen transcription factor.
histone_modification	When <i>mode: histone_modification</i> this parameter specifies the name of the chosen histone modification.
loc_chip_replicate_N loc_control_replicate_N	When <i>paired_end: FALSE</i> and <i>data_source: FILES</i> , this parameter indicates the path and file name of ChIP sample and, in case of <i>included_control: yes</i> , the control sample. N will take values from 1 to <i>number_of_replicates</i> .
chip_replicate_N control_replicate_N	When <i>data_source: DB</i> , this parameter specifies accession number of ChIP sample in GEO. In case of <i>included_control: yes</i> , <i>control_replicate_N</i> will specify the one for control sample in GEO.
loc_chip_replicate_left_N loc_chip_replicate_right_N loc_control_replicate_left_N loc_control_replicate_right_N	When <i>paired_end: TRUE</i> and <i>data_source: FILES</i> , this parameter indicates the path and file name of the ChIP samples containing the left and right reads, respectively. If <i>included_control: yes</i> , they also indicate this information for control samples.

The first step in our workflow loads the parameters file and generates the working directory including one folder where the samples are downloaded or copied, another one where the results are generated and a third one, called “logs”. In this last folder, several text files are generated containing messages written by the different processes in the pipeline that are useful to keep track of the important events. More interestingly, a different type of text file, called blackboard, is created and the computational processes in our workflow are given read and write permissions for it. Files functioning as blackboards are used for indirect communication between the computational processes in the parallel pipeline in order to synchronize them. In the next step, the pipeline forks into two different modes to process either RNA-seq or ChIP-seq data.

The fork of our pipeline dedicated to RNA-seq analysis is represented in Fig. 13. Depending on the raw data source, the first step consists of either downloading and extracting the corresponding fastq files from a database or copying them to the specified working directory. Next, a quality control of these raw data is performed and short reads are mapped to the reference genome using HISAT2 or kallisto. When HISAT2 is used, this step generates BAM (Binary Alignment Maps) files that contain the mapped reads. The following steps take this mapping as input and perform transcripts assembly and expression quantification using STRINGTIE (Pertea et al., 2015, 2016). In this step, GTF files with the assembled transcripts and CTAB files (chemical table files) with the expression quantification values are generated for all samples. However, when kallisto is used, transcripts are mapped and quantified in the same step, so a TSV (tabular separated values) file containing the result of the quantification is generated (Bray et al., 2016). These processing steps are carried out in parallel, simultaneously for each sample, being synchronized using a blackboard file. These parallel processes write on the blackboard when a goal is reached (for example, BAM files are generated) in order to keep track of their progress. When all parallel processes reach their goals, the following sequential steps of the pipeline are executed. They consist of a differential expression analysis carried out using the Bioconductor R packages Ballgown (Frazee et al., 2015; Pertea et al., 2016) and Limma (Ritchie et al., 2015). Specifically, DEGs are selected using the statistics based on a moderated t-student. Finally, a differentially expression gene report is generated, containing text files with activated and repressed genes, principal component analysis visualization, and several informative graphs, such as scatter plots, volcano plots, box-plots and bar-plots.

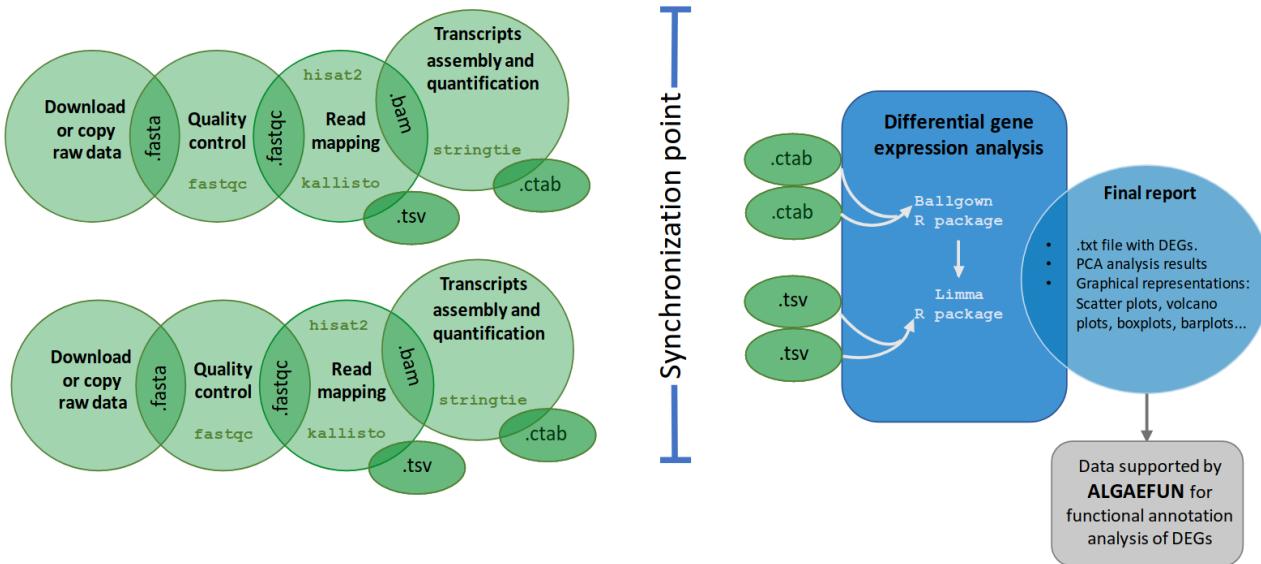


Figure 13: Workflow of the automatic pipeline for the analysis of RNA-seq data in MARACAS. The maracas-rna-seq pipeline receives as input a parameter file as described in Table 4. After data acquisition in fastq format, sequence quality analysis is performed using fastqc. Read mapping and gene expression quantification is then performed using HISAT2 and stringtie or kallisto depending on the user choice. A synchronization point ensuring the completion of all samples processing to quantify gene expression is necessary before identifying DEGs using the R packages ballgown and limma. Gene expression estimates measured as raw count, FPKM (Fragments per Kilobase of exon and Million of mapped reads) or TPM (Transcripts per Million) are stored in TSV files. Reports in html and pdf format are generated with details on sequence quality analysis, mapping process and normalization. Graphics for exploratory analysis such as principal components, box-plots, scatter-plots, volcano-plots and bar-plots of individual genes are also included. These reports include links to download gene expression estimates as well as lists of activated and repressed DEGs. The outputs of this pipeline are compatible with the input formats for ALGAEFUN in order to facilitate further functional enrichment analysis and visualization.

The second fork of our pipeline dedicated to ChIP-seq analysis (Fig. 14) shares with the above RNA-seq fork the first steps consisting of downloading or copying the fastq raw files for the ChIP samples (data corresponding to the chromatin immunoprecipitation condition) and control samples into the generated working directory and performing a quality control. Specifically, in this part of the workflow, short read mapping to the reference genome is executed using bowtie2 (Langmead & Salzberg, 2012) and the synchronization point requires that BAM files for every ChIP and control sample is generated. This synchronization point is also achieved using a blackboard. After synchronization, the last step takes as input all mappings in BAM format and performs peak calling using the software tool macs2

(Gaspar, 2018). This final step consists of the identification of genomic loci or regions significantly enriched with mapped reads, indicating the genomic occupation of the transcription factor or histone modification of interest. The output files of this pipeline consists of a BED (Browser Extensible Data) file, collecting the genomic loci or regions identified during the analysis, and a BW (BigWIG) file, containing the number of mapped reads or signal in each position of the genome.

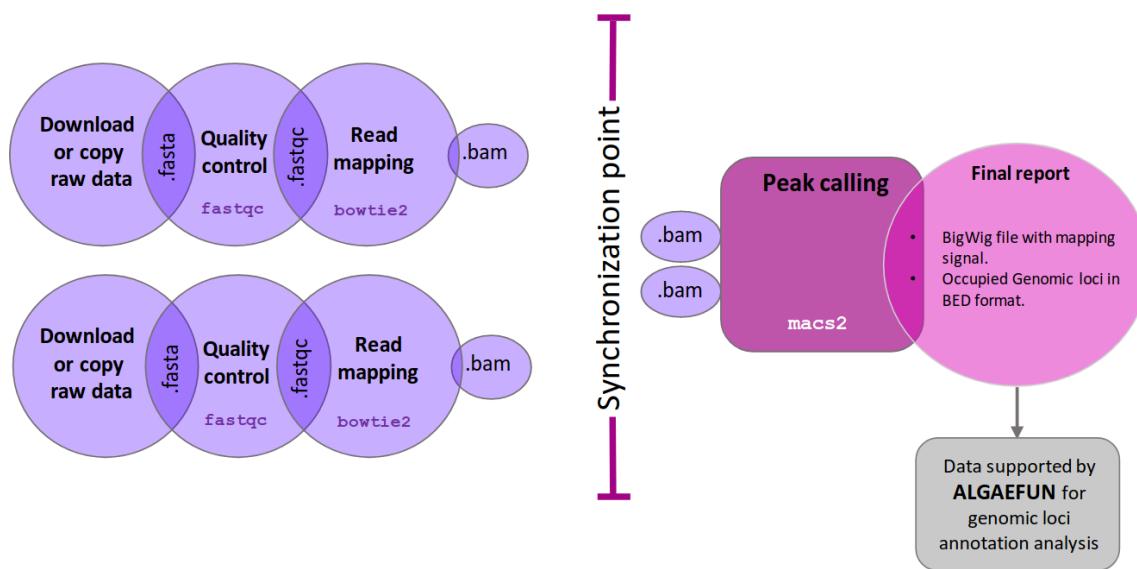


Figure 14: Workflow of the automatic pipeline for the analysis of ChIP-seq data in MARACAS. The maracas-chip-seq pipeline receives as input a parameter file as described in Table 5. After data acquisition in fastq format, sequence quality analysis is performed using fastqc. Read mapping to the reference genome is performed using bowtie2 and is stored in BAM format. A synchronization point ensuring the end of all replicates processing is necessary before carrying out peak calling with macs2. Reports in html and pdf format are generated with details on sequence quality analysis and mapping process. These reports include links to download the identified peaks in BED format and the genome wide mapping signal in bigwig format. These outputs are compatible with the input formats for ALGAEFUN in order to facilitate further annotation and visualization of the identified genomic loci significantly bound or occupied by the transcription factor or histone modification of interest.

ALGAEFUN implementation: functional annotation analysis.

The generated output from MARACAS, either sets of genes or genomic loci, can be functionally annotated using the next software tool in the portal, ALGAEFUN. Although this tool can also be used to functionally annotate genes sets or genomic loci generated independently from other tools.

The user interface of ALGAEFUN is shown in Fig. 15 and Fig. 16. Two different modes of operation, depending on the kind of input data to be analyzed, are implemented in ALGAEFUN.

The figure shows the ALGAEFUN user interface. At the top is a navigation bar with a green cartoon alga icon. Below it is a main title "ALGAEFUN with MARACAS" and a subtitle "microALGAE FUNctional enrichment tool for MicroAlgae RnA-seq and Chip-seq Analysis". A detailed description of the tool's purpose follows. The interface is divided into four numbered sections:

- (1)** Navigation bar: A sidebar containing links to Home, Gene Set Functional Analysis (selected), Genomic Loci Functional Analysis, MARACAS, MicroAlgae RnA-seq and Chip-seq Analysis, Funtree, Phylogenetic Analysis of Genes in Viridiplantae, Tutorials, GitHub repository, and Citation and Contact.
- (2)** Drop-down menu: "Choose your favourite microalga" set to "Ostreococcus tauri".
- (3)** Sidebar panel: "Choose your desirable analysis" (radio buttons for GO terms enrichment, KEGG pathways enrichment analysis, Both), "Choose gene ontology" (radio buttons for Biological process, Cellular Component, Molecular Function), and "Which will be your chosen p-value?" (text input field set to 0.05).
- (4)** Main panel: "Insert a set of genes" text input field, "Example" and "Clear" buttons, "Choose File with Gene Set to Upload" button (No file selected), "Would you rather use your own background set?" radio buttons (Yes, No), and a "Have fun!" button.

Figure 15: Microalgae Functional Annotation tool (ALGAEFUN) user interface for gene sets functional annotation. (1) Navigation bar for selecting the tool to use between the ones included in our web app; (2) Drop-down menu to select the microalga of interest; (3) Sidebar panel to select parameters for GO and/or KEGG pathway enrichment; (4) Main panel to input set of genes to analyze and select gene background.

In one of the modes, input data consists of sets of genes obtained, for instance, from an RNA-seq study. Here after selecting this analysis mode (Fig. 15-1), the user chooses the microalga of interest (Fig. 15-2) and the analysis to carry out, a GO term and/or KEGG pathway enrichment analysis at the selected significance level (Fig. 15-3). The set of genes to study can be inputted through a text box or uploaded from a file. Users can also choose between using their own background gene set or the entire microalga genome

(Fig. 15-4). In order to allow users to explore the functionalities of ALGAEFUN and to check the required gene id format, an example gene set for each microalga is included. This example can be accessed and inputted in the corresponding text box by clicking on the example button (Fig. 15–4). These examples were generated during the testing of MARACAS using previously published RNA-seq data sets and analysis and have in turn been used in the testing and validation of ALGAEFUN. The GO term and KEGG pathway enrichment analysis are carried out using the Bioconductor R package ClusterProfiler (Wu et al., 2021; Yu et al., 2012). This package implements statistical analysis and visualization of functional profiles for gene clusters or sets using the annotation packages developed for each microalga, integrating the systematic sources of functional annotation previously discussed. The outputs for the GO term and KEGG pathway enrichment analysis are presented in two different tabs. The first output in the GO enrichment tab consists of a table that summarizes the results from the GO enrichment analysis carried out over the input gene set. The user can find one row for each GO term and six columns that represent some relevant information about the enrichment. The first column shows the GO term identifier, followed by the second column where the user can find its description. Users can access more information about the GO term represented in a specific row by clicking on its identifier to be redirected to the web portal AmiGO (Carbon et al., 2009) where GO terms are described in detail. The third and fourth column represents the p-value and q-value, and the fifth one shows the enrichment value: $E = (m/n) / (M/N)$. The value “m” is the number of genes from the inputted gene set annotated with the corresponding GO term whereas “M” is the number of genes from the background annotated with the mentioned GO term. In a similar way, “n” is the number of genes with annotation from the gene set whereas “N” is the number of genes with annotation from the gene background. Finally, the last column shows the genes from the input set of genes annotated with each GO term. The user can click on them to get more information from the gene entry on the corresponding database from which the annotation was retrieved for the specific microalga under study. Furthermore, ALGAEFUN also generates several graphs that represent the GO term enrichment. Five visualization methods are used to illustrate the results:

- Acyclic graph: Each node stands for a GO term and their color indicates the level of significance (from grey, non-significant, to intense red, highly significant). An arrow

is drawn from GO term A to GO term B to represent that A is a more general GO term than B.

- Bar-plot: Each bar represents an enriched GO term whose length corresponds to the number of genes in the gene set annotated with the given GO term. Once again, the bar color shows the level of significance (from blue, less significant, to red, more significant).
- Dot-plot: Each dot represents an enriched GO term. The x-position of the dot corresponds to the ratio between the number of genes annotated with the corresponding GO term and the total number of annotated genes in the gene set. The dot color captures the level of significance (from blue, less significant, to red, more significant).
- Enrichment Map (emap-plot): Each node represents an enriched GO term and the size of each node is proportional to the number of genes annotated with the corresponding GO term in the inputted gene set. The node colors represent the level of significance (from less significant in blue to more significant in red). These nodes are connected by edges when the corresponding GO terms are semantically related.
- Gene-concept network (cnet-plot): The beige nodes represents GO terms and the grey nodes genes. An edge is drawn from a gene to a GO term when the gene is annotated with the corresponding GO term. The size of nodes representing GO terms is proportional to the number of genes annotated with the corresponding GO term.

The outputs shown on the KEGG pathway enrichment tab consist of:

- Table summarizing the result of the KEGG pathway enrichment analysis: Each row represents a pathway significantly enriched in the inputted gene set with respect to the selected gene background. The first column represents the KEGG pathway identifier and the user can click on it to read more information about the pathway. The second column contains its description. The third and fourth column present the p-value and q-value, and the fifth column displays the corresponding enrichment value E (m/n; M/N) as previously described. Finally, the last column shows the list of

genes from the inputted gene set assigned to the corresponding enriched pathway. KEGG pathways can be more informative than GO term since they are not general but specific to the corresponding organism of interest.

- KEGG pathway map: Users can choose a specific enriched pathway using a drop-down menu to generate the corresponding KEGG pathway map where genes from the inputted gene set are highlighted in red.
- Table summarizing the result of the KEGG module enrichment analysis: Each row represents a module significantly enriched in the gene set with respect to the selected gene universe or background. The columns in this table are organized in the same manner as the previously described. KEGG modules are distinct recurrent components of KEGG pathways in this respect they are more specific and can be more informative.

In the other mode, input data consists of genomic loci or regions obtained, for instance, from a Chip-seq study.

This analysis mode is selected from the side bar panel in Fig. 16-1. The microalga of interest can be selected using the drop-down menu from Fig. 16-2. Next, the distance around the transcriptional start site (TSS) that will be considered defining gene promoters must be specified. Gene features that will be considered when assigning gene targets to genomic loci or regions also need to be selected (Fig. 16-3). Genomic loci or regions to analyze can be inputted through a text box or uploading a file. Additionally, a BW file containing the number of mapped reads or signal in each position of the genome can be uploaded (Fig. 16-4). Similar to the previous mode, it is possible to explore the functionalities of this tool and check the required genomic loci or regions format, using an example included for each microalga by clicking on the example button (Fig. 16-4). These examples have been generated during the testing of MARACAS, using previously published ChIP-seq data sets and analysis, and have in turn been used in the testing and validation of ALGAEFUN. The functional annotation of the inputted genomic loci or regions is performed using the Bioconductor R packages ChIPseeker (Yu et al., 2015) and ChIPpeakAnno (L. J. Zhu, 2013). These package implements statistical analysis and visualization of genomic loci and re-

gions using the gene feature annotation packages generated for our tool. The outputs generated in this type of analysis consist of:

- A pie chart representing the distribution of the genomic loci or regions over the different type of gene features selected by the user such as the promoter, 3' UTR, 5'UTR, intron or exon.
- A table enumerating the target genes associated one generated when the data comes from a RNA-seq study. It represents each gene located in the enriched genomic loci and its different annotation terms. This set of genes can be downloaded and subsequently annotated functionally using ALGAEFUN.
- A visualization of the average level of signal around TSS and transcriptional end site (TES). For each individual gene, it generates a visualization of the signal and identification of DNA motifs recognized by transcription factors and regulators in microalgae.

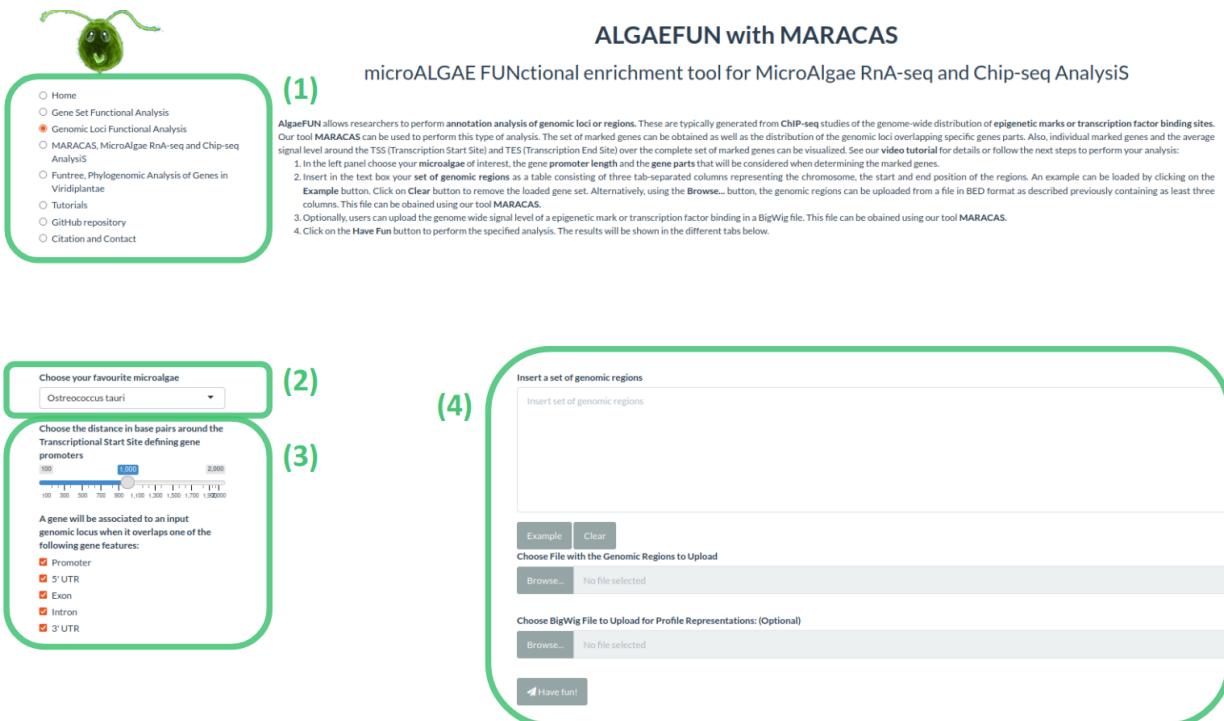


Figure 16: Microalgae Functional Annotation tool (ALGAEFUN) user interface for genomic loci annotation. (1) Navigation bar for selecting the tool to use; (2) Drop-down menu to select the microalga of interest; (3) Sidebar panel to select parameters for the identification of the promoter regions and the gene features or parts that will be considered to assign genes to genomic loci; (4) Main panel to input set of genomic loci and signal in bigWig format obtained from a ChIP-seq analysis.

Case study 1: from RNA-seq raw sequencing data to biological processes and pathways.

This case study is based on RNA-seq data generated in this thesis and illustrates the generation of relevant information suitable for publication. It consists of an RNA-seq study carried out using *Haematococcus*, a microalga of industrial interest for the bio-production of astaxanthin (Hoys et al., 2021). The analysis has been performed for vegetative *Haematococcus pluvialis* cells, grown both under N sufficiency and under moderate N limitation in order to unveil the transcriptomic program enhancing astaxanthin biosynthesis under N deprivation. This illustrates the type of information that ALGAEFUN with MARACAS is able to reveal from raw sequencing data.

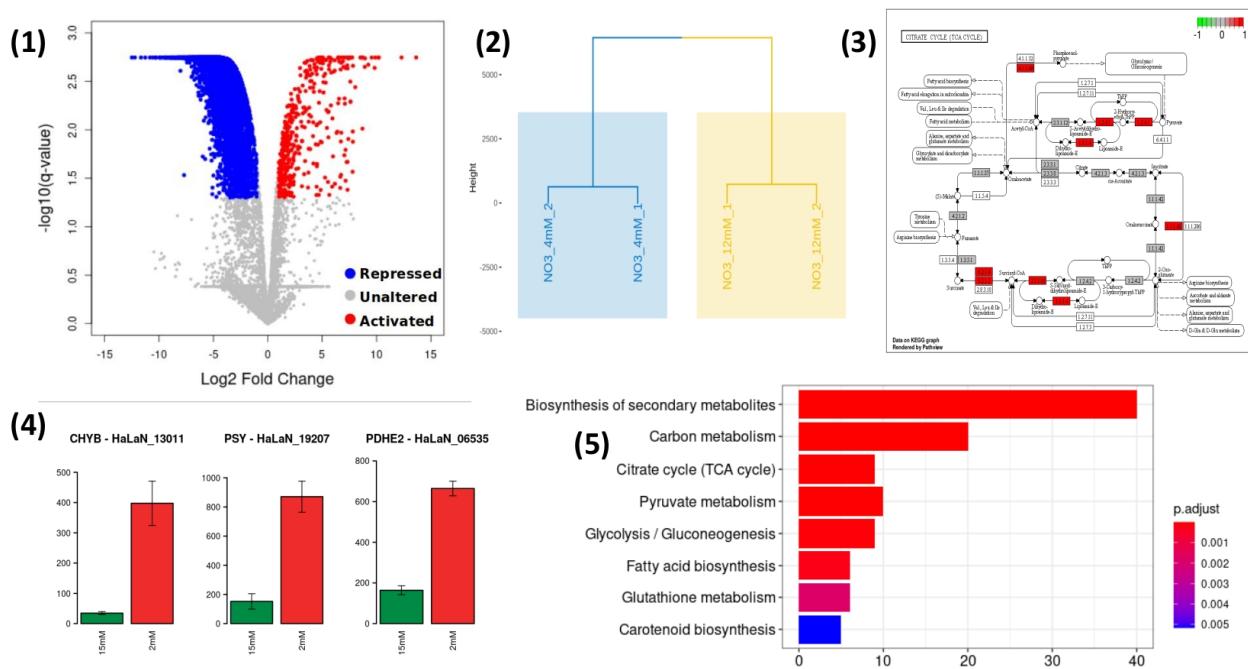


Figure 17: Results obtained from MARACAS final report and from ALGAEFUN analysis. (1) Volcano-plot generated by MARACAS showing the global effect over the transcriptome as well as the activated and repressed genes detected (in red and blue color, respectively); (2) Hierarchical clustering combined with PCA generated by MARACAS; (3) KEGG representation of one of the enriched pathways generated by ALGAEFUN, genes present in the list used as input are colored in red color; (4) Bar-plots representing individual gene expression levels of key enzymes, figure generated by MARACAS. Expression level of the selected gene under N sufficiency is represented in green color. Red color is used to represent moderate N deprivation conditions; (5) Vertical bar-plot representing the enriched biological process in the set of genes used as input, figure generated by ALGAEFUN. Gradient from red to blue color is used to represent p-value.

The high-throughput sequencing raw data in fastq format was processed using MARACAS in order to obtain the set of differentially expressed genes using a criterion based on a fold-change value of 2 and a q-value of 0.05. The report produced by MARACAS confirmed that all samples are of high quality and notified no problem during read mapping to the reference genome with mapping rates greater than 86%. Scatter plots comparing gene expression between samples were also produced in the MARACAS report. High Pearson correlations greater than 98% were identified between replicates of the same condition. Accordingly, automatically hierarchical clustering combined with Principal Components Analysis (PCA) that leads to the identification of more stable clusters, due to the noise reduction achieved with PCA, was performed by MARACAS (Fig. 17-2). It identified two clearly separated clusters constituted by the control (N sufficiency) and experimental condition samples (moderate N deprivation). Volcano plots comparing moderate N limitation with N sufficiency transcriptomes were used in the report to represent the repressed genes and activated genes. Moderate N limitation has shown a strong repressing effect over the transcriptome with respect to N sufficient conditions, identifying 414 activated and 5348 repressed genes (Fig. 17-1). These lists of genes can be then inputted into ALGAEFUN to determine significantly over-represented biological processes or pathways affected in the studied experimental conditions.

As described previously, ALGAEFUN can perform two different types of functional enrichment analysis when a set of genes from an RNA-seq analysis is used as input: GO terms and KEGG pathways enrichment. The GO terms analysis can identify, for example, specific biological processes enriched in the set of genes. One of the available graphical representations of the GO enrichment results in ALGAEFUN are the bar-plot included in Figure 17-5 that presents the biological processes enriched in the activated set of genes obtained from the *Haematococcus* RNA-seq study under moderate N limitation experiment. Reduced availability of nitrogen activates processes like biosynthesis of secondary metabolites, TCA cycle or pyruvate metabolism at a transcriptomic level. GO term analysis provide a general overview of the functional annotation of gene sets since they constitute universal functional terms not specific to a particular organisms. Complementary, KEGG pathway enrichment is specific to the corresponding organism and can be more informative in some cases. It allows to identify the transcriptomic activated enzymes in enriched path-

ways (Fig. 17-3). The genes involved in these processes can be further studied individually and their expression level in both conditions can be compared (Fig. 17-4).

ALGAEFUN with MARACAS analysis described differential expression of hundreds of genes affecting key pathways converging into astaxanthin biosynthesis and storage. The affected pathways were further studied at metabolic level and a massive cell reprogramming was verified for the cells growing under moderate N limitation. Furthermore, a major advance was made by discerning the underlying control mechanisms by finding differentially activated enzymes in the astaxanthin biosynthesis pathway. Moreover, the identification of significant DNA sequences in the promoters of these key enzymes was carried out and, for the first time, the family of transcription factors bHLH, was proposed as candidates for transcriptional regulators of these key enzymes in astaxanthin biosynthesis (Hoys et al., 2021).

Case study 2: From ChIP-seq raw sequencing data to marked genes.

As mentioned before, the development of ALGAEFUN with MARACAS has motivated our lab to generate transcriptomic as well as cistromic and epigenomic data. Although we have our own ChIP-seq data obtained during the study of epigenetic marks in *Ostreococcus tauri*, here we show, as an example of how the ALGAEFUN with MARACAS tool works, the re-analysis of raw data from a previously published epigenomic study in *Chlamydomonas*.

Histone modifications play a central role in gene expression control. The genome-wide distribution of the mark H3K4me3 associated to gene activation has been determined in *Chlamydomonas* (Ngan et al., 2015). After Chip-seq raw data was re-analyzed using MARACAS, as previously described, the 12,814 genomic loci identified as significantly occupied by H3K4me3 in the *Chlamydomonas* genome under standard growth conditions and the corresponding genome wide mapping signal file in BigWig format were uploaded in ALGAEFUN. The region of two kilobases around the TSS is considered as gene promoter and all the gene features are selected to determine the H3K4me3 marked genes. The outputs are presented in the graphical interface in different tabs. A downloadable table with the marked genes and their available annotation is generated. This gene list can, in turn, be analysed by ALGAEFUN to perform a GO term and/or pathways enrichment analysis. A total of 11,558 H3K4me3 marked genes are identified. Graphs representing the dis-

tribution of the genomic loci overlapping different gene features (Fig. 18-a) and the distribution of upstream and downstream signal around TSS and TES are also represented. In agreement with the previously published results, the 90.75% of the genomic loci occupied by H3K4me3 are located at gene promoters in *Chlamydomonas*.

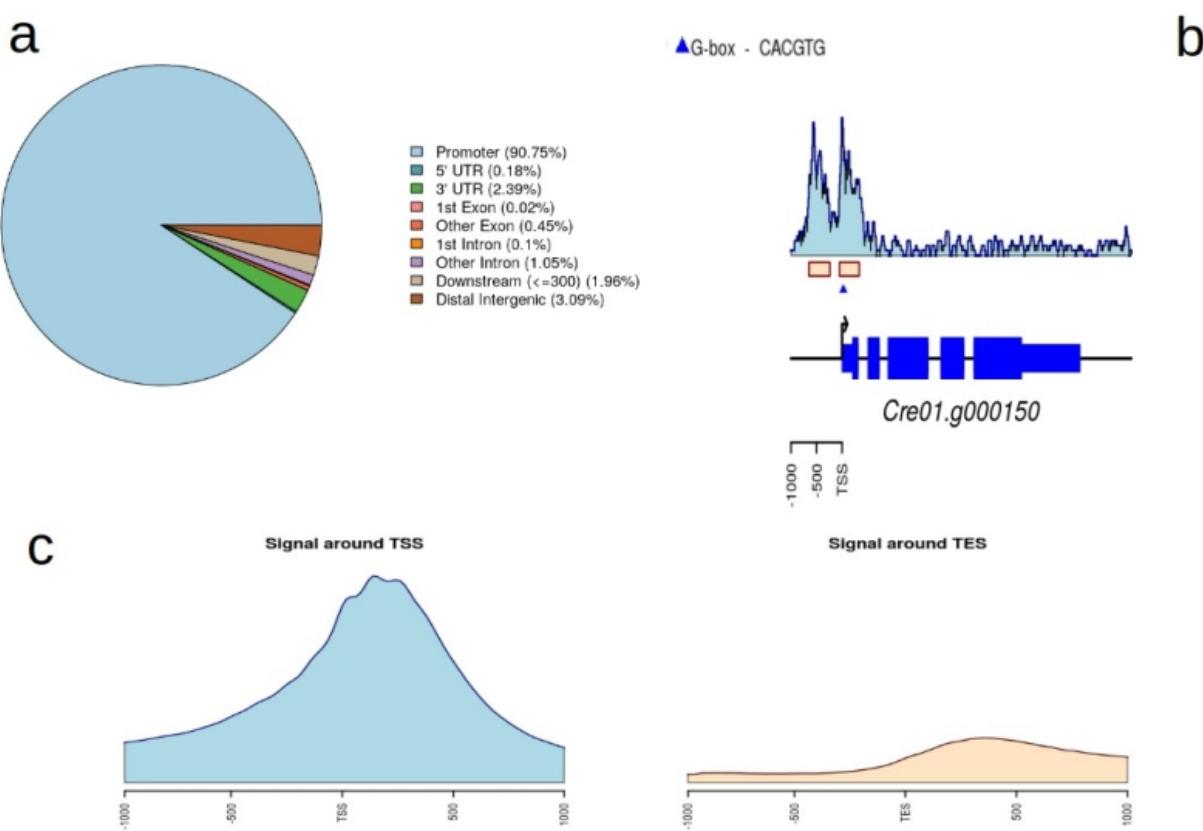


Figure 18: Summary of the outputs generated by ALGAEFUN when a genomic loci list is used as input. a) Pie chart representing the distribution of peaks or genomic loci over the different type of gene features such as the promoter, 3' UTR, 5' UTR, intron or exon; b) Visualization of the signal and identification of DNA motifs recognized by transcription factors and regulators in photosynthetic organisms for an individual gene; c) Visualization of the average level of signal around TSS and TES over the target genes.

As in this case study, when a BigWig file with the genome wide mapping signal is provided, specific marked genes can be selected to visualize the signal profile over their gene bodies and promoters. A gene example presenting two H3K4me3 peaks on its promoter is depicted to illustrate this functionality in Figure 18-b. Moreover, DNA motifs recognized by specific transcription factors and regulators in photosynthetic organisms can be identified in the promoter of the selected gene. Finally, a visualization of the average level of signal

around Transcriptional Start Site (TSS) and Transcriptional End Site (TES) across all marked genes is generated (Fig. 18-c). For the case of H3K4me3 in *Chlamydomonas* further evidence is obtained, showing that this epigenetic mark specifically and exclusively locates at the TSS of marked genes and not at the TES.

Contribution of ALGAEFUN with MARACAS to the field.

ALGAEFUN with MARACAS constitutes one of the first steps that has been taken for the development of tools that would enable the microalgae research community to exploit high throughput next generation sequencing data by applying systems biology techniques. The first difference between ALGAEFUN with MARACAS with respect to already existing tools consists in the wide range of supported microalgae species (Fig. 8). For the model microalgae *Chlamydomonas reinhardtii*, researchers can find several online tools to functionally annotate set of genes, such as Algal Functional Annotation Tool (López et al., 2011) and ChlamyNET (Romero-Campero et al., 2016). Only the online tool AgriGO (Tian et al., 2017) offers the possibility of analysing a restrictive number of different microalgae species beyond *Chlamydomonas*. The second biggest difference between ALGAEFUN and other tools is the annotation systems they use. Most available functional enrichment tools can only perform functional annotation of gene sets based exclusively on GO enrichment analysis. The identification of significantly enriched KEGG pathways in the inputted sets of genes is only supported by ALGAEFUN and Algal Functional Annotation Tool. A fundamental difference between ALGAEFUN and other tools consists of the statistical tests. Whereas AgriGO and ChlamyNET are based on Fisher's exact test, ALGAEFUN and Algal Functional Annotation Tool compute statistical significance according to Hypergeometric tests. It has been shown that, in general, the hypergeometric test has more statistical power than Fisher's exact and χ^2 (Masseroli et al., 2004). Moreover, none of these tools can be used as a complete and integrated tool to process high-throughput sequencing raw data from RNA-seq or ChIP-seq experiments, or functionally annotate genomic loci obtained from a ChIP-seq analysis. In this respect, ALGAEFUN with MARACAS improves and implements several novel functionalities of similar already existing software tools, Table 6.

Table 6: Comparison between ALGAEFUN with MARACAS and other functional enrichment analysis tools

	Algal functional annotation tool	AgriGo	ChlamyNET	ALGAEFUN with MARACAS
Gene sets as input	YES	YES	YES	YES
Genomic loci as input	NO	NO	NO	YES
GO enrichment	YES	YES	YES	YES
KEGG pathways enrichment	YES	NO	NO	YES
Several microalgae	NO	YES	NO	YES
Statistical test	Hypergeometric tests	Fisher's exact test	Fisher's exact test	Hypergeometric tests

ALGAEFUN with MARACAS is a constantly growing tool that will include new microalgae whenever their sequenced genomes are available. Also it has settle the bases to build numerous tools by other members of our laboratory aiming at generating an enabling technology for the microalgae research community doing systems biology studies.

Chapter 2: Transcriptomic analysis of diurnal and seasonal cycles in *Ostreococcus tauri*

High-throughput transcriptome sequencing produced approximately 10 million short reads per sample (Appendix 1). This allowed us to accurately estimate gene expression levels measured as FPKM (Fragments Per Kilobase of exon per Million reads mapped) in the transcriptomes corresponding to each data point of the time series. Indeed, out of the 7668 genes currently annotated in the *Ostreococcus tauri* genome (Blanc-Mathieu et al., 2014; Palenik et al., 2007), only 3 genes were never expressed and 260 genes never exceeded an expression level of 10 FPKM. This shows that practically the entire *Ostreococcus tauri* genome is expressed under seasonal and diurnal cycles. First, we focus in the 36 transcriptomes corresponding to the time points taken during three days under LD and SD conditions and perform a hierarchical clustering analysis in order to ensure the homogeneity of the samples taken. The transcriptomes corresponding to the same time points during the three different days clustered together (Fig. 19-A). This indicates a high circadian synchronization in the cultures. Moreover, these 36 transcriptomes assemble into three different groups (Fig. 19-A). The first cluster corresponds to midday, when irradiance is maximum, grouping the time points ZT4 and ZT8 under LD and ZT4 under SD. The second cluster conforms the dusk group. Here, the transcriptomes at time points ZT12 and ZT16 under LD and ZT8 under SD are grouped, coinciding with the end of the light period, when incident irradiance is low. The third cluster represents night/dawn and comprises the transcriptomes at time points ZT20, ZT0 under LD and ZT12, ZT16, ZT20 and ZT0 under SD. The transcriptomes at time points in the LD and SD nights or dark periods constitute two distinct groups suggesting noticeable differences in the transcriptomic responses during the night under LD and SD conditions. It is also noteworthy the higher similarity between the dusk, night/dawn transcriptomes when compare to the midday one (Fig. 19-A).

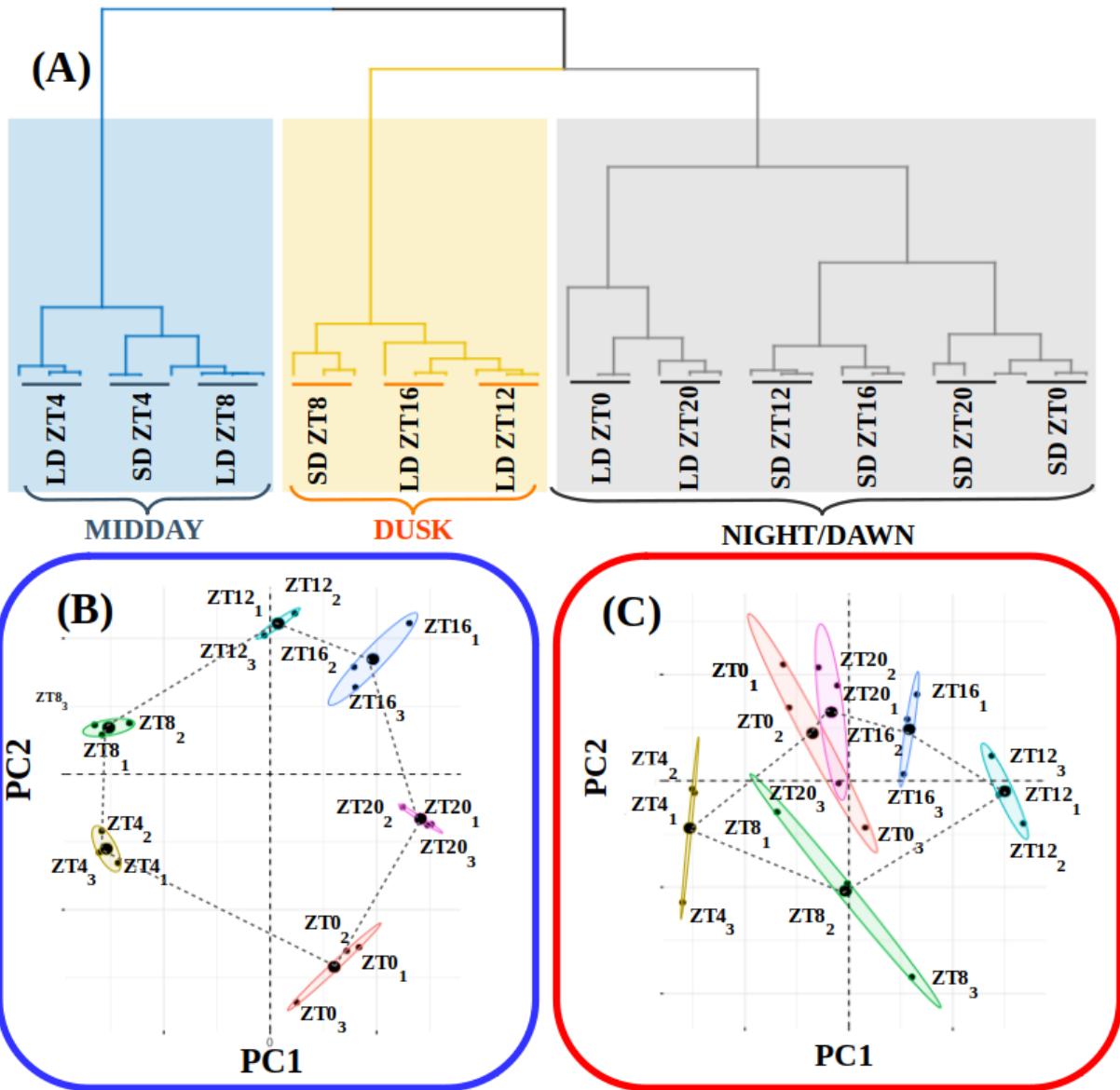


Figure 19: RNA reliability analysis. (A) Hierarchical clustering of the RNA-seq data corresponding to the 36 time points collected under alternating dark/light cycles simulating long and short day conditions. Three distinct clusters are observed corresponding to midday (blue rectangle), dusk (yellow rectangle) and night/dawn (grey rectangle). (B) Principal Component Analysis of the time point global transcriptomes under long day conditions. Small dots correspond to the 2D projection of each time point global transcriptome. Big dots correspond to the average of the three replicates 2D projections for each time point. Ellipses mark the 95% confidence regions corresponding to each time point global transcriptome. (C) Principal Component Analysis of the time point global transcriptomes under short day conditions. Points and ellipses are used as described before.

In order to obtain a deeper understanding of the underlying structure in these data we performed principal components analysis separately, over the LD (Fig. 19-B) and SD (Fig. 19-C) transcriptomes. Under LD condition, the transcriptomes corresponding to the same time

point in the three different days tightly cluster together globally, constituting a circular structure. Nonetheless, under SD conditions, more variability is observed and the time point transcriptomes form a structure resembling an ellipse. This could indicate that whereas in LD conditions gene expression in globally cycling precisely with a similar period, a more complex behavior is expected under SD conditions. In addition, it is remarkable the high similarity between the transcriptomes corresponding to ZT0 and ZT20 under SD conditions that is not present under LD conditions. This would suggest that the transcriptomic response at the end of a SD night is already preparing all molecular systems for the incoming light availability at dawn, whereas this anticipation is not so evidently observed under LD conditions. In overall, these results support that the experimental design grants a high level of synchronization in the data, allowing to proceed to the identification and comparison of genes exhibiting rhythmic expression patterns under LD and SD conditions.

Transcriptomic characterization of diurnal rhythmic expression profiles

Most genes in Ostreococcus tauri present diurnal rhythmic expression profiles under both photoperiods

Applying bioconductor R package RAIN (Rhythmicity Analysis Incorporating Non-parametric Methods) (Thaben & Westermark, 2014), genes exhibiting diurnal rhythmic expression patterns under both seasonal conditions have been identify. Specifically, time series consisting of three days with rhythmic light/dark periods were analysed. Independently from the photoperiod, more than 6000 genes comprising approximately 80% of the entire *Ostreococcus* genome present diurnal periodic rhythmic expression patterns. This result is in agreement with previous studies in *Ostreococcus tauri* (Monnier et al., 2010) under different photoperiods and other microalgae such as *Chlamydomonas reinhardtii* (Zones et al., 2015). The specific rhythmic genes under each photoperiod are practically coincident (Fig. 20-A) (Appendix Table 3).

The 20% of the genome of *Ostreococcus* that is apparently identified as non-rhythmic, present three time lower expression levels than rhythmic genes. This difference was significant according to a p-value of 1.45×10^{-4} computed using Mann-Whitney-Wilcoxon test (Fig. 20-B). Since the rhythmicity analysis method used requires high levels of expression to perform optimally (Laloum and Robison-Rechavi, 2020), it is possible that even these 20% of the genome is also rhythmic.

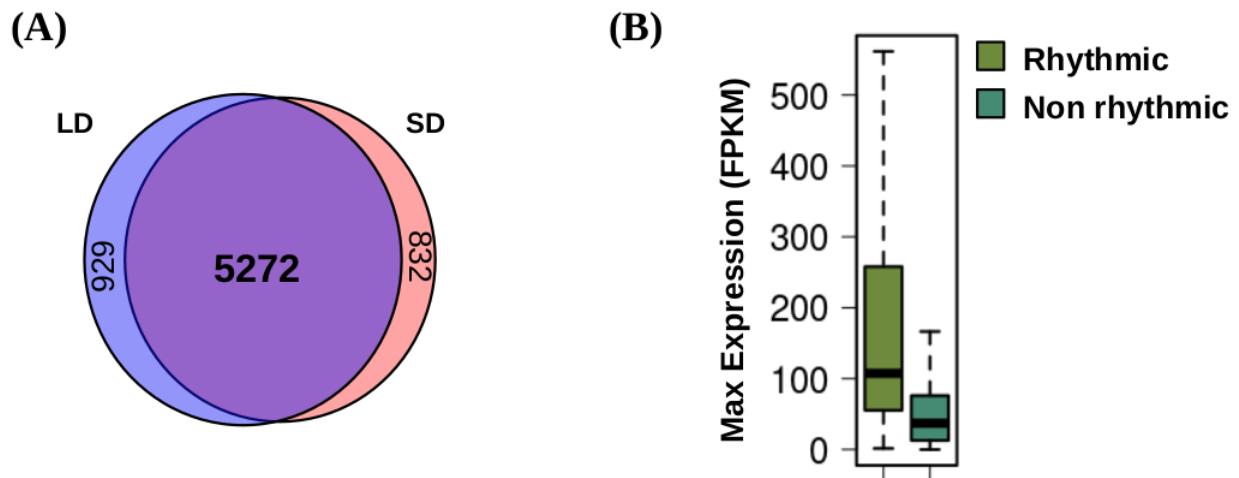


Figure 20: Diurnal rhythmic expression patterns detected in the transcriptome of *Ostreococcus tauri* under both photoperiods: (A) Venn diagram comparing rhythmic genes under LD conditions (light blue circle) and SD conditions (light red circle). (B) Boxplot representing the maximum expression level of rhythmic genes (light green) and non-rhythmic genes (dark green). Gene expression levels are measured as FPKM (Fragments Per Kilobase of transcript per Million fragments mapped).

Under free running conditions rhythmicity is maintained in different proportions depending on the photoperiod of entrainment

As it was described previously in the introduction, *bona fide* circadian processes are self-sustained and maintain their rhythmicity even when their specific zeitgeber, or synchronizing environmental signal, becomes constant. Following this definition, *bona fide* circadian genes can be identified and distinguished from light or dark responding ones from data generated under free-running conditions consisting in constant light (LL) and constant dark (DD). This also allows to identify rhythmic genes sets for which the main zeitgeber is light or dark as those maintaining rhythmicity under constant light (LL) or constant dark (DD) respectively. One of the main observations is that the maintenance of rhythmic expression profiles is dependent on the photoperiod of entrainment. This supports the key role played by seasonality in establishing the global state of *Ostreococcus* transcriptomes.

Although genes with rhythmic expression profiles under LD (6201 rhythmic genes) and SD conditions (6104 rhythmic genes) were almost coincident (Fig. 20-A), their rhythmicity was not maintained equally under free running conditions. Specifically, 2804 genes (36.57% of the entire genome) with a previous LD entrainment maintained their rhythmicity under constant light (LL) conditions (Fig. 23-A). The number of genes that after SD entrainment maintained their rhythmicity under LL conditions decrease to 1526 (19.9% of the entire genome) (Fig. 23-C). However, 3311 genes after LD entrainment and 4006 genes after SD entrainment maintained their rhythmicity under constant dark conditions (DD) (Fig. 23-A,C). This indicates that constant dark has a synchronizer effect over a larger part of the *Ostreococcus* transcriptome than constant light. In other words, that dark is the main zeitgeber for the expression of more genes than light. This was specially evident for rhythmic gene expression under SD conditions which was found very dependent on the presence of a dark period or skotoperiod.

Regulatory mechanisms are often composed of large networks influenced by a wide range of inputs. Circadian clocks are strongly influenced by external environmental signals but there exists a complex interplay between the clock and cell physiology as well (Mazzoccoli et al., 2020; Morris et al., 2020). Genes maintaining their rhythmic expression profiles only under LL or DD are partially regulated by diurnal changes in the circadian clock. They are influenced by other regulatory mechanisms as light (Fig. 23-B) or dark (Fig. 23-D).

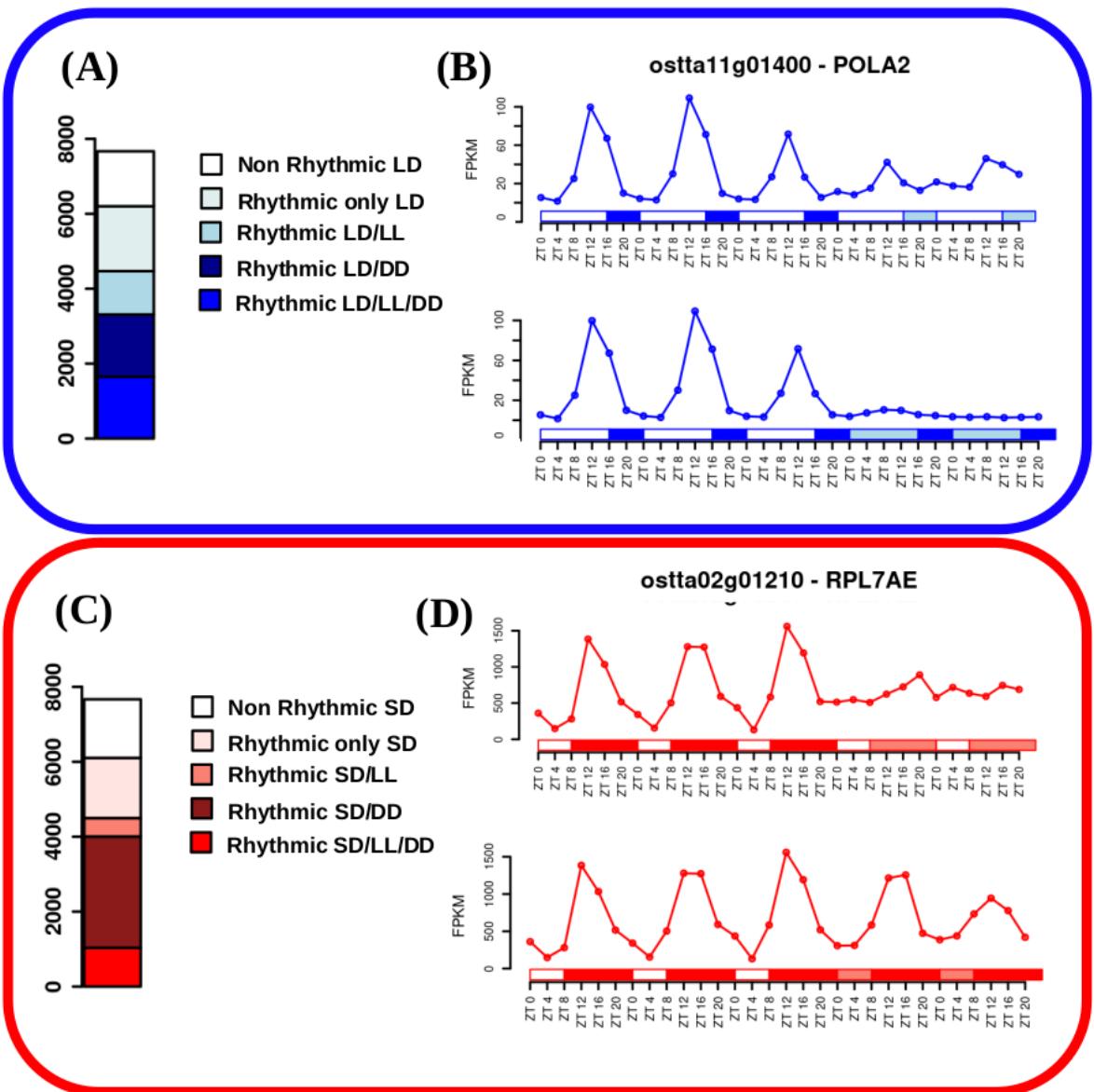


Figure 23: Rhythmicity maintenance under free-running conditions: (C) Barplot representing with blue colors different rhythmic gene sets under LD conditions. From bottom to top: circadian genes exhibiting rhythmicity under LD, constant light (LL) and constant dark (DD); rhythmic genes under LD and DD requiring a dark input; rhythmic genes under LD and LL requiring a light input; rhythmic genes only under LD and non-rhythmic genes. (D) Gene expression profiles under LD, LL and DD of DNA polymerase alpha subunit B (*ostta11g01400*, *POLA2*). (E) Barplot representing with red colors different rhythmic gene sets under SD conditions. From bottom to top: circadian genes exhibiting rhythmicity under SD, LL and DD; rhythmic genes under SD and DD requiring a dark input; rhythmic genes under SD and LL requiring a light input; rhythmic genes only under SD and non-rhythmic genes. (F) Gene expression profiles under SD, LL and DD of Ribosomal protein L7Ae (*ostta02g01210*, *RPL7AE*).

For example, one of the enriched GO biological processes in the set of genes that maintain their rhythmicity only under LL, for which light is the primary zeitgeber, was DNA repli-

cation (ANEXO). It agrees with previous cell cycle studies in microalgae like *Euglena* (Kato & Nam, 2021), *Chlamydomonas* (Donnan & John, 1983; Zones et al., 2015) and *Ostreococcus* (Moulager et al., 2007, 2010), suggesting that cell cycle have a strong circadian clock regulation, as well as G1 phase is light-dependent due to the need of light to grow in photosynthetic organisms. This provides evidence for the need of a light input to maintain rhythmicity in genes involved in DNA replication. Whereas, the main enriched GO biological processes in the set of genes that maintain their rhythmicity only under DD are RNA processing and ribosome biogenesis (ANEXO). These processes are known to have a complex regulatory mechanism influenced by the circadian clock as well as other regulatory machinery. They are commonly programmed at transcriptomic level to take place during the night, so translation of proteins can be achieved during the day (Merchant et al., 2017). It is logical that a dark input is needed to maintain rhythmicity of those genes since their activation is dark-dependent.

In addition, comparison of the gene sets maintaining rhythmicity under both DD and LL free-running conditions after both, LD and SD entrainment, allow us the identification of what is called *bona fide* circadian genes (Fig. 24-A). *Bona fide* circadian rhythms are the ones maintained under every condition (both photoperiods of entrainment and both free-running conditions) and are predominantly regulated by diurnal cyclic changes in the circadian clock. In our data, there are only 350 genes (comprising 4.6% of the entire *Ostreococcus* genome) that do not present either a flat or noisy profile under any of the studied conditions. Gene expression profiles of RuBisCO activase (ostta04g02510, RCA) under the different conditions exemplifies how *bona fide* circadian genes maintain their rhythmicity (Fig. 24-B).

In previous work, RuBisCO activase (RCA) and RuBisCO small subunit (RBCS) mRNA were quantified in *Arabidopsis* under a light dark cycle, constant light and constant darkness conditions (Pilgrim & McClung, 1993). Their results are in agreement with our transcriptomic data in *Ostreococcus*, where the gene expression profile of RuBisCO activase (ostta04g02510, RCA) under the different conditions exemplifies how *bona fide* circadian genes maintain their rhythmicity (Fig. 24-B).

A functional GO enrichment analysis over this set of genes showed that they are mainly involved in the biological processes photosynthesis, chlorophyll metabolic/biosynthetic process and chloroplast organization, among others (Fig. 24-C). Some of those processes

are known to present a circadian physiological activity in plants and microalgae like *Euglena*, but there is a lack of confirmation at the transcriptomic level in most cases like the one provided here for Ostreococcus (Cumming & Wagner, 1968; Noordally & Millar, 2015; Panter et al., 2019).

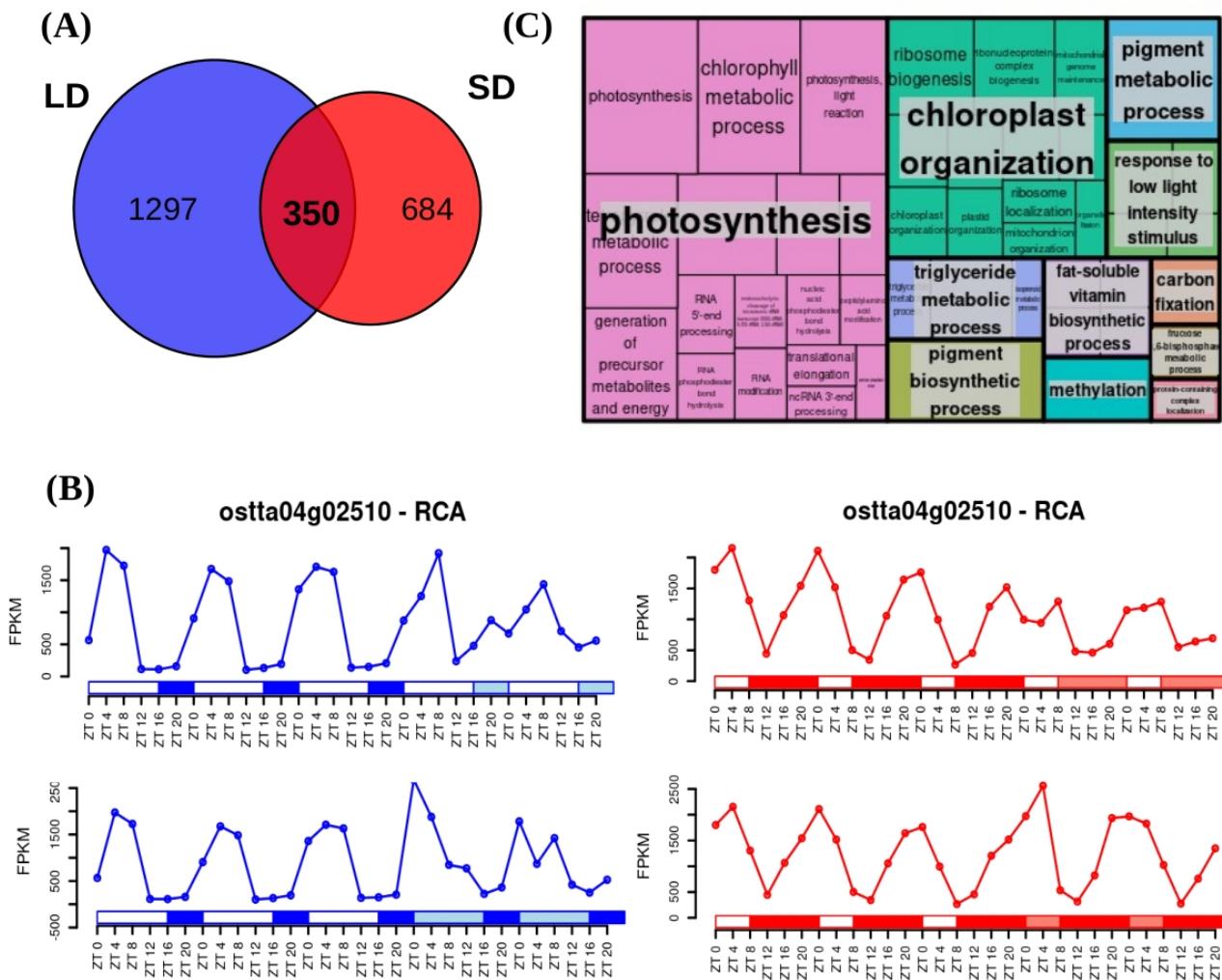


Figura 24: Identification of bona fide circadian genes and their functional enrichment analysis: (A) Venn diagram comparing circadian genes identified after LD entrainment (blue circle) and after SD entrainment (red circle). (B) Gene expression profiles under LD, SD, LL and DD of RuBisCO Activase (ostta04g02510, RCA). (C) Treemap summarizing the biological processes significantly enriched over the bona fide circadian genes or rhythmic genes under LD, SD, LL and DD. Rectangle sizes represent significance levels. Semantically similar biological processes are grouped together into the same colored rectangles. The most representative biological process is shown for each rectangle.

Constant light and constant darkness as free-running conditions have different effects over the transcriptome of *Ostreococcus*

In order to distinguish between oscillating genes predominantly regulated by the circadian clock, *bona fide* circadian genes, from those genes that exhibit oscillations as a response to the alternating light/dark cycles free running conditions consisting of constant light and constant dark is considered. The effect of the transition to constant light (LL) or dark (DD) on rhythmic gene expression patterns was performed using the co-sinusoidal parametric method implemented in the R package *circacompare*. Two rhythmic parameters, amplitude and phase, were compared between the expression profiles under LD or SD to the corresponding ones under LL and DD.

When comparing LD and LL gene expression patterns, an amplitude decrease in the rhythmic expression profiles was observed. Specifically, most LL rhythmic genes after LD entrainment (97.68%) that presented a decrease in amplitude when transferred to LL (Fig. 21-A), being significant in more than half of them. A positive (forward) phase shift (increasing phase) is observed under LL when compared to LD in 76.28% of LL rhythmic genes being significant in 14.98% of them. In contrast,, when comparing LD with DD gene expression patterns, the reduction in amplitude is less widespread (Fig. 21-A), being observed in 79.43% of the DD rhythmic genes with significance in 31.62% of them. Opposite to the LL effect, negative (backward) phase shifts (decreasing phase) were observed in DD rhythmic gene expression profiles. Specifically, 87.32% of the DD rhythmic genes after LD entrainment presented an anticipation in phase that was significant in 34.16% of them. Most of them present negative (backward) phase shifts, whereas under LL condition phase shifts are positive (forward) both between 0 and 5 h of difference (Fig. 21-B).

The global reductions of LL and DD amplitude in rhythmic gene expression profiles when compared to LD amplitude (Fig. 21-A) are significant with p-values 4.23×10^{-140} and 1.09×10^{-60} respectively. More precisely, the reduction in amplitude is significantly lower under LL than under DD with a p-value of 6.71×10^{-28} .

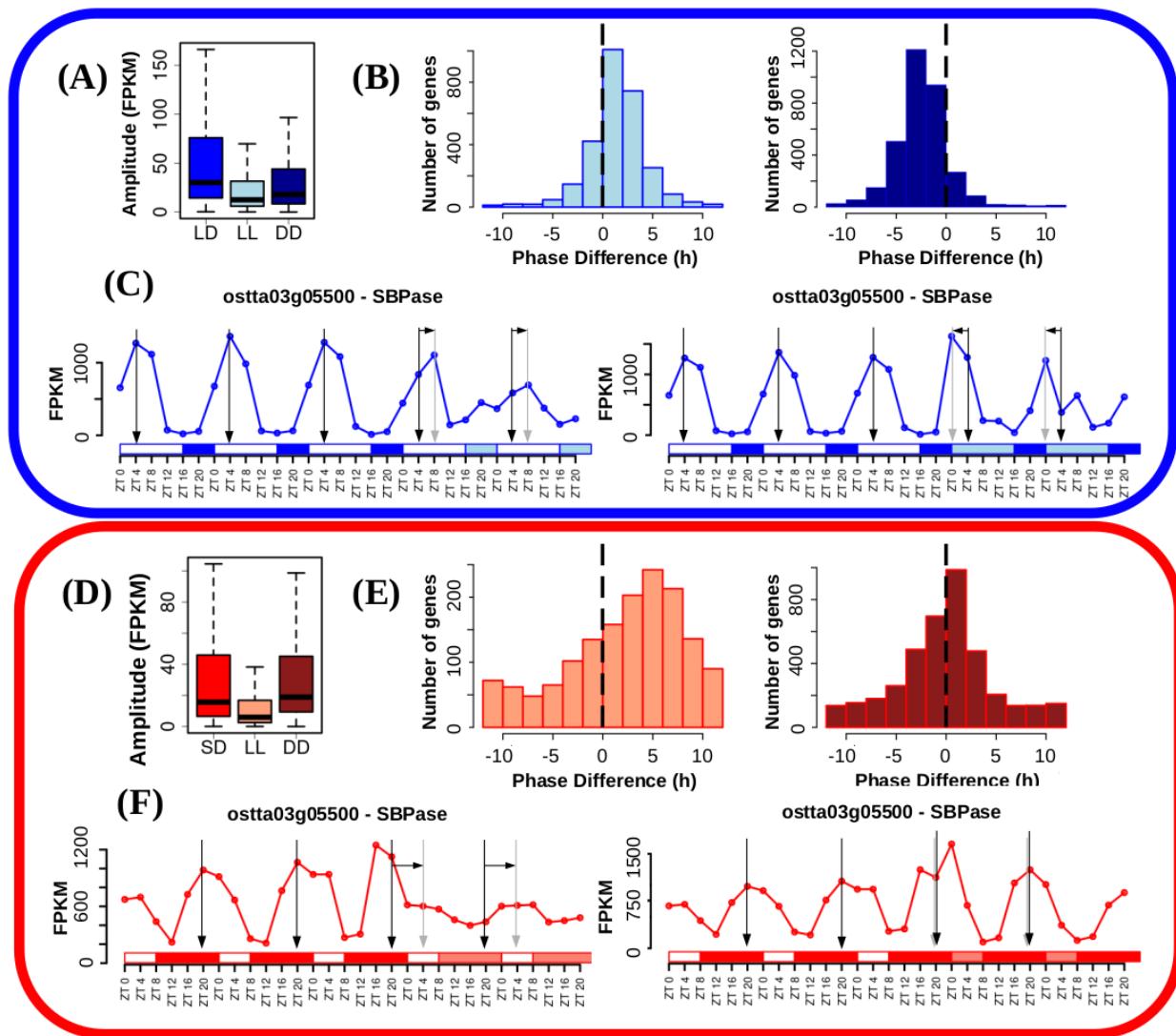


Figure 21: Free running conditions effects over gene expression profiles. (A) Boxplot representing rhythmic genes amplitude reached under long day conditions (blue), when cultures were kept under free running conditions consisting of constant light (light blue) and when cultures were kept under free running conditions consisting of constant dark (dark blue). (B) Histograms showing the distribution of the number of genes exhibiting positive and negative phase shifts under LL (light blue, left) and DD (dark blue, right) free running conditions when compared to LD. Vertical dashed lines mark no shift. (C) Gene expression profiles under LD, LL and DD of Sedoheptulose-bisphosphatase (*ostta03g05500*, SBPase). Vertical black arrows mark LD phases, vertical grey arrows mark LL and DD phases and horizontal black arrows represent phase shifts. (D) Boxplot representing rhythmic genes amplitude under short day conditions (red), when cultures were kept under free running conditions consisting of constant light (light red) and when cultures were kept under free running conditions consisting of constant dark (dark red). (E) Histograms showing the distribution of the number of genes exhibiting positive and negative shifts in phase or maximum expression level time point under LL (light red, left) and DD (dark red, right) free running conditions when compared to SD. (F) Gene expression profiles under LD, LL and DD of Sedoheptulose-bisphosphatase (*ostta03g05500*, SBPase).

Next we analyze free-running conditions effects over rhythmic genes with previous SD entrainment. First, comparing their expression profiles under SD and LL, it can be observed that amplitude decrease is more drastic than the one observed for LD entrainment. Specifically, most LL rhythmic genes (87.9%) presented a drastic decrease in amplitude when transferred to LL (Fig. 21-E), being significant in 34.6% of them. Similar to the behavior after LD entrainment, a positive (forward) phase shift around 5 h of difference (Fig. 21-F,G) was also observed in 68.28% of LL rhythmic genes after SD entrainment, being significant in 14.98% of them. However, when comparing SD and DD gene expression patterns, the reduction in amplitude was almost non-existent (Fig. 21-E), being observed only in 35.97% of the DD rhythmic genes with significance in 23.58% of them. Negative (backward) phase shifts were observed in 47.7% of the DD rhythmic genes after SD entrainment with significance in 28.83% of them. Nonetheless, globally most genes have a phase shift around 0 (Fig. 21-F), suggesting that phase is not drastically affected under DD after SD entrainment (Fig. 21-G).

The global drastic amplitude reduction in LL rhythmic gene expression profiles after SD entrainment was significant with a p-value 5.95×10^{-65} (Fig. 21-D). In contrast to what was observed after LD entrainment, a slight but significant increase in amplitude under DD was detected when compared to SD with a p-value of 2.50×10^{-8} (Fig. 21-D). This suggests a highly similarity between SD and DD conditions, since there are not significant differences between their maximum level of expression (amplitude) and the time when the maximum level of expression is reached (phase), probably due to long periods of dark entrainment.

Free-running conditions have been widely studied in nocturnal mammals (Bartoszewicz et al., 2010; Hundahl et al., 2012; Imai et al., 2020), plants (Edwards et al., 2010; Ohara et al., 2015) and other organisms (Biebach et al., 1991; Vatakis et al., 2018). The effect over the amplitude of biological rhythms has been previously observed at different biological levels and is commonly associated with a loss of synchrony (Paajanen et al., 2021; Vatakis et al., 2018) (Fig 22).

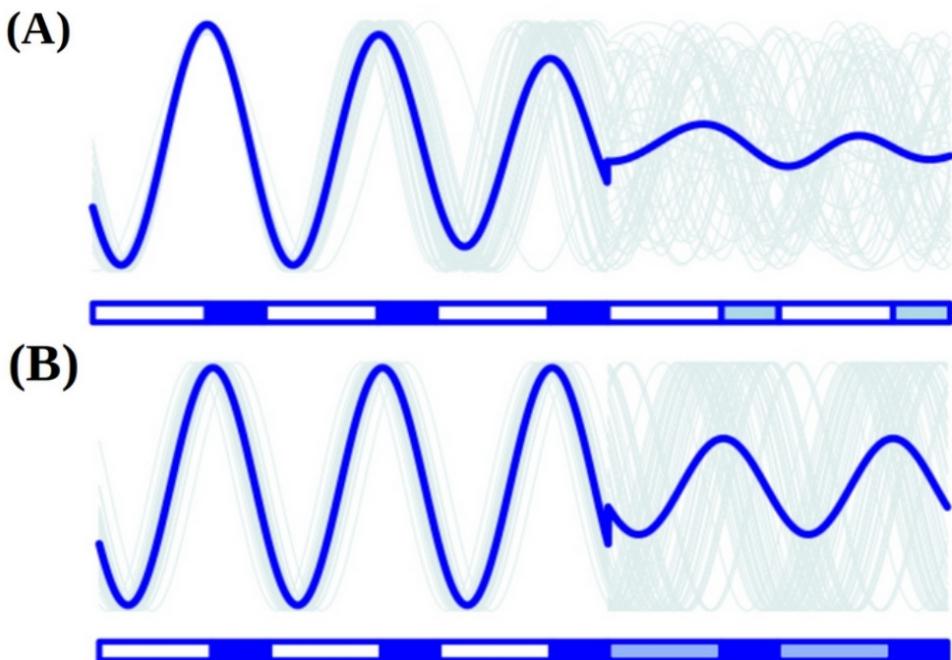


Figure 22: Reductions in amplitude under free running conditions can be explained by a decline in culture synchrony. (A) Culture average gene expression profile under LD and constant light (LL) is represented by a thick blue line. Examples of individual cell gene expression profiles under LD and LL are represented by thin grey lines. (B) Culture average gene expression profile under LD and DD is represented by a thick blue line. Examples of individual cell gene expression profiles under LD and DD are represented by thin grey lines.

Therefore, although *Ostreococcus* growth is dependent on photosynthesis and thus on light, LL conditions promote a larger loss of synchrony than DD conditions at the transcriptomic level. When cultures are transferred to LL, cells get strongly desynchronized and their individual gene expression profiles become out of phase. This results in a strong reduction in the amplitude of the culture average gene expression profile although individual gene expression profiles maintain the same amplitude. However, under DD condition, cells get moderately desynchronized causing a slight reduction in the amplitude of the culture average gene expression profile.

The free-running rhythms observed in *Ostreococcus* are possibly found in other photosynthetic organisms as well. There is a huge lack of research of this topic but positive and negative phase shifts under LL and DD, respectively, have been also observed in algal-coral symbiosis content in photosynthetic pigments (Sorek et al., 2013).

Light is currently considered the primary transcriptional zeitgeber or synchronizing signal in plants disregarding the relevance of dark periods in regulating diurnal rhythms (Wenden et

al. 2011 Light inputs shape the *Arabidopsis* circadian system, Wang et al. 2022 Circadian entrainment in *Arabidopsis*). Our results provide evidence of the importance of dark periods also in synchronizing diurnal cycles in photosynthetic organisms, since ~~in contrast, to other photosynthetic organisms~~ (Okada et al. 2017 Synchrony of plant cellular circadian clocks with heterogeneous properties under light/dark cycles), LL conditions exerted a stronger desynchronizing effect than DD conditions. Moreover, the LL and DD effects observed over the transcriptomes of *Ostreococcus* in this study (stronger desynchronization under LL, positive phase shifts under LL and negative phase shifts under DD) are typically described in nocturnal animals, where constant light is commonly used as a circadian disruption model. This suggest for the first time a strong dependence on dark periods in *Ostreococcus* to correctly regulate and programme its transcriptome.

Light is currently considered the primary transcriptional zeitgeber or synchronizing signal in plants disregarding the relevance of dark periods in regulating diurnal rhythms (Wenden et al. 2011 Light inputs shape the *Arabidopsis* circadian system, Wang et al. 2022 Circadian entrainment in *Arabidopsis*). Moreover, the effects of both free running conditions observed over the transcriptome of *Ostreococcus* in this study (stronger desynchronization under LL, positive phase shifts under LL and negative phase shifts under DD) actually agree with the activity circadian records of nocturnal animals, where DD conditions have been extensively studied. In fact, constant light is commonly used as a activity circadian disruption model in those organisms (Bartoszewicz et al., 2010; Hundahl et al., 2012; Imai et al., 2020; Vatakis et al., 2018), which agrees with the strongly desynchronizing effect over the transcriptome of *Ostreococcus*. This results provide evidence of the importance of dark periods also in synchronizing diurnal cycles in photosynthetic organisms for the first time, as well as a predominant nocturnal character of *Ostreococcus* transcriptome.

Transcriptomic characterization of seasonal effects over gene expression profiles

Seasonal changes induce changes in amplitude and phase over gene expression profiles.

Photoperiodic dependent changes in amplitude and phase shifts in biological rhythms have been already described in mammals (Messager et al., 2000; Sumová et al., 2003; Van Dongen et al., 1997; Wucher et al., 2022), as well as in plants and microalgae (Flis et

al., 2016; Panter et al., 2019; Serrano et al., 2009). Depending on the organisms under study increments or decrements in amplitude as a response to shortening or lengthening photoperiods have been reported. Specifically, when biological rhythms are studied globally in *Arabidopsis thaliana*, phase shifts as an adaptation to changes in photoperiod has been described but no changes in amplitude were observed (Flis et al., 2016). A similar effect is observed in *Chlamydomonas* in specific genes (Serrano et al., 2009). In both studies, differences in amplitude are only found in some biological rhythms when they are studied individually. For example, the rhythmic expression profile of the gene *CrCO* potential ortholog of the *CONSTANS* gene in *Arabidopsis thaliana* shows an increase in amplitude under SD conditions as well as a negative (backward) phase shift.

In *Ostreococcus* transcriptome, global changes affecting the amplitude in gene expression patterns have been found. Specifically, SD amplitudes are significantly reduced with respect to LD according to a p-value of $1.16e10^{-111}$ computed using Mann-Whitney-Wilcoxon (Fig. 25-A). A high number of rhythmic genes (2036 genes) exhibited a significant amplitude decrease in their rhythmic expression profiles, whereas, only 123 significantly increased their amplitude. This suggests a decline in culture synchrony under SD, accordingly to the previous similar synchronization observed between DD and SD conditions (Fig. 21-D).

Negative phase shift or phase anticipation were also globally observed over the transcriptome of *Ostreococcus*, in agreement with what has been described in *Arabidopsis* and *Chlamydomonas* for some specific genes. Specifically, 3424 genes comprising 64.95% of the rhythmic genes exhibited a significantly anticipated phase under SD condition when compared to LD condition. Phase anticipation were apparent under SD since gene phases are mostly reached around SD midnight (ZT12 to ZT16) whereas, under LD, phases are uniformly distributed from LD dusk (ZT12) to the end of the night (Fig. 25-B). Only a low number of rhythmic genes exhibit their phase or maximum level of expression during the light period in LD and SD. This indicates that the main activity at the transcriptomic level takes place during the night in *Ostreococcus*, which supports the nocturnal character of its transcriptome as described previously when analyzing the effects of free running conditions, namely, desynchronization and positive (forward) phase shifts under LL and increase in synchronization and negative (backward) phase shifts under DD.

Figure 25-C shows expression profiles of *Cyclin B* (*ostta01g06150*, *CYCB*) and *Delta-9 acyl-lipid desaturase 1* (*ostta01g00790*, *ADS1*) as two examples of genes exhibiting the typical phase anticipation and amplitude reduction under SD condition when compared to LD condition.

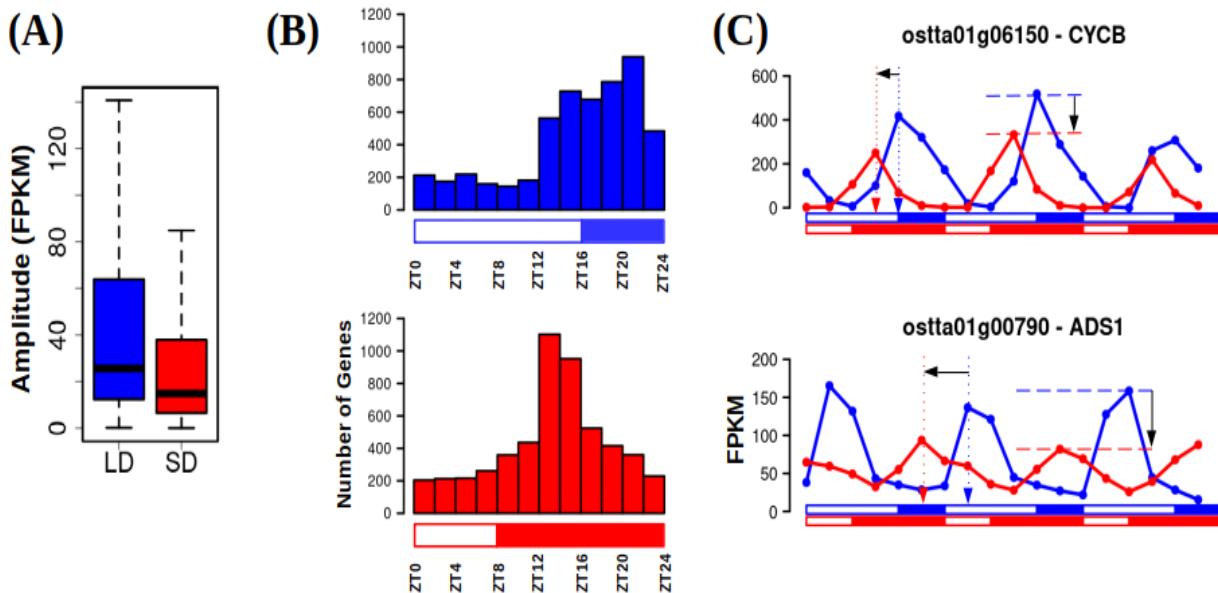


Figure 25: Photoperiod changes cause amplitude reductions and phase shifts over gene expression profiles. (A) Boxplot representing rhythmic genes amplitude or maximum expression level reached under LD and under SD. SD amplitudes are significantly reduced with respect to LD according to a p -value of 1.16×10^{-111} computed using Mann-Whitney-Wilcoxon test. (B) Histograms showing the distribution of the number of genes with phase or maximum expression level at specific time points during the day under LD conditions (blue, top) and SD conditions (red, bottom). (C) Gene expression profiles under LD (blue line) and SD (red line) of *Cyclin B* (*ostta01g06150*, *CYCB*, top) and *Delta-9 acyl-lipid desaturase 1* (*ostta01g00790*, *ADS1*, bottom). Blue and red vertical dotted arrows mark LD and SD phases. Horizontal black arrow represents backward phase shifts under SD when compared to LD. Blue and red horizontal dashed lines mark LD and SD amplitudes. Vertical black arrows represent the reductions in amplitude under SD with respect to LD.

Seasonal changes promote the emergence of 12 h period cycles

When comparing SD and LD conditions, another phenomenon has been identified in the transcriptome of *Ostreococcus* emerging as a response to photoperiod shortening. Under LD condition, almost every rhythmic gene (5825 genes covering 75.97% of the entire genome) reached its maximum level of expression once a day, presenting one single peak every 24h in its expression profile (Fig. 26-D, E). Under SD conditions the number of genes presenting one single expression peak per day decreases to 4249 (55.41% of the

entire genome). This is coupled to an increasing number of genes, namely 1855 genes, presenting a more complex rhythmic expression profile with two expression peaks per day (every 12 h) (Fig. 26-D, E).

Biological rhythms with 12 h periods (two peaks per day) have been described from marine organisms to mammals including humans. It is hypothesized that 12 h rhythms in gene expression and metabolism in terrestrial organisms is reminiscent of the ~ 12 h circatidal rhythms of coastal and estuarine organisms (Ballance & Zhu, 2021). The maintenance of 12 h rhythms after evolving to live on land is hypothesized to provide an advantage in the adaptation to metabolic stress that peak at transition periods during the diurnal cycle (Pan et al., 2020; B. Zhu et al., 2017).

Bimodal rhythms presenting two peaks per day not necessarily separated by 12h that are maintained under free running conditions have been observed in animals (Binkley & Mosher, 1985; Foà & Bertolucci, 2001; Kyorku & Brady, 1994; Prabhakaran & Sheeba, 2012; Watanabe et al., 2007), plants (Hayes et al., 2010; Van Gelderen, 2020) and some microalgae like Euglena (Mohabir & Edmunds, 1999). Nevertheless, they have been described only for specific processes, genes or compounds, not as a global response in the transcriptome to external signals. Seasonal cycles were thought to have some effect over these rhythms but, in the majority of the studies, different photoperiods were not studied or no difference between them was detected (Prabhakaran & Sheeba, 2012). There is only an observation of a bimodal biological rhythm detected in ruin lizards that is apparently strongly dependent on photoperiod length and is also maintained under free-running condition (Foà & Bertolucci, 2001).

Nonetheless, 12 h period rhythms in gene expression emerging under shorter photoperiods have gone mostly unnoticed and thus there is no hypothesis about its biological role so far. We have found this type of rhythmic gene expression when reanalyzing already published transcriptomic data. As an example, bimodal gene expression patterns were identified in a published microarray data set generated from cultures of *Ostreococcus tauri* under neutral day conditions (12 h light:12 h dark) (Monnier et al., 2010). Most of the rhythmic genes presented rhythmic expression profiles with a 24 hours period and, thus, a single expression peak. However, 1171 genes presented rhythmic expression pattern with an apparent period of 12 hours (two peaks of expression per day). This photoperiod can be

considered an intermediate step between the two extreme photoperiods studied in this thesis (LD and SD). Indeed, the data show an intermediate number of genes with two peaks of expression per day between the ones identified in this thesis for LD and SD conditions. Also, in agreement with our results, this type of rhythmic patterns can be found in data generated from other organisms like *Chlamydomonas* under neutral day (Zones et al. 2015). This suggests that, at least in *Ostreococcus* (and possibly other microalgae), there is an increasing number of genes that reach their maximum level of expression twice a day (every 12h) as the photoperiod get shorter although its biological role remains to be determined.

Tides play a central role in the dynamics of the natural environment of *Ostreococcus* as a marine organism. Tides take place following rhythms of approximately 12 h and can give rise to circatidal gene expression patterns. These rhythms must persists under free running conditions. This was discarded in our case since the observed 12 h period rhythmic gene expression profiles in *Ostreococcus* transcriptome under SD are not maintained under free-running conditions. Instead, bimodality disappears and only one of the expression peaks was maintained under constant light whereas the other one persisted only under constant darkness (Fig. 26-C).

Our results suggest that the seasonal effect observed over the transcriptome of *Ostreococcus* is not a self-sustained 12 h rhythm, but a combination of two distinct rhythmic profiles: one dependent on the photoperiod (maintaining its rhythmicity only under LL) and another one sustained by the skotoperiod (maintaining its rhythmicity only under DD). This was supported by a decomposition performed over the bimodal gene expression profiles present under SD conditions into two co-sinusoidal unimodal profiles. Indeed, each one of the profiles was coincident with the one maintained under LL and DD respectively. Moreover, this decomposition produces a possible explanation for the presence of bimodality under SD conditions and its absence under LD conditions. Whereas the light-dependent expression profile does not change as the photoperiod shortens the dark-dependent expression profile is shifted as the skotoperiod lengthens. In this way, under LD both profiles are coincident in phase and they cannot be distinguished from one another. Nevertheless, as the skotoperiod lengthens the phase of the dark-dependent profile is shifted. Therefore, both profiles become out of phase under SD conditions unveiling a joint but independent

regulation exerted by the photoperiod and skotoperiod in the expression of the corresponding gene. (Fig. 26-C).

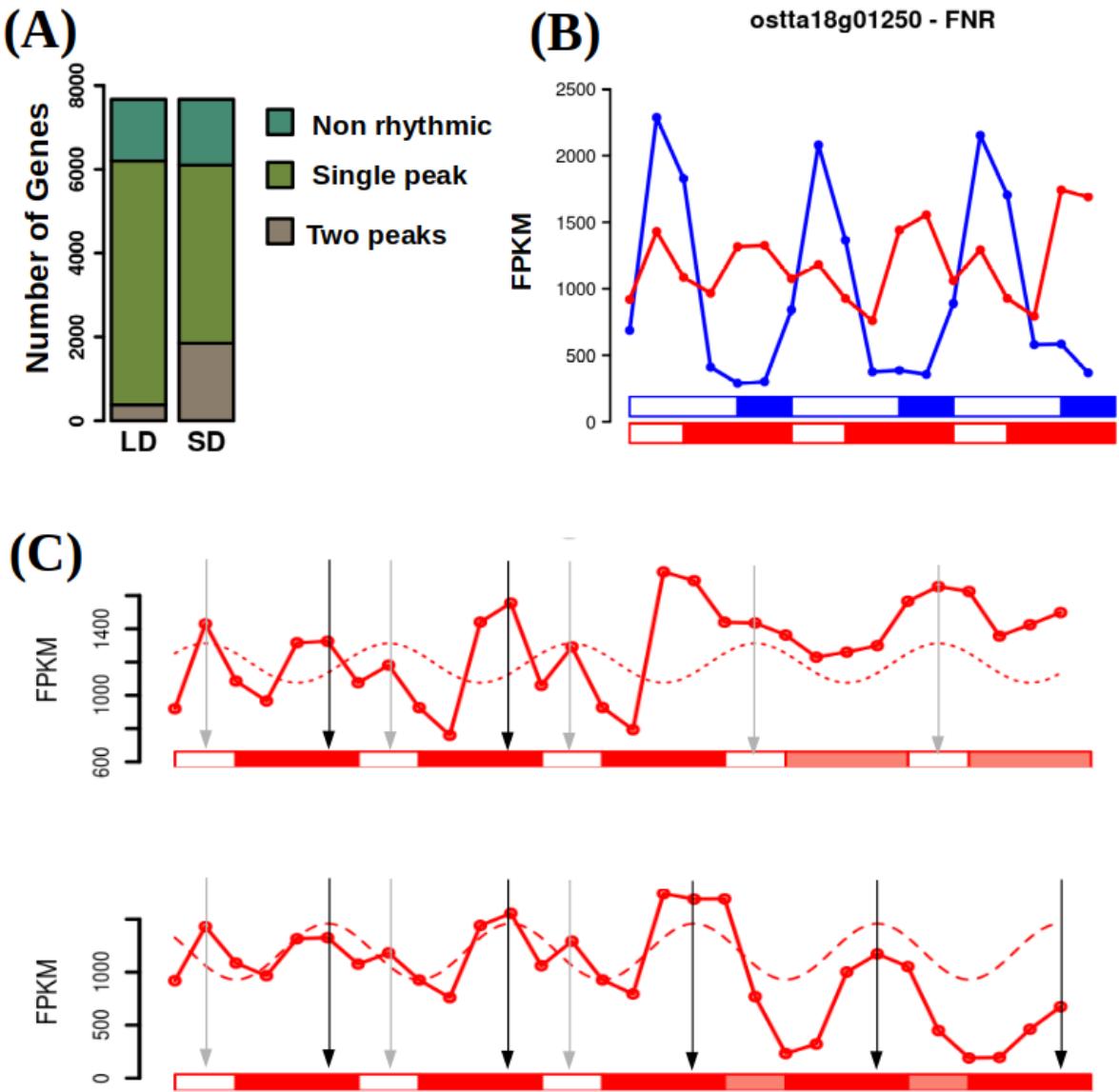


Figure 26: Emergence of 12 h period rhythmic expression profiles under SD. (A) Barplot representing in different green colors the number of non rhythmic, single peak rhythmic and two peaks rhythmic genes under LD and SD conditions. (B) Gene expression profiles under LD (blue line) and SD (red line) of Ferredoxin-NADP⁺ reductase (*ostta18g01250*, FNR). (C) Gene expression profiles under SD and free running conditions consisting of constant light (LL) or constant dark (DD) of Ferredoxin-NADP⁺ reductase (*ostta18g01250*, FNR). This gene exemplifies how two peaks expression patterns under SD conditions could emerge as the combination of two distinct rhythmic profiles. One depending on the photoperiod (dotted line) with phase marked with a grey vertical arrow maintaining its rhythmicity only under LL (top). Another expression profile is apparent depending on the skotoperiod (dashed line) with phase marked with a black vertical arrow maintaining its rhythmicity only under DD (bottom).

Seasonal cycles induces distinct temporal transcriptional programs organizing biological processes during diurnal cycles

In order to determine the temporal organisation of the transcriptional program in *Ostreococcus* under summer LD and winter SD conditions, genes were clustered depending on their phase or time of maximum expression level. A functional enrichment analysis of the resulting gene clusters revealed the cellular processes activated at the transcriptomic level in each temporal point (Fig. 27-28). Focusing on the most enriched processes in each time point, a transcriptional temporal map of *Ostreococcus* under each photoperiod is illustrated in Fig. 29.

RNA-associated protein 14 (*ostta04g00770*, *Utp14*) and the *Ribosome Biogenesis Factor BMS1* (*ostta05g01080*, *BMS1*) (Supp 8). During the first part of the morning (ZT4) *Ostreococcus* transcriptome is almost completely focused in genes involved in translation, such as *eukaryotic Initiation Factor 2* (*ostta03g02100*, *elf2*) and *translation elongation factor P* (*ostta03g03015*, *YelP*). The genes mainly involved in photosynthesis but also in carbohydrate metabolism reach their maximum expression level during midday, when irradiance is maximum (ZT8). Some of those genes are subunits of both photosystems: *ostta01g03170* (*PsbP*), *ostta02g00580* (*PsaL*), *ostta02g02560* (*PsbX*), *ostta02g03860* (*PsaE*), *ostta04g01790* (*PsaF*), *ostta05g04560* (*PsbR*), etc. During the afternoon (ZT12), the activation of genes involved in DNA replication takes place. Some minichromosome maintenance proteins *ostta01g02580* (*MCM6*) and *ostta05g01680* (*MCM9*) are found, as well as the *proliferating cell nuclear antigen* *ostta06g02890* (*PCNA*) which is central to the DNA replication process. Intracellular transport and cellular respiration are the two most prominent biological processes whose genes reach their maximum expression level at dusk (ZT16) under LD conditions. Genes like *lysophospholipases* *ostta01g04440* (*CLC*) or *nucleoporins* *ostta14g02210* (*Nup133*) are some examples for this time point. Finally, during midnight, *Ostreococcus* focuses on expressing genes involved mainly in cellular amino acid metabolic process, like *3-Deoxy-D-arabinoheptulosonate 7-phosphate () synthase* *ostta06g03270*, (*DAHP*) which encodes the first enzyme in the biosynthesis of the amino acids phenylalanine, tyrosine and tryptophan.

During summer days (Fig. 27), *Ostreococcus* activates genes involved in RNA processing and ribosome biogenesis at dawn (ZT0). Examples for such genes are *U3 small nucleolar RNA-associated protein 14* (*ostta04g00770*, *Utp14*) and the *Ribosome Biogenesis Factor*

BMS1 (*ostta05g01080*, *BMS1*) (Supp 8). During the first part of the morning (ZT4) *Ostreococcus* transcriptome is almost completely focused in genes involved in translation, such as *eukaryotic Initiation Factor 2* (*ostta03g02100*, *eIF2*) and *translation elongation factor P* (*ostta03g03015*, *YeIP*). The genes mainly involved in photosynthesis but also in carbohydrate metabolism reach their maximum expression level during midday, when irradiance is maximum (ZT8). Some of those genes are subunits of both photosystems: *ostta01g03170* (*PsbP*), *ostta02g00580* (*PsaL*), *ostta02g02560* (*PsbX*), *ostta02g03860* (*PsaE*), *ostta04g01790* (*PsaF*), *ostta05g04560* (*PsbR*), etc. During the afternoon (ZT12), the activation of genes involved in DNA replication takes place. Some minichromosome maintenance proteins *ostta01g02580* (*MCM6*) and *ostta05g01680* (*MCM9*) are found, as well as the *proliferating cell nuclear antigen* *ostta06g02890* (*PCNA*) which is central to the DNA replication process. Intracellular transport and cellular respiration are the two most prominent biological processes whose genes reach their maximum expression level at dusk (ZT16) under LD conditions. Genes like *lysophospholipases* *ostta01g04440* (*CLC*) or *nucleoporins* *ostta14g02210* (*Nup133*) are some examples for this time point. Finally, during midnight, *Ostreococcus* focuses on expressing genes involved mainly in cellular amino acid metabolic process, like *3-Deoxy-D-arabinoheptulosonate 7-phosphate () synthase* *ostta06g03270*, (*DAHP*) which encodes the first enzyme in the biosynthesis of the amino acids phenylalanine, tyrosine and tryptophan.

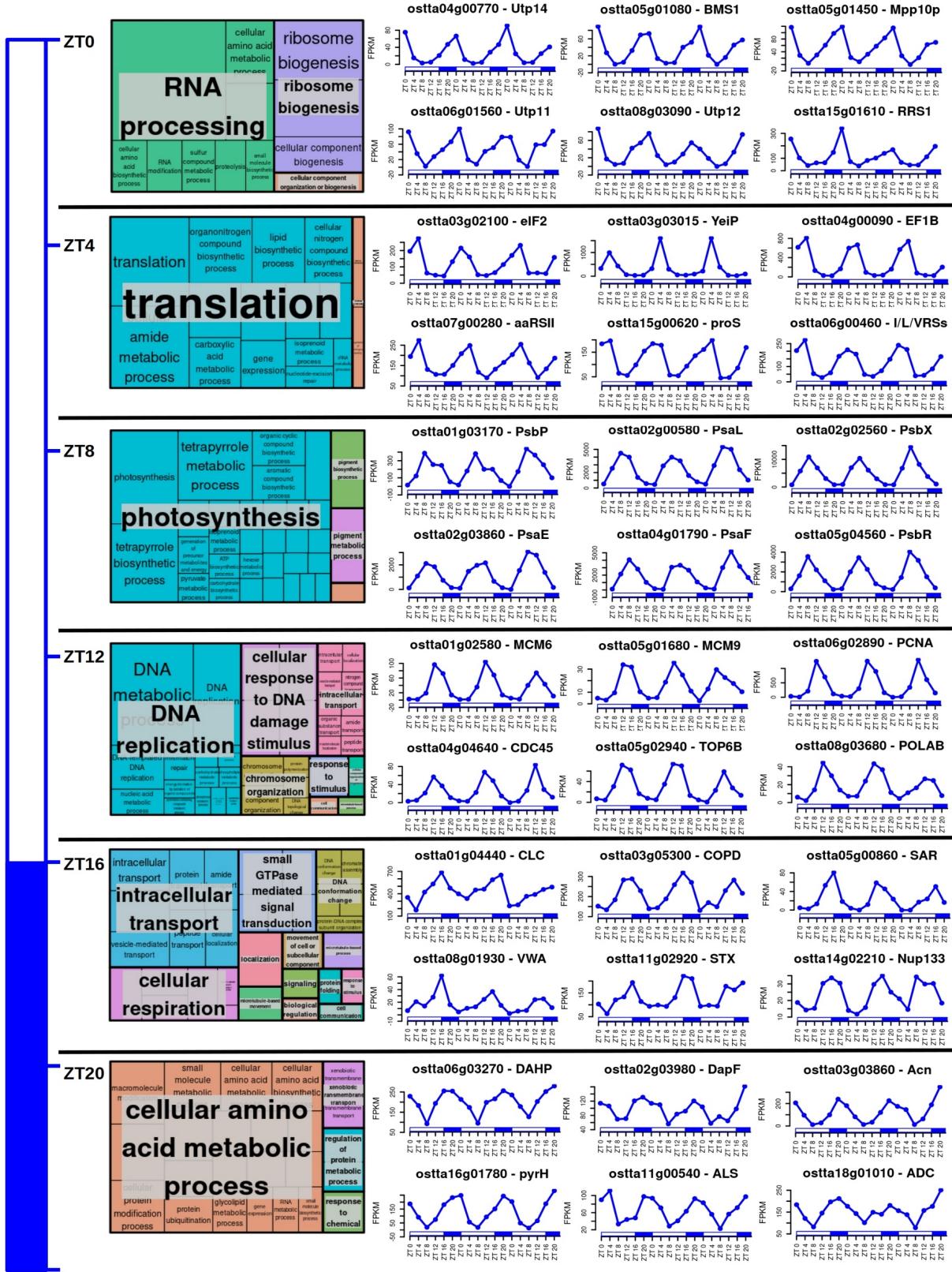


Figure 27: Cellular processes transcriptomically activated in each time point under LD conditions. For each cluster of genes with phase in the different time points, there are: a treemap summarizing the biological processes significantly enriched and expression profiles representation of relevant genes.

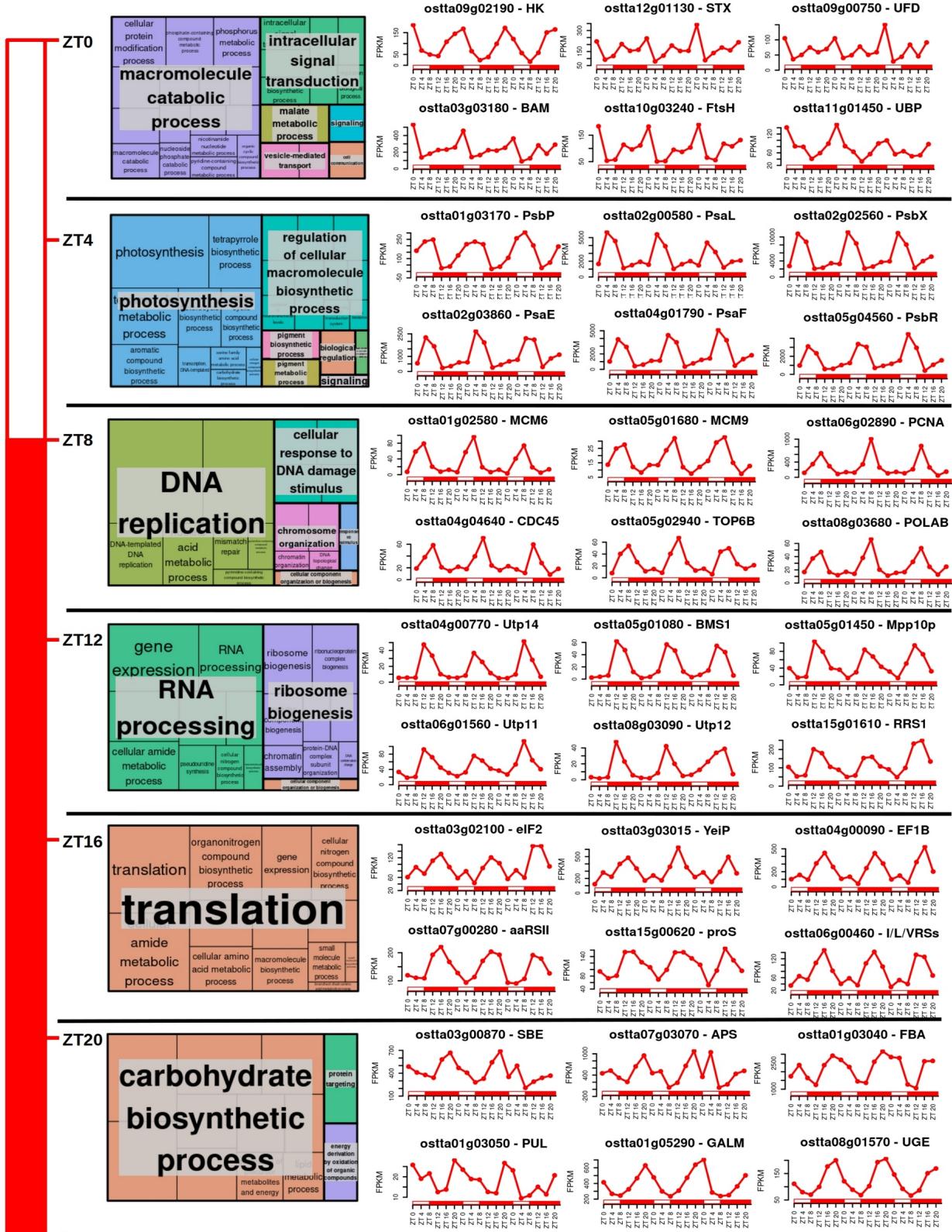


Figure 28: Cellular processes transcriptomically activated in each time point under SD conditions. For each cluster of genes with phase in the different time points, there are: a treemap summarizing the biological processes significantly enriched and expression profiles representation of relevant genes.

During winter days, the most prominent biological process whose genes reach their maximum expression level at dawn (ZT0) is protein catabolism. Examples for such genes are *Signal transduction Histidine Kinase ostta09g02190 (HK)*, or *Ubiquitin Fusion Degradation protein ostta09g00750 (UFD)*. In agreement with what was observed in summer, genes having their peak of expression during midday (which in winter takes place at ZT4, maximum irradiance) are involved in photosynthesis. Once again, genes encoding photosystems subunits are clustered in this time point. Under SD conditions, dusk takes place at ZT8. Genes involved in DNA replication show their maximum level of expression during this time point. The same genes used as an example in LD are found also in SD but anticipating their peak of expression 4h. Then, RNA processing and ribosome biogenesis are the two most prominent biological processes early during the night (ZT12), showing an anticipation of 12h compared with LD temporal program. In addition, genes involved in translation also show a 12h anticipation reaching its maximum expression level during midnight (ZT16). Under LD photoperiod, genes involved in aminoacids biosynthetic processes reach their maximum expression level at ZT20, 8h before genes involved in translation show their expression peaks (ZT4). However, under SD photoperiod genes involved in both processes seems to reach their maximum expression level at the same time. Genes involved in photosynthesis and carbohydrate metabolism reach their maximum expression level at the same time under LD (ZT8), but they take place at two different times under SD. In this condition, genes involved in photosynthesis maintain their maximum level of expression at the maximum irradiance time (which correspond to ZT4 in SD). However, genes involved in carbohydrate metabolism reach their maximum expression level 12 h later (after midnight at ZT20). This suggests that phase shifts globally observed over the transcriptome of *Ostreococcus tauri* as an adaptation to photoperiods, not only consist in anticipating or delaying processes but also in a rearrangement of the complete temporal program.

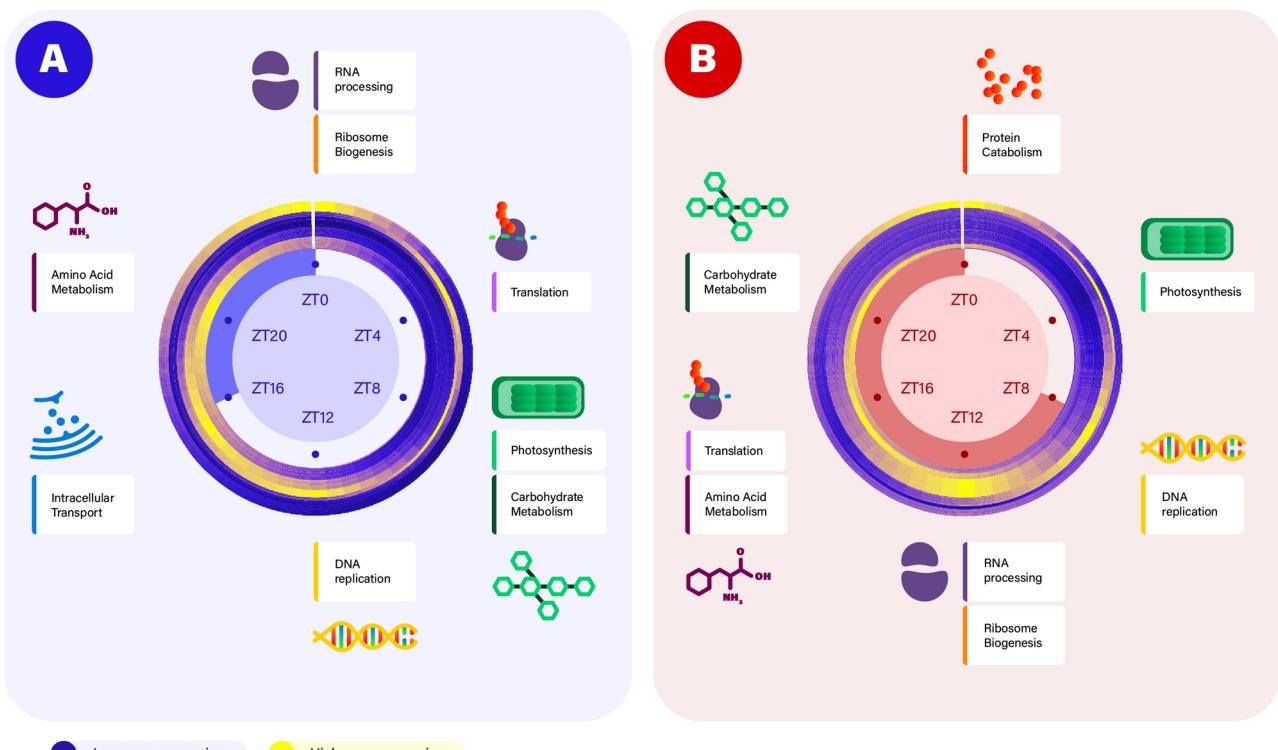


Figure 29: Summary of the transcriptional temporal program of *Ostreococcus tauri* under LD and SD conditions. (A) Circular heatmap representing the temporal organization of gene expression profiles under LD conditions. Dark blue stands for low expression whereas yellow represents high expression. Genes are clustered depending on their phase or time of maximum expression where genes with phase at ZT0 are located in the outer circle and genes with phase at subsequent ZTs are placed sequentially into inner circles. Biological enriched processes in the gene set with phase at each specific time point are depicting capturing the temporal transcriptional program over diurnal cycles under LD conditions. (B) Similarly, circular heatmap representing the temporal organization of the diurnal transcriptional program under SD conditions. A shift as well as a re-arrangement is observed in the SD diurnal transcriptional program when compared to the one inferred for LD.

Chapter 3: Proteomic analysis of diurnal and seasonal cycles in *Ostreococcus tauri*

SWATH proteomics allow us not only to quantify but also to identify proteins in *Ostreococcus tauri* proteome. Our proteomics analysis has reached a proteome coverage of 40%, detecting proteins from all the different cell components (Fig. 28).

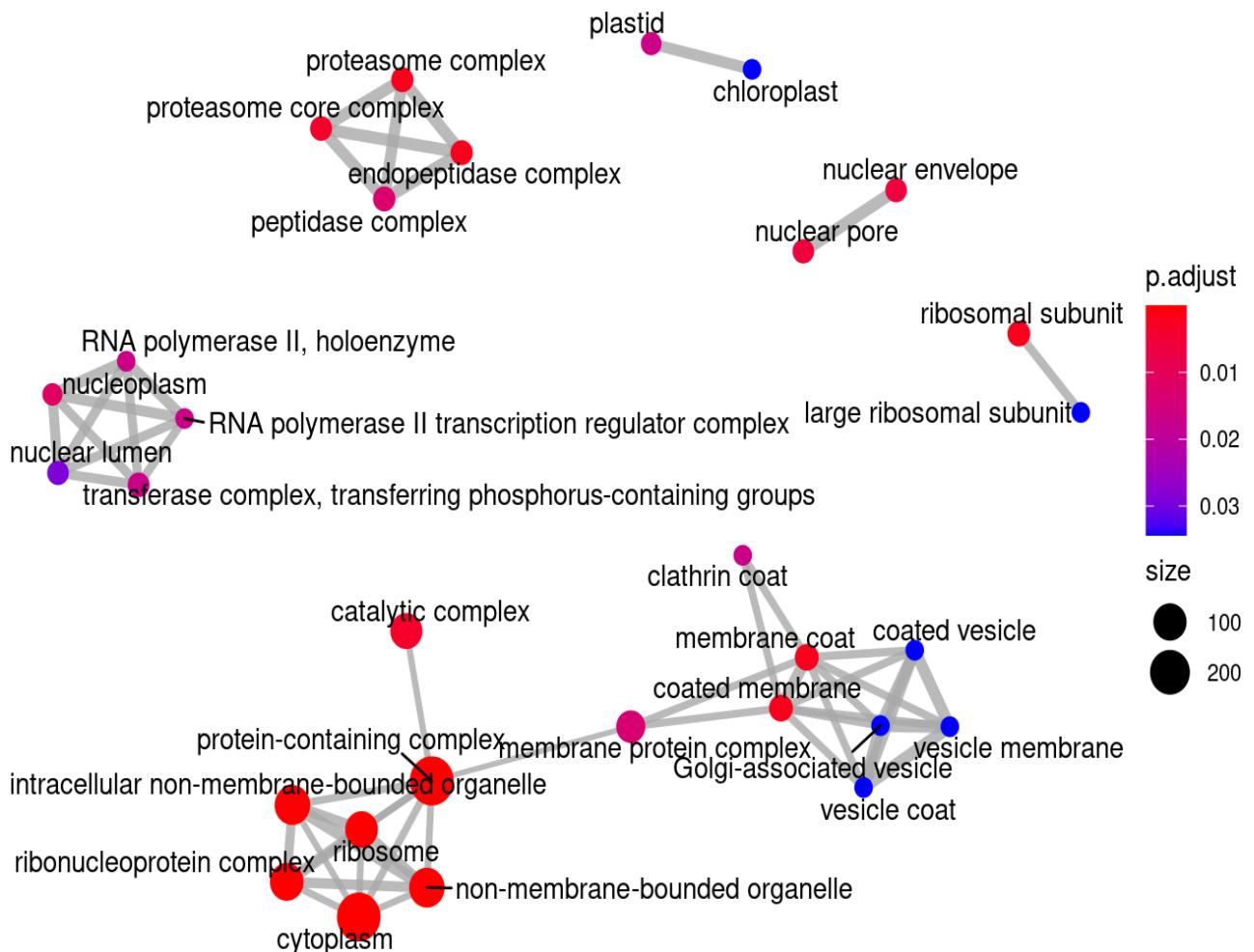


Figure 28: **Our proteomics data cover all cell components.** Enrichment map representing the cellular components or organelle significantly covered by the proteins detected in our data. Dots or nodes sizes represent the number of proteins identified located in the corresponding organelle. A blue to red gradient is used to represent the level of significance. Lines or edges link dots or nodes representing related organelle.

Furthermore, since our data is obtained from 3 technical replicates of each biological replicate (3 light/dark cycles), it can be observed that the global distribution of each group of 3 technical replicates is similar (Fig. 29-A). The differences observed over the global distribu-

tion of different groups of technical replicates are solved with log₂ quantile normalization of the data, ensuring data comparability (Fig. 29-B).

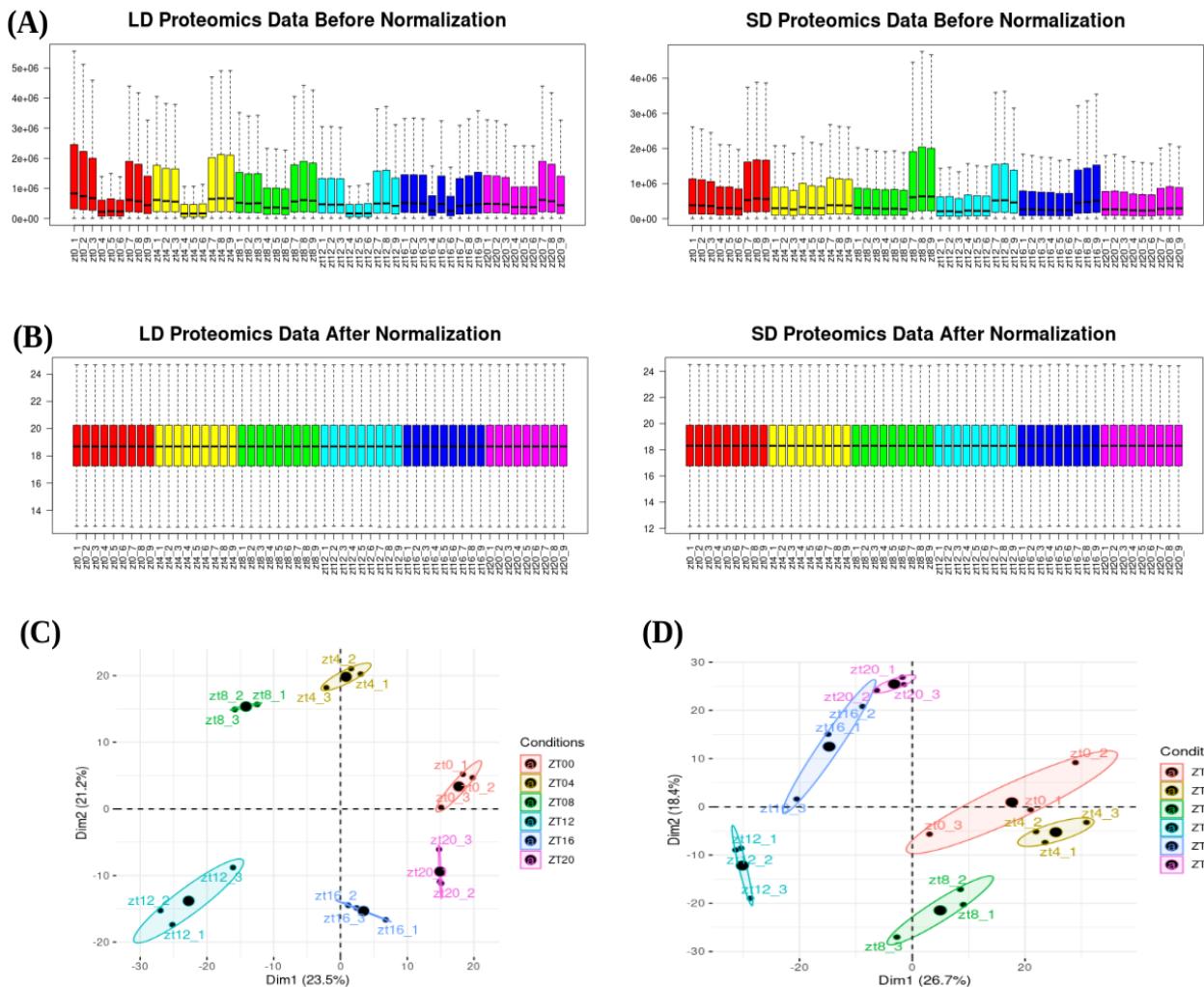


Figure 29: Proteomic data normalization and reliability. (A) Boxplots representing global distributions (before normalization) of protein abundances over the three technical replicates of the six time points corresponding to three consecutive days under long day conditions on the left and under short day conditions on the right. (B) Boxplot representing global distributions after normalization. (C) Principal Component Analysis of the time point global rhythmic proteomes under LD conditions. Small dots correspond to the 2D projection of each time point global rhythmic proteome. Big dots correspond to the average of the three replicates 2D projections for each time point. Ellipses mark the 95% confidence regions corresponding to each time point global rhythmic proteome. A clear circular distribution emerges capturing the cycling structure in diurnal rhythmic LD proteomes. (D) Principal Component Analysis of the time point global rhythmic proteomes under SD conditions. Points and ellipses are used as described before.

In order to obtain a deeper understanding of the underlying structure in our data a principal components analysis was performed separately over the LD (Fig. 28- C) and SD (Fig. 28-

D) rhythmic proteomes. Under LD conditions, the proteomes corresponding to the same time point in the three different days tightly cluster together globally constituting a circular structure. Nonetheless, under SD conditions more variability is observed but the circular structure is still maintained, in contrast to what was observed with SD transcriptomes. Also, the high similarity between the transcriptomes corresponding to ZT0 and ZT20 under SD conditions that is not present in SD proteomes corresponding to those time points. This suggests that during the night starts the transcription of genes which encoding proteins are needed during light , while at the proteomic level, those proteins are present during the light period and, thus, there isn't a clear similarity between night and morning proteomes.

In overall, these results support that our experimental design grants good quality data and a high level of synchronization in our rhythmic proteomic data allowing us to proceed to the identification and comparison of proteins under LD and SD conditions.

Proteomic characterization of diurnal rhythmic abundance profiles

Phase offsets and a decrease in rhythmicity are observed when proteomic and transcriptomic data are compared

The same analysis executed with transcriptomic data, using the R package RAIN, is also executed with proteomic data. In long day proteomic data, only 928 proteins (comprising approximately 32% of the total proteins detected) present diurnal periodic rhythmic expression patterns (FIGX-A). An increase in rhythmicity is observed in short day proteomic data, detecting 1442 rhythmic proteins comprising 44% of the proteins detected (FIGX-B). However, independently from the photoperiod, the rhythmicity levels present in the proteome are much lower than the ones observed in the transcriptome of *Ostreococcus tauri*.*(en el paper de gerben tienen un 67% de proteinas ritmicas en ND, lo menciono?)* Transcripts rhythmic expression profiles do not seem to always lead to a rhythmic protein abundance profile. This phenomenon highlight the relevance of multiomics integration, since transcripts behavior cannot be extrapolated to proteins or cellular processes, neither vice versa. Rhythmic transcripts which encoding proteins maintain their abundance levels constant among diurnal changes have been a paradox in chronobiology research. Currently, protein degradation process is commonly used to unify transcriptomic and proteomic data(citar rhythmic degradation y quizas el de gerben degradation). This hypothesis sug-

gest that rhythmic transcripts are needed to maintain a constant abundance level of the encoding protein when its degradation process is rhythmic. In that way, cells have to cyclic supply new transcripts to balance protein synthesis/degradation ratio.

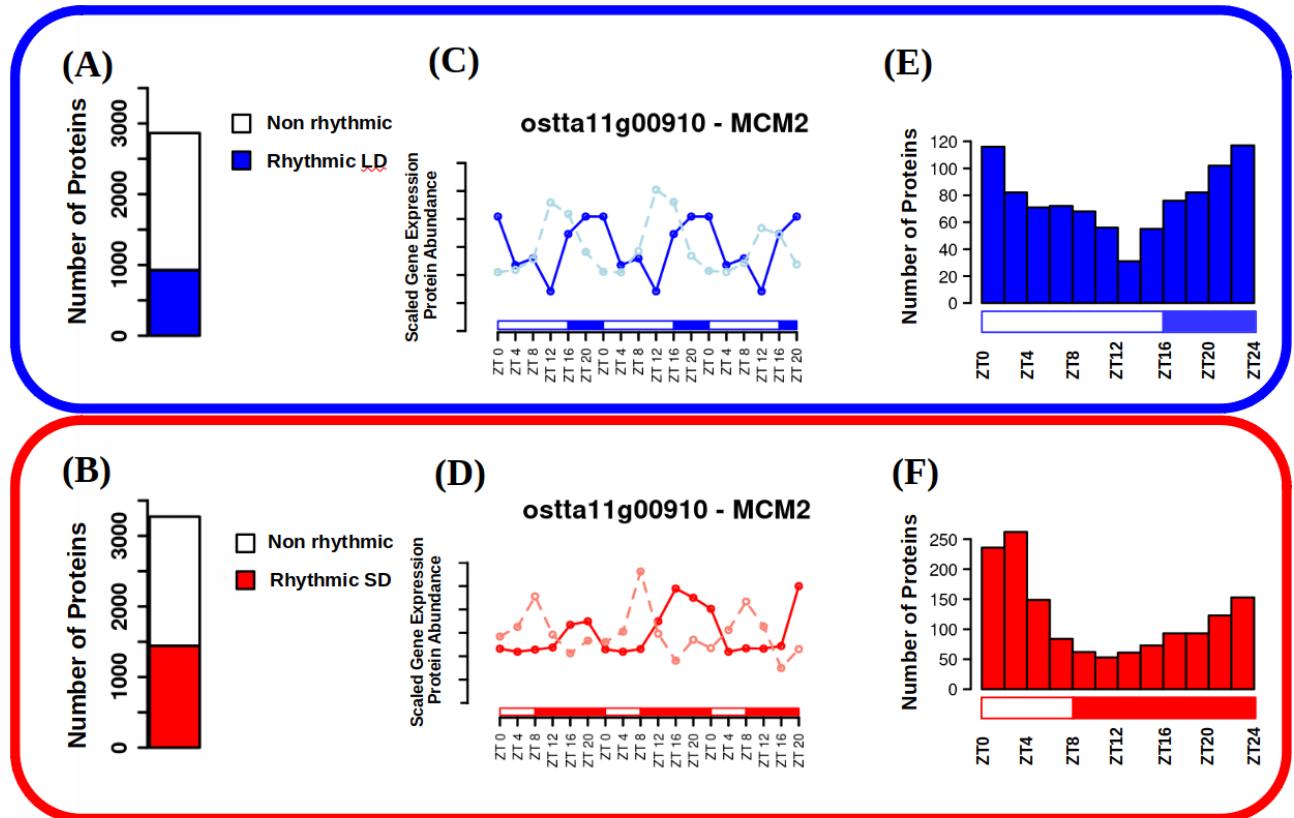


Figure 30: Proteome rhythmicity under alternating light/dark cycles and integration with the transcriptome. (A-B) Barplots representing the number of identified proteins under long day conditions and under short day conditions. The number of rhythmic proteins under LD conditions is represented in blue and under SD conditions in red. Non rhythmic proteins are represented in white. (C-D) Protein abundance profiles under LD (blue) and SD (red) conditions represented together with gene expression profiles under LD (light blue) and SD (light red) conditions for Minichromosome Maintenance 2 (*ostta11g00910*, MCM2). (E-F) Histograms showing the distribution of the number of proteins with phase or maximum abundance at specific time points under LD conditions (blue) and SD conditions (red).

In agreement with previous chronobiology results in other organisms (mismo paper), when protein rhythmic abundance profiles are compared with transcripts rhythmic expression profiles, it is observed a clear phase offset (FIG-C). It suggests that protein translation takes place few hours after gene expression. In our data, while the majority of the transcripts have their maximum expression level during night hours in both photoperiods, the majority of the rhythmic proteins present their maximum abundance level during light hours (FIGX-D). It suggests that the transcriptome of *Ostreococcus tauri* present a nocturnal ac-

tivity while its proteome present a clear diurnal activity. This observation is in agreement with the effects of free-running conditions over the transcriptome of *Ostreococcus tauri*, which were similar with the ones described in the activity of nocturnal organisms.

Proteomic characterization of seasonal effects over the protein abundance profiles

The effects of seasons over the wave parameters in the transcriptome are also found in the proteome of Ostreococcus tauri

As it was executed in the transcriptomic analysis, the R package circacompare is used to study the global effect of the photoperiod over the amplitude and phase of rhythmic abundance profiles of proteins as well. In the previous chapter, it was mentioned that changes in amplitude and phase offsets of biological rhythms dependent of photoperiods have been already described in a wide range of organisms. The effects observed over the transcriptome are also found on the proteome of *Ostreococcus tauri*.

In contrast to our transcriptomic data, when LD and SD rhythmic proteomes are compared globally, there is not a clear increase in the amplitude of the waves described by proteins abundance profiles. However, 57% of the detected rhythmic proteins (that maintain their rhythmicity under both photoperiods) present an increase in amplitude, being significant on 45% of them. This could be directly caused by the decline of synchrony observed over SD transcriptome and/or a decline of synchrony of post-traductional rhythms under shorter photoperiods.

Negative phase shift or phase anticipation is observed over the proteome of *Ostreococcus*, in agreement with what has been described in our transcriptomic data. Specifically, 53% of the detected rhythmic proteins exhibit an anticipated phase under short day conditions when compared to long day conditions, being significant in 60% of them. Phase anticipation are apparent under SD since protein phases are mostly reached around SD light period (from ZT0 to ZT8) whereas, under LD, phases are more uniformly distributed from LD night (ZT16-ZT20) to the end of the maximum irradiance hours (ZT4-ZT8) (Fig. 30-E,F).

These results suggest that seasonal effects over amplitude and phase are more severe over the transcriptome than the proteome of *Ostreococcus tauri*. However, the same

changes are found in a considerable portion of the rhythmic proteins detected. In order to further study this results, the correlation between rhythmic gene expression profiles and proteins abundance profiles is tested (Fig. 31). Whereas the original rhythmic protein abundance and gene expression profiles are negative correlated with a value of -0.34 between, a high positive correlation of 0.84 is observed between the phased aligned profiles, globally (Fig. 31-A). It suggests that the rhythmic wave described by a gene expression and its encoding protein abundance are almost identical, except for their phase offsets (Fig. 31-B,C).

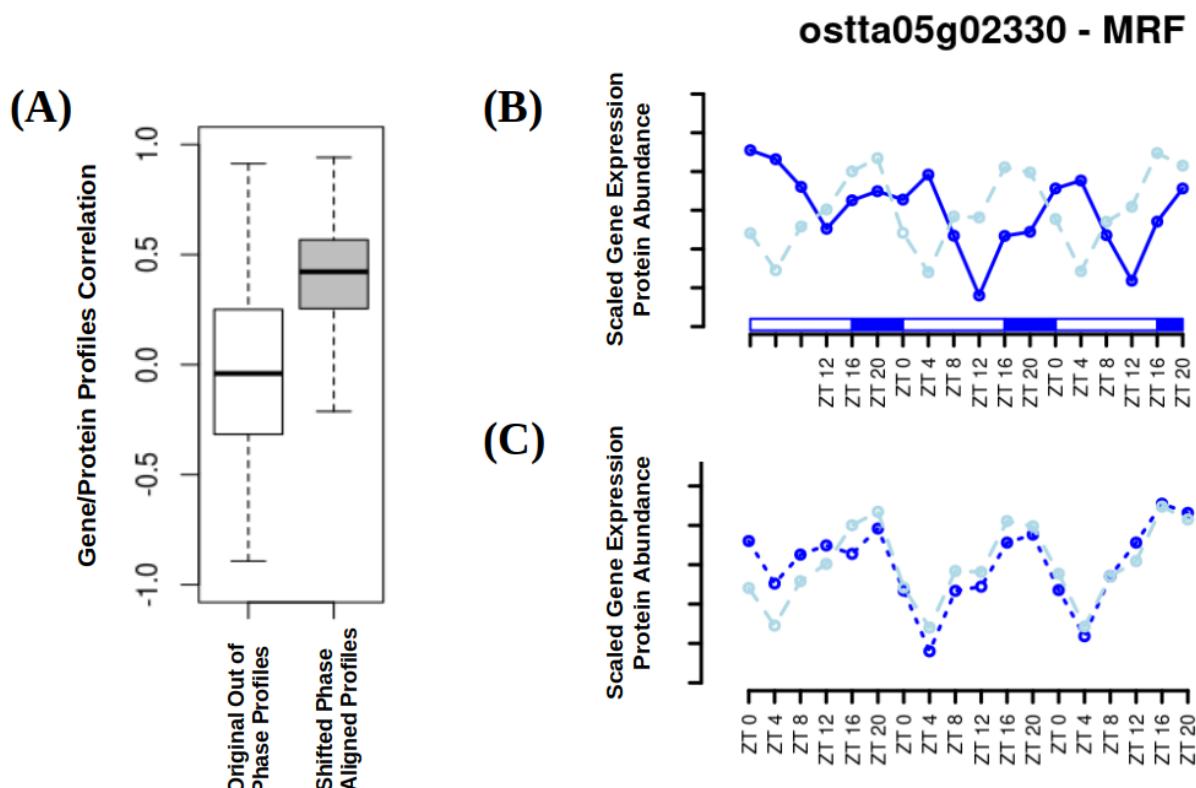


Figure 31: Protein/gene correlation analysis. (A) Boxplots representing the global distribution of the correlations between protein abundance and gene expression profiles (white box) and shifted aligned profiles with coincident phases (grey). (B) Protein abundance (continuous blue line) and gene expression (dashed light blue line) profiles under LD conditions for MA3 domain-containing translation Regulatory Factor (*ostta05g02330*, MRF). (C) Phase aligned protein abundance (dotted blue line) and gene expression (dashed light blue line) profiles.

Phase offsets between genes and proteins involved in the same biological process are adjusted by seasons

In order to obtain a deeper understanding of gene/protein phase offsets, an extensive analysis of its correlation with different factors and protein properties is executed.

One of the possible hypothesis is that gene/protein offsets depend on proteins properties computed from their sequences such as amino acid composition, charge and hydrophobicity. However, no relation is apparent between any protein index and protein/gene offsets (ANEXO).

Another of the possible hypothesis is that gene/protein offsets depend on the time when the maximum level of expression of the encoding gene is reached (gene phase). So, for example, a gene that reaches its maximum level of expression during the morning could take less time to translate than a gene which peak of expression takes place in the middle of the night. However, in our LD data, no significant differences are found when comparing gene/protein offsets of different genes sets with specific phases (Fig. 32-A). Whereas, in our SD data, genes with phases during the skotoperiod (dark period, ZT8, ZT12, ZT16 and ZT20) present significantly longer offsets when compared to those genes with phases during the photoperiod (light period, ZT0 and ZT4) according to Mann-Whitney-Wilcoxon test (Fig. 32-B).

Furthermore, gene/protein offsets affected by seasons cycles is considered as a possible phenomenon. In fact, global protein/gene offsets are significantly longer under SD conditions with respect to LD conditions according to a p-value of 1.2×10^{-9} computed using Mann-Whitney-Wilcoxon test (Fig. 32-C). This phenomenon can also be easily noticed when comparing gene/protein offsets of gene sets involved in the same biological function. For example, following GO terms functional annotation, the genes and proteins involved in DNA replication (associated with the term GO:0006260), show a significant increase in the offset observed between expression and translation under SD conditions (Fig. 32-D). Although the increase in this offset is observed globally, it does not affect equally to every gene/protein, as it is represented in (Fig. 32-D). It suggests that seasonal cycles adjust the offset between gene expression and translation depending on the function they are involved in.

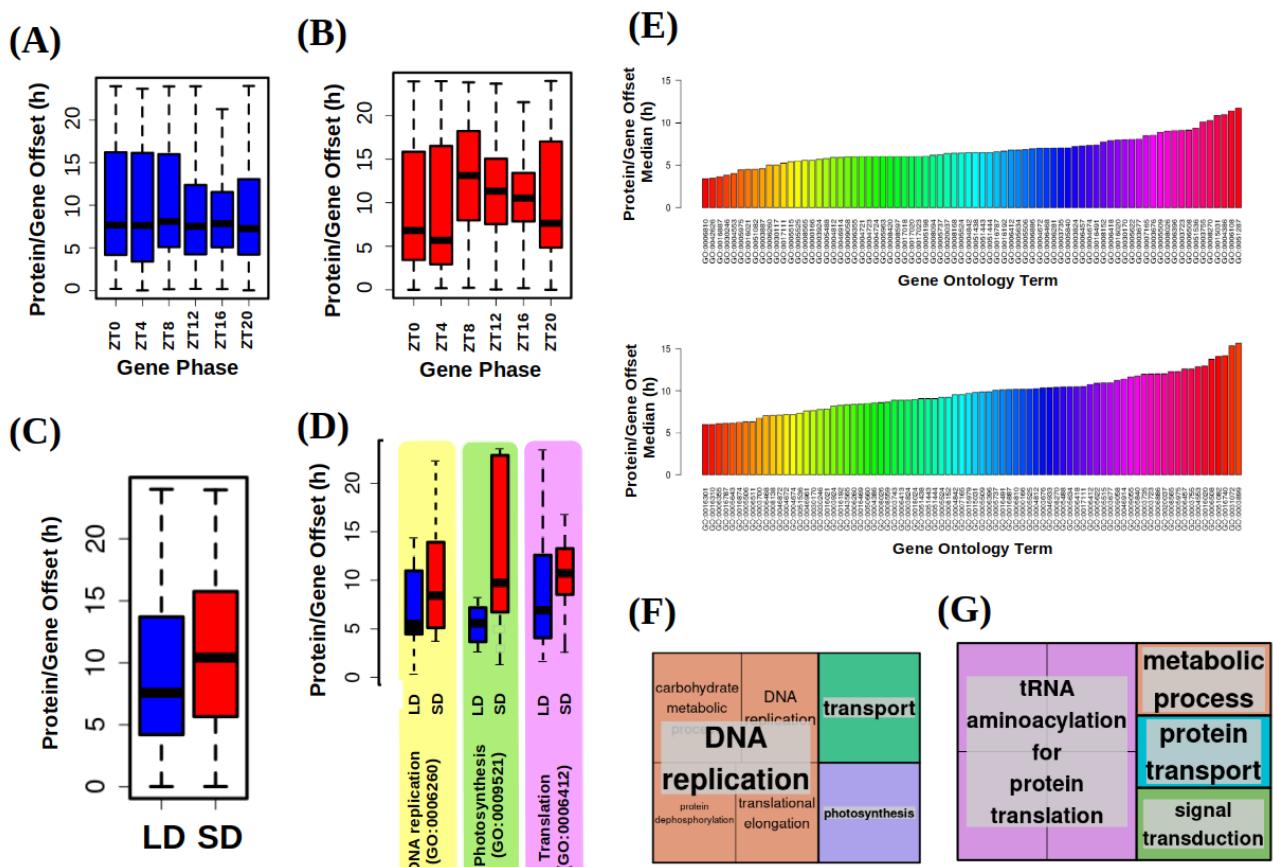


Figure 32: Gene/protein offsets analysis. (A) Boxplot representing protein/gene offsets under LD conditions for different gene sets with specific phases or maximum expression time points. There is no significant differences. (B) Boxplot representing protein/gene offsets under SD conditions for different gene sets with specific phases or maximum expression time points. Differences are significant according to Mann-Whitney-Wilcoxon test. (C) Boxplot representing the offset in hours between protein abundance and gene expression phases under LD (blue) and SD (red) conditions. Differences are significant according to a p-value of 1.2×10^{-9} computed using Mann-Whitney-Wilcoxon test. (D) Boxplot illustrating how genes involved in different biological processes according to their gene ontology (GO) annotation present distinct protein/gene offsets that are longer under SD (red) than LD (blue) conditions. DNA replication (GO:0006260), photosynthesis (GO:0009521) and translation (GO:0006412) are chosen as examples exhibiting short and long protein/gene offsets. (E) Median protein/gene offset for gene sets annotated with the same Gene Ontology (GO) term under LD conditions (top) and SD conditions (bottom). Different biological processes identified by specific GO terms present distinct short or long protein/gene offsets. (F) Treemaps summarizing the biological processes with shortest protein/gene offsets and (G) with longest protein/gene offsets. Semantically similar biological processes are grouped together into the same colored rectangles. The most representative biological processes are shown for each rectangle.

A deeper analysis of gene/protein offsets for gene sets annotated with the same GO term, shows how the different biological functions present distinct short or long offsets under both photoperiods (Fig 32-E). For example, one of the biological processes with the long-

est time between gene expression and translation is related with translation (Fig. 32-F). This result is in agreement with the transcriptional temporal program of *Ostreococcus tauri* illustrated in the previous chapter. Genes involved in translation reach their maximum level of expression during midnight in SD conditions, 8h before dawn. *Ostreococcus tauri* severely reorganize its transcriptional temporal program taking in count not only the current photoperiod but also the different gene/proteins offsets that each biological function presents. In that way, it ensures that proteins will be ready in the exact time they are needed. This could be a possible explanation of why the LD and SD transcriptional temporal programs are not just shifted, but differently arranged.

Furthermore, one of the processes with the shortest time between gene expression and translation is photosynthesis. Some proteins involved in photosynthesis present a high rate of change (CITA?), which can be preserved with short times between gene expression and translation. Also, once again in agreement with our transcriptomic data, genes involved in photosynthesis reach their maximum of expression during high irradiance hours under both photoperiods, so a short delay between gene expression and translation is needed to get the proteins ready when they are needed the most. Photosynthesis and other processes are further analyzed in the next chapter, where an integration of transcriptomics, proteomics and physiological data is presented.

Chapter 4: Diurnal and seasonal multi-omic integration with physiological data.

In the last two chapters, an in-depth analysis and discussion have been carried out on biological rhythms described by proteins and mRNAs. Distinct behaviors between the proteome and the transcriptome, with evident time offsets and even instances of completely divergent patterns have been identified. These observations emphasize the significance of both transcriptional and post-translational regulation in governing biological rhythms. In order to attain a comprehensive understanding of how biological processes are regulated by diurnal and seasonal cycles, it is imperative to integrate not only the above mentioned multi-omic data but also the dynamics of physiological processes. Such integration serves not only as a biological validation of computational omics analysis but also as a crucial component in unraveling the intricate puzzle that biological systems present.

Cell Division Cycle (CDC) of *Ostreococcus tauri* under diurnal and seasonal cycles

The cell division cycle (CDC) is a highly regulated sequence of processes that govern cell proliferation, ranging from unicellular organisms to tissue renewal, and is conserved across eukaryotes. The impact of diurnal cycles on cell division has been studied in various phyla, including plants and microalgae such as *Chlamydomonas*, *Euglena* and *Gonyaulax* (Bruce, 1970; Edmunds & Laval-Martin, 2019; Fung-Uceda et al., 2018; Homma & Hastings, 1989) as well as in mice and humans (Fu et al., 2005; Matsuo et al., 2003).

Nevertheless, the confirmation that circadian regulation controls cell division has been a topic of controversy in some organisms, such as the common microalgae model organism, *Chlamydomonas reinhardtii*. While some studies have concluded that the cell division cycle of this microalgae is subject to circadian regulation (Bruce, 1970), others have proposed that the observed periodicity is linked to cyclic changes in energy status, resulting from circadian regulation of photosynthesis (Spudich & Sager, 1980).

Currently, it is widely accepted that this biological rhythm provides evidence of direct regulation by the circadian clock in nearly all organisms, including photosynthetic ones. It persists under free-running conditions and presents the ability to synchronize with different

photoperiods (Roenneberg & Merrow, 2005), regardless of photosynthetic capacity. The cell division cycle, therefore, possesses a complex regulatory mechanism comprising robust circadian clock regulation as well as light-dependence in photosynthetic organisms, since light serves as their primary energy source. (Goto & Johnson, 1995; Hagiwara et al., 2002; Moulager et al., 2007, 2010). In line with the results presented in Chapter 2, the expression patterns of DNA replication genes remained rhythmic under constant light (ANEXO), while rhythmicity was disrupted (Roenneberg & Merrow, 2005) under constant darkness. This observation supports the notion that a light stimulus is necessary to sustain rhythmicity under free-running conditions, which is in agreement with the complex regulatory mechanism that the cell division cycle (CDC) present in photosynthetic organisms.

The CDC of *Ostreococcus* follows the typical phases of a simple binary fission. Initially, there is a Gap 1 (G1) phase that is dependent on the light-energy status, during which the cell undergoes growth and commitment occurs (Moulager et al., 2007). In cell division cycle studies, the term commitment refers to the moment when the cell, taking into consideration its energy status, decides whether is ready or not to continue with the progression of the cell division cycle. Once cells are committed, cell division is not impaired by darkness. Consequently, if commitment is achieved, G1 phase is followed by the DNA Synthesis or S phase where DNA replication takes place. The initiation of the S phase is typically timed several hours after sunrise (Moulager et al., 2007, 2010). After DNA replication is completed, cells enter the final Gap 2 and Mitotic (G2|M) phase, where they prepare for cell division (G2) and undergo mitosis (M). These two phases are often considered together since they are the shortest and most challenging to distinguish using common techniques.

In all eukaryotes, the progression of cells through the cell division cycle is controlled by cyclins and cyclin-dependent kinases (CDKs). *Ostreococcus tauri* possesses an extremely limited set of cyclins and CDKs, with only a single copy of each gene (Robbens et al., 2005). Additionally, the genome of *Ostreococcus* contains a canonical cell division control protein 25 (CDC25), which is not present in plants (Khadaroo et al., 2004), and a plant-specific CDKB (Corellou et al., 2005).

In the preceding chapters, genes and proteins involved in DNA replication (S phase) have been highlighted several times. Now, in order to validate these observations, an estimation of the distribution of cells in each phase over diel cycles is conducted. This integration un-

veils the adaptation of the cell division cycle in *Ostreococcus* to different seasons and contributes to unraveling the molecular mechanisms of circadian regulation of cell division in microalgae.

Temporal program of cell division cycle under summer and winter photoperiod

The phases of the cell division cycle were determined by estimating the DNA content of cells through flow cytometry, and the division of chloroplasts was observed using fluorescence microscopy, as outlined in Materials and Methods section. The rhythmicity analysis was conducted using data obtained from samples collected over three days in a row under light-dark cycles.

During the summer photoperiod, the G1, S, and G2|M phases exhibit noteworthy rhythmic profiles, with p-values of 2.96×10^{-6} , 3.84×10^{-4} and 0.017, respectively. Conversely, under the winter photoperiod, only the G1 and S phases demonstrate significant rhythmicity, with p-values of 3.08×10^{-3} and 0.067, respectively. Consistent with the findings from transcriptomic and proteomic analyses, a decline in synchronization, characterized by a reduction in amplitude, is evident under the winter photoperiod (Fig. 33-A). Notably, the reduction in amplitude is so substantial in the G2|M phase that the RAIN package used in the rhythmicity analysis fails to detect a rhythmic profile. These observations suggest that the cell division cycle of individual cells within the culture is more synchronized during the summer photoperiod, which aligns with the higher synchronization during summer photoperiod that has been also found at the transcriptomic level in Chapter 2. The few hours of light also seem to have a direct effect on the progression of the cell cycle, since a significant reduction of approximately 24% in the number of committed cells is observed under winter photoperiod. Additionally, significant backward shifts of approximately 4 hours were observed in the cell cycle phases under winter photoperiod (Fig. 33-A). These observations are consistent with the observed backward shifts in the time points when transcript and protein abundances reach their maximum levels in response to photoperiod shortening.

The mean percentage of cells involved in G1, S and G2|M phases was calculated for each time point, allowing the characterization of the temporal progression of the cell division cycle in *Ostreococcus* under different photoperiods (Fig. 33-B,C). Under summer photoperiod, G1 phase predominantly occurs during the light hours, with the maximum percentage

of cells in this phase coinciding with the maximum irradiance hours (around ZT8). After commitment, the percentage of cells in G1 phase gradually decreases, while the percentage of cells in the S phase increases. The majority of cells are in the S phase around sunset and the first part of the night (ZT16-ZT20). Subsequently, the percentage of cells in the G2|M phase gradually rises as DNA duplication is successfully accomplished. The transition from the G2|M to G1 phase, which indicates the completion of cell division, takes place during the first part of the morning (ZT4). This indicates that cell division in *Ostreococcus* mainly takes place after sunrise in summer photoperiod (Fig. 33-B).

During the winter photoperiod, consistent with the observations made in the summer photoperiod, the G1 phase coincides with maximum irradiance hours (ZT4 in winter photoperiod) and the S phase occurs 4 hours after sunset (around ZT8). However, the G2|M phase not only adapts to the photoperiod but also undergoes a reorganization to anticipate the limited daylight hours ahead. In the winter photoperiod, the G2|M phase exclusively occurs during the night-time. As soon as the sun rises, cell division is completed, allowing cells to undergo growth in the morning. These findings suggest that the cell division cycle is strongly influenced by the circadian clock and can anticipate cyclic changes, such as the reduced duration of light during the winter photoperiod. To ensure this anticipation, the circadian clock ensures that all cells enter the G1 phase precisely at sunrise, maximizing the utilization of available daylight hours (Fig. 33-C). This anticipation is also observed in chloroplast division. Under summer photoperiod, chloroplast duplication is achieved during the latter part of the night (ZT20) (Fig. 33-D), while under the winter photoperiod, it takes place at ZT16. Before sunrise, a substantial number of cells already possess only one chloroplast during the winter photoperiod (Fig. 33-E).

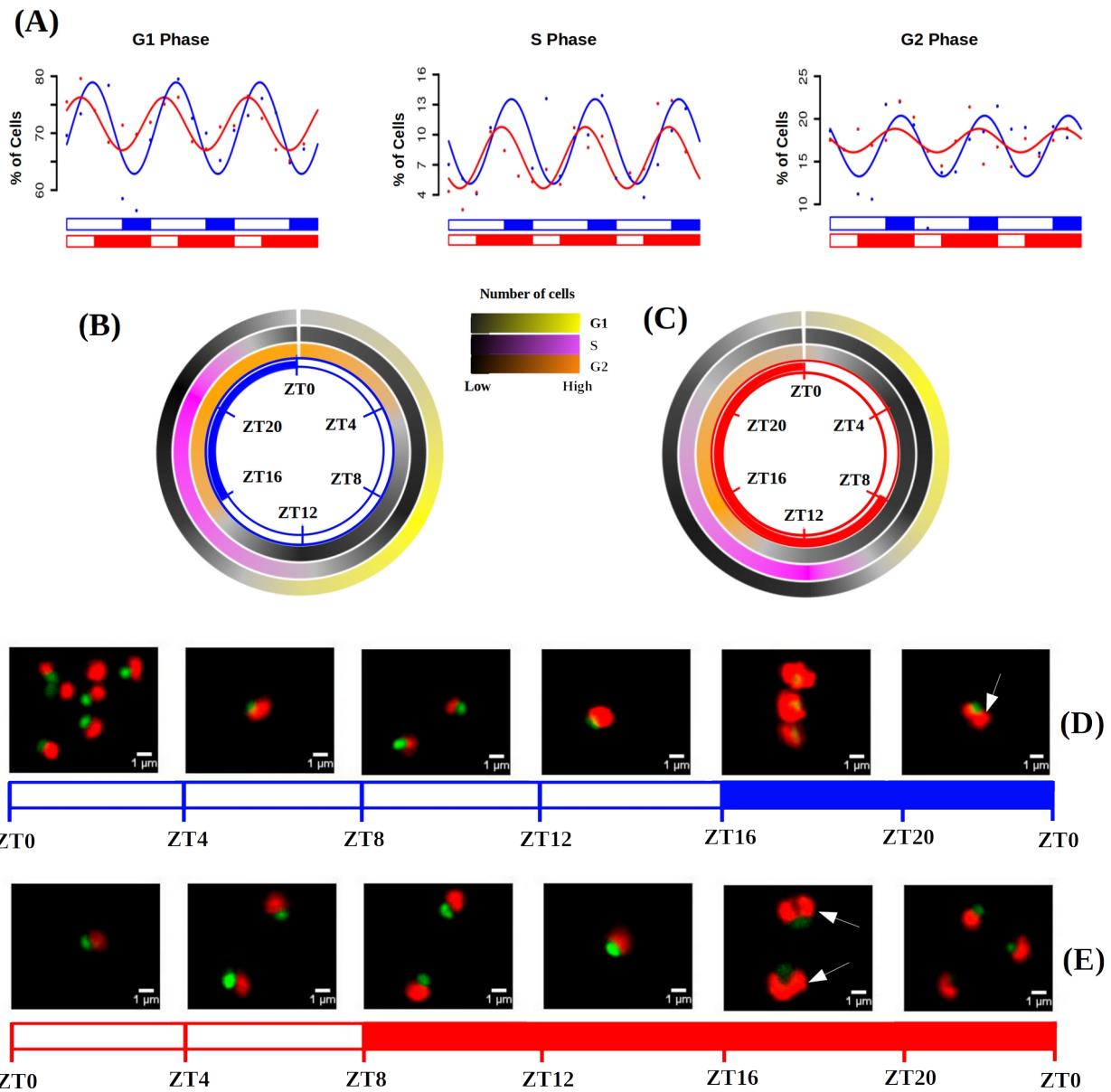


Figure 33. Cell division cycle (CDC) of *Ostreococcus tauri* under summer and winter photoperiod. (A) Percentage of cells in G1, S and G2|M phases during the three days of sampling. Points correspond to real data and lines represent waves approximations made by Circacompare during the rhythmicity analysis. (B-C) Circular heatmap representing mean percentage of cells in G1, S and G2|M phases during summer and winter photoperiod, respectively. (D-E) Photographs of cells under the fluorescence microscope. Each photograph corresponds to a different time point of summer and winter photoperiod, respectively. Nucleus are dyed and they fluorescence in green, chloroplast in red. White arrows point cells that posses two chloroplasts due to chloroplast division prior to cell division.

Integration of CDC program with transcriptomic and proteomic data.

Ostreococcus tauri annotated genes involved in cell division cycle were organized in three different groups in order to mark in which phase of the cell cycle they are present, according to the current cell cycle model in plants (Carneiro et al., 2021) (Table. 7, 8, 9).

*Table 7: Proteins that are present during the G1 phase of the cell division cycle in *Ostreococcus* and the corresponding genes that encode them.*

Gene IDs	Protein names
ostta18g01420	CYCD
ostta04g00110	CDKA
ostta04g01050	CDC6
ostta16g02100	Rb
ostta02g01950	E2F
ostta10g01190	Dp
ostta08g02940	DEL
ostta04g05220	ORC1
ostta15g02820	ORC2
ostta11g01820	ORC3
ostta03g02760	ORC5

*Table 8: Proteins that are present during the G2|M phase of the cell division cycle in *Ostreococcus* and the corresponding genes that encode them.*

Gene IDs	Protein names
ostta01g06150	CYCB
ostta15g00670	CDKB
ostta06g02700	APC1
ostta11g00730	APC2
ostta06g00360	APC3
ostta02g03470	APC4
ostta06g04290	APC5
ostta01g01000	APC6
ostta07g02520	APC8
ostta10g02910	APC10
ostta11g03040	APC11
ostta04g04580	Cdc20
ostta02g03080	Cdc25
ostta13g02370	CDH1
ostta08g02230	FTSZ1
ostta07g01610	FTSZ2

Table 9: Proteins that are present during the S phase of the cell division cycle in Ostreococcus and the corresponding genes that encode them.

Gene IDs	Protein names
ostta02g00150	CYCA1
ostta18g01570	CYCA2
ostta11g00910	MCM2
ostta02g00690	MCM3
ostta14g01050	MCM4
ostta11g01760	MCM4-2
ostta04g00450	MCM5
ostta01g02580	MCM6
ostta02g02010	MCM7
ostta12g01020	MCM8
ostta14g01690	GINS1
ostta02g00490	PSF2
ostta16g02410	GINS3
ostta04g00390	SLD5
ostta02g00500	RFC1
ostta02g00555	RFC2
ostta09g00990	RFC4
ostta07g02710	RFA
ostta02g03430	DNA polymerase II
ostta05g00150	DNA polymerase III γ
ostta08g00710	DNA polymerase α
ostta11g01400	DNA polymerase α
ostta11g01400	DNA polymerase α
ostta07g02610	DNA polymerase δ
ostta08g03680	DNA polymerase α/ε
ostta12g00110	DNA polymerase α/ε
ostta11g01840	DNA polymerase ε
ostta06g02890	PCNA
ostta08g01110	DNA primase
ostta11g00940	DNA primase
ostta13g02040	DNA primase
ostta08g02290	XPG
ostta10g00340	DNA helicase
ostta10g00640	DNA ligase
ostta13g01140	RPA replication protein A
ostta10g00670	Wee1
ostta07g01870	Cks

Cyclin D and CDKA are present specifically during the first part of the G1 phase. Therefore, they are considered essential proteins associated with G1, facilitating the transition between G2|M to G1 phase and, thus, the progress of the cell cycle. Transcription factors like E2F and Dp as well as other proteins (Rb, cell division control protein 6 and ORCs)

are also involved in G1 phase, playing a crucial role in the activation of genes associated with the S phase (Table 7).

In order to start the S phase is required the presence of the Cyclin A (CYCA). Concurrently, there is a coordinated synthesis of polymerases and replication-related proteins (such as MCM complexes, replication factors, PCNA, primases, helicases, ligases, and others) that are essential for DNA replication (Table. 9). The onset of the G2|M phase is marked by the presence of Cyclin B (CYCB), along with subunits of the anaphase-promoting complex (APC) and cell division control proteins (CDC20 and CDC25) (Table. 8) (Carneiro et al., 2021; Moulager et al., 2007, 2010; Robbins et al., 2005).

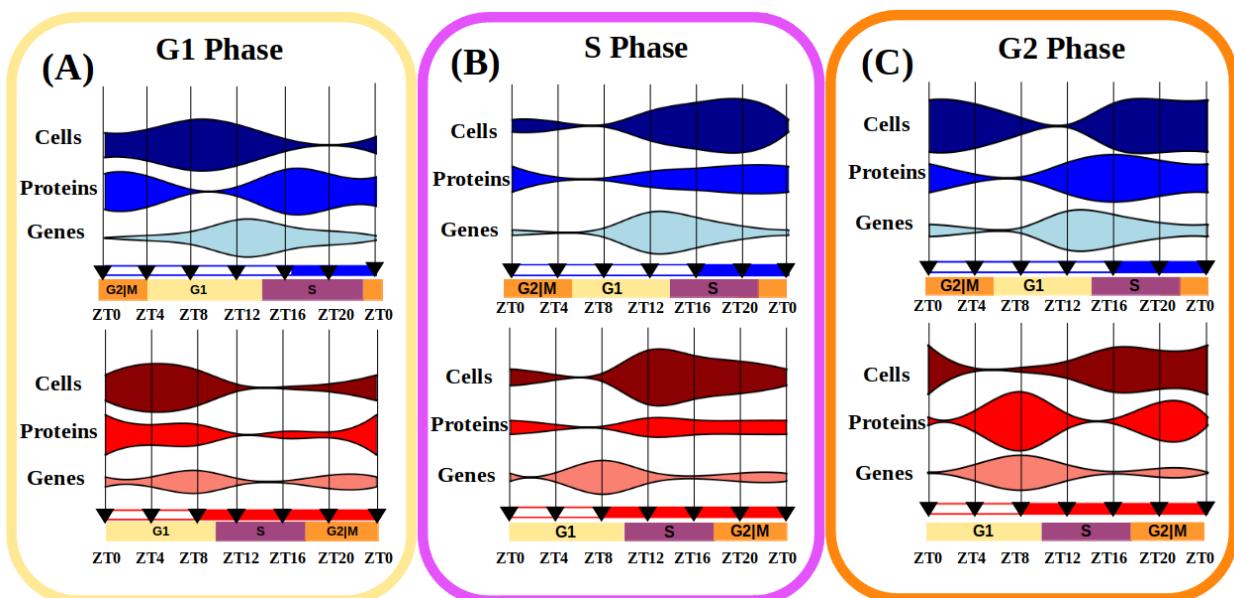


Figure 34: Integration of gene expression, protein abundance and cell population profiles for each phase of the cell cycle. Violin plots represent the three biological levels studied: “Genes” for transcriptomic data, “Proteins” for proteomic data and “Cells” for DNA content estimation by flow cytometry. (A) Involves G1 phase related data, (B) S phase and (C) G2 phase.

The gene expression and protein abundance profiles are compared with the percentage of cells in the respective phase of the cell cycle in which these genes and proteins are present (Fig. 34). In addition to the globally observed gene-protein offset, an overall offset is also observed between protein abundance and the execution of their physiological role. Notably, this offset appears to be more pronounced in certain phases such as G1 or G2|M (Fig. 34-A,C). However, during the S phase, once the proteins are available, the corresponding biological processes are promptly executed, resulting in a shorter offset between protein and physiological execution (Fig. 34-B). It could be explained by the experimental

design followed, since proteins included in the S phase set are directly involved in DNA replication and flow cytometry directly detect cell division phases based in the estimation of DNA content.

The transcriptomic and proteomic data for cyclins and CDKs found in *Ostreococcus* (Fig. 35) align with the current cell cycle model for plants (Carneiro et al., 2021). In both summer and winter photoperiods, the transcription of the CYCA, that will trigger the start of the S phase, occurs during the G1 phase and it is the first cyclin to be activated. It has been suggested that Cyclin A is predominantly regulated by the circadian clock, as it has been shown to be independent of metabolic status (Moulager et al., 2010). Consistent with those findings and previous transcriptomic analyses (Carneiro et al., 2021; Moulager et al., 2007), the expression of Cyclin A (during G1 phase) present a strongly rhythmic expression profile that is followed by the expression of Cyclin B (during the S phase) (Fig 35-A).

Cyclin proteins were not detected in our proteomic analysis, but CDKA and CDKB protein abundance profiles are detected and their abundance profiles align with the proposed model (Carneiro et al., 2021). The expression of CDKA encoding gene reaches its maximum level during G1, preceding an increase in CDKA protein abundance during the latter part of the G1 phase (Fig. 35-B). This increase, in conjunction with Cyclin A, facilitates the progression of the cell cycle into the S phase. During the S phase, the abundance of CDKB protein reaches its maximum level (Fig. 35-C), coinciding with Cyclin B transcript levels. The transcript levels of Cyclin D are relatively low under both photoperiods, but their peak of expression aligns with the G2|M phase (Fig. 35-A). Genes involved in chloroplast division, such as Filamentous Temperature-Sensitive Z (FtsZ, ostta07g01610), play central roles in the G2|M phase. It represents a crucial protein that has been conserved from its cyanobacterial ancestors (TerBush et al., 2013). FtsZ present protein peaks reached during the transition S/G2|M phase (at sunrise, ZT0, in summer photoperiod and during the night, ZT16, in winter photoperiod) (Fig. 35-D). Confocal microscopy images validated these findings by identifying cells with two chloroplasts as a result of recent divisions at ZT20 under LD conditions and ZT16 under SD conditions (Fig. 33-D,E).

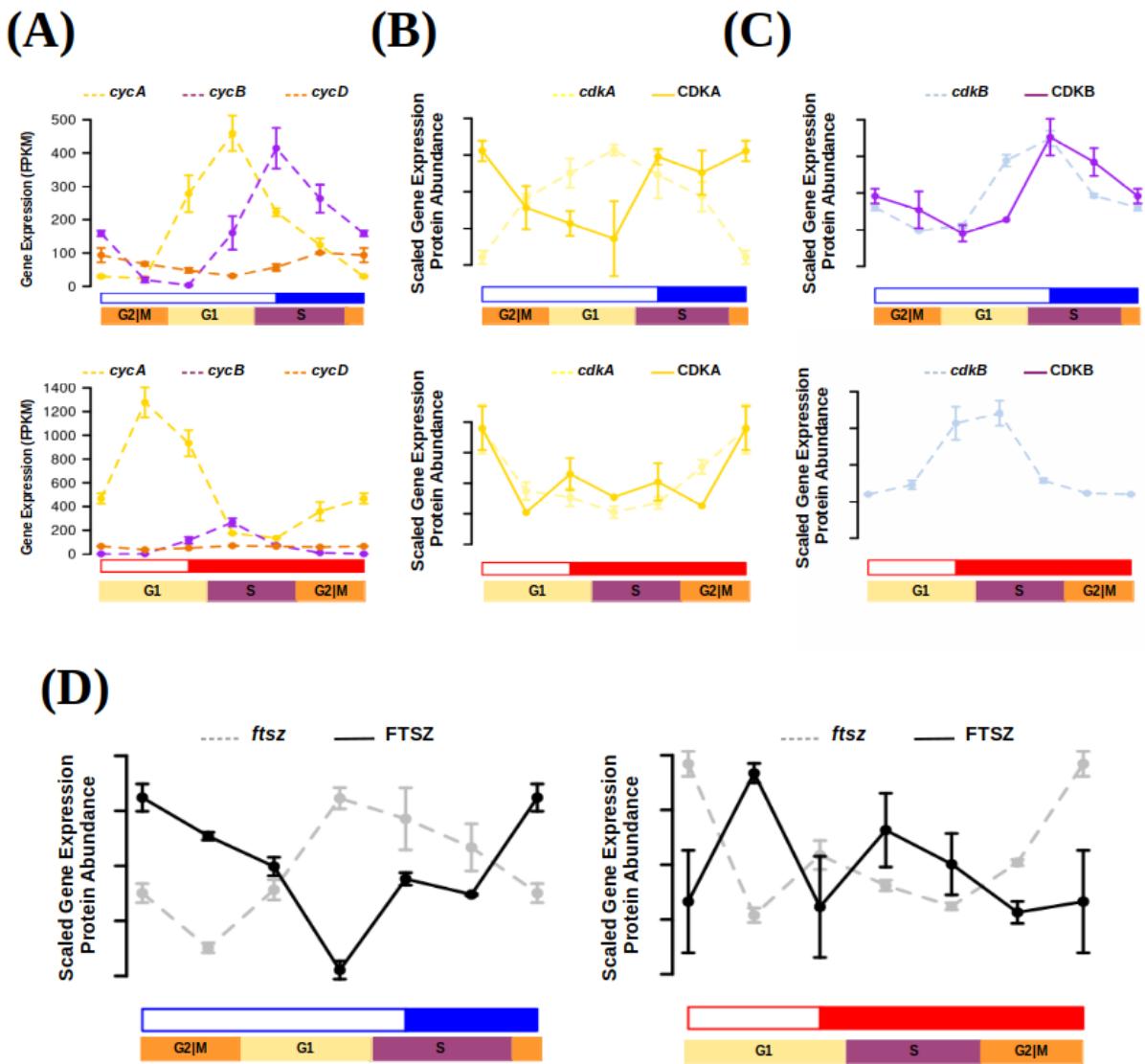


Figure 35: Transcript and protein abundance profiles of the main cell cycle proteins under summer and winter photoperiod in *Ostreococcus tauri*. (A) Expression level of cyclins A (yellow), B (purple) and D (orange) genes are represented with discontinued lines under summer (blue) and winter (red) photoperiods. Transcript (discontinued line) and protein (solid line) abundances of CDKA are represented in yellow (B), CDKB in purple (C) and FTSZ in black (D).

Diurnal and seasonal rhythm of photosynthesis in *Ostreococcus tauri*

Photosynthesis constitutes a fundamental process for plants, wherein oxygen (O_2), adenosine triphosphate (ATP), and reduced nicotinamide adenine dinucleotide phosphate (NADPH) are generated from water (H_2O) and light. The generated oxygen is released into the atmosphere, while ATP serves as an essential energy source for cellular processes. Moreover, NADPH plays a crucial role as a reducing agent, facilitating assimilatory pro-

cesses such as the Calvin cycle, which mediates the assimilation of atmospheric carbon dioxide (CO_2) into organic carbon compounds.

Within the genome of *Ostreococcus tauri*, all the indispensable proteins implicated in the electron transport chain of photosynthesis and carbon fixation are present. However, the number of copies in *Ostreococcus* appears to be lower when contrasted with plants and other microalgae. Notably, the composition of light-harvesting complexes in *Ostreococcus* exhibits distinct characteristics. While light-harvesting complex proteins associated with photosystem I (LHCl) are present, LHCII are lacking. Instead, specific chlorophyll-binding proteins unique in prasinophytes are identified in *Ostreococcus* (Blanc-Mathieu et al., 2014; Derelle et al., 2006). This observation suggests the presence of LHCl within the green lineage from an evolutionary stage prior to *Ostreococcus* ancestors (Six et al., 2005).

Understanding how photosynthesis is adapted to diurnal and seasonal cycles can contribute to develop systems enhancing plants productivity, which is a relevant topic in agriculture. The circadian regulation of photosynthesis was initially documented in marine algae, with the observation of circadian oscillations in oxygen production (Dodd et al., 2014; Sweeney & Haxo, 1961). After that discovery, circadian oscillations in various physiological phenomena associated with photosynthesis, including chloroplast ATP concentration, electron transport rate, starch content, and photosynthetic efficiency, have been observed in microalgae (Mackenzie & Morse, 2011; Ral et al., 2006; Sorek et al., 2013; Sweeney & Haxo, 1961). Furthermore, circadian oscillations in photosynthesis have also been documented in agriculturally relevant plant species (Feugier & Satake, 2013; Lonergan, 1981; Tucker et al., 2004). Nowadays, the application of omics techniques has enabled the elucidation of 24-hour rhythmic oscillations in genes involved in photosynthesis and carbon fixation in both microalgae and plants (Ferrari et al., 2019).

In addition, this thesis represents the first instance of describing the maintenance of rhythmic expression profiles of genes involved in photosynthesis and carbon assimilation under varying photoperiods and free-running conditions (constant light and constant darkness) in *Ostreococcus tauri*. The genes involved in photosynthesis have been described as *bona fide* circadian genes in Chapter 2 due to their sustained rhythmic expression profiles during both summer and winter photoperiods, as well as under free-running conditions. Addi-

tionally, in Chapter 3, it has been observed that photosynthesis exhibits one of the shortest offset between gene expression and translation in *Ostreococcus tauri*. These findings, derived from multi-omics analyses, are now validated with photosynthetic efficiency measurements under winter and summer photoperiods. This integration sheds light on the adaptation of photosynthesis, as well as other interconnected processes such as carbon fixation and starch metabolism in *Ostreococcus*, to accommodate varying seasonal conditions. Furthermore, it provides valuable insights about the conserved mechanisms governing circadian regulation and ultimately influencing photosynthetic productivity within the green lineage.

Rhythmic oscillations of photosynthetic efficiency under summer and winter photoperiod

Photosynthesis efficiency has been calculated using Pulse-Amplitude-Modulation (PAM) by estimating maximum quantum efficiency (F_v/F_m), a widely accepted metric to assess the overall integrity and efficiency of Photosystem II (PSII). To determine the rhythmicity of photosynthesis, F_v/F_m measurements were obtained from samples collected under 3 consecutive light-dark cycles. Detailed explanations of data generation and analysis are provided in the Materials and Methods section.

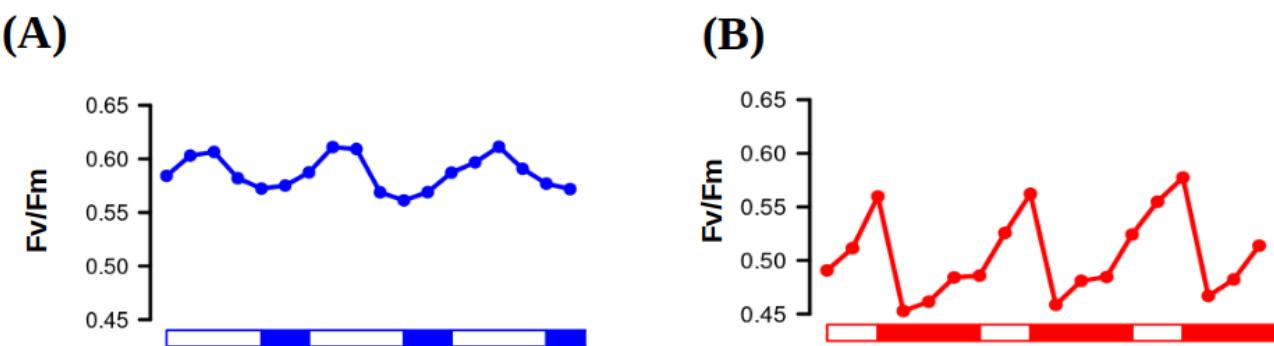


Figure 36. Photosynthetic efficiency rhythmic oscillations. F_v/F_m oscillating values used as an estimation of PSII performance and, thus, photosynthetic efficiency under three consecutive days of summer (A) and winter (B) photoperiods.

Under summer photoperiod, the F_v/F_m parameter exhibits an evident rhythmic profile with a 24 h period, displaying a significant p-value of 7.07×10^{-6} (Fig. 36-A). The maximum F_v/F_m value consistently occurs every 24 h during the maximum irradiance hours, around ZT8. This observation indicates that the photosystems operate at their highest efficiency during that specific time of the day, consequently enhancing photosynthetic efficiency. In

contrast, the Fv/Fm profile under the winter photoperiod displays a rhythmic pattern with two peaks every 24 h (Fig. 36-B). Both peaks demonstrate significant periodicity every 12 hours, supported by a p-value of 2.02x10-8. One of the peaks aligns with the Fv/Fm rhythmic profile observed under the summer photoperiod, occurring at ZT8 as well. Whereas the second peak represents a smaller increment in the Fv/Fm value, occurring more than 8 hours prior to sunrise. This finding suggests that the photosynthetic machinery in *Ostreococcus tauri* exhibits a circadian response, preparing itself in advance during the night to anticipate the limited daylight hours of the winter photoperiod. These outcomes underscore the ability of *Ostreococcus tauri* to anticipate cyclic variations in photoperiod, which is a widely accepted signal of robust circadian regulation (Roenneberg & Merrow, 2005).

Integration of photosynthesis efficiency rhythmic profile with multi-omics data

Photosynthesis is a complex process involving numerous proteins and different metabolic pathways strongly depend on its execution. Photosynthesis entails an electron transport chain consisting of three major protein complexes: Photosystem II, cytochrome b6f, and Photosystem I. These complexes facilitate the transfer of electrons, generated through the cleavage of the water molecule to their final acceptor, NADP, generating the NADPH required for biosynthetic reactions. The energy derived from this transport enables the pumping of protons into the chloroplast lumen, ultimately leading to ATP synthesis as they return to the stroma via ATP synthase. NADPH and ATP are needed to fix CO₂ and generate carbon compounds within the Calvin cycle which can be stored as starch reserves (Fig. 37-A). In higher plants, the circadian clock participates in coordinating different physiological processes like photosynthesis, carbon fixation and starch biosynthesis (de los Reyes, Romero-Campero, Teresa Ruiz, et al., 2017; Farré & Weise, 2012; Graf et al., 2010). The Fv/Fm rhythmic profiles presented are now integrated with the proteomics and transcriptomics data of genes associated with the PSII, which are listed in Fig. 37-B. This integration provide a holistic understanding of how these processes respond to and anticipate to seasonal and diurnal cycles in *Ostreococcus tauri* (Fig. 37-38).

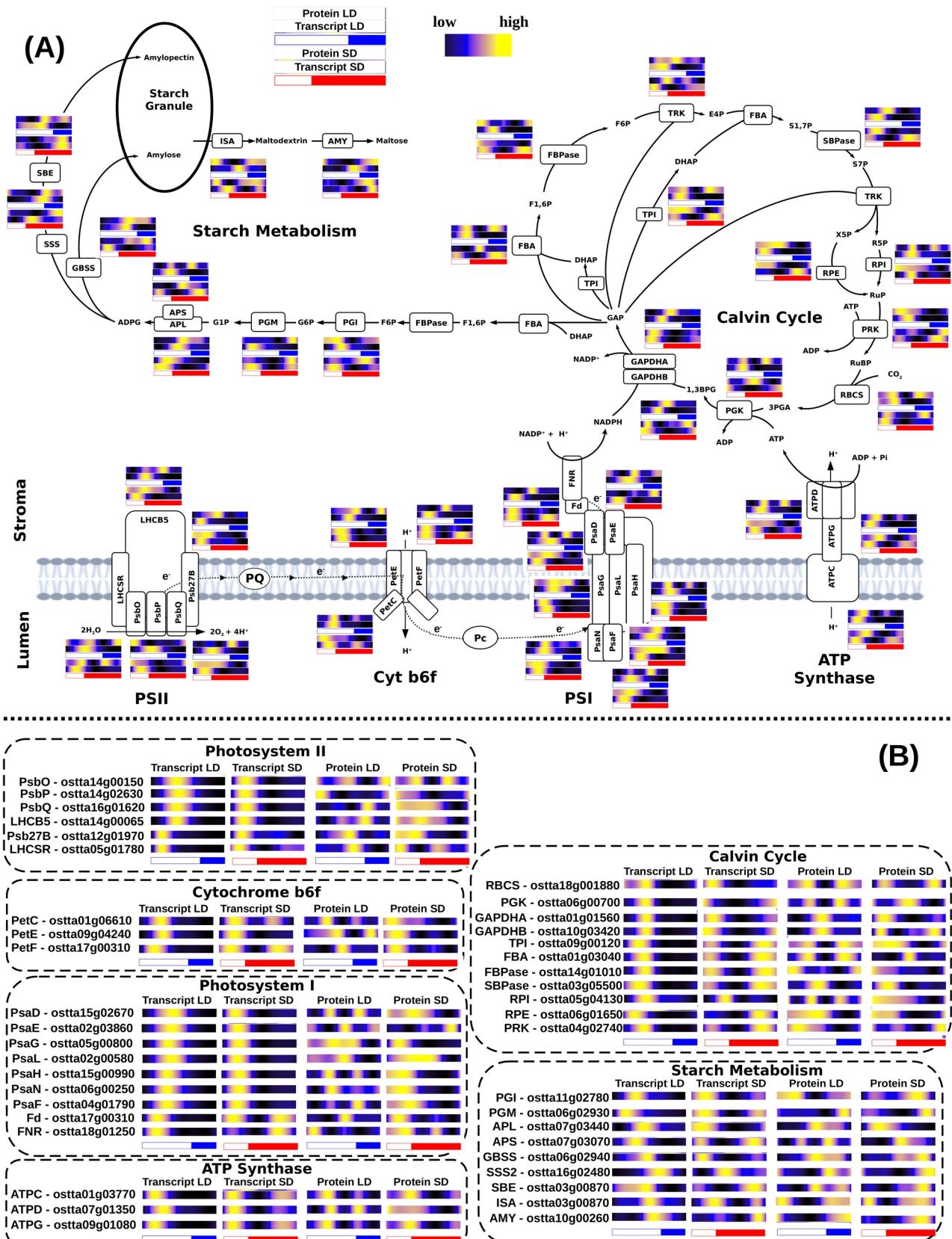


Figure 37. Integration of multi-omic data of enzymes and proteins involved in photosynthesis, Calvin cycle and starch biosynthesis. (A) Schematic representation of the mentioned metabolic pathways and (B) organized list of genes IDs involved in them, including protein abundance and gene expression profiles of each enzyme.

Under summer photoperiod, no temporal offsets were observed between the time points of maximum transcript and protein abundances and the highest Fv/Fm values (Fig. 38-A). The observations suggest that during the summer photoperiod, genes are promptly translated as soon as they are transcribed, leading to an increase in photosynthetic efficiency through *de-novo* protein synthesis. Conversely, during the winter photoperiod, as it was discussed in Chapter 3, there is a short gene-protein temporal offset (Fig. 38-B).

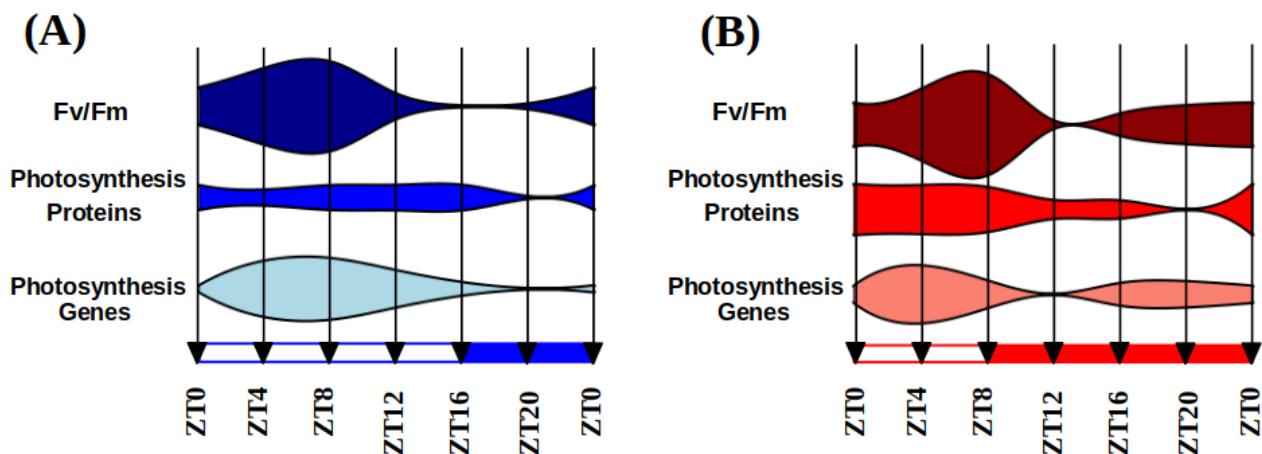


Figure 38. Integration of Fv/Fm oscillations with multi-omic data. Fv/Fm measurements are integrated with multi-omic data from proteins and genes related with photosynthetic efficiency under summer (A) and winter (B) photoperiod.

Consequently, the maximum photosynthetic efficiency occurs a few hours after the maximum gene expression level (Fig. 38-B). For example, the early increase in gene expression at the beginning of the night, observed in genes encoding components of the Oxygen Evolving Complex PSII subunits O, P and Q (PsbO, ostta14g00150; PsbP, ostta14g02630; PsbQ, ostta16g01620), among others, (Fig. 37-B) resulted in a corresponding increase in protein abundance and Fv/Fm values during the second half of the night with a ~4 h offset (Fig. 38-B).

Rhythmic profiles with a 12-hour period (two peaks every 24 hours) are observed not only in Fv/Fm but also in the gene expression profiles under the winter photoperiod (Fig. 38-B). Profiles with two peaks under SD conditions were found in genes such as Protein Electron Transfer C (PetC, ostta01g06610), Ferredoxin (Fd, ostta17g00310) and ATPase delta subunit (ATPD, ostta07g01350) coding for key components of Cytochrome b6f, Photosystem I and ATP Synthase (Fig. 37-B). These genes constitute examples of the emergence of two peaks under SD conditions (Fig. 26-A), one induced by the photoperiod maintained only

under LL and the other one induced by the skotoperiod maintained only under DD. Specifically in photosynthesis, one expression peak occurs during the early morning, enhancing photosynthetic efficiency during the hours of maximum irradiance. The second expression peak takes place during the night, inducing the anticipation of the photosynthetic machinery before sunrise. These findings suggest that the response of the photosynthetic machinery in anticipation to the photoperiod is transcriptionally regulated in *Ostreococcus tauri* and likely established since early in the green lineage.

In general, genes involved in photosynthesis electron transport chain and Calvin cycle are consistently expressed early in the morning, under both summer and winter photoperiods (Fig. 37-B). The expression of those genes seems to be unaffected by the photoperiod, since it has been also observed in *Ostreococcus* under a neutral photoperiod, consisting in 12h of light and 12h of dark cycles (Monnier et al., 2010). In fact, genes coding for key components on the Calvin Cycle such as Glyceraldehyde-3-phosphate dehydrogenase A (GAPDH, ostta01g01560) and Fructose-1,6-bisphosphate aldolase (FBA, ostta01g03040) are examples of *bona fide* circadian genes exhibiting rhythmicity under both summer and winter photoperiods, as well as free-running conditions of constant light and constant darkness (Table bonafide?).

Not only the genes coding for PSI, PSII and antenna complexes exhibit 12 h period expression profiles during winter photoperiod. Numerous genes, involved in Calvin cycle and starch metabolism exhibit these expression profiles as well (Fig. 37-B). It suggests that the transcriptomically regulated anticipation to the short light hours during winter photoperiod is not limited to photosynthesis efficiency. Instead, it is found systematically in the complete metabolic interaction of all processes related with photosynthesis, including not only the electron transport chain but also the Calvin cycle and starch metabolism.

In addition, protein abundance rhythmic profiles with 12h period are observed under summer photoperiod systematically along numerous genes listed in Fig. 37-B. The secondary peaks observed in these proteins are not induced by an increment in the transcription level of their corresponding gene (Fig. 37-B). This phenomenon has been previously observed in RuBisCO small subunit (RBCS) protein abundance profile in plants during neutral photoperiod. Multi-omics integration of the results presented in this thesis shows that this phenomenon is present not only in RBCS but systematically all Calvin cycle enzymes, as well

as some proteins from the photosynthetic electron transport chain (Fig. 37-B). These 12h period profiles are not transcriptomically regulated as the ones described in winter photoperiod, since its encoding transcripts describe one peak of expression per day (24 h period) (Fig. 37-B). The mRNA rhythmic profiles of enzymes from the Calvin cycle are in agreement with previously published data in plants, both showing a 24 h period (Pilgrim & McClung, 1993). It suggest that a strong post-translation regulatory mechanism is causing this 12h period profile in proteins involved in photosynthesis during long photoperiods (including summer and neutral ones) in both microalgae and plants.

Integration of starch content diel oscillations with multi-omic data

There exists an influx of carbon compounds, obtained from the Calvin cycle, to starch. The accumulation and degradation of starch have been described to be circadian regulated, since periodic oscillations of its content is observed in photosynthetic organisms as *Chlamydomonas* and *Arabidopsis*, as well as a rhythmic gene expression profile of the enzymes involved in the process (Flis et al., 2019; Geigenberger, 2011; Kötting et al., 2010; Ral et al., 2006; Smith et al., 2004; Sulpice et al., 2014). *Chlamydomonas* reaches its maximum starch content few hours after sunset (Ral et al., 2006), while *Arabidopsis* plants does it exactly at sunset (Feugier & Satake, 2013; Kötting et al., 2010). In both organisms, starch starts to accumulate during the light hours, until amylases (AMY) are activated and its degradation starts. Starch content is a result of a controlled balance between its degradation, by AMY, and its synthesis, ADP-glucose pyrophosphorylase with its small and large subunit genes (APS and APL) (Fig. 37-A).

Starch content in *Ostreococcus tauri* under diurnal cycles is also rhythmic with a 24h period (Fig. 39-A) with a p-value < 0.05, which align with the periodic oscillations described in *Arabidopsis* and *Chlamydomonas*. However, *Ostreococcus* do not reach its maximum starch content around sunset as they do. Instead, the maximum starch content aligns with the high irradiance hours and it decreases gradually after that point, under both photoperiods (Fig. 39-A). These results suggest that there is not a conserved starch content temporal program throughout the green lineage, since its degradation starts at different times of the day depending on the organism.

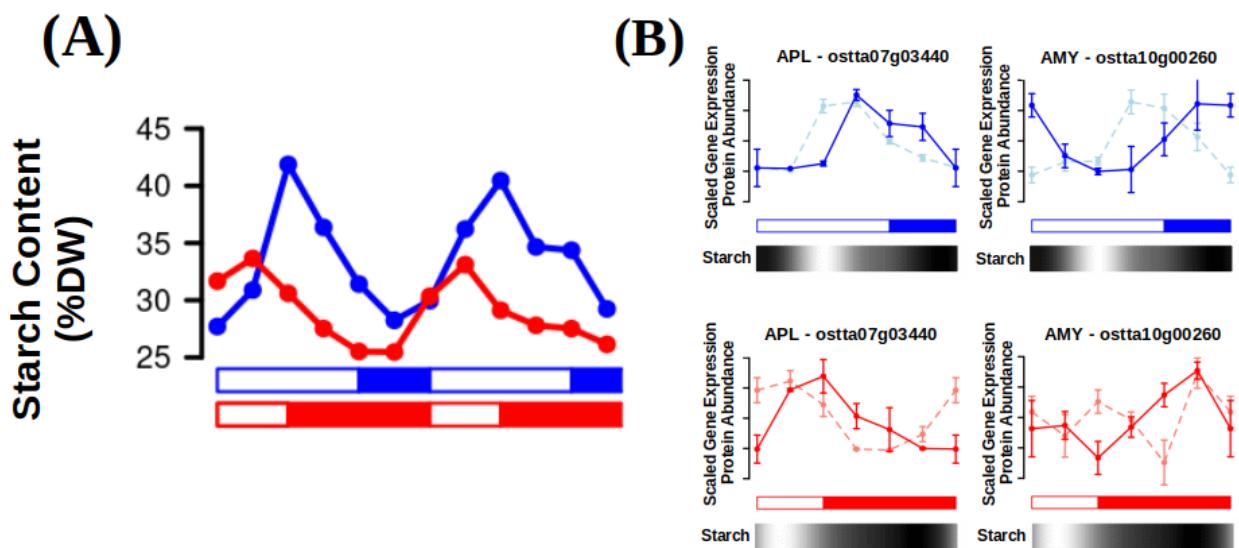


Figure 39. Multi-omics integration of starch periodic oscillations. (A) Starch content profile under summer photoperiod (blue) and winter photoperiod (red). (B) Scaled gene expression profiles (lighter dashed lines) and protein abundance profiles (darker solid lines) of the main enzymes involved in starch synthesis (glucose-1-phosphate adenylyltransferase, APL - ostta07g03440) and degradation (amylases, AMY - ostta10g00260). Starch content is represented as a color block that gradually changes from black (low starch content) to white (high starch content).

However, which seem to be conserved are the differences between photoperiods. In *Ostreococcus*, starch degradation during winter photoperiod seems to be executed more slowly than during summer photoperiod and a higher content in starch is reached during summer photoperiod, as it has been also described in plants (Feugier & Satake, 2013; Geigenberger, 2011; Kötting et al., 2010; Sulpice et al., 2014),

The starch temporal program observed in *Ostreococcus tauri* (Fig. 39-A) aligns with the transcriptomics and proteomics data generated and analyzed in this thesis. Under both summer and winter photoperiods, starch content reached its maximum at midday, despite the abundance of enzymes involved in starch biosynthesis, like APL (ostta07g03440), peaking several hours later, toward end of the day (Fig. 39-B). The halt in starch increase and the subsequent decrease in its content can be attributed instead to the activation of the genes encoding enzymes involved in starch catabolism or degradation, like AMY (ostta10g00260). The consequently increase in abundance of the corresponding proteins during the second half of the day under both photoperiod is, thus, coincident with the decrement in starch content (Fig. 39-B).

In chapter 3, gene-protein offsets were hypothesized to be affected by the photoperiod of entrainment and the biological process where those proteins are involved. Our findings show how temporal offsets of genes involved in the same biological process increase uniformly under short photoperiods, indicating a common regulation under seasonal cycles. In this metabolic pathway, offsets of APL (~4 h under both photoperiods) and AMY (8 h in summer and 12 h in winter) are different and they increment in a differently range under short photoperiod (Fig. 39-B). This support previous results in plants, that suggest the hypothesis that synthesis and degradation of starch are complex processes that are regulated by different mechanisms (Geigenberger, 2011; Hartman et al., 2023; Kötting et al., 2010). In addition, both APL and AMY protein abundance profiles are strongly coincident with their gene expression profile except for the described offset (Fig. 39-B), showing that at least transcriptomic regulation is one of the mechanisms that may contribute to starch synthesis-degradation balance in *Ostreococcus tauri* as it has been observed previously with *Arabidopsis* and other plants as well (Finegan et al., 2022; Geigenberger, 2011; Kötting et al., 2010; Sorokina et al., 2011).

Other metabolic pathways of *Ostreococcus tauri* showing periodic oscillations under diurnal and seasonal cycles

Carotenoids biosynthesis in Ostreococcus tauri under diurnal and seasonal cycles

Carotenoid are a group of isoprenoid pigments that are widely distributed among various organisms, including microalgae and plants. Some of these pigments are associated with light-harvesting complexes and perform a crucial role in photosynthesis by efficiently absorbing light energy and transferring it to reaction centers. In addition, carotenoids exhibit antioxidant properties, safeguarding the organism against potential harm induced by excessive light exposure and environmental stress (García-Plazaola et al., 2017; T. Sun et al., 2022). Remarkably, the expression of genes associated with carotenoid biosynthesis is intricately regulated by the circadian clock in both plants and algae. This circadian regulation ensures that the production and accumulation of carotenoids aligns with the physiological demands and environmental conditions. Optimal timing of carotenoids production maximize their effectiveness in light absorption, energy transfer, and antioxidant protection.

(Covington et al., 2008; García-Plazaola et al., 2017; Pan et al., 2009; T. H. Sun et al., 2010; Zhang et al., 2022).

In addition to their role in photosynthesis, carotenoids have considerable nutritional value for humans. Certain carotenoids, including β-carotene, possess the capacity to be converted into vitamin A, a vital nutrient essential for maintaining vision and bolstering the immune system. Furthermore, specific carotenoids, such as astaxanthin, have exhibited promising potential in promoting health, as they have been associated with reducing the risk of certain cancers and cardiovascular diseases. (Eggersdorfer & Wyss, 2018).

Microalgae have emerged as a promising source for large-scale production of carotenoids. However, the full potential of this technology has not been reached due to limited understanding of the molecular mechanisms underlying carotenoid biosynthesis. Over the past two decades, numerous research groups have been studying growth conditions, microalgae metabolism and optimizing photobioreactors design to maximize carotenoid production while minimizing associated costs (Del Campo et al., 2004; Hoys et al., 2021; Sierra et al., 2008).

Industrial-scale cultivation of microalgae is predominantly achieved in outdoor settings. Therefore, comprehending the oscillation patterns of carotenoid biosynthesis under diurnal and seasonal cycles becomes crucial for ensuring the maximum carotenoid content at harvesting time, as well as identifying potential gene and protein targets for further optimization. To understand the adaptive nature of carotenoid content to seasonal variations in diel cycles and its implications for optimizing light energy capture, photoprotection and, thus, its possibilities of industrial optimization, we examined the transcript and protein abundance profiles of carotenoid biosynthesis genes in *Ostreococcus*. In addition, these profiles were integrated with carotenoid content profiles as biological validation of the results (Fig. 40).

Integration of multi-omics data with oscillations described by carotenoids content in *Ostreococcus tauri* under diurnal and seasonal cycles

Ostreococcus tauri is rich in widely distributed carotenoids like violaxanthin, antheraxanthin or zeaxanthin. Although specific carotenoids of Mamiellophyceae like micromonal, uriolide or prasinoxanthin are also found in this prasinophyte, being prasinoxanthin the most abundant one (Egeland et al., 1995; Guyon et al., 2018; Six et al., 2009). Its genome presents

genes encoding for the Methylerythritol 4-phosphate (MEP) pathway (Derelle et al., 2006; L. Zhao et al., 2013), which derives pyruvate to the production of geranyl pyrophosphate (GPP), the main carotenoid precursor (Fig. 40-A). Most genes encoding enzymes involved in the MEP pathway peaked at sunrise under summer photoperiod and during the last part of the night under winter photoperiod, preceding the corresponding protein abundance peaks by 4 hours or less (Fig. 40-B). Similar patterns were observed in the first enzymes of the preceding carotenoid biosynthesis pathway or carotenogenesis. This pathway starts with the production of phytoene by the phytoene synthase (PSY), that will finally result in lycopene (Fig. 40-A). The progression of the MEP and carotenogenesis pathways allow the cell to produce the main precursors needed for carotenoids production and seems to be transcriptomically regulated in a similar way. In both pathways, enzymes are transcribed during the night under winter photoperiod ensuring the presence of its encoding proteins during the day (Fig. 40-B). From this point, the pathway diverges in two different branches, due to the Lycopene α/β cyclase (LCY α/β , ostta14g00700) that seems to be regulated in specific ways depending of the biological function of the carotenoids produced: β -branch, including the xanthophylls cycle; and α -branch, including the main antenna carotenoids in prasinophyte (Fig. 40-A) which biosynthesis pathways are still unknown (Guyon et al., 2018; Six et al., 2009).

Carotenoids content during diurnal cycles under both summer and winter photoperiods have been estimated from HPLC profiles, as described in Materials and Methods. The rhythmicity analysis detected rhythmic abundance profiles with periods of 24h in all carotenoids under both photoperiods. With the exception of lutein and violaxanthin, that do not maintain their rhythmicity under winter photoperiod. In general, fluctuations on carotenoids content during winter photoperiod are less drastic, resulting in a lower wave amplitude. (Fig. 40-A).

The xanthophyll cycle, the interconversion between violaxanthin, antheraxanthin and zeaxanthin as a response to light intensity (CITA), was especially active under summer photoperiod, although its activity was also detected under winter photoperiod. The changes in these xanthophylls coincided with the accumulation of transcripts and proteins encoded by genes associated with the xanthophyll cycle, with short temporal offsets (Fig. 40-A). Under summer photoperiod, the maximum protein abundance of violaxanthin de-epoxidase (VDE, ostta16g00660) (Fig. 40-B) match the increasing zeaxanthin content during the light

hours (Fig. 40-B) match the increasing content of violaxanthin during the dark hours (Fig. 40-C). These enzymes are transcribed sequentially with a clear temporal regulation. β CH gene is expressed early in the morning, sequentially followed by ZEP and VDE in that specific order under both photoperiods (Fig. 40-B). However, there is not a significant amount of zeaxanthin being accumulated during winter photoperiod. Instead, a high level of violaxanthin without drastic variations is maintained during diurnal cycles (Fig. 40-A). It suggests that xantophylls cycle is not enhancing the production of zeaxanthin due to the limited daylight hours during winter. There are some studies regarding irradiance stress in *Ostreococcus* and other prasinophytes that present variations in xantophylls contents in a similar way to the ones observed in this thesis due to short light periods (Böhme et al., 2002; Guyon et al., 2018; Six et al., 2009).

In general, the enzymes of the β -branch pathway seem to present a strong transcriptomic regulation to sequentially achieve their roles at the right time. However, these transcriptomic regulation does not reach the physiological level under winter photoperiod (Fig. 40-A), where xantophylls contents are similar to the ones observed in low irradiance stress experiments (Böhme et al., 2002; Guyon et al., 2018; Six et al., 2009). Suggesting that despite the strong temporal transcriptomic regulation that is also reflected in proteins abundance profile (Fig. 40-C), there are other regulatory pathways that balance xantophylls production under certain conditions like winter photoperiod.

Regarding pigment of the α -branch biosynthesis pathway, a lack of lutein during winter photoperiod is observed (Fig. 40-A). However, during summer photoperiod lutein is accumulated during the light hours, which is followed by the increment of prasinoxanthin content after sunset (Fig. 40-C). Lutein and prasinoxanthin contents behavior under summer photoperiod are similar to the results obtained in irradiance stress experiments in other prasinophytes. For example, a *Mantoniella squamata* study linked the accumulation of lutein to irradiance stress and its following conversion to prasinoxanthin when the stress condition is over (Böhme et al., 2002).

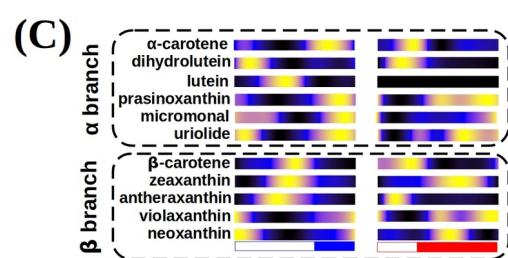
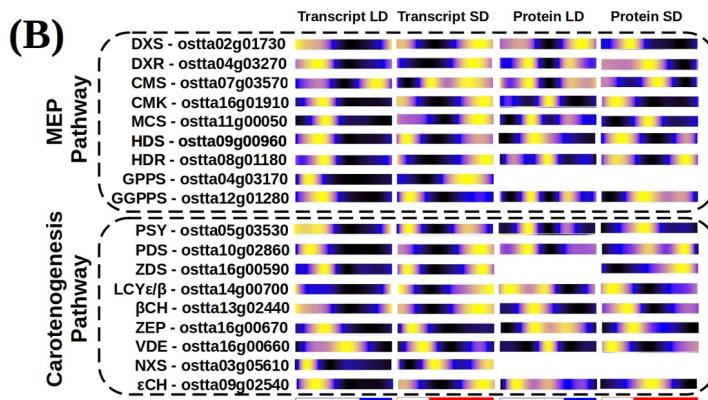
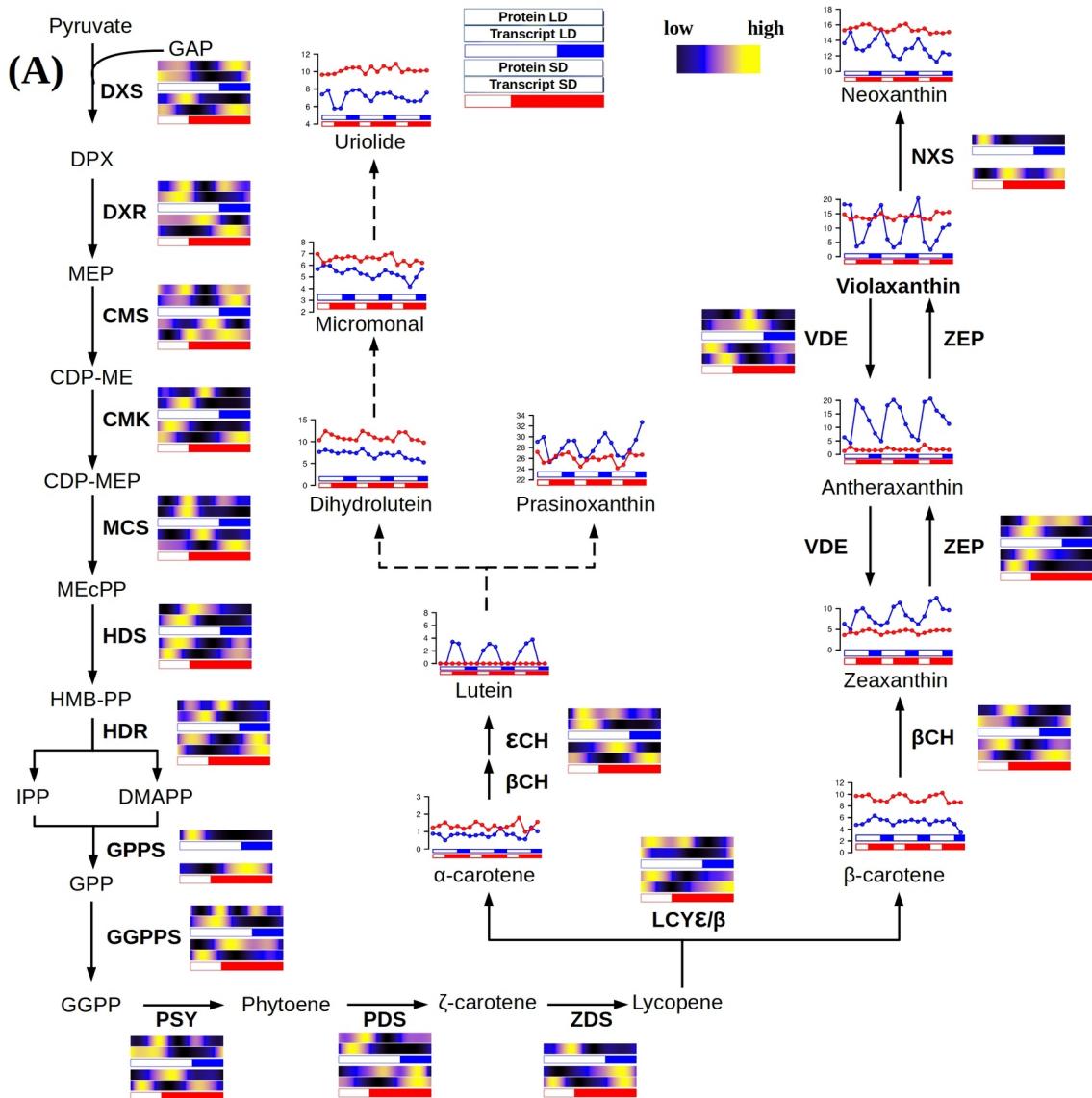


Figure 40. Integration of multi-omics data from the complete carotenoids biosynthesis pathway and carotenoids content of *Ostreococcus tauri*. (A) Schematic MEP pathway and carotenogenesis pathway according to (Egelund et al., 1997). (B) Organized list of the gene IDs involved in these pathways including multi-omic data. (C) Organized visualization of content oscillations of each carotenoid from the β and α branch.

Although the α -branch biosynthesis pathway is still unknown there are some hypothesis about the inter-conversion of lutein-prasinoxanthin (Egeland et al., 1997). This could be supported by our results in *Ostreococcus* since the contents of lutein and prasinoxanthin seem to be linked, not only under irradiance stress as it has been described in previous published studies, but under different photoperiods as well. However, the enzymes involved in the interconversion of these carotenoids remain to be identified, and a comparison of their gene expression and protein accumulation was not feasible in this study.

In summary, *Ostreococcus tauri* carotenogenesis present the common characteristics of a process regulated by the circadian clock, as being able to adapt to different photoperiods and presenting an anticipation to diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in *Arabidopsis* leaves I cyclic changes. However, content in carotenoids seem to also depend on other regulatory pathways besides the circadian clock, as some of the changes observed under the photoperiods studied align with high and low irradiance experiment results.

Nitrate assimilation under diurnal and seasonal cycles in Ostreococcus tauri

The macroelement nitrogen is also an essential component in biomolecules of great importance for all living beings. In the atmosphere, N₂ is the most abundant form of nitrogen. This gas is dissolved in water ecosystems but is inaccessible for microalgae, it can only be used by fixing bacteria like *Synechococcus*. In fact, nitrogen is a major limiting nutrient of marine phytoplankton (Barros et al., 2005; Mittag, 2001; Sanz-Luque et al., 2015).

O. tauri have developed competitive mechanisms to ensure nitrogen assimilation in the marine ecosystem. It can grow on nitrate, ammonium, and urea, and complete sets of genes allowing transport and assimilation of these substrates have been identified in its genome (Blanc-Mathieu et al., 2014; Derelle et al., 2006). Specifically, in the growth media used during this work, nitrate was the main nitrogen source (Table 2). In the previous chapters of this thesis, nitrate assimilation has been identified as one of the biological processes which genes and proteins present significant rhythmic profiles under diurnal cycles, as well as one of the processes with larger offset between gene expression and translation. In this section, enzymatic activities of two of the main enzymes involved in the nitrate

assimilation pathway are integrated with the transcript and protein abundance profiles of the complete pathway. This results give new insights on the adaptive response of this assimilation process to seasonal variations in diel cycles and its implications for optimizing nutrient uptake and metabolism.

Integration of key enzyme activities from nitrate assimilation pathway with multi-omic data

Nitrate is first transported into the cell by Nitrate Transporters 2 and 3 (NRT2, ostta10g00950 and NRT3, ostta10g00940), followed by its reduction to nitrite by Nitrate Reductase (NR, ostta10g00920). Nitrite is further reduced to ammonia by Nitrite Reductase (NIR, ostta10g00930). The central part of nitrate assimilation is played by the Glutamine Synthetase (GS, ostta01g05020) and Glutamine Oxoglutarate Aminotransferase (GOGAT, ostta14g01900) cycle, which converts inorganic nitrogen, ammonia, into glutamine and glutamate (Fig. 41-A), a central precursor for the biosynthesis of nitrogen-containing compounds such as amino acids and nucleotides. (Sanz-Luque et al., 2015).

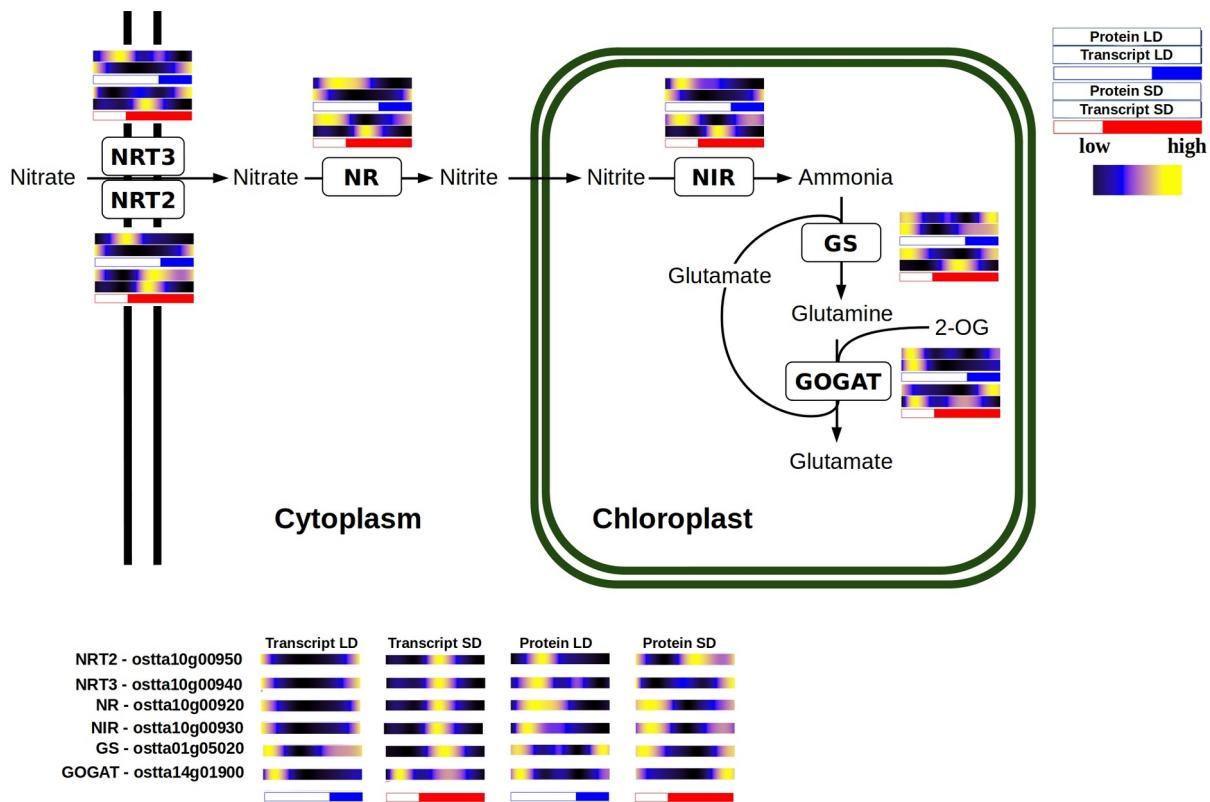


Figure 41: Multi-omics integration of nitrate assimilation pathway. Schematic nitrate assimilation pathway and list of the gene IDs involved including protein abundance and gene expression profiles of each enzyme.

During summer photoperiod, gene expression profiles of NRT2/3, NR and NIR reached their maximum at dawn (ZT0), while their protein abundances peaked 8 hours later at mid-day (ZT8), coinciding with the time point of maximum light irradiance. GS and GOGAT gene expression and protein abundance profiles are almost coincident, reaching their maximum at the beginning of the day (ZT4) without noticeable temporal offsets between them. However, a slight increase in protein abundance was detected at the end of the day (ZT12) for both GS and GOGAT (Fig. 41).

In contrast, under winter photoperiod, all genes encoding the transporters and enzymes involved in nitrate assimilation showed their maximum expression level during the first part of the night (ZT12-ZT16), preceding their protein abundance peaks at dawn (ZT0) or mid-day (ZT4) by 8 hours or more. Notably, GOGAT gene expression displayed a bimodal pattern under SD conditions, maintaining the peak observed under LD at ZT4 besides the new peak at ZT20. Therefore, GOGAT gene expression constitutes an example of the

emergence of complex expression patterns under summer photoperiod consisting of two gene expression peaks per day (Fig. 41).

Circadian oscillations in expression and activity of the first enzyme of this pathway (NR) have been described in *Arabidopsis* and other crop plants as maize or tomato (Lillo et al., 2001; Lillo & Ruoff, 1989; Tucker et al., 2004; Z. Yang & Midmore, 2005). In fact, light is apparently an important factor for NR to maintain its rhythmic behavior. Rhythms in NR activity or NR gene expression profiles were shown to persist only in continuous light in plants (Lillo et al., 2001; Lillo & Ruoff, 1989). These results are in agreement with the transcriptomic data obtained in this work, where NR gene expression rhythmic profiles, as well as other enzymes involved in this pathway, are maintained only under light-dark cycles and constant light.

To validate these results, the enzymatic activities of NR and GS are measured throughout complete diel cycles under summer and winter photoperiods. These measurements presented a significant rhythmic profile with a p-value lower than 0.05 and an almost non-existent offset between their protein abundance profiles and their activity profiles was found (Fig. 42).

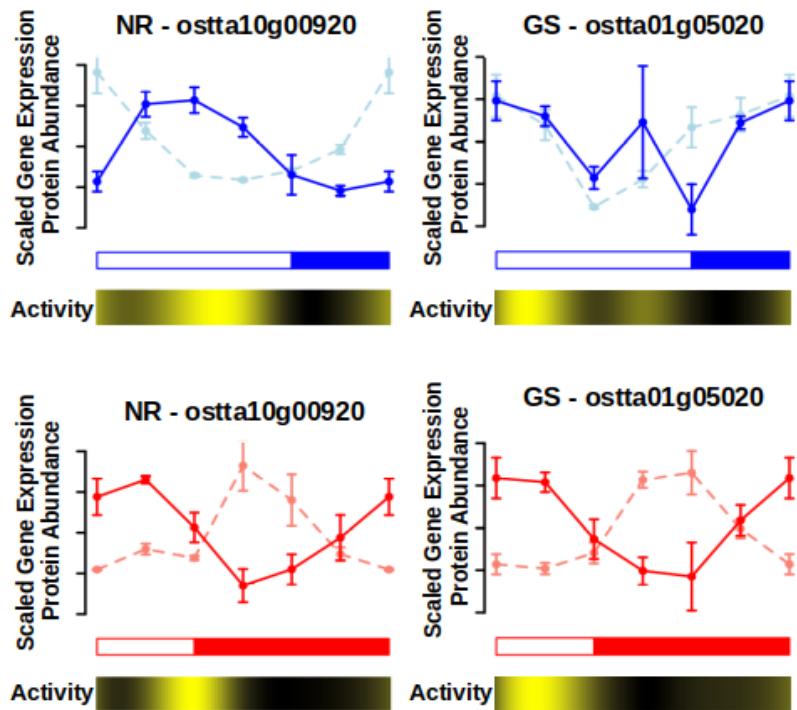


Figure 42. NR and GS rhythmic activity compared with its proteomic and transcriptomic data generated. Transcript (lighter color) and protein (darker color) abundance profiles for Nitrate reductase (NR, left) and Glutamine Synthetase (GS, right) under LD conditions (top, blue) and SD conditions (bottom, red). Heatmaps are incorporated below to represent the changes in enzymatic activity of these enzymes. Black represents low activity and white high activity.

The huge transcriptomic anticipation observed is adjusted by the clock taking in count the large offset between gene expression and translation described by the enzymes involved in this pathway. In fact, although genes are transcribed at different times under winter and summer photoperiod, proteins reach their maximum abundance level at a similar time of the day in both cases (Fig. 41, 42). This is a clear example of how *Ostreococcus* adjust its transcriptional program in order to ensure the presence of proteins at the exact right time, in spite of their specific translation offset.

Conclusions

- ¿Como puedo añadir algo sobre el chapter1? Copiado del chapter1: Since molecular systems biology has much to contribute to microalgae research, we hope that ALGAEFUN with MARACAS will reach the hands of many research groups and will be as useful to them as it has been to us. Editar y meter visitas.
- Around 80% of the *Ostreococcus* transcriptome present rhythmic expression patterns under diurnal cycles, but bona fide circadian genes represent only the 4% of the transcriptome.
- Although *Ostreococcus* is a photosynthetic organism, its transcriptome strongly depends on dark periods to maintain rhythmic expression patterns described under light-dark cycles. The effects of the free-running conditions observed over the transcriptome (greater desynchronization under LL, positive phase shifts under LL and negative phase shifts under DD) agree with the ones described in activity circadian records of nocturnal organisms.
- Seasonal cycles induced changes in gene expression profiles consisting of the emergence of 12 h period cycles, phase shifts and amplitude reductions.
- The 12 h period cycles (2 peaks every 24h) emerged under winter photoperiod are not a self-sustained rhythm, but a combination of two distinct rhythmic profiles: one depending on the photoperiod and another depending on the skotoperiod. These

rhythmic profiles are coincident under long photoperiods, becoming out of phase as photoperiod gets shorter.

- Only 25% of the *Ostreococcus* proteome present rhythmic protein abundances under diurnal cycles.
- The transcriptome of *Ostreococcus tauri* present a nocturnal activity (the maximum expression levels take place during the night hours) while its proteome present a clear diurnal activity (the maximum protein abundance levels take place during the light hours).
- There exist a temporal offset between gene expression and translation. The rhythmic wave described by a gene expression level and its encoding protein abundance are almost identical, except for their phase offsets.
- The offset between gene expression and translation increases during winter photoperiod and shows a different increase level depending on the physiological function in which they are involved. Therefore, *Ostreococcus tauri* severely reorganize its transcriptional temporal program taking in count not only the current photoperiod but also the different gene/proteins offsets that each biological function presents.
- There exist a temporal offset between the proteome of *Ostreococcus tauri* and the execution of their physiological processes.
- Cell division cycle is strongly influenced by the circadian clock in *Ostreococcus*, it can anticipate cyclic changes like the short time of light in winter photoperiods. To anticipate it, the circadian clock ensures that all cells have completed the division phase before sunrise, so any hour of light is wasted. The cell cycle temporal program observed is in agreement with the transcriptomic and proteomic data generated.
- **Photosynthetic** efficiency rhythmic oscillations present a 12 h period cycles under winter photoperiod, which is again in agreement with the transcriptomic and proteomic data generated. Photosynthesis machinery anticipation to photoperiod is transcriptionally regulated in *Ostreococcus tauri* and, probably, since early in the green lineage.

- Starch content in *Ostreococcus tauri* present a rhythmic 24 h cycle, reaching its maximum starch content at the high irradiance hours under both summer and winter photoperiods. Both APL and AMY protein abundance profiles are strongly coincident with their gene expression profile with a ~4h offset, showing that starch circadian synthesis-degradation balance is possibly transcriptomically regulated.
- *Ostreococcus tauri* carotenogenesis and nitrate assimilation pathway present the common characteristics of processes regulated by the circadian clock (at a transcriptomic, proteomic and physiological level), as being able to adapt to different photoperiods and present an anticipation to diurnal cyclic changes.

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