Universidad de Oviedo

Departamento de Biología de Organismos y Sistemas
Programa Oficial de Doctorado en Biogeociencias

Caracterización funcional e implicaciones biotecnológicas de los mecanismos moleculares de respuesta a estrés térmico y agentes xenobióticos en *Chlamydomonas reinhardtii*

Functional characterization and biotechnological implications of the molecular mechanisms of response to thermal stress and xenobiotic agents in *Chlamydomonas reinhardtii*

Tesis doctoral/*Doctoral thesis*

María Carbó Muñoz

Oviedo 2023

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Justificación

Oviedo, a 15 de Diciembre de 2022

Presidente de la Comisión Académica del Programa de Doctorado

Fdo.: María Aida González Díaz

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Contra la presente Resolución, podrá interponer recurso de alzada ante el Rectorado, en el plazo de un mes, a partir del día siguiente al de la presente notificación, de conformidad con el art. 122 de la Ley 39/2015, de 1 de octubre, de Procedimiento Administrativo Común de las Administraciones Públicas

SR. DIRECTOR DEL CENTRO INTERNACIONAL DE POSTGRADO

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Caracterización funcional e implicaciones biotecnológicas de los mecanismos moleculares de respuesta a estrés térmico y agentes xenobióticos en *Chlamydomonas reinhardtii*	Inglés: Functional characterization and biotechnological implications of the molecular mechanisms of response to thermal stress and xenobiotic agents in *Chlamydomonas reinhardtii*

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RESUMEN (en español)

Las microalgas están expuestas a diversos agentes estresantes, incluidos algunos de origen antrópico. Esta tesis tiene como objetivo el estudio de un estrés natural, el frío, y uno de origen antrópico, el Bisfenol A (BPA). El BPA se acumula en los ecosistemas acuáticos, siendo uno de los contaminantes más abundantes y peligrosos, dando lugar a trastornos endocrinos que en mamíferos derivan incluso en distintos tipos de cáncer. Sin embargo, y a pesar de esta evidencia, los efectos xenobióticos del BPA sobre las plantas y las microalgas aún son poco conocidos a nivel molecular. Para entender la respuesta, caracterizamos la respuesta fisiológica y proteómica de *Chlamydomonas reinhardtii* durante una exposición prolongada a BPA mediante el análisis de parámetros fisiológicos y bioquímicos combinados con proteómica. El BPA desequilibró la homeostasis del hierro y redox, lo que interrumpió por completo la función celular y desencadenó ferroptosis. Curiosamente, las microalgas tienen la capacidad de recuperarse de la exposición a este contaminante, tanto a nivel molecular como fisiológico, mientras acumula almidón a las 72 h de exposición a BPA. En este trabajo abordamos los mecanismos moleculares involucrados en la exposición al BPA, demostrando por primera vez la ocurrencia de ferroptosis en un alga eucariota, y cómo esta situación fue revertida por mecanismos de desintoxicación de ROS y otros reordenamientos proteómicos específicos. Estos resultados son de gran importancia no solo para comprender la toxicología del BPA o explorar los mecanismos moleculares de la ferroptosis en microalgas, sino también para definir

nuevos genes diana para el desarrollo eficiente de cepas de biorremediación de microplásticos.

La mayoría de los ecosistemas habitados por microalgas sufren intervalos de frío periódicos que impactan el ciclo de vida de las microalgas. El frío también es una fuente de estrés importante, pero también un fenómeno natural que marca el ritmo circadiano para las plantas terrestres a través de ritmos internos a cargo del establecimiento de las diferentes etapas del desarrollo (por ejemplo, floración, maduración del fruto, brote de las yemas). La comprensión de los mecanismos básicos que impulsan la percepción, señalización y aclimatación a bajas temperaturas en las microalgas podría darnos pistas sobre el origen del mecanismo más complejo observado en las plantas hoy en día. Entre los procesos celulares involucrados en la respuesta al frío, la epigenética parece ser un actor importante. Sin embargo, los mecanismos epigenéticos que tienen lugar en Chlamydomonas son poco conocidos. Por ello, cultivamos este microorganismo con 5 epi-drogas con capacidad de modular rutas epigenéticas. Bajo estrés por frío, en combinación con los epi-drogas, se midió el crecimiento de Chlamydomonas durante 72 h. La reducción de la acetilación en histonas condujo a una disminución del crecimiento. Además, la reducción de la metilación en adenina en condiciones de frío indujo la muerte celular tras 72 h de crecimiento. Sin embargo, la reducción del contenido de 5mC no induce la disminución de la biomasa celular como la inhibición de 6mA, mientras que la inducción de la metilación general mejora ligeramente el crecimiento.

Gracias a los hallazgos proporcionados por el enfoque de epi-drogas, investigamos el papel de la 6mA (marca característica de los procariotas). Primero, cultivamos Chlamydomonas en frío más la combinación de inhibición 6mA y agentes que promueven la metilación. Este tratamiento combinado recuperó parcialmente el crecimiento de Chlamydomonas en comparación con aquellos con 6mA reducidos. Además, descubrimos que después de 6 h de tratamiento con el agente reductor de 6 mA en frío, Chlamydomonas no pudo recuperarse incluso retirando la droga de los cultivos. Para obtener información sobre los mecanismos de metilación relacionados con la aclimatación al estrés por frío, analizamos la epigenómica y la transcriptómica de cultivos refrigerados en tratamientos de control y con el agente reductor de 6mA. Los resultados mostraron que el epi-droga utilizado redujo la metilación de la adenina en las primeras 5 horas de tratamiento. La metilación de adenina acumula mayores diferencias entre los

controles y los tratamientos con pyrimidinedione. Finalmente, definimos cómo Chlamydomonas 6mA es clave en la modulación de genes de respuesta para enfrentar el estrés por frío y la supervivencia.

RESUMEN (en Inglés)

Microalgae are exposed to diverse stressing agents including those some of anthropic origin. This thesis targets the study of one natural stressor, cold, and one of anthropic derived, Bisphenol A (BPA). BPA accumulates in the aquatic environments, being one of the most abundant and dangerous, leading to endocrine disorders deriving even in different types of cancer in mammals. However, despite this evidence, the xenobiotic effects of BPA over plantae and microalgae are still poorly understood at the molecular level. To fill this gap, we characterized the physiological and proteomic response of *Chlamydomonas reinhardtii* during a long-term BPA exposure by analyzing physiological and biochemical parameters combined with proteomics. BPA imbalanced iron and redox homeostasis, which completely disrupted cell function and triggered ferroptosis. Intriguingly, this microalgae defense against this pollutant is recovering at both molecular and physiological levels while starch accumulation at 72 h of BPA exposure. In this work, we addressed the molecular mechanisms involved in BPA exposure, demonstrating for the first time the induction of ferroptosis in a eukaryotic alga and how this situation was reverted by ROS detoxification mechanisms and other specific proteomic rearrangements. These results are of great significance not only for understanding the BPA toxicology or exploring the molecular mechanisms of ferroptosis in microalgae, but also for defining novel target genes for microplastic bioremediation efficient strain development.

Most of the ecosystems inhabited by microalgae underwent periodic chilling intervals which impact the microalgae life cycle. Cold is also a major stressor but also a zeitgeber for land plants entraining internal rhythms in charge of the setting of developmental milestones (e. g. flowering, fruit maturation, budding). The understand of the basic mechanisms driving the perception, signaling and acclimation to low temperatures in microalgae could give us clues about the origin of the more complex mechanism observed in plants nowadays. Among cellular processes involved in cold response epigenetics seen to be a major player. However, epigenetic mechanisms that take place in Chlamydomonas are poorly

understood. Because of that reason we cultured this microorganism with 5 epi-drugs with the capacity of modulating epigenetic pathways. Under cold stress, in combination with the epi-drugs, Chlamydomonas growth was measured during 72 h. Reducing acetylation led to a growth decreasing. Moreover, impairing adenine methylation under cold condition induced the cell death after 72 h of growth. However, reducing 5mC content does not induce the cell biomass decrease as 6mA inhibition, while inducing general methylation slightly improves the growth.

Thanks to the findings provided by the epi-drugs approach, we aimed to investigate the role of the 6mA (characteristic of prokaryotes). We first cultured Chlamydomonas under cold plus the combination of inhibition 6mA and promoting methylation agents. This combined treatment partially recuperated the Chlamydomonas growth comparing with those with 6mA reduced. Additionally, we discovered that after 6 h of 6mA reducing treatment under cold, Chlamydomonas couldn't recuperate even retiring the drug from the cultures. To get insights the methylation mechanisms regarding to cold stress acclimation, we analyzed the epigenomic and the transcriptomics of chilled cultures in control and 6mA reducing treatments. Results showed that the epi-drug used reduced the adenine methylation in the first 5 hours of treatment. Adenine methylation in accumulated higher differences between controls and pyrimidinedione treatments. Finally, we defined how Chlamydomonas 6mA is key when modulating responsive genes for facing cold stress and survival.

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Abbreviations

ABA	Abscisic acid
ACSL	Acyl-Coa synthetase long-chain
AKR1C	Aldoketoreductase type 1C
BCA	Bicinchoninic acid
BPA	Bisphenol A
CAH	Carbonic anhydrase
CAT	Catalase
CcrM	Cell cycle–regulated methyltransferase
CEF	Cyclic electron Flow
ChIP	Chromatin immunoprecipitation
CKIN	Chlamydomonas sucrose non-fermenting related kinases
COX2	Cyclooxygenase 2
CRISPR	Clustered regularly interspaced short palindromic repeats
$_{dd}H_2O$	Double distilled water
DHODH	Dihydroorotate dehydrogenase
DME	DEMETER
DMSO	Dimethyl sulfoxide
DNMT	DNA methyltransferase
Epi-drugs	Epigenetic drugs
FDR	False discovery rate
FEA1	Low iron-inducible periplasmic protein 1
FEA2	Low iron-inducible periplasmic protein 2
Fer-1	Ferrostatin-1
Fer1	Ferritin heavy chain 1
Fer2	Ferritin 2
FLC	Flowering locus C
FSP1	Ferroptosis Suppressor protein 1
GPX4	Glutathione peroxidase 4
GSH	Glutathione
GSS	Glutathione Synthase
GST	Glutathione S-transgerase
HAC	Histone acetylase
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HDM	Histone demethylase
HK	Histone kinases
HMT	Histone methylase

HSP90	Heat shock protein 90 chaperone
KDM	Lysine demethylase
KOD	Kiss of Death
LIP	Lipid iron pool
LOX	Lipoxygenase
MAPK	Mitogen activated protein kinase
MeDIP	Methylated DNA inmunoprecipitation
MDA	Malondialdehyde
miRNAs	microRNAs
MnSOD	Manganese Superoxide Dismutase
MS	Mass spectrometry analysis
MSTFA	N-Methyl-N-trimethylsilyl-trifluoroacetamide
PacBio	Pacific Biosciences
PCD	Programmed cell death
PEPC	Phosphoenolpyruvate carboxylase
PGD1	Plastid galactoglycerolipid degradation 1
PHB	poly-β-hydroxybutyrate
PpSnRK	*Pinus pinaster* SnRK
PSII	Photosystem II
PTMs	Post-translational modifications
PUFAs	Polyunsaturated free fatty acids
Pyrimidinedione	Pyrimidinedione,1-(4 bromophenyl)-5-(2-furylmethylene)-3-phenyl-2-thioxodihydro-4, 6 (1H,5H)-pyrimidinedione
RdDM	RNA-directed DNA methylation
ROS	Reactive Oxygen Species
ROS1	Repressor of silencing 1
SAM	S-adenosylmethionine
SHMT	Serine hydroxymethyltransferase
siRNA	Small interfering RNA
SMRT	Single molecule real time
SnRK	Arabidopsis sucrose non-fermenting related kinases
SOD	Superoxide Dismutase
sPLS	Sparse Partial Least Squares
sRNAs	Small RNAs
TAGs	Triacylglycerols
TCA	Tricarboxylic acid cycle
TE	Transposable elements
TGS	Transcriptional gene silencing
THF	Thylakoid formation protein
TSA	Trichostatin A
TSS	Transcription start site

TSS	Total Soluble Sugars
VDAC1	Voltage-dependent anion channel 1
VDAC2	Voltage-dependent anion channel 2
VIPP1	Vesicle inducing protein in plastids 1
WGBS	Whole genome bisulfite sequencing
5-aza-2-deoxy	5 -Aza-2′-deoxycytidine
5hmC	5 hydroximethylcytosine
5mC	5 methylcytosine
6mA	6 methyladenosine

General index

CHAPTER 1

General introduction, approach & objectives

CHAPTER CONTENTS

Microalgae as source of biomolecules and bioremediation strategies

Plants are well-known as high added value biomolecules producers and good bioremediatory organisms. For millenniums plants were used to obtain fiber, feedstock, timber or medicine drugs. In the last century, the need for novel environmentally friendly strategies for alimentary, cosmetic, pharmaceutical, chemistry or textile industries raised new needs to be addressed by the green biotechnology [1]. Plants and microalgae have been revealed as a good alternative as they can turn their primary carbon metabolites into secondary metabolites demanded by industry, in fact we can control de accumulation of some of them by applying different stresses [1]. Furthermore some photosynthetic

organisms are able to uptake heavy metals, plastics, or other pollutants being useful for bioremediating environments. Phytoremediation is considered economically viable with a high acceptance rate by the public and reduced risk of spreading the contamination and can be used simultaneously for remediation of more than one type of pollutant (e.g., heavy metals and microplastics) [2] while producing biomolecules of interest. Good examples are the antimalarial artemisinin production by *Artemisia annua* while uptaking As from soil [3,4] or the capacity of some aquatic plants as *Landoltia punctata*, and *Azolla filiculoides* to produce bioenergy while retiring nitrates and phosphates from textile industry wastewaters [5]. Despite their success, phytoremediation and plant derived biomolecules production are commonly associated to some negative aspects, as lack of sustainability, land usage or compromised food security [6]. To solve associated disadvantages, non-crop species have been proposed as source of high-value molecules and bioremediation tools, being the microalgae the most promising organisms to substitute them [7].

Microalgae have many advantages over plants, such as their rapid growth rate, high biomass yield and high rate of atmospheric CO_2 capture, together with no direct competition for agricultural land usage [7]. In addition, it is important to integrate microalgae high added-value compounds production with phytoremediation to cost effectively remove contaminants from water through biomolecules production. These organisms are capable to fix carbon into lipids and carbohydrates, that are being extensively used in cosmetic, nutraceuticals, pharmaceutical industry while are considered the third-generation biofuels. They are a sustainable source of carotenoids, vitamins, omega-3-fatty acids or biohydrogen [8]. In this line the concept of biorefinery was coined to descript the process in which the microalgae produce these bioproducts while utilizing nitrates, phosphates, carbon and microelements from wastewater to biomass enhancement. This concept covers from the upstream and the downstream process of the microalgae biorefineries. The upstream process involves the strain, the nutrient source supply and the illumination source. The downstream process covers from the harvesting to the bioproducts purification [9]. Because of all these reasons, microalgae biorefinery seem to be a sustainable way to produce high value biomolecules.

However, the kind of biomolecule accumulated by microalgae is generally related to the applied stressor. For example, *Chlamydomonas reinhardtii* accumulates starch under cold stress [10] and low UV-C doses [11], sugars and glycerol under osmotic stress [12] or lipids under S [13] or N deprivation [14]. Other species such as *Dunaliella* sp. or *Chlorella vulgaris* increase lipid accumulation under high salinity [15].

Moreover, microalgae have the capacity to bioremediate pollutants from wastewater, and this property has been also evaluated by many researchers for simultaneous wastewater treatment and biofuel generation [16]. For example, *Scenedesmus quadricauda* has been used for Cr bioremediation while stimulated lipid and carbohydrate production [17], *Haematococcus pluvialis* stimulates astaxanthin production while removing Cu from the culture media [18]. *Chlorella regularis* is capable to remove B meanwhile produce lipids and starch [19]. *C. vulgaris* was efficiently employed for textile wastewater bioremediation while lipid production [20]. Although there are several publications about heavy metals bioremediation and their consequent stress biomolecules of interest production, just a few are published about the use of other type of pollutants as plastic components. Plastic components are widely distributed in wastewaters from both industry and landfills. Microalgae has been revealed as promising organisms to promote the investigation in this field, as some assays have described their capacity [2]. However, there is still a scarce information in this field, creating the necessity to fill this gap.

While stress successfully modulates algal biomass composition, yield is generally reduced. This fact, together with the energy consumption from stress application increase the production cost. For these reasons, the sustainability concept of biorefinery starts to be far to be the expected, decreasing the interest of the industry in microalgae biorefineries. Although the associated disadvantage, the microalgae potential as bioproducers and bioremediators incentive the research about possible solutions, starting with the improvement of culture management for growth condition optimization, and the abiotic stress knowledge generation for metabolic engineering.

Microalgae-based bioremediation of microplastics

Today, plastics represent the most widespread environmental pollutant: their production exceeds 350 million tons per year [21], with plastic fragments and waste detected in every environment, including mount Everest to the Mariana Trench's depths [21,22]. Once plastics end up in the environment, their degradation can take up to several hundred years, making them persistent pollutants. Degradation of plastics has different routes depending on the environmental matrix in which they are found and plastics' chemical composition. The most common process is light-induced photodegradation (UV radiation; [23]), but it can also include mechanical, thermal, and biological degradation [24].

Plants have been historically discarded for plastic bioremediation as these biological systems as they were not affected by micro and nanoplastics, but contrary to these beliefs it was recently demonstrated that plants cannot absorb plastic particles. Microplastics

usually cannot be absorbed by plant root systems due to their size, but some reports indicate they might enter plant tissues through stomata. On the other hand, nanoparticles can enter plant root systems, and reports of their transport via xylem to upper plant parts have been recorded. Bioaccumulation of nanoplastics in upper plant parts is still not confirmed. The prospects of using biosystems for the remediation of soils contaminated with plastics are still unknown [19]. However, algae could be used to degrade plastic particles in water systems [19,25].

Chlorella and Scenedesmus bioremediation study showed that positively charged particles are adsorbed on the surface of algae more than negatively charged ones [26]. This is the first step in microalgal absorption, but even this initial adsorption step is harmful for algae, as it can reduce the photosynthetic rates affecting ocean carbon stock, and to the environment as adsorbed plastics in microalgae are later eaten by zooplankton and sea macrofauna [2,27]. In a second step, plastics can be introduced within the cell following active or passive transportation of the pollutant into the cell. Nano and microplastics (diameter < 10 μm) can passively cross eukaryotic membranes and use non-specific channels [28]. Active assimilation involves specific carriers crossing the cytoplasmic membrane, and are usually coupled to catabolic pathways to oxidize plastics for energy and biomass production. Alternatively, plastics can be imobilized within algal cell walls or in the vacuole to prevent toxic effects on the cell [29]. Furthermore, microalgae can form biofilms over plastics, actively inducing cracking and increasing its surface/volume and secrete degradative enzymes, which also helps reduce the plastics' molecular weight, facilitating the introduction of the molecules within the cell.

When plastic interacts with algae, the accumulation of plastic particles can result in reduced photosynthetic capacity of the organism due to the reduction of the amount of light passing to the algae, resulting in reduced survival, and increased oxidative stress. Similarly, Casado *et al.* (2013) and Bergami *et al.* (2017) [30,31] observed a reduction in the growth of *Pseudokirchneriella subcapitata*, *Dunaliella tertiolecta*, and *Artemia franciscana*. In contrast, Long *et al.* (2017) [31] observed no effect on the physiology of the algae *Chaetoceros neogracile*, *Tisochrysis lutea*, and *Hormophysa triqueta* in case of particles larger than 1 μm but instead noted a tendency of particles to aggregate [2]. Sjollema *et al.* (2016) [32] found no effect on photosynthesis but a dramatic reduction in *D. tertiolecta*. Laboratory research on *Scenedesmus obliquus* [33] and *Scenedesmus costatum* [34] demonstrated a reduction of chlorophyll content, but the concentrations of plastic particles in the experiment exceeded the concentrations that are recorded in aquatic ecosystems. When particle concentrations were closer to the environmental ones,

the effects are more contained, and in some cases, enhanced growth is recorded [32]. However, in experimental setups, exposure times are short compared to environmental exposure of algae to plastic pollution, and obtained results must be taken with caution. In vivo, prolonged exposure of algae to even lower particle concentrations would reduce growth rate and, in some species, chlorophyll content and photosynthetic rate. Plastic biodegradation exhibited by microalgae occurs in four essential steps, as described by Dussud and Ghiglione (2014): biodeterioration (microalgal biofilm over plastic surface, increasing pore size and provoking cracking, which may be helped with modifying the pH inside plastic pores), biofragmentation (extracellular enzymes which reduce the molecular weight of polymers in order to be assimilated), assimilation (use plastics as carbon source) and mineralization as the ultimate step excreting completely oxidized metabolites (CO_2, N_2, CH_4 and/or H_2O). Not all microalgae species can perform these four steps, neither all plastics can be processed, so a wide range of metabolic responses can be observed ranging from a carbon storage compound enhancing cell growth (poly-β-hydroxybutyrate (PHB) in the model cyanobacterium *Synechocystis* [35]) to a near-complete metabolic disruption (Bisphenol A in *Mycrocystis aeruginosa* [36]). As an example, the bioassimilation involves several enzymatic steps, for instance, the metabolic incorporation of PHB only requires two steps for being processed by ß-oxidation enzymes, and four to reach the form of acetyl-CoA while polystyrene requires 12 steps to be transformed into succinyl-CoA [37]. The toxic effects of plastic particles on algae largely depend on the characteristics of the algae membranes and their species-specific physiology and the type of polymer considered [34]. Further research is necessary to fully understand these effects, as to how the different nanoplastics disrupt metabolism is still not clear.

For this reason, the importance of investigations oriented towards plastic pollution solutions cannot be emphasized enough. Some algal species show potential for phycoremediation due to the presence of polyethylene terephthalate-degrading enzyme (PETase) such as *Chlamydomonas reinhardtii, Cylindrotheca closterium* and *Phaeodactylum tricornutum* and could be considered for biodegradation processes. Use of algae should be further investigated for uses as phytoremediators of plastic pollution from the aquatic ecosystems.

Microalgae as biofuel resource

Fossil fuels are strongly associated to global warming, pollution, and global greenhouse effects. Therefore, there is a wide concern about the search of environmentally-friendly

substitute of fossil fuels capable of satisfying the growing global energy demand. Among all the alternatives, biofuel present relevant advantages between all the options [38].

Plant biofuels has been hard researched and implemented. For example, crops as sugarcane and palm oil have been yet used for fossil fuels replacement, being the first generation of biofuels. The second generation was emerged from the use of lignocellulosic feedstock, the non-edible parts from food crops that are usually discarded such as stems and leaves. Despite these generation of biofuels can supply a high demand for fossil fuels substitution, are associated with numerous negative aspects for society. Between them are competition with alimentary crops for land and the extensive land usage [38]. Therefore, the solution came from the third generation of biofuels, made by microalgae. Microalgae based biofuels have several advantages over the previous generations, raising the research in this field.

These microorganisms are capable to efficiently uptake CO_2 and light and convert into lipids and starch, the precursors of biofuels, in a more efficient way than plants used for previous based biofuels due to their rapid biomass production rate [16,38]. These species do not compete with food crop production and don't require fresh water for their growth, as they are capable to grow in wastewater [16,38]. Microalgae produce primary metabolites as carbohydrates, lipids, proteins along with a range of commercially important products i.e., phycobilins, carotenoids, sterols, vitamins, etc. [16]. From these, neutral lipids, primarily triacylglycerols (TAGs), are used to produce biodiesel and are a promising source of edible oils. These lipids are produced in the chloroplast and accumulated in droplets. Starch formed by microalgae, is structurally similar to those from higher plants, and can be used to produce bioethanol, biohydrogen and biobutanol under controlled conditions [39]. Despite molecules triggering biofuels in these species are produced under environmental conditions, wild-type microalgae are not capable of producing enough lipids to satisfy the global energy demands [38].

To solve this problem, with the knowledge that starch and lipids are energy storage molecules the strategy of their accumulation induction is being applied. The main chemically stimuli for starch and/or lipid production are the lack of essential nutrients as nitrogen [16] or sulfur [40], and the major physical stimuli are temperature [16] and light intensity [41]. Nitrogen deprivation lead to accumulation of oil bodies in Chlamydomonas [14], Chlorella and Nannochloropsis [42]. Systems biology integration of proteomics and metabolomics revealed that cell growth and N metabolism are linked by the branched chain amino acids, suggesting an important role under N deprivation stress. Lipid

accumulation was also tightly correlated to the COP II protein, involved in vesicle and lysosome coating, and a major lipid droplet protein [14]. Sulfur deprivation triggers starch accumulation in Chlamydomonas [13] and lipids in Phaeodactylum [43]. Response to S depletion include the cessation of cell division, the accumulation of storage starch, and a decrease in metabolic processes including photosynthesis [44]. Specific responses include elevated SO_4^{2-} transport activity and the synthesis of enzymes required for efficient S assimilation. Osmotic stress in Chlamydomonas is key to stimulate sugar accumulation by a complete proteome remodeling to achieve metabolic changes through signalling redox, phosphorylation and epigenetic mechanisms [12]. Starch enhancement is also triggered under low UV-C doses, which misbalances redox homeostasis, and triggers reactive oxygen species (ROS) scavenging and protein damage repair/avoidance elements upregulated along with others related to the modulation of photosynthetic electron flux, carbon fixation and C/N metabolism [11]. The stress triggering more starch accumulation in Chlamydomonas is cold. Low temperatures activate gluconeogenesis and starch biosynthesis pathways leading to a pronounced starch and sugar accumulation, while growth rate is significatively inhibited [10].

Nevertheless, all characterized stress application to microalgae for enhancing biomolecules production have in common that reduce the growth rate significatively. This low productivity and production high cost associated to stress application make difficult this biorefineries processes to be scaled up and commercialized. Improvement of photosynthetic efficiency for productivity enhancement is a must to reduce cultivation cost. Therefore, solutions include strain improvement and culturing process optimization. Genetic engineering would improve starch and/or lipid production without compromising growth and removing stress application. To carry out this solution, further understanding of biosynthesis pathways and regulatory mechanisms of microalgae starch and lipid accumulation under stress is required [39].

Chlamydomonas reinhardtii: the microalgae model between plants and animals

Chlamydomonas reinhardtii is a single-celled green microalgae. Various features make this chlorophyte an excellent research model. Its characteristic fast cell cycle (sexual and asexual) (*Figure 1, Figure 2*), its rapid growth, and the ability to grow under darkness condition with a C source (mixotrophic growth) while maintaining photosynthesis apparatus make this species perfect to work with (*Figure 1*) [45,46]. Moreover, this species has motile cilia with the same structure proteins as the mammals (*Figure 1*) [45,46]. This

wide research in both mammal and plant fields, allowed the nuclear [47], chloroplast and mitochondrial sequencing [48–50]. The most remarkable from its sequenced genome, is that revealed the evolution of key animal and plant functions [47]. Because of that, classically, has been studied as a photosynthesis model, but also for cilia research, characteristic of mammals [45,46]. Chlamydomonas use as laboratory model allowed the development of transformation and isolation of mutants protocols, the construction of a genome-wide library of mapped, indexed insertional mutants and recently, the CRISPR-mediated gene disruption protocols [46].

Chlamydomonas has been used in laboratory field for many decades, but nowadays, with the increasing necessity to look for renewable resources and bioremediate pollutants from water, it is also used in industry research. For further understanding the accumulation of biomolecules of interest and the bioremediation capacities of this species, omics studies are being carried out in the last decade.

Chlamydomonas stress acclimation response

Green organisms are commonly exposed to environmental stresses and, because of that, are prepared to efficiently cope with several stresses by modifying their physiology and development [52]. The capacity to cope with the different stressors determine not only survival, but also growth, reproduction, and in the case of crops, yields. Consequently, the acclimation stress responses have been widely studied, being the most common stress-responses the production of ROS, photosynthesis decrease and development arrestment. Intense stresses can also lead to programmed cell death (PCD), hypersensitive reaction, or autophagy, as it was described in Arabidopsis [53–55] but, however, in Chlamydomonas are still under study.

Like land plants, Chlamydomonas has the mechanisms to face environmental stresses. Chlamydomonas strategies to cope with abiotic stress rely on the intensity of the stress (*Figure 3*). Under moderate stress, acclimation mechanisms are triggered to better resist the stress (stress level 1). This is for example the effect of a first low UV-C stress, which induces the resistance to a second stress shock of single oxygen induced by rose bengal [11]. A more intense stress, induce the aggregation of cells, forming multicellular structures (stress level 2). Several abiotic stresses induces cell aggregation, as nitrosative, paraquat, heat, rose bengal [56] and polyestyrene microplastics [57]. If more intense conditions are applied, this species has the ability of self-destruction, releasing metabolites to the medium that triggers stress response mechanisms in cells nearby (stress level 4). This is the case

of the induction of PCD, as caused by acetic acid [58]. If a higher intense stress is applied, cells can be destroyed via necrosis (stress level 5) [59] as triggered by Cd stress [60].

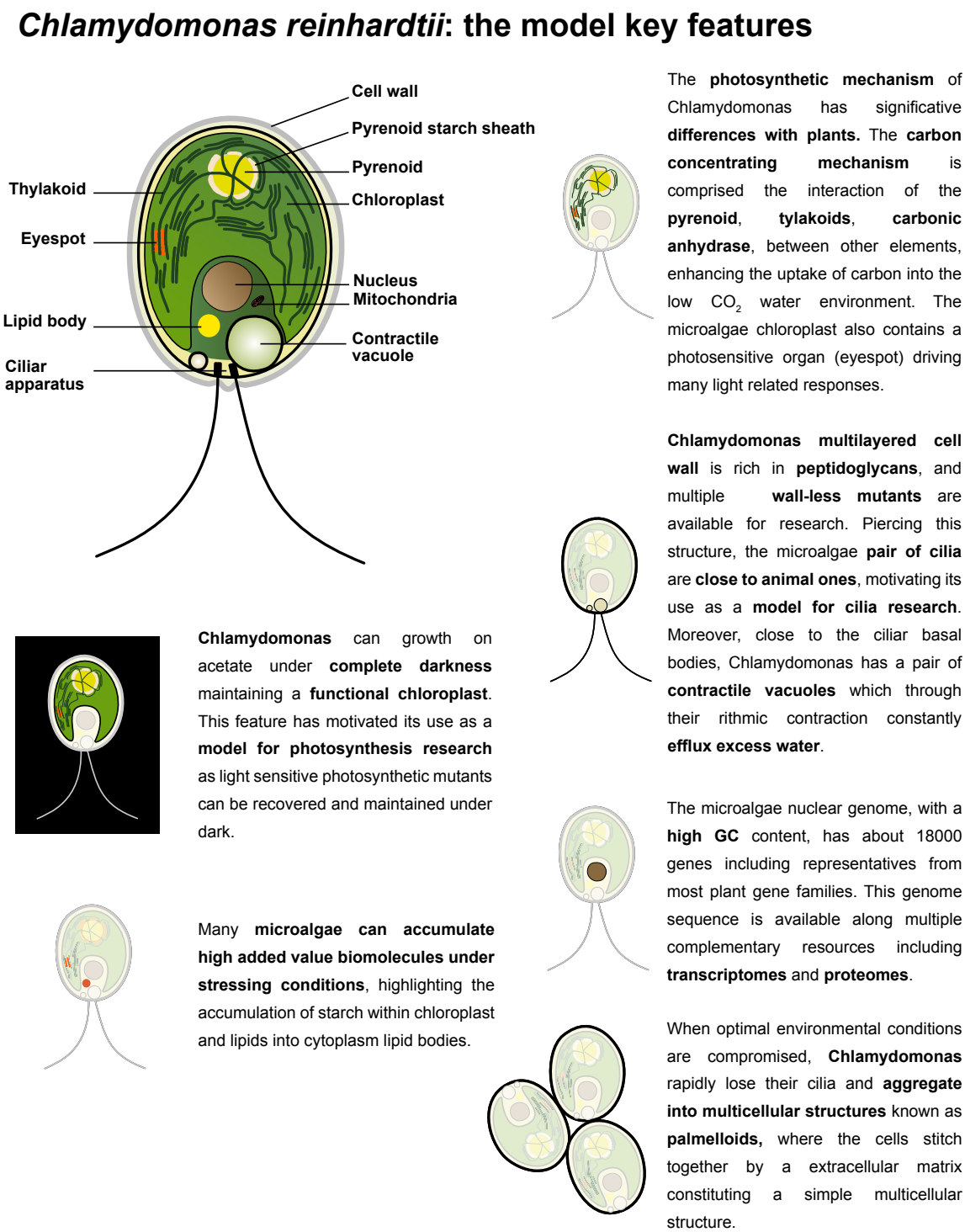

Figure 1. Main structural and functional features of the microalgae model *Chlamydomonas reinhardtii* which have motivated the use of the fast-growing organism as a research model for different organisms including close microalgae species with industrial interest, plants and animals (Modified from Colina (2019) [51]).

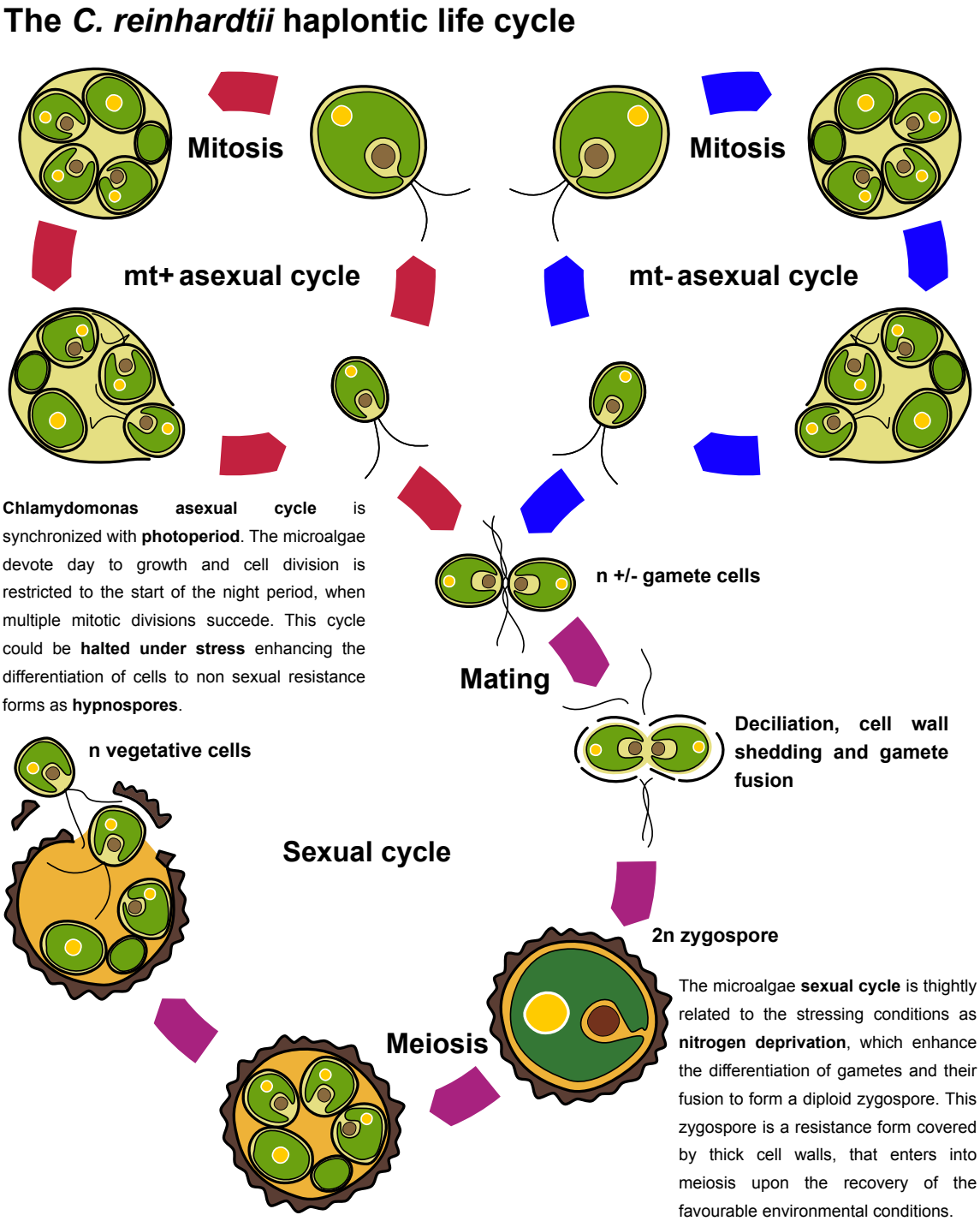

Figure 2. Life cycle of *Chlamydomonas reinhardtii*. This species divides by mitosis only into its haploid phase and upon the start of the night period. Duging the day, Chlamydomonas grow as a single cell, and after, this species could divide into as much as 30 cells by rapidly succeding mitotic cycles. However, under stress, different mating type but isogamous ciliated cells (gametes), fuse to create a diploid cell called zygospore. When optimal growth conditions restablished, zygospore enters into meiosis giving to haploid and ciliated vegetative cells (Modified from Colina (2019) [51]).

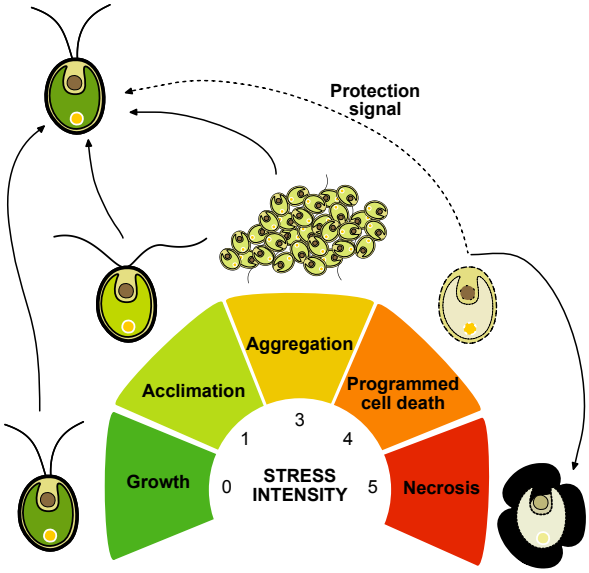

Figure 3. Chlamydomonas strategies to cope with stress, modified from [59].

Chlamydomonas specific molecular responses to abiotic stress induces ROS production, and consequently oxidative stress cell damage while promotes defense signaling responses [61]. Therefore, the first line of defense of this microorganism relies in the reactive oxygen species mitigation by production of alleviation enzymes and metabolites, the disturbance of photosynthesis and consequently cell cycle arrestment [59,61]. Besides all abiotic stress produces this common cell damage, every stress induces specific responses.

One of the stress that has a great of interest to study specific responses is the cold induction. This stress is one of the environmental factors limiting plant growth and development. The capacity to respond and acclimate to cold is shared from single celled to higher land plants. Thus, the knowledge of how this cold adaption mechanisms evolve from microalgae to higher plants would help to engineer crops and species of biomolecules of interest production such are microalgae.

Cold stress induces specific responses in Chlamydomonas, as it has been described by a systems biology approach (Valledor *et al.,* 2013). Low temperature implies stopping cell division and a dramatic change of cell morphology. Cell size increases with time while nucleolus density decrease. Chloroplast changes its shape and the starch shell around the pyrenoid and the vacuoles size increase. Ciliary apparatus is lost after 72 h and chloroplast disorganized and starch granules appear after 120 h of cold exposition. Chlamydomonas photosynthetic capacity is altered, Fv/Fm decreases and the light

harvesting complex proteins and b6f cytochromes decline. Protecting enzymes such are the vesicle inducing protein in plastids 1 (VIPP1), the thylakoid formation protein (THF), and the monogalactosyldiacylglycerol-specific lipase PGD1 (plastid galactoglycerolipid degradation 1) are accumulated. Proteins involved in oxidative metabolism such as the tricarboxylic acid cycle (TCA) and the electron transport chain are repressed, while glycolysis enzymes maintained and those regarding to pentose-phosphate pathway increased, indicating energy generation. Most of the proteins related to oxidative phosphorylation and CO_2 fixation enzymes remained unchanged. In this scenario, where the photosynthesis is decreased, while metabolism is active and growth and cellular structure is reorganizing, the supply of carbon to central metabolism should be guaranteed. Because of that, carbonic anhydrases (CAH) and phosphoenolpyruvate carboxylase (PEPC2) increased. Besides, polyunsaturated fatty acids increased suggesting this as a mechanism of maintaining membrane fluidity and for preserving cell energy states. Gluconeogenesis and starch biosynthesis pathways are activated leading to a pronounced starch and sugar accumulation. All these mechanisms linked central metabolism, autophagy, and epigenetic related mechanisms [10,62]. Thanks to this analysis, novel implied protein families are discovered to have a key role in Chlamydomonas stress acclimation response, such are the sucrose non-fermenting related kinases (CKIN in Chlamydomonas, SnRK in Arabidopsis) [10].

This protein family plays an important role in plant metabolism and stress response. Arabidopsis SnRK allow the crosstalk between metabolic and stress signaling, by directly activation of transcription factors, including bZIPs, general stress response elements, and histone modification leading to epigenetic changes [10]. These proteins enable plants to adapt to stress conditions through metabolic changes, for example, by the sugars and polysaccharides interchanging [63]. Due to these proteins are described as key role in not only to cold and other stresses response but also in metabolic processes and their evolutive situation, in our laboratory we have characterized the Chlamydomonas CKIN family in this species [64]. These genes show different grades of responsiveness depending on the stress applied. Thus, presenting specialization but also pleiotropy between some family members. This family regulatory pathway differs from land plants, because SnRK sequences show regulatory features, being some of them sensitive to ABA, despite conserved receptors (PYR/PYL/RCAR) and regulatory domains for this hormone are not present in Chlamydomonas. Land plants and core Chlorophytes have divergent stress signaling, but this gene family has the same role in stress response and adaption and therefore survival, including specific biomolecules accumulation. This fact places the Chlamydomonas CKIN family as good candidates for genetic engineering strain

improvement for studying sugars, lipids and secondary metabolites accumulation, while providing new findings in the evolution of ABA-signaling mechanisms and stress biology [10]. Because of the importance of the ABA-signaling mechanisms evolution, together with SnRK contribution to stress response, the knowledge of this family members in not only microalgae and angiosperms but also in gymnosperm species is of vital importance. Between gymnosperms, conifers have a great of importance even their phylogenetic position would reveal key evolutionary and stress response acclimation questions. Because of that, we recently have described the SnRK family in *Pinus pinaster* [65]. This species has forestry relevance into northwestern Mediterranean area and will provide the ideal example to explore SnRK evolution history [66]. Moreover, this family study will provide information about reductions in plant biomass yield related to regional increasing abiotic stress due to climate change, which are also a main constraint for other Pinus species [65].

Nonetheless the study about evolution of stress response mechanisms in all plant species are of vital importance. Thougth Chlamydomonas general and specific responses to abiotic stress are described, fundamental research is needed to identify what genes and molecules are involved in the dialogue controlling key processes in stress response such are the autophagy, the PCD, the multicellularity or the epigenetic mechanisms. This knowledge will contribute not only to cell biology but also to search for genetic engineering targets for strain improvement.

Epigenetics in Chlamydomonas

As previously mentioned, epigenetic changes have a key role in acclimation stress response, as well as in cell cycle and development. Epigenetic attempts to understand the processes behind the inheritance of traits that cannot be attributed to changes in DNA sequence [67] (*Table 1*). Because of that reason it is of vital importance understand how these mechanisms act, and how to are modulated under abiotic stress.

Table 1. Basic concepts in epigenetics.

Basics of epigenetics
Histone modifications: chemical posttranslational modifications of N-termini of histones composition. The most prominent modifications are the phosphorylation of serine, acetylation and methylation of lysine, and methylation of arginine residues. These modifications alter the DNA accessibility, thus being repressive or active marks. Writers, readers, and erasers enzymes are several, being the most studied histone

acetyltransferases (HATs), histone methylases (HMTs), histone kinases (HKs), histone deacetylases (HDACs), histone demethylases (HDMs) and lysine demethylases (KDMs) [67].

DNA methylation: addition of a methyl group in cytosine (5-methylcytosine, 5mC) or adenine (6-methyladenosine, 6mA) with the consequence of gene expression alteration. This type of modification is present in all kingdoms. Writers of DNA methylation are DNA methyltransferases (5mC: DNMT in human , MET1 in plants [67]; 6mA: N6AMT1 in human [68], not determined in Arabidopsis [69]) for that use S-adenosylmethionine (SAM) to transferee methyl groups to nucleotides [67]. Elimination of this mark occurs by successive rounds of DNS replication due to lack of DNMT and by enzymatic reactions performed by ten-eleven translocation (TET) family of proteins [70] in mammals and DEMETER (DME) and repressor of silencing (ROS1) in higher plants for 5mC and ALKBH1 in human [68] for 6mA (no determined in Arabidopsis). In plants, DNA methylation enzymes are divided between their activity of *de novo* or *maintenance* methylation [71].

smallRNAs (sRNAs): noncoding RNAs (ncRNAs) of about 20–24 nucleotides that mediate mediate transcriptional and posttranscriptional gene silencing via the RNA-directed DNA methylation (RdDM) pathway (*de novo* methylation). Among them, **microRNAs (miRNAs)** and **small interfering RNAs (siRNAs)** have crucial role in plant epigenetics [71].

The most studied epigenetic mechanisms is the 5mC mark which is characteristic of eukaryotes and is well-characterized model organisms, especially in human (5-8 % of total C abundance) and Arabidopsis (5-25 % of total C abundance). In the mammalian genomes GC nucleotides are in less frequency than expected, but these nucleotides are reunited in the frequency expected in the CpG islands. These are associated to gene promoters and normally devoid of any 5mC, except in certain physiological situations, causing transcriptional repression [67]. Moreover, in human, C are normally methylated in the CG dinucleotides context. In the case of Arabidopsis, C is methylated in the contexts CG, CHG, and CHH (H=A, T, C) [71]. Plant 5mC mark is highly enriched over heterochromatic transposable elements (TEs) and repeats, where it plays a prominent role in silencing their expression at the transcriptional level (Transcriptional Gene Silencing, TGS) [72]. Abundance of 5mC is higher in flowering plants than in lower plants and in monocots than in dicots, suggesting a taxonomic significance for 5mC. Plant methylation mechanism is more complex than the mammals, and it is great of importance in natural variation [71]. In

both, mammals and plants this mark has a key role in biological processes, such are development, imprinting, flowering and stress response acclimation [67,71]. However this 5mC prevalence in eukaryotes, some laboratory organisms are revealed to have a very low content. The microalgae *Chlamydomonas reinhardtii*, has a very low content of 5mC (0.75 %) [73,74]. Comparing to the animals and plants genomes, Chlamydomonas has a estrange methylation pattern. This microalgae 5mC mark does not change during the different life stages and occur in the same contexts as in plants. Moreover, 5mC higher density is located in regions containing high levels of repeats and few protein-coding genes [74,75]. Not only Chlamydomonas has this 5mC features, other important model species such are the fly *Drosophila melanogaster* [76] has low content of 5mC or is totally absent as in the nematode *Caenorhabditis elegans* [77]. Even, *C. elegans* doesn't present homologs to C methyltransferases [77]. By contrast, these organisms have recently revealed to have the 6mA mark, most prevalent in prokaryotes. Chlamydomonas 6mA mark (0.4 % of total A abundance) is enriched around the transcription start site (TSS), marks active genes, correlates with nucleosome positioning and exclusively marks DNA linkers between adjacent nucleosomes around TSS [73].

Even though the methods to elucidate 5mC at nucleotide resolution are well-developed, those regarding to 6mA sequencing are just developed with the single molecule real time sequencing (SMRT) raising (*Table 2*). Because of that reason, the 5mC mark role is well-characterized and those of 6mA is still starting to be elucidated.

Table 2. Location analysis of epigenetic marks.

Location analysis of epigenetic marks
Chromatin immunoprecipitation (ChIP): DNA is cross-linked to histone using formaldehyde, when chromatin is in its native form within cells. Subsequently, cells are lysed, and chromatin is sheared either by ultrasound treatment or partial digestion with nucleases. Chromatin that is associated with a specific histone modification or protein is enriched by immunoprecipitation with a specific antibody for a specific mark. After purification, the chromatin associated DNA can be analyzed by dot blot, gene specific PCR, and next generation sequencing [67].
Mass spectrometry (MS) analysis: this technique allows the detection of the global amount of DNA methylated in the genome. Despite this technique is very useful, it does not provide single base resolution.

Methylation DNA immunoprecipitation sequencing (MeDIP): methylated DNA is precipitated and enriched through antibodies specific to 5mC or 6mA and then studied through dot blot, PCR, and sequencing. This process has low resolution for identifying methylation sites [71].

Genome wide bisulfite sequencing (GWBS): treatment of DNA with sodium bisulfite that converts unmethylated cytosines to uracil, while 5mC remain unchanged. Then, a PCR followed to sequencing can reveal 5mC positions in the genome at single base [67,71]. This method the most common to 5mC mark reveal but does not provide 6mA information.

Single molecules real time (SMRT) sequencing: part of the third-generation sequencing technology, **Oxford nanopore (ONT)** sequencing allow to detect all DNA modifications at base resolution. This method uses voltage to drive molecules through nanopores and monitors how the ionic current through the nanopore changes as single molecules pass through it. Different nucleotides passing through nanopores generate different electric currents, which can be measured and designated to the corresponding nucleotides or modified nucleotides [71]. This method is the most useful developed until now.

Epigenetic modifications revealed as crucial in biological processes and stress acclimation response all above the clades. Plants growth in dynamic environments, and sessility make their development influenced by both biotic and abiotic factors [78]. In addition to the various regulatory mechanisms to combat different stresses and to adapt to the changing environmental conditions, plants also have the ability to memorize the stress and even transmit it to the next generation. In flowering plants, cold has been evaluated through vernalization studies, an epigenetic regulatory system that prevents flowering in unfavorable time. In Arabidopsis, premature flowering is prevented by active synthesis of the transcription factor flowering locus C (FLC), that represses floral integrator genes. The exposure to prolonged cold at winter inactivates FLC through a mechanism involving 5mC methylation. Also, low-temperature stress decreases the amount of 5mC methyltransferase in corn, which, in turn, might reduce the level of genomic 5mC methylation [71]. Methylation under cold stress have been studied in flowering plants, but microalgae epigenetic mechanisms knowledge is still scarce, even some elements appeared involved [10]. Reducing the 5mC and histone methylation variability with some epigenetic drugs (epi-drugs), reduce the adaption to salt, high CO_2 and phosphate starvation stresses in Chlamydomonas. Transgenerational epigenetic effects play a role

in adaptive evolution and suggest that the relationship between changes in 5mC methylation patterns and differences in evolutionary outcomes, at least for quantitative traits such as cell division rates, is complex [79]. However, and even the 6mA are active marks, there is no study about how it contributes to stress response acclimation and finally adaption.

Advances in scientific knowledge along with the help of SMRT sequencing (*Table 3*) have made it possible to improve the identification of the alterations of genes controlling epigenome in response to stress. Moreover, these recent developed methods would allow to understand not only the well-known 5mC epigenetic mark but also 6mA. Therefore the analysis of epigenomics of Chlamydomonas will not only help to answer several intriguing questions, including those related to Chlamydomonas development, regulation of metabolism and evolution, but also to enhance the economic value of the biomass yield and composition.

Characterization of cellular responses based on a multiomics approach

For stress understanding from a basic point of view as well as for bioproduction efficiency improvement, omics seem the best approach. Omics and their integration by systems biology are used for understanding the regulation and network integration for biosynthesis/degradation of metabolic precursors, intermediates, end products, and identifying the key elements regulating metabolic flux [80]. Therefore this approaches allow to understand how organisms cope with stress and thus, facilitate the comprehension of physiological processes and will allow the genetic engineering of strains to improve cultures [80].

In the last decades, transcriptomics has raised as the most common used omics, followed by genomics, proteomics and metabolomics. Thanks to the cost reduction in the next generation sequencing methods, the number of genomes as well as transcriptomes number available increase a lot. Currently, the best algae genome annotated is *Chlamydomonas reinhardtii*, firstly published in 2007 [47], and nowadays under its 5.6 version [81], already sequenced with SMRT) of Pacific Biosciences (PacBio). Genomic sequencing and downstream bioinformatics allowed the elucidation of species-specific particularities in multiple metabolic pathways as the nitrogen or iron assimilation among others. Information from genomics is usually complemented with transcriptome analyses. Transcriptomics was greatly stimulated with the development of a powerful and innovative sequencing tool in Human Genome Project (2001). RNA-Seq (*Table 3*) provides accurate

information about gene expression, quantifying tens of thousands of genes in a single run, with costs being reduced each year, and these experiments are now affordable for a large number of researchers. RNA-Seq improves the detection and assignment of peptides in proteomics experiments following a so-called proteogenomic approach since using databases generated from cDNA that contain fewer irrelevant entries, noncoding sequences, and incorrect splice variants compared to DNA [82]. Remarkably, proteomics (*Table 3*) gives a further understanding about the protein accumulation in certain stress situation. Nowadays, proteins can be easily identified and quantified by using a bottom-up proteomic approach in many plant systems). The use of next-generation spectrometers such as Orbitrap or qTOF, coupled to liquid chromatography separation systems, allowed peptide characterization without precedent in terms of speed, resolution, dynamic range, and accuracy, being possible the analysis of full proteomes [82]. Label-free shot-gun proteomics integration with metabolomics not only give a comprehension about the physiological status of the microalgae but also allow to find molecules of interest and which pathways are triggering their enhancement during stress. Following this approach we have yet characterized the N deprivation, osmotic, low doses of UV-C and cold stresses [10–12,14] in Chlamydomonas. Thanks to the previously described omics studies, and as an example, our research group has characterized the protein family Sucrose non-fermenting related kinases (SnRK), key in the response and acclimation in microalgae response [64]. Taking this into account, this family is a good candidate for genetic engineering.

All of these omic layers form the called algomics (*Figure 4*), covered in the last reviews in the field [20,25,80,83,84]. Likewise the number of papers published in the recent employing algomics to further understand how microalgae response and acclimate to stress are increasing to cover the gap in microalgae research. But, curiously, none of them include the layer of epigenomics, even in the rest of the -omics sets appear epigenetic related mechanisms as key for stress recovering. For this reason, there is a necessity not only to increase the research on algomics but also to explore the epigenetic mechanisms elements in microalgae. At the end, all knowledge generated will be employed to generate new genetic engineered strains useful in terms of production efficiency.

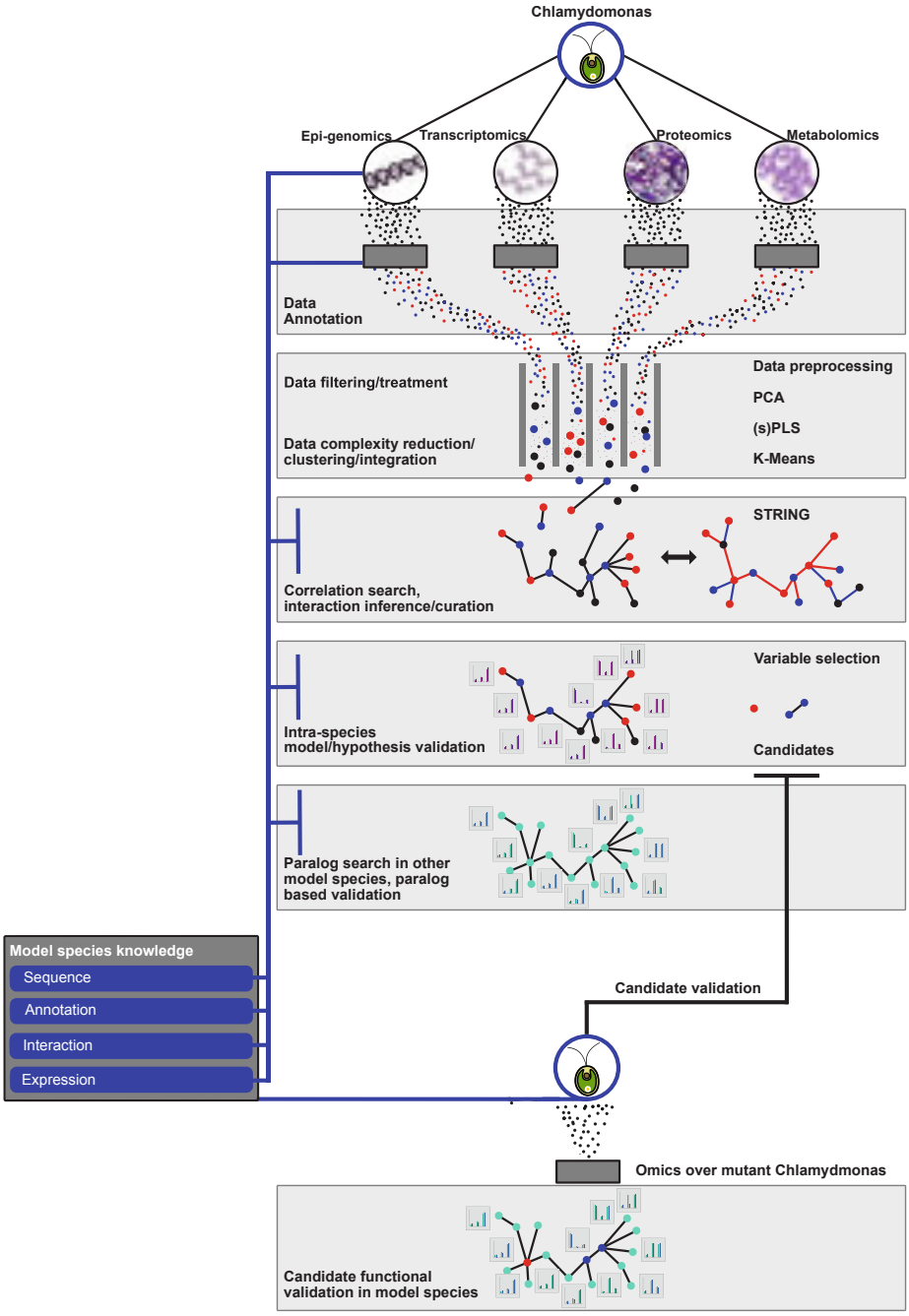

Figure 4. Algomics process. Epi-genomics, transcriptomics, proteomics and metabolomics are performed. Then, data annotation is performed followed by data complexity reduction. From this data, correlation search is performed to candidate selection. These candidates are search for paralogues in other model species, and then their functional validation and final omics through mutants is performed.

Following the previously described workflow, in our lab we further study the abiotic stress in plants and microalgae. Concretely in this thesis we employ a multi-omics approach for the study of xenobiotics induced stress and epigenetic related responses to cold stress in Chlamydomonas and finally, to contribute to evolution studies about stress response proteins.

Table 3. High throughput technologies and approaches included in algomics

Tecgnologies and approaches included in algomics
High resolution mass spectrometry (HRMS) involves a series of MS approaches driven by diverse mass analyzing platforms (TOF, Orbitrap and FT-ICR) providing a high mass accuracy and resolution analysis. This approach is specially advantageous for complex sample matrices rich in unknow compounds (e.g. complex solutions of peptides or metabolites purified from biological tissues).
Shotgun proteomics, (also sometimes called bottom-up proteomics) is a mass spectrometry-based technique allowing the inferential analysis of proteoforms using peptide proxies produced by enzyme-catalyzed hydrolysis of entire proteomes. Along HRMS this approach has recently allowed the inference of more than 18.000 proteoforms from the proteome of *Arabidopsis thaliana*.
Illumina sequencing is a next generation sequencing (NGS) by synthesis platform allowing the parallel sequencing of short stretches (~250 bp) of thousands of DNA (or cDNA) molecules. This approach has been intensively used during the past 15 years to sequence whole genomes and transcriptomes. Requires an amplification step and a reverse transcription (RT) step for RNA-seq, both introducing biases in quantification. Moreover, the reduced read length can produce issues in the assembly steps. This approach is unable to detect DNA modification unless used in bisulfite sequencing pipelines.
RNA-seq involves the inference of a transcriptome (transcript species and abundance) out of sequence data. This approach is based in the mapping to reference genome/transcriptome assemblies sequence data generated by NGS/third generation sequencing approaches from cDNA (Illumina, SMRT) or RNA (ONS) libraries allowing the relative quantitation of the different transcripts species out of the abundance of their reads.
Systems biology is a computational and mathematical approach targeting the modelling of the emergent features of biological systems (e.g. a cell, an organism, a group of organisms, …) out of their individual constituents (abundance and features of their diverse transcripts, proteins and metabolites, phenotypical characteristics,…). Omic approaches are an outstainding source of high density and multilevel system

information for these approaches fostering the observed abundance of works combining omics and systems biology.

Approach and objectives

Microalgae are exposed to diverse stressing agents including those some of antropic origin. This thesis targets one natural stressor (cold) and one of anthropic origin, the plastic component bisphenol A. The pollutant BPA concentration is being enhanced in the aquatic environments. However despite the great interest of the impact of this compounds in mammals, and specially humans, the impact on aquatic plants is mostly unknow. Because of that reason, the microalgae Chlamydomonas reinhardtii represents a suitable and convenient model to study the BPA associated toxicity.

Most of the ecosystems inhabited by microalgae underwent periodic intervals of cold which impact the microalgae life cycle. Low temperatures are also a major stressor but also a timer for land plants entraining internal rithms in charge of the setting of developmental milestones (flowering, fruit maturation, budding). The understandment of the basic mechanisms driving the perception, signalling and acclimation to low temperatures in microalgae could give us clues about the origin of the more complex mechanism observed in plants nowadays. Among cellular processes involved in cold response epigenetics is a major player.

Therefore, the main goals of this thesis is the characterization of the Chlamydomonas stress response to bisphenol A and the characterization of the epigenetic responses of this species in response to cold stress. This general objective can be divided into the following four:

1. Description of the Chlamydomonas molecular responses to Bisphenol A stress through the integration of physiological and proteomic measurements by using an integrative systems biology approach.

2. Characterization of the effect of epigenetic drugs over the cold-stress responses in *Chlamydomonas reinhardtii*.
3. Analysis of site-specific epigenetic modifications ocurring during cold-stress acclimation by using ONT-SMRT.
4. Integration of transcriptomic and epigenetic datasets towards the understanding of the regulatory mechanisms that mediate cold-stress response and acclimation in Chlamydomonas.

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