TESIS DOCTORAL

Mimetización de mecanismos de respuesta al estrés abiótico en la mejora de la producción de biomasa vegetal

Improvement of plant biomass production by mimicking natural responses to abiotic stresses

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Tesis doctoral/*Doctoral thesis*

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RESUMEN DEL CONTENIDO DE TESIS DOCTORAL

1.- Título de la Tesis	
Español/Otro Idioma: Mimetización de mecanismos de respuesta al estrés abiótico en la mejora de la producción de biomasa vegetal.	Inglés: Improvement of plant biomass production by mimicking natural responses to abiotic stresses.

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RESUMEN (en español)

El cultivo extensivo de plantas como fuente de biomoléculas de interés industrial amenaza la seguridad alimentaria y el entorno natural, lo que ha motivado la búsqueda de alternativas como el uso de microalgas. Estos microorganismos comparten características con las plantas sin muchos de sus inconvenientes como fuentes de biomasa, sin embargo, la producción de compuestos a partir de microalgas depende de estímulos estresantes que también reducen su rendimiento en biomasa y, dado al alto coste energético de su aplicación, la rentabilidad de los productos derivados. Por estas razones, la generación y/o selección de cepas de microalgas capaces de acumular biomoléculas sin necesidad de inducción por estrés es de gran interés. Muchas de estas biomoléculas de interés industrial participan en la respuesta a distintos estreses y, por tanto, lograr su acumulación de forma independiente al estrés requerirá de la caracterización y posterior modulación del sistema de respuesta a estrés. Múltiples trabajos han iniciado la caracterización de los complejos sistemas de respuesta de la microalga modelo *Chlamydomonas reinhardtii* a diferentes estreses, revelando la implicación de miles de variables. Algunas de estas, destacan por su función en la integración/modulación de diferentes rutas de señalización y respuesta a estrés, incluyendo la acumulación de biomoléculas, constituyéndose como elementos prometedores para la modulación del sistema.

El objetivo principal de esta tesis es la caracterización del sistema de respuesta a estrés de *C. reinhardtii* frente a los estreses osmótico y por radiación ultravioleta B y C, poco descritos en microalgas, pretendiendo identificar nodos centrales del sistema, especialmente aquellos implicados en la modulación de la producción de biomasa bajo estas condiciones. Para alcanzar este objetivo, se ha elegido una aproximación metodológica incluyendo ómicas (proteómica y metabolómica) basadas en espectrometría de masas y acopladas a análisis englobados en la biología de sistemas. Estas metodologías fueron complementadas con técnicas de minado y edición del genoma de la microalga, útiles para la descripción y caracterización de los candidatos derivados de los análisis ómicos.

Esta tesis describe los sistemas de respuesta de *C. reinhardtii* a estrés osmótico y ultravioleta B y C evidenciando las similitudes de estos con los de plantas y levaduras, así como la importancia del ajuste en la composición de la biomasa durante la respuesta a estrés. Bajo estrés osmótico la acumulación de glicerol y distintos azúcares dirigieron la recuperación del equilibrio osmótico, la protección frente al estrés oxidativo y la homeostasis redox; mientras que los cambios en la composición de la biomasa bajo estrés ultravioleta se centraron el bloqueo de la radiación incidente, aunque también en el estrés oxidativo y la homeostasis redox. La variación en la composición de la biomasa celular durante la exposición a ambos estreses fue el reflejo de la modulación del proteoma celular, destacando su importancia en la respuesta al estrés ultravioleta donde la reparación y el recambio de proteínas dañadas, especialmente las fotosintéticas, fue clave en la adaptación de *C. reinhardtii* a este estrés. Además, la aproximación de biología de sistemas empleada pudo identificar múltiples nodos señalizadores centrales como la MAP quinasa seis (MAPK6) y múltiples fosfatasas 2C (PP2Cs) así como nodos efectores como la subunidad seis de la ATP sintasa mitocondrial (ATP6) bajo

estrés osmótico y una nueva DYRK quinasa, probablemente implicada en la modulación de la acumulación de biomasa, bajo estrés ultravioleta.

La descripción de las respuestas a estos estreses apuntó también al papel central de las quinasas de la familia *Sucrose-non-Fermenting Related Kinases* (SnRK) en el sistema de respuesta a estrés de *C. reinhardtii*. Esta tesis incluye la descripción de los miembros de esta familia tanto en la microalga modelo como en especies próximas, así como el estudio de la sensibilidad a diferentes estreses abióticos y mediadores en la respuesta a estrés como el ácido abscísico en la familia de *C. reinhardtii* designados como CKIN. Esta aproximación identificó una gran familia de quinasas en la microalga que siendo sensibles a estrés/ácido abscísico y mostrando múltiples puntos en común con la familia SnRK de plantas presentaba múltiples elementos exclusivos de microalgas. Estos resultados motivaron el inicio de la caracterización de la función de algunos de estos elementos incluyendo distintas CKINs como *CKIN2.12*, CKIN$\beta\gamma$ y *CKIN*γ así como de elementos relacionados con la respuesta al estrés osmótico como *ATP6* mediante la modulación de su expresión. Debido a las características de algunos de estos candidatos (redundancia funcional, letalidad) fueron necesarias diferentes metodologías de expresión génica cuya aplicación requirió de la construcción de un nuevo vector. Esta aproximación ha permitido hasta el momento confirmar el papel de *ATP6* en la respuesta a estrés osmótico y ha apuntado a la función clave de CKIN exclusivas de microalgas como *CKIN2.12.*

A pesar de lo preliminar de los resultados de caracterización, la validación de la función de uno de los candidatos derivados de los análisis ómicos, *ATP6*, aclara la capacidad de la metodología empleada para identificar elementos clave en la respuesta a estrés en *C. reinhardtii*. Esta idoneidad también es avalada por la identificación de otros nodos previamente relacionados con la respuesta a estos estreses en plantas y microalgas como la quinasa MAPK6 o la proteína fotosintética de respuesta a estrés ultravioleta LHCSR1. Tanto estos elementos como otros cuya implicación se sugiere por primera vez en esta tesis son de gran interés en la continuación del estudio de la respuesta a estrés en la microalga y al mismo tiempo puntos prometedores en la generación de variedades mejoradas. Adicionalmente, los paralelismos existentes tanto en los mecanismos de respuesta como en las familias génicas de muchos de los señalizadores implicados exponen el potencial del uso de las microalgas como modelo de investigación aplicado a la solución de problemas de plantas, como la reducción global en la producción de biomasa vegetal que se pronostica bajo el contexto actual de cambio climático.

RESUMEN (en Inglés)

The threat for environment and food security that poses the extensive farming of plants as providers of biomass and high value-added biomolecules has motivated the search for alternatives as the use of microalgae species. Microalgae share most plant advantages without most of their flaws as biomass providers, however, the production of value-added compounds by these organisms relies on stressing stimuli. Stress reduces microalgae biomass yield and, due to the high energetic cost of its application, the profitability of their derived products. These issues have motivated the generation and/or selection of enhanced microalgal strains able to accumulate biomolecules without stress induction. Most of these value-added biomolecules have key roles into the response to different stresses, thus, the generation of these enhanced strains would need from the characterization and modulation of the stress response system. Different works have already started the characterization of the response to different stresses into the microalgae model *Chlamydomonas reinhardtii*, discovering highly complex systems involving thousands of different level variables. Some of these variables have been highlighted as promising elements for the modulation of the microalgae response systems due to their central roles into the integration/modulation of different stress signaling and response pathways, including the accumulation of stress related biomolecules.

The main objective of this thesis is the characterization of the *C. reinhardtii* stress response systems to ultraviolet and osmotic stresses, still poorly characterized, aiming to identify and characterize central signaling nodes related to the modulation of the biomass production under stress. The accomplishment of this objective involved a methodological approach including different omics (proteomics and metabolomics) based into mass spectrometry techniques and coupled to systems biology analyses. This approach would be followed by different genome mining and engineering techniques to descript and characterize the omics derived candidates.

This thesis describes the *C. reinhardtii* response systems to osmotic and ultraviolet B and C stresses evidencing their likeliness to those of land plants and yeasts, and the relevance of the

fine tuning of biomass composition into the stress responses. The accumulation of glycerol and different sugars drove the recovery of the osmotic equilibrium and the control of oxidative stress and redox homeostasis under osmotic stress. On the other hand, biomass profile under ultraviolet stress was more focused on ultraviolet shielding compounds although reactive oxygen species scavengers and redox homeostasis modulating compounds were also relevant under this stress. The modulation of biomass composition observed under these stresses was tightly coupled to the turnover of the microalgae proteome. This turnover was especially important into ultraviolet stress response, more focused on the avoidance and repair of protein damage, specially to the photosynthetic proteins. Moreover, the used systems biology approach allowed the identification of central signaling nodes as the Mitogen Activated Protein Kinase 6 (MAPK6) and different Phosphatases 2C (PP2Cs), and effector nodes as the subunit 6 of the mitochondrial ATP sinthase (ATP6) into osmotic stress and a novel DYRK kinase possibly involved into the modulation of biomass under ultraviolet stress.

Many of the findings about these responses point to the central role of the family of Sucrose-non-Fermenting Related Kinases (SnRK) into the *C. reinhardtii* stress responses to the tested stresses. Thus, this thesis also covered the identification of the microalgae SnRK family members (designated as CKIN) and the SnRK families of close microalgae species, and the description of the CKIN elements sensitivity to different stresses and stress signalers as abscisic acid. This approach identified a large and stress/abscisic acid sensitive kinase family which although has common points to their plant orthologs also displayed many microalgae specific features. These results motivated the functional characterization of many of these elements including CKINs as *CKIN2.12*, *CKIN*$\beta\gamma$ and *CKIN*γ, along the osmotic stress effector *ATP6*, through the modulation of their expression. The issues associated to the function of these candidates (functional redundancy, lethality) required from the use of different genome edition methodologies whose application needed from the construction of a new vector frame. This approach did confirm the role of ATP6 into the response to osmotic stress and pointed to the key role of the microalgal exclusive *CKIN2.12.*

Besides the preliminary characterization results, the validation of the *ATP6* function into osmotic stress supports the power of the chosen methodological approach to identify key elements into the *C. reinhardtii* stress response. This power was also supported by the identification of other nodes previously related with the response to the studied stresses both into plants and microalgae as the MAP kinase 6 or the UV stress response related photosynthetic protein LHCSR1. These nodes and others whose implication in theses stress responses is suggested for the first time into this thesis would be valuable into the continuation of the microalgae stress response characterization and/or the generation of enhanced microalgae strains. Moreover, the broad parallelisms observed between plants and microalgae stress response mechanisms and gene families of stress signalers/effectors, showed the potential usage of microalgae as a convenient model for plant research.

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Abbreviations

ABA	Abscisic acid
ACX	ACYL-CoA OXIDASE/DEHYDROGENASE
ADK3	ADENYLATE KINASE
AGPase	ADP-GLUCOSE PYROPHOSPHORYLASE
AGT2	ALANINE GLYOXILATE TRANSAMINASES
AKIN	ARABIDOPSIS SUCROSE NON-FERMENTING RELATED KINASE 1
AMA2	ALPHA-AMYLASE
AMP	Adenosine Monophosphate
AMPK	AMP-activated protein kinase
APX	Ascorbate peroxidase
APX2	L-ASCORBATE PEROXIDASE
AREB/ABFs	ABA RESPONSIVE-ELEMENT BINDING PROTEIN/ABRE BINDING FACTORS
Ath	*Arabidopsis thaliana*
ATP6	MITOCHONDRIAL F1F0 ATP SYNTHASE SUBUNIT 6
CAM1	CALMODULIN
CBL	CALCINEURIN BETA-LIKE
CCMs	Carbon Concentrating Mechanisms
CDJ1	CHLOROPLASTIC DNAJ-LIKE PROTEIN
CDK/CDPK	CALCIUM DEPENDENT PROTEIN KINASES
CDPKK2	Calcium/calmodulin dependent protein kinase kinase 2
CDS	Coding Sequence
CEF	Cyclic Electron Flow
CFP	CYAN FLUORESCENT PROTEIN
CGL134	PHYTOL KINASE
CGL86	FORKHEAD-ASSOCIATED DOMAIN-CONTAINING PROTEIN
Chla/b	Chlorophyll a/b ratio
CHLI2	MAGNESIUM CHELATASE SUBUNIT I
CHX	Beta-Carotene Hydroxylase
CKIN	CHLAMYDOMONAS SNRK
CPLD58	GLUTATHIONE S-TRANSFERASE homolog
Cre	*Chlamydomonas reinhardtii*
CRK	CISTEINE-RICH RECEPTOR-LIKE KINASES
Csu	*Coccomyxa subellipsoidea*
CsuCsKIN	*Coccomyxa subellipsoidea* Sucrose non-Fermenting Related Kinases
Cva	*Chlorella variabilis*
CvaCvKIN	*Chlorella variabilis* Sucrose non-Fermenting Related Kinases
CYG29	ADENYLATE/GUANYLATE CYCLASE

CYN38	CYCLOPHILLIN TYPE PEPTIDYL-PROLYL CIS-TRANS ISOMERASE
DGTT1	Diacylglycerol acyltransferase
DMSO	Dimethyl sulfoxide
Dsa	*Dunaliella salina*
DsaDKIN	*Dunaliella salina* Sucrose non-Fermenting Related Kinases
DW	Dry weight
DYRK	DUAL SPECIFICITY TYROSINE REGULATED KINASES
EI	Electron Impact
EIF1a	EUCARIOTIC TRANSLATION INITIATION FACTOR 1A
ELIP	EARLY LIGHT INDUCIBLE PROTEINS
FA	Fatty Acids
FAP121	Flagellar associated protein
FAP204	FLAGELLAR ASSOCIATED PROTEIN 204
FBP1	FRUCTOSE-1,6-BISPHOSPHATASE
FHY3/FAR1	FAR-RED ELONGATED HYPOCOTYLS 3/FAR-RED-IMPARED RESPONSE 1
FKB16-3	PEPTIDYLPROLYL ISOMERASE
FLC	FLOWERING LOCUS C
FNR1	FERREDOXIN-NADP REDUCTASE
FNR1	FERREDOXIN NADP REDUCTASE
FTSH	FILAMENTOUS TEMPERATURE SENSITIVE H like
FTSY	CHLOROPLAST SRP RECEPTOR
FW	Fresh weight
GA	Gibberellin
GC	Gas Chromatography
GPM2	PHOSPHOGLUCOMUTASE
GRIK1	GEMINIVIRUS REP INTERACTING KINASE 1
GRX2	CPYC TYPE GLUTAREDOXIN
GSK3	GLYCOGEN SYNTHASE KINASE 3
GSR1	GLUTATHIONE REDUCTASE
GUN4	TETRAPYRROLE-BINDING PROTEIN
H3K27	HISTONE H3 lysine 27
H3K9/K14	HISTONE H3 Lys 9/Lys 14
H3K9/K14	HISTONE H3 Lys 9/Lys 14
H4R3	HISTONE H4 arginine 3
HAP	HEPES-Acetatate-Phosphate
HCD1	3-HYDROXYACYL-CoA DEHYDROGENASE
HK	Histidine Kinase
HMM	Hidden Markov Model
HMO1	HEME OXYGENASE
HOG	High-osmolality Glycerol Response Pathwaw
HOG1	MITOGEN-ACTIVATED PROTEIN KINASE HOG1

HR	Homologous recombination
HSP/RBCS2	Heat Shock Protein/rubisco small subunit gene
HSP70	Heat Shock Protein 70
HSP70C	HEAT SHOCK PROTEIN 70C
HSP70E	HEAT SHOCK PROTEIN 70E
HTA14	transcription/RNA HISTONE H2A
HTA14	Histone H2A
HXK	Hexokinase
ICL1	ISOCITRATE LYASE 1
ICL2	ISOCITRATE LYASE
ICL2	ISOCITRATE LYASE 2
ICL3	ISOCITRATE LYASE 3
IDA5	Actin
IDH1	NAD DEPENDENT ISOCITRATE DEHYDROGENASE
IDH2	TCA ISOCITRATE DEHYDROGENASE
ISA2	ISOAMYLASE-TYPE STARCH DEBRANCHING ENZYME
JA	Jasmonates
KASIII	B-KETOACYL-[ACYL-CARRIER-PROTEIN] SYNTHASE III
KO	Knock out
LAL1	LA PROTEIN
LC	Liquid Chromatography
LEA	LATE EMBRYOGENESIS ABUNDANT
LEF	Linear Electron Flow
LEU3	ISOPROPYLMALATE DEHYDROGENASE
LHCB4	CHLOROPHYLL A-B BINDING PROTEIN
LHCI	Light harvesting complex I
LHCII	Light harvesting complex II
LHCSR1	STRESS-RELATED CHLOROPHYLL A/B BINDING PROTEIN 1
LW	Lipid weight
MAPK	Mitogen Activated Protein Kinases
MBBH	Homology based multiple bidirectional best hit
MCS	Multicloning Site
MET1	METHYLTRANSFERASE 1
METC	CYSTATIONINE BETA LYASE
MGE1	GrpE PROTEIN HOMOLOG
MIGS	miRNA-induced gene silencing
MINA53	MYC INDUCED NUCLEAR ANTIGEN
MIP1	AQUAPORIN
miRNA	micro RNA
ML	Maximum likelihood
MME5	NADP MALIC ENZYME 5

MME5	NADP MALATE DEHYDROGENASE
MME6	NADP MALIC ENZYME 6
MS	Mass Spectrometry
MSD2	Mn SUPEROXIDE DISMUTASE
MSD3	CHLOROPLAST Mn SUPEROXIDE DISMUTASE
MtATP6	ATP synthase Fo subunit 6
NDA1	MITOCHONDRIAL TYPE-II NADH DEHYDROGENASE
NDA5	TYPE II NADH DEHYDROGENASE
NGS	Next Generation Sequencing
NHEJ	Non-homologous end joining
NIT1	Nitrate Reductase 1
NIT2	Nitrate Reductase 2
NITp	*Nitrate Reductase 1 promoter*
NO	Nitric oxide
NTRC	THIOREDOXIN REDUCTASE C
NUOS6	NADH:UBIQUINONE OXIDOREDUCTASE 16.3 kDa SUBUNIT
OEC	Oxygen Evolving Complex
Olu	*Ostreococcus lucimarinus*
OluOKIN	*Ostreococcus lucimarinus* Sucrose non-Fermenting Related Kinases
PANK	PANTOTHENATE KINASE
PAT1	PHOSPHATE ACETYLTRANSFERASE
PC1	Principal Component 1
PC2	Principal Component 2
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PCY1	PLASTOCYANIN
PDC1	MITOCHONDRIAL PYRUVATE DEHYDROGENASE COMPLEX, E1 COMPONENT, ALPHA SUBUNIT
PEG	Polyethileneglycol
PETC	RIESKE IRON-SULFUR SUBUNIT OF THE CYTOCHROME b6f COMPLEX
PETC	CYTOCHROME b6f RIESKE IRON-SULFUR CENTER SUBUNIT
PETO	CYTOCHROME b6f COMPLEX SUBUNIT V
PFK2	PHOSPHOFRUCTOKINASE 2
PGP2	PHOSPHOGLYCOLATE PHOSPHATASE
PKL1	PROTEIN PHOSPHATASE 1 BETA
PP1K	Protein phosphatase type 1 isoform K
PP2A	PHOSPHATASE 2A
PP2Cs	Phosphatases 2C
pPIKIN	*Pinus pinaster* Sucrose non-Fermenting Related Kinases
PPM	Protein phosphatase Mg2+/Mn2+ dependent
PPOX	Arabidopsis PROTOPORPHYRINOGEN IX OXIDASE

PPX	Chlamydomonas PROTOPORPHYRINOGEN IX OXIDASE
PPX rs-3	Chlamydomonas PROTOX resistance mutant
PPX1	PROTOPORPHYRINOGEN OXIDASE
PRMT10	PROTEIN-ARGININE N-METHYLTRANSFERASE
PRMT2	HISTONE-ARGININE N-METHYLTRANSFERASE
PRP39	NUCLEAR PRE-mRNA SPLICING FACTOR
psaB	P700 CHLOROPHYLL A APOPROTEIN A2
psAD	Photosystem I protein D
PSAG	PHOTOSYSTEM I REACTION CENTER SUBUNIT V
psaG	PHOTOSYSTEM I REACTION CENTER SUBUNIT V
PSAN	PHOTOSYSTEM I REACTION CENTER SUBUNIT N
PSB28	PHOTOSYSTEM II SUBUNIT 28
psbD	PHOTOSYSTEM II D2 PROTEIN
PSBH	PHOTOSYSTEM II REACTION CENTER PROTEIN H
PSBP4	LUMENAL PSBP-LIKE PROTEIN
PSBP6	LUMEN TARGETED PROTEIN
PSI	Photosystem I
PSII	Photosystem II
PYK1	PYRUVATE KINASE
PYK2	PYRUVATE KINASE
qPCR	Quantitative Polymerase Chain Reaction
RBCL	RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE LARGE SUBUNIT
RBCL	RuBisCO large subunit
RBCMT1	RBCL methyltransferase
RBCS1	RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE SMALL SUBUNIT 1
RBCS1	RuBisCO small subunit isoform 1
RBCS2	RuBisCO small subunit isoform 2
RCA1	RuBisCO ACTIVASE
RCK1	Receptor of activated protein kinase C1
RFK2	RIBOFLAVIN KINASE
RFK2	RIBOFLAVIN KINASE
RNA-Seq	RNA-Sequencing
ROS	Reactive Oxygen Species
RPL23a	RIBOSOMAL PROTEIN L23a
RPL7A	RIBOSOMAL PROTEIN L7A
RPS19	RIBOSOMAL PROTEIN S19
RPT2	26S PROTEASOME REGULATORY SUBUNIT
RT-qPCR	Reverse Transcription real time Polymerase Chain Reaction
RWC	Relative water content
SA	Salicylic Acid
SAC1	Phosphoinositide phosphatase SAC1

SAM MTase	S-ADENOSYL-L-METHIONINE-DEPENDENT METHYTRANSFERASE
SAT3	SERINE ACETYLTRANSFERASE
SBE3	STARCH BRANCHING ENZYME
SBP1	SEDOHEPTULOSE 1,7 BISPHOSPHATASE
SCA1	CHLOROPLAST-ASSOCIATED SecA PROTEIN
SCL1	SUCCINYL-CoA LIGASE ALPHA CHAIN
SCY1	PREPROTEIN TRANSLOCASE secY SUBUNIT
SEC13	COP-II COAT SUBUNIT
Ser	Serin
SGA1	SERINE GLYOXILATE TRANSAMINASES
SHMT1	SERINE HYDROXYMETHYLTRANSFERASE 1
SHMT2	SERINE HYDROXYMETHYLTRANSFERASE 2
SHMT3	SERINE HYDROXYMETHYLTRANSFERASE 3
siRNA	small interference RNA
SKP1	E3 UBIQUITIN LIGASE
SLN1	OSMOSENSING HISTIDINE PROTEIN KINASE SLN1
SNF/SnRK	SUCROSE NON-FERMENTING/SUCROSE NON-FERMENTING RELATED KINASES
SNF1	Saccharomyces SnRK1/AMPK ortholog
SNF1	YEAST/MAMMALS SUCROSE NON-FERMENTING RELATED KINASE 1
SnRK1	SUCROSE NON-FERMENTING RELATED KINASE 1
SnRK2s	SnRK subfamily 2
SnRK3/CIPK	SUCROSE NON FERMENTING RELATED KINASE 3/CALCIUM INTERACTING RELATED KINASES
SPL1	SF3A1 SPLICING FACTOR SUBUNIT 1
sPLS	Sparse Partial Least Squares Analysis
SPP	Sucrose Phosphate-Phosphatase
STA2	GRANULE BOUND STARCH SYNTHASE
SWI/SNF	SWITCH/SUCROSE NON-FERMENTING
SYK1	LYSYL-tRNA SYNTHETASE
T6P	Threalose-6-P
TAG	Triacylglycerols
TAP	Tris-Acetate-Phosphate
TBE	FELSESTEIN´S BOOTSTRAPPING FBP, and TRANSFER BOOTSTRAP EXPENTANCY
TCS	Transitive Consistency Score
TEF14	THYLAKOID LUMINAL FACTOR
Thr	Threonin
TIM22B	MITOCHONDRIAL INNER MEMBRANE TRANSLOCASE
TLA1	CHLOROPHYLL ANTENNA SIZE REGULATORY PROTEIN
TOR	TARGET OF RAPAMYCIN

TORC1	TARGET OF RAPAMYCIN COMPLEX 1 KINASE
TRXm	THIOREDOXIN M
TSL1	LEUCYL-tRNA SYNTHETASE
TSM2	METHIONYL tRNA SYNTHETASE
TUB	Tubulin
UAP56	SUBUNIT OF EXON JUNCTION COMPLEX
UBQ	Ubiquitin
UCP1	Thermogenin-like
UGP1	UDP-GLUCOSE PYROPHOSPHORYLASE
UV	Ultraviolet
UVR8	ULTRAVIOLET-B RECEPTOR
Vca	*Volvox carteri*
VcaVKIN	*Volvox carteri* Sucrose non-Fermenting Related Kinases
VTE1	TOCOPHEROL CYCLASE
VTE5	PHYTOL KINASE 1

General index

CHAPTER 1

General introduction

CHAPTER CONTENTS

I. Stress, an inductor of biomolecule accumulation across Plantae kingdom

Plants are providers of biomass (timber, fibers, feedstock), hydrogen and different chemicals including pigments, polymers and diverse pharmacological products. These are valuable goods for alimentary, pharmaceutical, chemistry, textile and cosmetic industries with lipid rich Elaeis guineensis (Norhaizan *et al.* 2013), Hevea brasiliensis and other rubber producing species (van Beilen & Poirier 2007) and conifer terpene rich oleoresins (Rodrigues-Corrêa, de Lima & Fett-Neto 2012) as good examples of this value.

Despite the successful historical use of plants as bioproducers, most plant-based strategies are compromised in terms of sustainability, land usage and food security (Rulli, Bellomi, Cazzoli, De Carolis & D'Odorico 2016; Vijay, Pimm, Jenkins & Smith 2016). Thus, different solutions have been proposed, as the use of non-crop species (van Beilen & Poirier 2007) or the more efficient

biomass processing through coextraction procedures (biorefineries) involving a better use of waste materials (Ali, Othman, Shirai & Hassan 2015).

Microalgae have several advantages over the plant based bioproduction solutions, maintaining plant biomolecule production potential but with shorter life cycles, higher biomass yields, lower culture requirements and a low to negligible land usage. Besides the advantages of these minute organisms, their use as biofactories is still associated to high energy costs which have limited their application to few cases involving easy processing (microalgae as feed in aquaculture) or the extraction of high value compounds from high yield strains (astaxanthin production). The induction of the accumulation of pigments, lipids or sugars in microalgae and plants is commonly linked to a stressing stimulus. Although stress is an effective biomass modulator, it also limits biomass increase and raises the microalgae culture energy input to be added to the culture harvesting, product extraction and purification costs. Ultimately, the high associated costs reduce the industry interest into most microalgae derived products (Khan, Shin & Kim 2018).

Besides associated problems, the microalgae potential as bioproducers has motivated the development of different solutions, addressing specific problems from the culture management to the engineering of the microalgae metabolic pathways. These solutions include the separation of biomass production from stress induction into batch culture systems and the optimization of growing conditions (Remmers, Wijffels, Barbosa & Lamers 2018), the modulation of biomass accumulation through phytohormone and oxidative stress related compounds treatments (Lu & Xu 2015; Yu, Chen & Zhang 2015), the fractionation of biomass into biorefineries (Chew *et al.* 2017) and the selection or improvement of microalgae strains. Both microalgae and management focused solutions are devised to be implemented together although the currently unavoidable stress dependence of microalgae biomass accumulation highlights the greater need for microalgae based solutions (Remmers *et al.* 2018).

Microalgae improvement strategies are mainly based on the selection of high yield species and within each species on screenings over natural/induced high yield mutants (Li *et al.* 2016), or into the directed editing of genome to increase yield or ease biomass harvesting processes (Sun, Ren, Zhao, Ji & Huang 2018; Li *et al.* 2019). Many of the genome editing strategies have focused in enzymes directly related to biomass production obtaining reduced production increments. Lipid accumulating starchless mutants are a good example of this where the blockage of starch synthesis has allowed big but limited increase on lipid yields. The limits to yield increase found using conventional genome edition strategies along the known effect not only of stress but of different known stress response intermediaries as phytohormones and oxidative stress over biomass production has clarified the thigh and complex regulation under which these biomass related genes are. These outcomes highlight both the complex modulation of biomass producing enzymes and its current poorly understanding. As example, some microalgae strains keep under laboratory conditions for long times have evolved into rapidly growing high yield strains accumulating hundreds on mutations not only on biomass related genes but also on signaling related ones and untranslated regions (Shin *et al.* 2017).

Thus, stress response mechanisms characterization is key into the search for new solutions over stress induction. However, stress signaling mechanisms and their interaction with metabolic modulation, part of stress response, is a complex system requiring from high throughput approaches for its elucidation. Omics have already identified key gene targets within stress response which have been successfully modulated driving to enhancements into microalgae biomass production (Chen, Hu & Liu 2018b). However, there is no doubt stress response elements have the key as many successful high yield or high resistance mutants from microalgae and plant come from the modulation of stress related transcription factors or effectors (Ma *et al.* 2018; Sun *et al.* 2018). Besides this, omics alone fail short for the understanding of these complex traits, thus systems biology approaches are allowing standing on omic data the detailed reconstruction of the systems given not only targets but functional elements integrated into the cell system. These system models also highlights

the importance of more complex genome edition approaches based on the modulation of multiple elements at once (Remmers *et al.* 2018) and/or their conditional regulation with the use of more sophisticated expression modulation elements.

Besides the greats steps being made into the deciphering of the cell system, most of the microalgal stress response characterization have been done over nutrient deprivation (-N, -P) or salt stress responses where other common and promising stressors as osmotic and UV stresses have been less covered. Osmotic stress can induce the accumulation of sugars, polyols and simple C3 molecules as glycerol into different microalgae species (Gustavs, Eggert, Michalik & Karsten 2010). UV stress is also after a wide metabolic modulation focused on valuable polyunsaturated fatty acids (Paliwal *et al.* 2017).

II. Osmotic stress

The modulatory effects of osmotic stress over biomass are part of the cell response to this stress and came after a complex cascade of stress signaling mechanisms. These processes are mainly triggered by different osmosensitive relays (Zhang & Bartels 2018) and directed towards the quick avoidance and repair of osmotic and associated oxidative stress damage and to the long-term acclimation through the recovery of homeostasis and growth.

Most of the description of these osmorelays came from works over yeast and Arabidopsis (Osakabe, Yamaguchi-Shinozaki, Shinozaki & Tran 2013; Tatebayashi *et al.* 2015) being Histidine Kinase (HK) receptors the primary osmosensors. In yeast, HK triggers High-osmolality Glycerol Response (HOG) pathway controlling the accumulation of osmolytes as glycerol and trehalose after an osmotic shock (Saito & Posas 2012). Other key stress regulatory elements as TARGET OF RAPAMYCIN (TOR) and SUCROSE NON-FERMENTING/SUCROSE NON-FERMENTING RELATED KINASES (SNF/SnRK), also control yeast osmotic response in a HOG independent manner (Muir, Roelants, Timmons, Leskoske & Thorner 2015) along $Ca2+$, which also peaks as a consequence of this stress mainly activating Reactive

Oxigen Species (ROS) mediated signaling pathways (Kurusu, Kuchitsu & Tada 2015).

Land plants as Arabidopsis share signaling mechanisms with yeast; despite this, abscisic acid (ABA), a plant-unique central response hub (Yoshida, Mogami & Yamaguchi-Shinozaki 2014), plays a key role into these species stress response. ABA mediated regulation requires an interplay between different elements as kinases from the SnRK subfamily 2 (SnRK2s), phosphatases 2C (PP2Cs), and transcription factors as ABA RESPONSIVE-ELEMENT BINDING PROTEIN/ABRE BINDING FACTORS (AREB/ABFs) (Coello, Hey & Halford 2011; Rodriguez *et al.* 2014; Yoshida *et al.* 2015). The accumulation of this phytohormone can elicit the synthesis of osmoprotective/antioxidant proline and sugars during osmotic stress (Abraham *et al.* 2003; Thalmann *et al.* 2016). This enhancement of carbohydrate and lipid metabolism is part of the phytohormone role into the deployment of stress acclimation through its binding to specific receptors. These receptor elements can activate different pathways modulating the expression of multiple effector genes (Golldack, Li, Mohan & Probst 2014).

The early started synthesis and accumulation of osmoprotective/ osmoregulatory compounds, as glycerol and trehalose, allow the cell to reach osmotic equilibrium and avoid further damage to its structures. Osmotic readjustment give way to late acclimation measures as the storage of sugars, cell growth resumption or DNA synthesis, as stressed cells exit mitotic cycle and start a mitosis independent DNA replication process (Skirycz *et al.* 2011). Late acclimation responses are after a wide stress dependent gene expression modulation triggered by many different signals, including ABA, through different genetic and epigenetic mechanisms (Forestan *et al.* 2016) which can not only trigger acclimation responses but enhance organism performance under future stress expositions.

Knowledge on microalgae osmotic stress response shows a similar response based on the accumulation of compatible osmolites, osmoprotectors and storage compounds to avoid water loss and ROS damage under mild

dehydration (Gustavs *et al.* 2010). However, microalgae response mechanisms are much faster than those of land plants (Ben-Amotz & Avron 1973). As into plants, osmolyte accumulation could be also regulated by a common osmosensitive HK receptor (Suescún-Bolívar & Thomé 2015) and Ca2+ signaling, but with mechanisms and response times closer to those of animals (Brownlee & Colin 2008; Bickerton, Sello, Brownlee, Pittman & Wheeler 2016), or potentially ABA-dependent mechanisms. Potentially because, although microalgae can synthetize ABA (Hauser, Waadt & Schroeder 2011), adding ABA to cultures improves stress response and tolerance (Yoshida, Igarashi, Mukai, Hirata & Miyamoto 2003) and its genome has ABA-related PP2C and SnRKs (Gonzalez-Ballester, Pollock, Pootakham & Grossman 2008; Colina *et al.* 2019), the absence of recognizable ABA boxes into the microalgae SnRKs or plant-like ABA receptors suggest an alternative ABA stress response path in these species (Colina *et al.* 2019).

Mitogen Activated Protein Kinases (MAPK), part of the yeast HOG pathway and accumulating under osmotic stress in microalgae, are known and conserved stress hubs related in microalgae to the different osmotic stress responses including cell cycle regulation, glycerol (Zhao, Ng, Fang, Chow & Lee 2016) and lipid accumulation (Yang, Suh, Kang, Lee & Chang 2018). Moreover, MAPK as yeast MITOGEN-ACTIVATED PROTEIN KINASE HOG1 (HOG1) and ABA and PP2C related SnRK kinases, both osmotic stress signaling hubs, are recruiters of the SWITCH/SUCROSE NON-FERMENTING (SWI/SNF) nucleosome remodeling complex under stress and thus related to transcriptome and epigenetic changes (Proft & Struhl 2002; Peirats-Llobet *et al.* 2016). Thus, although involving a different timing as microalgae responses are faster, microalgae stress signaling pivots over HOG like, Ca2+ and potentially ABA/SnRK linking stress perception with response through the modulation of multiple genes at epigenetic level.

III. UV-B/C stress

The characterization of the mechanisms related to UV response have gained new interest on its effects over plant and microalgae biomass production but also on its effects over stress resistance and development in land plants. UV

treatments induce more complex metabolite patterns than osmotic stress characterized by the accumulation not only of storage compounds as triacylglycerols (TAG) but also sterols and different UV shielding and antioxidant compounds as flavonoids and carotenoids into microalgae and plants (Forján *et al.* 2011; Ahmed & Schenk 2017; Xu *et al.* 2017, 2019).

Besides the common metabolic modulation effects, different UV radiations come associated to different signalling mechanisms. While UV-A/B and other biotic/abiotic stressors effects converge in the generation of ROS and derived macromolecule damage, UV-B/C can also directly damage DNA and proteins (Stingele, Habermann & Jentsch 2015; Mullenders 2018). These similar but different stresses involve specific signalers as damaged DNA (Mullenders 2018), ROS (Urban, Charles, de Miranda & Aarrouf 2016), salicylic acid (SA), and jasmonates (JA) (Takeno 2016; Xu, Zhang & Wu 2018). These signalers are shared between UV-B and UV-C responses (Besteiro, Bartels, Albert & Ulm 2011; Dakup & Gaddameedhi 2017; Zhang *et al.* 2017), modulating UV stress responses including, the enhancement of DNA repair and protein turnover and the biomass modulation towards the accumulation of UV absorbing and ROS scavenging compounds.

Besides damage-based UV sensing and response mechanisms, plants can directly sense UV-A/B through specific receptors as UV-B specific ULTRAVIOLET-B RECEPTOR (UVR8), and blue/UVA specific photoreceptors. The excitation of UVR8 by UV-B in Arabidopsis thaliana and Chlamydomonas can directly trigger UV-B acclimation responses (Tilbrook *et al.* 2016; Liang, Yang & Liu 2018). UVR8 modulates gene expression trough the enhancement of HISTONE H3 Lys 9/Lys 14 (H3K9/K14) acetylation over its regulated gene loci (Velanis, Herzyk & Jenkins 2016). Moreover, UVR8 functions overcome the activation of UV-B stress signalling cascade as it has been proposed as the input for UV-B signals into plant circadian oscillators (Oakenfull & Davis 2017). This UV-mediated clock adjustment is key for the regulation of the intensity of UV-B stress responses to variable UV intensity in plant and animal systems (Horak & Farré 2015; Dakup & Gaddameedhi 2017) and might be related to other UVR8 mediated responses as shade avoidance

response and flowering time (Hayes, Velanis, Jenkins & Franklin 2014; Dotto, Gómez, Soto & Casati 2018).

All these complex processes into which UV is involved shows how this radiation has been engaged during plant evolution into the control of complex growth/development and cycle regulation processes. Conversely, UV-C effect on UVR8 is unclear (Christie *et al.* 2012; Jiang, Wang, Olof Björn, He & Li 2012) although signaling elements related to UVR8 have been associated to UV-C signaling in Arabidopsis thaliana (Xie, Xu, Cui & Shen 2012). Moreover it has been related to the modulation of flowering time into a stress like response involving SA signalling (Takeno 2016), and to the enhancement of pathogen tolerance (Lee *et al.* 2016; Chen *et al.* 2018a; Xu *et al.* 2019). Differences come at the signaling level with the involvement of JA and SA into UV-C signaling both for these processes.

Besides this, other candidates might be mediating the metabolic modulation under both UV-B and UV-C highlighting SUCROSE NON-FERMENTING RELATED KINASES/ CHLAMYDOMONAS SNRK (SnRK/CKIN) and related DUAL SPECIFICITY TYROSINE REGULATED KINASES (DYRK) kinases. Both have been related to carbon fluxes control under stress and SnRK/CKIN have been found sensitive to UV-B/C in Chlamydomonas (Schulz-Raffelt *et al.* 2016; Colina *et al.* 2019).

The different UV responses rely on radiation properties with UV-A/B inducing promising photomorphogenic effects while UV-C enhance biotic stress resistance. All wavelengths also induce specific metabolic modulation. These involve promising UV applications on the modulation of biomass production in microalgae, the control of plant development or the reduction of pesticide usage.

IV. SnRK, a plant-microalgae common stress regulatory family, may hold the key of the biomass problem

Both for microalgae and plants the perception, response and final acclimation to stress is a multigenic, multilayer and partially redundant system interacting

with the remaining cell ones. The whole plant cell works as a sensor where the stressors either through the activation of diverse protein sensors or through the alteration of cell structures and macromolecules can trigger different signaling processes related to different intermediaries as Ca2+, ROS, lipid derivatives, Nitric Oxide (NO), Adenosine Monophosphate (AMP), SA and ABA. These messengers in turn act over other signaling nodes highlighting MAPK, TOR and CDPK-SnRK kinases which ultimately can integrate different signaling pathways into the regulation of different effector gene sets expression (Zhu 2016).

The modulation of biomass composition and production, related to the maintenance of ROS/redox homeostasis, the shield from ionizing radiation and the recovery of the osmotic equilibrium is between the commonest and quickest stress response mechanisms. The importance of biomass fluxes for the maintenance/regulation of cellular processes as photosynthesis and respiration/fermentation, and the complexity of the multiple signaling pathways modulating them are the main problems for the strategies directed towards the enhancement of biomass production in microalgae and plants. Thus, deciphering these systems complexity is a needed first step, allowing the identification of the key factors related to the modulation of biomass production under stress. The regulation/edition of these key elements would allow the retrieval of more productive/stress resistant plants and high biomolecule yield algal strains even under non-stressing conditions.

Plant and microalgae share many stress sensing, signaling and stress effector mechanisms which allow the exchange of research knowledge between both groups. Besides this, these groups also have important differences as the speed of calcium signaling, faster in Chlorophytes (Bickerton *et al.* 2016; Edel, Marchadier, Brownlee, Kudla & Hetherington 2017), and the reduced/simpler role of ABA and other phytohormones in microalgae as Chlamydomonas. lacks homologs for many of the ABA related elements into land plants. On the other hand, microalgae have unique structures and pathways, highlighting their diverse fermentative metabolisms and the presence of characteristic carbon concentrating structures as the pyrenoid.

Besides its differences, both groups share kinases with a conserved central role into stress response, highlighting the CALCIUM DEPENDENT PROTEIN KINASES-SUCROSE NON-FERMENTING RELATED KINASES (CDPK-SnRK) superfamily of protein kinases. These kinases share a common serin/threonine kinase domain associated to different family/subfamily-specific regulatory domains. For CALCIUM DEPENDENT PROTEIN KINASES (CDK/CDPK) family Ca signaling EF hands are the regulatory domains, allowing their involvement into Ca signaling during ionic stress response. This function is shared with SUCROSE NON FERMENTING RELATED KINASE 3/CALCIUM INTERACTING RELATED KINASES (SnRK3/CIPK) subfamily, within SUCROSE NON FERMENTING RELATED KINASE (SnRK) family, with CALCINEURIN BETA-LIKE (CBL) domains able to interact with calcium binding proteins similar to calcineurin.

Also within SnRK family, SnRK1 and 2 subfamilies link different stress inputs with biomass production. SnRK1 forms a protein complex with a catalytic subunit and several regulatory subunits involved in substrate specificity (beta subunit) and its AMP based regulation (gamma subunit). Besides its broad conservation, plants have an extra regulatory subunit (betagamma) with functions similar to those of animal gamma and a gamma subunit whose interaction with the catalytic subunit has not been identified. Plantae gamma has more identity with saccharomyces SDS23, not involved into the fungi SNF1 (Saccharomyces SnRK1/AMPK ortholog) complex. SnRK1 complex have key role into the carbon metabolism adjustment to the cell energetic status sensible to the exposition to stressing factors. Moreover, SnRK2 derived from SnRK1 and plantae exclusive along SnRK3 are elements sensible to ABA and osmotic stress and related to the accumulation of biomolecules linked to stress response as proline and lipids. Besides its parallelisms SnRK1 modulation seems to be related to trehalose in land plants while this sugar signaling seems not to be present in microalgae.

High SnRK conservation between evolutively distant species and its role over biomass production under stress has made SnRK a highly interesting target towards the enhancement of stress response and more specifically the

enhancement of biomass accumulation under stress. Along these kinases others as hexokinase (HXK) and DIRK also highly conserved and linked to biomass production and accumulation under stress have been also considered as interesting targets. Moreover, microalgae features, closer to plantae common ancestor, can help into the identification of land plant exclusive mechanisms evolution but also facilitate the identification of targets into more complex systems as vascular plants.

Despite SnRK prominent role into stress response and links with biomass production regulation, studying SnRK functions has proven to be complex. These proteins are part of a big family in plants coming from recursive SnRK1 duplication events. Within Arabidopsis, SnRK2 and 3 elements have highly analogous sequences and most share same stress sensitivity or activates upon the same factor (ABA). SnRK1 subfamily is the opposite example. Most species display only one SnRK1 and its disruption is lethal. These have complicated SnRK functional characterization in Arabidopsis. For SnRK1 the use of inducible promoters allowed the controlled blockage of the kinase expression and its functional characterization. For SnRK2, the generation of multiple mutants was the only way to link these kinases with osmotic stress response. For these reasons both the description and the characterization of the stress perception and response mechanisms and the generation of enhanced varieties relies on the development of new genetic engineering strategies directed towards the efficient modulation of target gene expression both in microalgae and agroforestal species.

V. The model microalgae *Chlamydomonas reinhardtii*

The Chlorophyte *Chlamydomonas reinhardtii* is a unicellular biflagellate commonly used as microalgae research model due to its short generative time, its haplodiplontic life cycle, and ability to growth in darkness with a carbon source. These features allow a reduced growing time, ease the recovery of mutant lines due to the haploidy of the vegetative cells and allow the recovery of light sensitive photosynthesis mutant lines when growth in the dark. Moreover, the use of the microalgae research model has progressively

increased the available research resources for Chlamydomonas as a sequenced genome and diverse bioinformatic tools (Figure 1A, B).

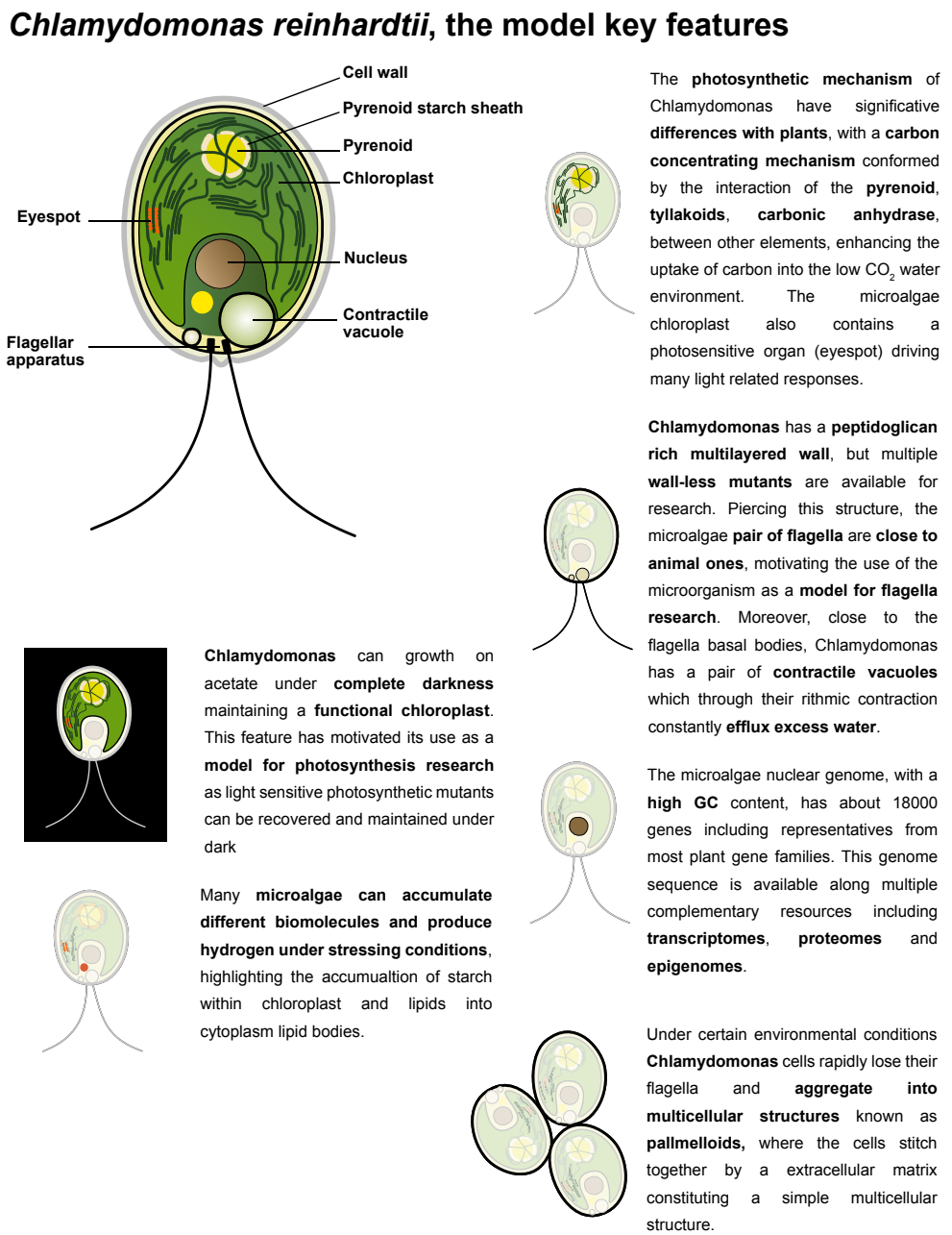

Figure 1A. Main structural and functional features of the microalgae model *Chlamydomonas reinhardtii* which have motivated the use of the fast-growing organism as a research model for different organisms including close microalgae species with industrial interest, plants and animals.

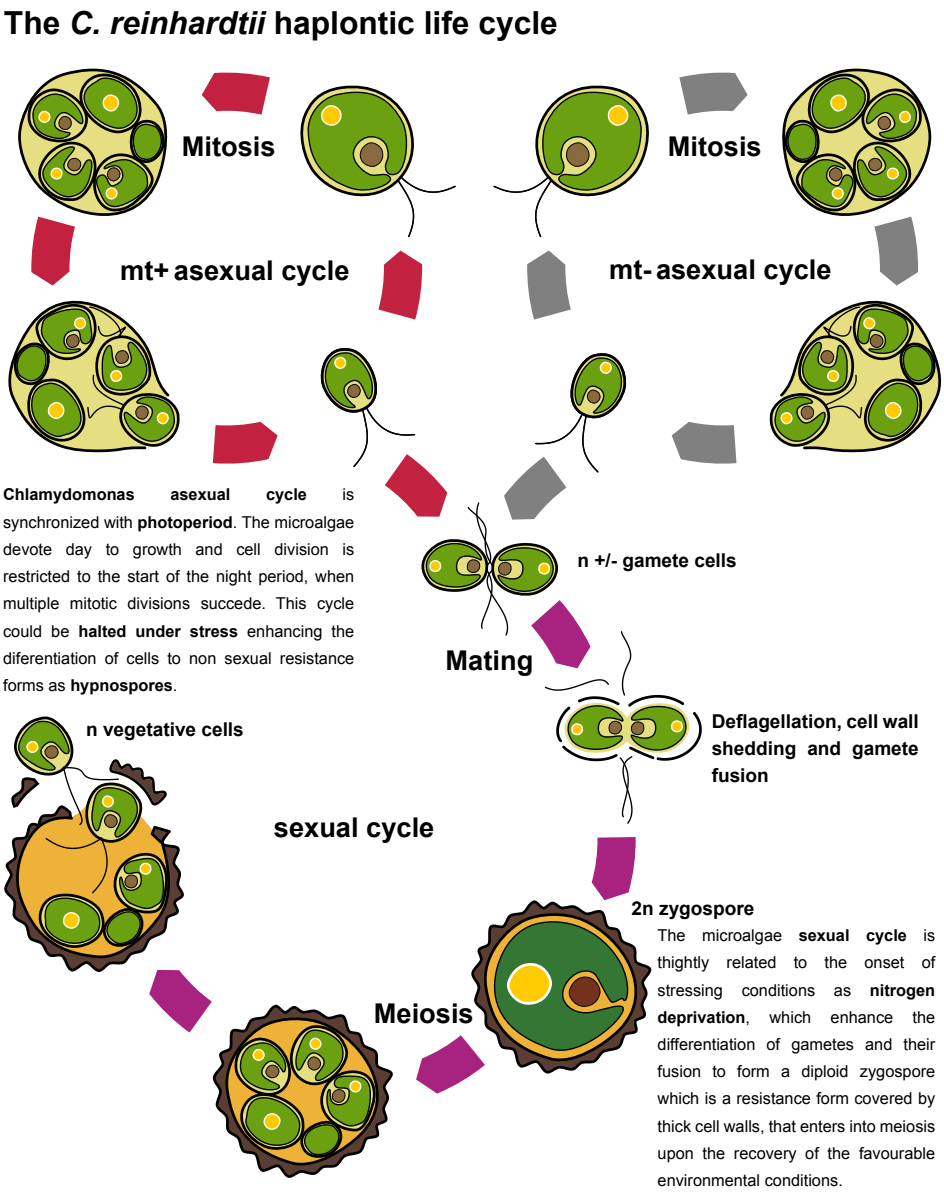

Figure 1B. Haplontic life cycle of Chlamydomonas. The microalgae divides by mitosis only into its haploid phase and upon the start of the night period. After growing during the day a single cell could divide into as much as 30 cells into rapidly succeding mitotic cycles. Under stressing conditions different mating type but isogamous flagellated cells (gametes) can fuse to form a diploid cell, the zygospore, which upon the recovery of the favourable environmental conditions enters meiosis giving way to haploid and flagellated vegetative cells.

The use of Chlamydomonas for research started on its advantages for photosynthesis research, its closeness to land plants and the similarity between

animal and microalgae flagella with multiple works have focused on the characterization of the microalgae flagella and photosystems (Merchant *et al.* 2007). More recently the microalgae has focused diverse omic works covering UV-B stress (Tilbrook *et al.* 2016), UV-C related SA and JA treatments effects (Lee *et al.* 2016; Xu *et al.* 2018), osmotic related salt stress (Wang *et al.* 2018b), oxidative stress (Blaby *et al.* 2015), cold stress (Valledor, Furuhashi, Hanak & Weckwerth 2013), nitrogen and Sulphur deprivation stresses (González-Ballester *et al.* 2010; Valledor, Furuhashi, Recuenco-Muñoz, Wienkoop & Weckwerth 2014b), and iron deficiency (Urzica *et al.* 2012). Moreover, other omics works have covered the light dependent dynamics of the different Chlamydomonas genes under normal growth conditions (Zones, Blaby, Merchant & Umen 2015; Strenkert *et al.* 2019), and the importance of redox (Perez-Perez *et al.* 2017) and phosphorylation (Roustan & Weckwerth 2018), or epigenetics (Lopez *et al.* 2015) into the regulation of the microalgae genome and proteome.

The wealth of data coming from the use of the Chlorophyte as a research model, the Chlorophyte common features with land plants and animals, and its closeness to industry relevant microalgae species have placed this organism into a central position. Knowledge generated into the microalgae can thus be transferred to plant and animal species where research is more complex due to a higher structural complexity, generative times and more complex response/signaling systems.

VI. Gene duplication, a flood of novel genes driving Plantae evolution

The comparison of common elements between different groups as microalgae and land plants must consider their divergent evolutive history. Land plants and Chlorophytes are part of a monophyletic group originating out of the same original gene pool with a 700 mya divergence in between. This divergence is easily observed when comparing their genomes gene family diversity and size with the richness in small gene families into Chlorophytes genomes, and the greater gene count per family into plants (Guo 2013). This

is observable into the increase of the kinase gene family members from 426 genes in *C. reinhardtii* to the 2532 genes found in the Embriophyte angiosperm *Eucaliptus grandis* (D. & Shin-Han 2012).

Gene duplication is the main force before the increase into the size of plant gene families involving different processes as genome/tandem duplication and transposable elements. Genome duplication is the most powerful of these processes rapidly creating big clusters of duplicated genes which under certain conditions can confer increased mutational robustness, higher resistance to stressors and higher adaptivity potential. Rice and Arabidopsis poliploids show higher stress resistance than diploid counterparts due to enhancements into the phytohormone and stress related genes expression, and their metabolic fluxes associated to their higher gene counts. Moreover, multiple predicted ancient poliploization events are close to past catastrophic events (Van De Peer, Mizrachi & Marchal 2017).

Polyploidy along the other duplication sources have not only contributed to the short term adaption to fast environmental changes but also contribute to long term adaption. Most duplicated genes are rapidly lost, but some are retained following a biased pattern where transcriptional regulation, signal transduction, and stress response genes tend to have paralogs while those involved in essential functions, such as genome repair, replication, and organelles, tend not to. The bias in retainment is influenced by multiple factors including the function and number of interactants of its derived protein, dosage balance, dosage increase effects, sub and neofunctionalization (Panchy, Lehti-Shiu & Shiu 2016).

The preferential retention of duplicated stress and transcription regulation related elements would explain some of the complexity gains into the plant lineage, specially within angiosperms where multiple ancient duplication events have been detected affecting genes related to flowering time and development. These interrelated regulatory elements are preferentially retained after genome duplications due to a dosage balance effect where the duplication of these

linked genes is viable exclusively if all of the system ones are duplication at once, maintaining the balances between their products (Van de Peer 2011).

Despite whole genome duplication have had a great impact on Embriophyte genomes from Bryophytes to Angiosperms, this seems not to be the Gymnosperms case. No recent genome duplication events are recorded into the Gymnosperms highly conserved genomes excepting for the disputed evidences of ancient duplications at the origin of seed plants and at the divergence between Gymnosperms and Angiosperms. However, its genomes are between the richest in transposable elements due to low efficiency recombination mechanisms for its removal. High transposon content would have contributed to the current relatively low diversity observed between Gymnosperms, but probably represents the main force driving gene number change and regulation into these species (Fedoroff 2012; Nystedt *et al.* 2013). Transposable elements could also be source for novel genes, as the transposase derived transcription factors FAR-RED ELONGATED HYPOCOTYLS 3/FAR-RED-IMPAIRED RESPONSE 1 (FHY3/FAR1) observed in Arabidopsis (Tang *et al.* 2012).

For stress response related genes, transposon dependent and independent tandem duplication might have had a more prominent role with the example of the tandem duplication of desiccation tolerance related EARLY LIGHT INDUCIBLE PROTEINS (ELIP) genes. Higher ELIP gene number translate to higher ELIP transcripts and proteins, generating a dosage increase effect before the dessication tolerance in angiosperms (VanBuren, Pardo, Man Wai, Evans & Bartels 2019). Duplication effect is also observed into the gymnosperm secondary metabolism diversification through the amplification of the diterpene synthase gene family involved into oleoresin biosynthesis. These genes underwent duplication events followed by sub and neofunctionalization processes diverging them from their original role in gibberellin (GA) biosynthesis (Hall *et al.* 2013).

Despite the positive effect described for many duplication events other situations remain where duplicated genes have been retained without much

sequence change and no evident adaptive advantage. This is well exemplified by the large and apparently redundant Arabidopsis SnRK2 gene subfamily. The independent mutation of these genes does not produce phenotypic effect or change into the stress response of the plant and effects are only observed after blocking the expression of all them at once (Fujii, Verslues & Zhu 2011). These retentions would be either related to the selection of lineages more resistant to deleterious mutation effects (genetic buffereing), which is a controversial hypothesis, or the allowance of adaptions that have been unnoticed due to the apparent lack of phenotype of the mutant individual. However, they might also be duplication outcomes that have not go yet through a divergence or elimination process (Panchy *et al.* 2016).

Despite the effects of duplication, the divergence of resulting sequences through point mutations or transposon insertion has also a key adaptive role. Point mutations into the promotor sequences of seed desiccation related genes created desiccation responsive cis regulatory elements allowing the expression of these genes into the vegetative tissues and their desiccation tolerance (Singh, Jung, Satpathy, Giarola & Bartels 2018) have allowed dehydration tolerance into a regulatory co-option process. Co-option is also observed into ABA-SnRK2 integration into stomatal regulation and cell cycle regulation changes driving to multicellularity into the Volvox lineage evolution (Hanschen *et al.* 2016; McAdam *et al.* 2016). Transposons might have facilitated gene regulation co-option through their insertion into promoter sequences (Negi, Rai & Suprasanna 2016).

Thus, different process driving adaption and evolution explain the divergence of gene family and number between Chlamydomonas and Pinus, supporting the conservation of central regulatory elements but also its duplication and redundancy.

VII. Genome wide identification and description of gene families across plantae

The first step into the functional characterization of signalling elements as the SnRK gene family into a certain species is the description of the family members within the species genome. Multiple works have already characterized different gene families into different plant and microalgae species highlighting the characterization of gibberellin signalling related GRAS family members in *Citrus sinensis* (Zhang *et al.* 2019), biotic stress related CISTEINE-RICH RECEPTOR-LIKE KINASES (CRK) in *Gossypium barbadense* (Li *et al.* 2018), HEXOKINASE and desiccation related LATE EMBRYOGENESIS ABUNDANT (LEA) gene families in *Brassica napus* (Liang *et al.* 2016; Wang *et al.* 2018a) and bHLH transcription factor family in *Solanum lycopersicum* (Sun, Fan & Ling 2015).

Most of these works have been directed towards elements related to development and stress response elements also covering the SnRK family and their related elements. The SnRK related bzip transcription factor family has been characterized into different plant and microalgae species (Corrêa *et al.* 2008) along the SnRK family into *Gossypium hirsutum* (Li *et al.* 2018) and rice (Kobayashi, Yamamoto, Minami, Kagaya & Hattori 2004) and the partial characterization of SnRK2 subfamily into *Chlamydomonas reinhardtii* (Gonzalez-Ballester *et al.* 2008).

All these works start from a common workflow including homology and Hidden Markov Model (HMM) assisted searches through BLAST and HMMER (Clements, Eddy & Finn 2011) tools over custom databases. These searches could be complemented with HMM based online tools as Inter Pro Scan (Jones *et al.* 2014), Prosite (de Castro *et al.* 2006), SMART (Letunic & Bork 2017), more complex PANTHER db (Mi, Muruganujan & Thomas 2013) and other platforms devoted to the identification of conserved motifs as MEME (Bailey *et al.* 2009). These tools allow the filtering of resulting sequences through the characterization of its domain architecture and its

comparison with the canonical domain distribution of the family to characterize.

The identification and filtering steps could also be complemented with other available databases allowing the classification of plant kinase and transcription factors with iTAK (Zheng *et al.* 2016) and the identification of cis regulatory elements with PlantCARE (Lescot *et al.* 2002) and PLACE (Higo, Ugawa, Iwamoto & Korenaga 1999) dbs. Other databases as wolf psort can be used for the prediction of signal peptides along others predicting the presence of regulatory motifs including protein-protein interaction and posttranslational modifications as phosphorylation or glycosylation.

Sequence description step is coupled into most works to the description of the sequence expression pattern under different conditions, using different available RNAseq databases and qPCR data. These results are usually presented through clustering algorithms showing expression patterns and clustering contributing to the phylogenetic ones. Complementing this expression data, family description will also include the prediction of protein interactions based on known protein-protein interaction databases as STRING (Szklarczyk *et al.* 2015).

Family description also include the analysis of its evolution involving the identification of ancient duplication, retainment and divergence events. This is performed through the identification of the gene position within chromosomes or their localization within synteny blocks. The divergences between family members are easily observable through their alignment using different alignment algorithms as MUSCLE (Edgar 2004), clustal Ω (Sievers *et al.* 2011) or meta aligners as M-coffee relying on different aligners consistence for alignment curation through specific algorithms as TCS (Notredame, Higgins & Heringa 2000).

Alignments evidence conserved regions into found sequences along predictions methods but also allow the reconstruction of the family evolutive history and the clustering of their members using different phylogeny approaches relying on different platforms and packages as RAxML (Stamatakis

2014), PhyML platform (Guindon *et al.* 2010) or mega and geneious suites. Different clustering methods are used according to the specific data sets coupled to different tree consistency curation including classical FELSESTEIN´S BOOTSTRAPPING FBP, and TRANSFER BOOTSTRAP EXPENTANCY (TBE) bootstrapping for large trees (Lemoine *et al.* 2018). Multiple platforms are used for tree and alignment edition and analysis including the online platform iTol (Letunic & Bork 2019) and the suites Geneious and MEGA.

Other works go ahead expanding the description of family sequences and to multiple species in an attempt to reconstruct a broader image of the evolutionary history of a certain family or a signaling pathway. The identification and description of the SnRK1 subfamily and related signalling elements along the life tree (Roustan, Jain, Teige, Ebersberger & Weckwerth 2016) is a good example of this along the comparison of all gene families size and sequence features within Viridiplantae species (Guo 2013). These works involve greater data sizes and require for high throughput methods for the identification of thousands of orthology relationships between species including tools as HMM based HaMStR-OneSeq (Ebersberger *et al.* 2014) and Homology based multiple bidirectional best hit (MBBH) (de los Reyes, Romero-Campero, Ruiz, Romero & Valverde 2017). Other high-troughput strategies include the retrieval of predicted gene families and ortology groups available into different databases as Plaza (Van Bel *et al.* 2017) and orthDB (Kriventseva *et al.* 2015).

These broader description works evidence the phylostratification of the organism's genes highlighting common elements and novel acquisitions which would be practical into the identification of candidate genes for different purposes. Moreover, this protein/gene phylogenetic structure is easily mergeable to proteomic or transcriptomic interaction networks, enriching the outputs of these analysis. These integrations would ease the comprehension of complex biological systems while providing new rules for network construction and processing into system biology (Guo 2013; Chen, Ho,

Huang, Juan & Huang 2014; Lei, Steffen, Osborne & Toomajian 2017; Ruprecht *et al.* 2017).

VIII. Using Omics into the characterization of stress response

Omic based techniques are able to describe the change of thousands of variables within a biological system covering a wide spectra of high throughput monitorization techniques of different variable types, highlighting phenomics, metabolomics, proteomics and transcriptomics. Phenomics include platforms addressing into a high throughput and synchronous manner multiple phenotypic traits over thousands of plants (Tardieu, Cabrera-Bosquet, Pridmore & Bennett 2017), Chlamydomonas cultures and even Chlamydomonas single cells (Fujita, Matsuo, Ishiura & Kikkawa 2014; Breker, Lieberman & Cross 2018; Rühle, Reiter & Leister 2018).

The use of bottom-up proteomic assays and next-generation spectrometers coupled to liquid chromatography separation systems, has allowed the rapid analysis of complete proteomes with high resolution, dynamic range, and accuracy (Nagaraj *et al.* 2012). In these analysis, proteins are first digested into peptides and then identified based on their masses and fragmentation patterns which are compared to sequence databases. These ions corresponding to the peptides are also quantified, and proteins present in the sample are then reconstructed based on the identified peptides (Zhang, Fonslow, Shan, Baek & Yates 2013). Multiple platforms have been developed for data acquisition and validation in proteomics highlighting popular proprietary software proteome discoverer (Thermo Fisher) and open source MaxQuant (Cox & Mann 2008).

As in proteomics, advances in mass spectrometry have enabled the analysis of cellular metabolites in an unthinkable scale (Patti, Yanes & Siuzdak 2012). However, unlike proteins, which are usually easy to extract from tissues, the major challenge of metabolomics is related to the chemical complexity of the metabolome, the biological variance inherent in most living organisms and the

dynamic range limitations of most instrumental approaches (Sumner, Mendes & Dixon 2003). To reduce and/or to circumvent these problems is mandatory the use of selective extraction and parallel analyses using a combination of technologies to obtain the most comprehensive visualization of the metabolome (Gehlenborg *et al.* 2010). Metabolites are also quantified by chromatographic peak height or area and identified by comparing retention time and mass to standards, or by making an interpretation of MS fragmentation patterns. Different softwares are available for the acquisition and identification of metabolomics data highlighting proprietary LC-quan (Thermo Fisher) and compound discoverer, and open source MZmine (Pluskal, Castillo, Villar-Briones & Oresic 2010).

RNA-Seq is other of the most relevant omics, quantifying the expression of tens of thousands of genes in a single run. This technique is based on the fragmentation and retrotranscription of the sample RNA followed by the multiplexed sequencing of the resulting fragments through NGS platforms. Each sequence is then aligned to a reference genome/transcriptome or, for non-sequenced species, ensembled through de novo assembly pipelines and then quantified by counting the number of reads per gene.

Acquisition allows the reduction of initially heterogenous data to integrable continuous (intensity, concentration, counts) or discrete (presence/absence) variables. Acquired data needs to be preprocessed through empty variable removal, lost value imputation and normalization (van den Berg, Hoefsloot, Westerhuis, Smilde & van der Werf 2006; Fukushima, Kusano, Redestig, Arita & Saito 2009; Gardinassi, Xia, Safo & Li 2017). Furthermore, overlapping of the data coming from different omics into the same organism can further complement validation steps. RNA-Seq improves the detection and assignment of peptides in proteomics experiments following a so-called proteogenomic approach (Bryant, Patole & Cramer 2016; Zargar *et al.* 2017; Zhu *et al.* 2017) since using databases generated from cDNA reduces the number of confounding entries as noncoding sequences and incorrect splice variants compared to genomic DNA references (Armengaud *et al.* 2014).

Sistems biology work towards the unraveling of the biological systems, through the comprehension of its individual elements contribution into the system emergent features, the system nonlinear behaviors (Weckwerth 2011; Fukushima, Kanaya & Nishida 2014; Großkinsky, Syaifullah & Roitsch 2018). For this it relies on statistical methods to establish models driven by multilevel data coming from the integration of the different system omic layers. Integrative analyses are usually depicted as interaction networks which allow establishing relationships based on clustering algorithms to find out co-expression patterns among the different omic levels studied (Moreno-Risueno, Busch & Benfey 2010) and/or correlations as sparse partial least square (sPLS) implemented in mixOmics R package (Rohart, Gautier, Singh & Lê Cao 2017).

These networks usually end into bulky high information density archives benefiting from network analysis and visualization tools. One of the most extended is Cytoscape (Shannon *et al.* 2003). Coexpression and correlations ultimately protagonizing integrative analisis require from curation, or into a network topographical environment pruning. Cytoscape offers as extra plug-ins access to known protein interaction databases as STRING (Jensen *et al.* 2009) and BiNGO (Maere, Heymans & Kuiper 2005). These allow to integrate known interaction data into the frame of the integrative model interacting elements in order to identify how well the mathematical model resolves known interactions and thus the overall model performance.

Although systems biology has largely benefited from high throughput methodologies and data analysis, the quality, comparability and analysis of these data have turned also into major issues. High biological variability and molecular fluctuation between sample replicates often introduce biases affecting the final models. Thus, final model value starts at the extraction process which must be designed towards the final integrative aim. Multiple extraction methods are good examples of integration focused methods with different ones developed to date (Weckwerth, Wenzel & Fiehn 2004; Xiong *et al.* 2011; Roume *et al.* 2013; Gunnigle, Ramond, Frossard, Seeley & Cowan 2014; Valledor *et al.* 2014a; Green & Sambrook 2018). These are able to obtain multiple molecular layers from the same sample while reducing the

requirements in sample size (Weckwerth *et al.* 2004; Roume *et al.* 2013). Valledor et al. (2014a) protocol is one of the most complete ones extracting protein, RNA, DNA and metabolite layers from same sample and has been tested into algae (Chlamydomonas) and multiple plant species. Moreover, this protocol can also be coupled to the acquisition of different phenotypic variables for Chlamydomonas as the biomass or the pigment content.

IX. Genetic engineering

Omic/systems biology works commonly highlight multiple candidates within the characterized biological systems. These candidates are frequently unrelated to the existing paradigmas about the biological systems function coming from reductive approaches. However, the mathematical assumptions into which their selection and function/interaction assignment is supported must be validated into the studied biological system. Validation involves the interrogation of the organism genome about the candidate function. Genome interrogation requires the modulation of candidate gene expression, the localization of its derived proteins or the identification of its interactions. Omics are also powerful tools for the characterization of those candidates, specially to the identification of its interactants and the elucidation of their function through the description of its effects (Nukarinen *et al.* 2016).

Multiple strategies have been devised for the engineering of the C. reinhardtii genome with different levels of success (Jinkerson & Jonikas 2015). These strategies efficiency is largely dependent on C. reinhardtii recombinant protein expression efficiency and DNA double strand break repair mechanisms preference, determining how the genetic modules integrate into its genome. C. reinhardtii DNA repair is highly shifted towards non-homologous end joining (NHEJ) over homologous recombination (HR), thus most fragments are inserted randomly into its genome. This feature makes homologous recombination, a common strategy in other microalgae species as Nannochloropsis sp. allowing the fast generation of knock out lines (Kilian, Benemann, Niyogi & Vick 2011), a very low efficient technique in C. reinhartii.

Other promising strategy for the direct editing of Chlamydomonas genome is the nuclease Cas9. Until 2017 no stable transformants with the nuclease were recovered in the microalgae (Greiner *et al.* 2017). Unique successful events were limited to the transfection of guide RNA and CAS9 protein to the microalgae through electroporation. These strategies have low transformant colony yield and require from the direct selection of the transformed genes being this only possible for a reduced gene group (Shin *et al.* 2016).

In the absence of efficient protocols towards the direct editing of C. reinhardtii genome and taking advantage of the NHEJ preference, construction of insertional libraries has become usual for the generation of KO lines (Gonzalez-Ballester *et al.* 2011; Zhang *et al.* 2014; Li *et al.* 2016; Cheng *et al.* 2017). A less time-consuming alternative for the reduction of target gene expression into the microalga is the generation of silenced lines trough interference RNA. Most successful and efficient strategy include the generation of artificial miRNA out of native Chlamydomonas miRNAs (Molnar *et al.* 2009). Other RNA strategies based in siRNA instead of miRNA, as MIGS strategy (de Felippes, Wang & Weigel 2012) are simple and successful in plants (Zheng *et al.* 2018) but its functionality in the microalgae have not yet been proven.

Independently of the different efficiencies of these methodologies all of them rely in similar elements, including simple genetic modules as constitutive expression promoters as HSP/RBCS2 or psAD and inducible promoters as HSP70 and NIT1. These latter drive the gene conditional expression based on the presence of certain factors into the culture medium and have proven to be handy for study of letal genes in Chlamydomonas (Koblenz & Lechtreck 2005; Breker *et al.* 2018) and Arabidopsis (Nukarinen *et al.* 2016). Other key elements are the resistance markers allowing for the efficient selection of transformants, with multiple of these already optimized for Chlamydomonas. Within this group are included metabolic markers originating defective mutants and resistance markers to different antibiotic and herbicides, including AphVIII, AphVII and PPXrs3, giving resistance to paromomycin, hygromycin and to the herbicide oxyfluorfen respectively (Jinkerson & Jonikas 2015).

All these techniques have specific features requiring also from dedicated solutions. Insertional mutation needs from the efficient screening of the insertion sites, allowed though the development of sequencing protocols involving the addition of specific restriction sites to the sides of the inserted cassette, making possible the recovery of the genomic sequence flanking the inserted cassette (Gonzalez-Ballester *et al.* 2011; Zhang *et al.* 2014; Li *et al.* 2016; Cheng *et al.* 2017). All expression systems also pursue the efficient expression of their genetic modules using different strategies including autocatalytic sequences allowing for the combined resistance marker and the gene of interest combined expresion and intron insertion enhancing transcript processing (Jinkerson & Jonikas 2015; Baier, Wichmann, Kruse & Lauersen 2018). Other engineering techniques are focused into the identification of protein interactants (Wodak, Vlasblom, Turinsky & Pu 2013), protein location, or towards the targeting of proteins to specific locations (Zedler, Mullineaux & Robinson 2016). These require the coupling of the sequence of the gene of interest to fluorescent protein sequences (many are optimized for Chlamydomonas), purification tags (histidine and antibody tags) and signal sequences.

All these common or specific genetic modules could be inserted alone into the cell as single (Zorin, Hegemann & Sizova 2005) or double strand sequences through electroporation or glass beads transformation. However, most works rely in vectors for the delivery of these modules into Chlamydomonas. Vectors allow for the highly efficient low error prone copy of specific sequences within bacteria, avoiding mutations due to the more error prone polymerases into PCR. Although vectors are convenient supports for genetic transformation, many studies require for the sinchronous insertion of multiple elements: different miRNAs, multiple genes to be overexpressed. This usually require from the mix of different genetic modules: promoters, resistance genes purification tags. Commonly these elements come from repositories where they are dispersed between different backbones which are not compatible.

The evolution of genetic engineering techniques based in recombination have largely removed the need for restriction sites diversity into the vector. Selection of restriction sites could be complicated as many inserts are rich in restriction motifs and sometimes avoiding this problem requires the costly editing of the vector backbone. Recombination based techniques remove this inconvenience flexibilizing the vector construction. However, constructing whole vectors merging different parts is still a time-consuming task were many PCR amplification for adapting specific sequences to target vectors and further recombination or restriction cloning steps are needed. Thus, Multiple works have initiated a standardization labor for facilitating the construction ensemble. These include constructions allowing for the fast production of miRNAs for Chlamydomonas (Zorin *et al.* 2005). Other more modular constructions as pOptimized include easily interchangeable fluorescent and luminescent proteins, signal peptides and different antibiotic selection markers (Lauersen, Kruse & Mussgnug 2015). The most advanced modular system is the MoClo toolkit including 119 exchangeable sequences including promoters, UTRs, resistance markers, tags, terminators, reporters and introns. These would allow not only for the faster edition of Chlamydomonas genome but they are aimed towards turning the microalgae into a new efficient chassis for synthetic biology and gene function validation (Crozet *et al.* 2018).

X. Approach and objectives

Plants and microalgae are key biomass providers, but biomass production is tightly entangled to stress with different mostly adverse consequences. Although stress can trigger biomolecule accumulation in plants and microalgae it does so at a high cost including the increase of the required energy input for microalgae cultures and the limitation of bulk biomass yield in plants and microalgae. Thus, providing effective answers to the stress problem becomes crucial to stablish microalgae as reliable and rentable future biofactories. Moreover answers obtained in microalgae would contribute to enhance the stress resistance of crops and forestry species under the current context of climate change.

Under the limited power of traditional management and enhancement strategies against the complex traits/systems behind stress resistance and stress biomass modulation, and the evident links between stress signalers and biomass production, characterizing the stress response system becomes essential to reach both resistance and production needs. Omics and systems biology are the best suited available tools to cope with the expected data complexity into the stress signalling and response system, coupled to genomic data mining and gene edition strategies.

Besides their advantages, the needs of these techniques, specially the time requirements of gene edition into long-lived forestry species evidences the need for a link between the both sides of the stress effects coin. The study of stress response over microalgae species as Chlamydomonas reinhardtii could not only contribute to microalgae biomass modulation strategies but due to their common ancestry with land plants, simpler response systems and short generative time could also contribute to accelerate plant stress research, especially for those species with long life cycles.

Therefore, the main goal of this thesis is the characterization of Chlamydomonas reinhardtii stress response system to UV and Osmotic stress, known biomass modulators in microalgae, aiming to identify and characterize central nodes related to the modulation of the biomass production under stress and conserved with land plants. This general objective can be subdivided into five partial objectives:

1. Description of the C. reinhardtii osmotic stress response system through the integration of proteometabolomic changes measured under the stress exposition using an integrative systems biology approach directed towards the selection of candidate genes related to the accumulation of biomolecules under stress and/or to the stress response enhancement.

2. Description of the C. reinhardtii UV-B/C stress response system through the integration of proteometabolomic changes measured under the stress exposition using an integrative systems biology approach directed

towards the selection candidates related to the biomolecule accumulation under stress and/or the stress response enhancement.

3. Identification of orthologs to the Arabidopsis thaliana SnRK protein kinase family into C. reinhardtii and other microalgae species covering Chlorophyta and mamiellales diversity using a homology and domain (HMM) assisted search of these microalgae available proteomes, to describe C. reinhardtii orthologs sequences and evolutive context.

4. Description of the expression profiles of C. reinhardtii CKIN/SnRK family sequences under different stressing conditions through qPCR and available RNAseq data. CKIN/SnRK sequence clustering by their expression profiles. Prediction of the CKIN/SnRK interactions through the use of available sequence interaction databases.

5. Identification of effective strategies for the modulation of the expression of candidate CKIN/SnRK genes in C. reinhardtii including its overexpression and the blockage of its expression.

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CHAPTER 2

Systemic osmotic stress adaptation in *Chlamydomonas reinhardtii*

CHAPTER CONTENTS

I. Introduction

Microalgae inhabit diverse environments, adapting to different water availabilities. As mainly poikilohydric organisms, the avoidance of dryness and the tolerance to desiccation are the principal countermeasures against severe water scarcity employed by these species (Holzinger & Karsten 2013).

Desiccation tolerance implies the resistance of the cell to an almost complete reduction of its water content. This process is fairly well studied in plants (Pressel & Duckett 2010; Holzinger & Karsten 2013) but, surprisingly, this interest is not extensible to most microalgae. Despite desiccation tolerance is common between aeroterrestrial species, microalgae inhabiting aquatic mediums are exposed to dehydration rather than desiccation. Dehydration is associated with tides and temperature related water osmolarity changes (Holzinger & Karsten 2013).

Dehydration, involving a limited water loss of about 30-40 % of the plant relative water content (RWC), is a milder stress than desiccation (Zhang & Bartels 2018). This water loss reduces cell volume, altering cell structure, and increase cytoplasmic osmolarity, disturbing macromolecule structure. Consequently, enzymes function and metabolic yields are altered while oxidative damage increases. As a result of these damages, osmosensitive relays activate to trigger signaling and response processes directed not only to quick response, but also to long-term acclimation (Zhang & Bartels 2018). Quick responses are mainly focused on the accumulation of osmolytes, Reactive Oxygen Species (ROS) detoxifying enzymes and scavengers, the degradation or repair of misfolded/damaged proteins, and the delay of cell cycle by reducing cell division rate (Wang, Vinocur, Shoseyov & Altman 2004; Skirycz et al. 2011; Anjum et al. 2017). Plants rapidly modulate their primary metabolism upon osmotic stress imposition, allowing the mobilization of their starch reserves to generate sugars and osmolytes that can activate downstream components in the stress response cascade (Thalmann & Santelia 2017).

Long-term acclimation in turn, modulates primary and secondary metabolism to avoid the formation of ROS while maintaining cell activities. This metabolic rearrangement keeps the synthesis of osmoprotective/osmoregulatory compounds as glycerol and trehalose while redirects inputs towards storage of sugars, cell growth and DNA synthesis, stressed cells exit mitotic cycle and start a mitosis independent DNA replication process (Skirycz et al. 2011).

To date, several osmorelays have been described in yeast and Arabidopsis (Osakabe, Yamaguchi-Shinozaki, Shinozaki & Tran 2013; Tatebayashi et al. 2015) being Histidine Kinase (HK) receptors the primary osmosensors. In yeast, High-osmolality Glycerol Response (HOG) controls the accumulation of glycerol (Saito & Posas 2012) and possibly trehalose (Babazadeh et al. 2017) after an osmotic shock. TARGET OF RAPAMYCIN (TOR) and SUCROSE NON-FERMENTING/SUCROSE NON-FERMENTING RELATED KINASES (SNF/SnRK), key abiotic-stress responsive nodes in plants and animals (Baena-González 2010), also control yeast osmotic response in a HOG independent manner (Muir, Roelants, Timmons, Leskoske & Thorner 2015). Ca^{2+} also peaks as a consequence of this stress activating signaling pathways, specifically ROS mediated ones (Kurusu, Kuchitsu & Tada 2015). Despite Arabidopsis can also adopt these strategies, also accumulating stress related metabolites like phosphatidyl inositol (Munnik & Vermeer 2010), ROS, trehalose, or chlorophyll synthesis, the key response mechanisms are regulated by abscisic acid (ABA), a plant-unique central response hub (Yoshida, Mogami & Yamaguchi-Shinozaki 2014). ABA triggers stress acclimation and specifically carbohydrate and lipid metabolism at gene level through their binding to specific receptors (Golldack, Li, Mohan & Probst 2014). ABA mediated regulation requires an interplay between different elements as kinases from the SnRK subfamily 2 (SnRK2s), phosphatases 2C (PP2Cs), and transcription factors as ABA RESPONSIVE-ELEMENT BINDING PROTEIN/ABRE BINDING FACTORS (AREB/ABFs) (Yoshida et al. 2014). This phytohormone also elicit other plant responses as osmoprotective/antioxidant proline and sugars accumulation during osmotic stress (Thalmann & Santelia 2017). Interestingly and despite in plants trehalose is only accumulated at trace amounts under osmotic stress, threalose-6-P (T6P) acts as SnRK1 inhibitor in Arabidopsis controlling growth and development (Gazzarrini & Tsai 2014).

Microalgae, a less evolved plant species, also accumulate compatible osmolites, osmoprotectors and storage compounds to avoid water loss under mild dehydration (Casais-Molina, Peraza-Echeverria, Echevarría-Machado & Herrera-Valencia 2016), being these mechanisms much faster than in land plants (Ben-Amotz & Avron 1973). This accumulation could be regulated by a

common osmosensitive HK receptor (Suescún-Bolívar & Thomé 2015), Ca^{2+} signaling, with mechanisms and response times closer to those of animals (Bickerton, Sello, Brownlee, Pittman & Wheeler 2016), or potentially ABA-dependent mechanisms. Potentially because, although microalgae can synthetize ABA (Hauser, Waadt & Schroeder 2011), adding ABA to cultures improves stress response and tolerance (Yoshida et al. 2014) and its genome has ABA-related PP2C and SnRKs, the absence of recognizable ABA boxes into the microalgae SnRKs or plant-like ABA receptors suggest an alternative ABA stress response path in these species (Colina et al. 2019).

Mitogen Activated Protein Kinases (MAPK), part of the yeast HOG pathway and accumulating under osmotic stress in microalgae, are known and conserved stress hubs related in microalgae to the different osmotic stress responses including cell cycle regulation, glycerol (Zhao, Ng, Fang, Chow & Lee 2016) and lipid accumulation (Yang, Suh, Kang, Lee & Chang 2018). Moreover, MAPK as yeast MITOGEN-ACTIVATED PROTEIN KINASE HOG1 (HOG1), and ABA and PP2C related SnRK kinases, both osmotic stress signaling hubs, are recruiters of the SWITCH/SUCROSE NON-FERMENTING SWI/SNF nucleosome remodeling complex under stress and thus related to transcriptome and epigenetic changes (Proft & Struhl 2002; Peirats-Llobet et al. 2016).

Adaption, a long-term acclimation, is the consequence of specific changes at genome and proteome levels, which are triggered by different signals which modulate gene expression through genetic and epigenetic mechanisms. Some of these changes are mitotically inheritable, enhancing the organism response capability to future stressful events (Carbó et al. 2019). The proteogenomic changes driving to acclimation have the controlled modulation of the cell proteome, specifically on photosynthesis and respiration related proteins, as their main consequence. These changes built over early response metabolome modulation, allow acclimation and thus cell homeostasis and growth recovery under the initially disturbing hyperosmotic conditions partially at the expense of the accumulation of protective and storage compounds.

In consequence, this work aims to characterize the mechanisms behind the accumulation of storage compounds and other biomolecules under osmotic stress in microalgae, as a first step towards the understanding of how plants evolved and adapted to environments with limited water availability. To this end Chlamydomonas reinhardtii, the model microalgae, is an excellent platform for understanding drought responses and acclimation processes, since they share the same regulatory elements with land plants, but with simpler gene families and a reduced number of elements, with unique presence of plant-animal features found into its genome (Merchant et al. 2007). Moreover, plant-algae closeness and algae exclusive features make this group not only convenient models but needed into the enhancement of plant systems stress resilience and biomass production. The integration of algae metabolic and stress signaling pathways into the model plant A. thaliana and crop species as Canola have already enhanced their biomass production and response to stressors (Foresi et al. 2015; Walsh et al. 2016). However, algae metabolism still holds greater prospects for plant stress response and biomass production improvement, needed under a changing climate (Sharwood 2017). Considering the far-reaching consequences of microalgae research on industry and agronomy, multiple omic levels were studied to characterize Chlamydomonas response to osmotic stress in a short time frame (24 h) by employing an integrative systems biology approach. This approach will contribute to the comprehension of how stress induces the accumulation of industry demanding biomolecules, a key step for production improvement both in microalgae and plants.

II. Material and methods

Strains, Cultures and Physiological Measurements

Chlamydomonas reinhardtii CC-503 cw92 cultures were grown on a culture chamber [25 °C, 120 rpm, continuous light and 85 μE m^2 s^{-1} light intensity provided by Sylvania GroLux lamps] in HEPES-Acetatate-Phosphate (HAP) culture media (Valledor, Furuhashi, Hanak & Weckwerth 2013). HAP was formulated from Tris-Acetate-Phosphate (TAP) (Harris 2009), substituting Tris for HEPES as a MS compatible buffer. Osmotic stress was induced through

the addition of 20% w/v Polyethileneglycol (PEG) 4000 to the culture medium. Initial culture was prepared 48 h before experiment start diluting twentyfold stock culture originated from a single colony. After 48 h, cells were harvested and diluted to $1 \cdot 10^5$ cell/mL in their respective mediums (control or PEG supplemented) splitting the resulting culture volumes between three controls and three osmotic stress flasks.

Flasks were sampled twice per experimental time (0, 5, and 24 h) by centrifuging 50 mL of media at 4000 x g. Pellets were weighted for obtaining fresh weight (FW) and immediately frozen in liquid nitrogen. Samples were then freeze dried for 72 h and re-weighted for obtaining dry weight (DW). One of the two pellets was employed for obtaining proteins and metabolites according to Valledor et al. (Valledor et al. 2014a) and the other for quantifying starch and total lipids, and pigments according to Smith & Zeeman (2006), Valledor et al. (2013), and Sims & Gamon (2002), respectively. Three biological replicates were analyzed at each sampling point.

Quantitative Proteome Analysis (GeLC-LTQ-Orbitrap MS)

Protein sample preparation for MS/MS analysis was performed as recommended by (Valledor & Weckwerth 2014). After protein digestion, ten micrograms of digested peptides were loaded per injection into a one-dimensional nano-flow LC-Orbitrap/MS and resolved in a 90-min gradient from 5 to 40% (v/v) acetonitrile/0.1% (v/v) formic acid using a monolithic C18 column (Chromolith RP-18r, Merck, Darmstadt, Germany). MS analysis was performed on an Orbitrap LTQ XL mass spectrometer (Valledor et al. 2013).

Raw data coming from Orbitrap were searched with SEQUEST algorithm present in Proteome Discoverer version 2.1 (Thermo) as previously described (Valledor, Recuenco-Munoz, Egelhofer, Wienkoop & Weckwerth 2012) and employing a label-free quantification based on precursor's areas. Three different databases were employed for identifying proteins, Chlamydomonas 5.5 (18750 accessions), Chlamydomonas chloroplast & mitochondria (84 accessions) and Swissprot-viridiplantae (36097 accessions). Only high

confidence proteins (at least one significant peptide, XCorr > 1.8, FDR 5%) present in all the three biological samples of at least one treatment were considered for this analysis.

Metabolite GC-MS Analysis

Polar fraction analysis were carried out following previously developed procedures (Valledor, Furuhashi, Recuenco-Muñoz, Wienkoop & Weckwerth 2014b) with some minor changes on a triple quad instrument (TSQ Quantum GC; Thermo). In brief, one 1 μL of sample was injected, and GC separation was performed on a HP-5MS capillary column (30 m 9 0.25 mm 9 0.25 mm) (Agilent Technologies). Oven temperature was increased from 80 °C to 200 °C at 3 °C per min and then reduced to 25 °C at 10 °C per min and maintained at 25 °C for 3 min. Post run conditions were maintained at 30 °C for 4 min. The mass spectrometer was operated in electron impact (EI) mode at 70 eV in a scan range of m/z 40–600. Metabolites were identified based on their mass spectral characteristics and GC retention times through comparison with the retention times and spectral characteristics of standards available in our in-house library and in Golm Metabolome Database (Kopka et al. 2005).

Biostatistical analyses

Statistical procedures described above were performed using the software R v3.3 (R Core Team, 2019) core functions and pRocessomics package (available at https://github.com/Valledor/pRocessomics). Missing values of proteomics and metabolomics datasets were first imputed employing a sequential K-Nearest Neighbor algorithm. This procedure was performed only in those cases in which one value per sampling point was missing. Protein and metabolite abundances were then re-estimated following a sample-centric approach (individual peak areas divided by total peak area per sample). Data was then subjected to univariate (one-way ANOVA; 5% FDR followed by a Tukey HSD post-hoc) and multivariate analyses (principal component analysis, heatmap clustering and sparse partial least squares regression analysis).

Multivariate models, based on sPLS (Lê, Rossouw, Robert-Granié & Besse 2008), were employed to build correlation networks employing the R package

mixOmics using proteins as predictors and metabolites and physiological parameters response. Networks were visualized and processed in Cytoscape V3.6.1 (Shannon et al. 2003). STITCH platform (Szklarczyk et al. 2016) was used to build known protein-protein/protein-metabolite interaction networks from previous correlation network. Cytoscape plug in StringApp (Doncheva, Morris, Gorodkin & Jensen 2019) was used for STICH network import, setting interaction confidence to 0.4 (medium confidence).

III. Results

Physiological characterization of Chlamydomonas response to non-dessicating osmotic stress

Osmotic stress initially halted cell growth until, at least, 5 hours of culture (Figure 1A). After this time cells started to grow again but at lower rates than controls. Chlorophylls were also affected, with Chla/b ratio increased two folds after 24h (Figure 1B). Osmotic stress also induced starch but not lipid accumulation. This polysaccharide accumulated rapidly between 5 and 24 h after stress start (Figure 1C).

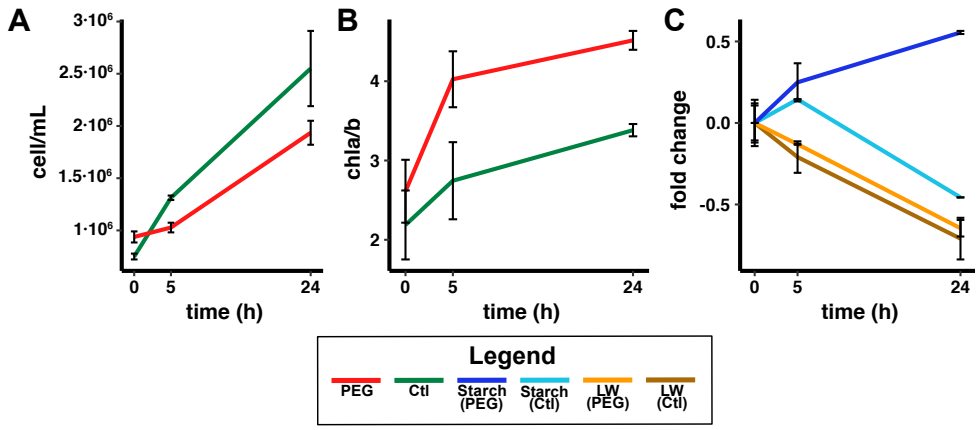


Figure 1. Changes in cellular density (A), chlorophyll a/b ratio (B), and lipids (LW) and starch content per cell (C) between control and stressed Chlamydomonas cultures measured in three time points (T0, 5, 24 h) into a 24 h timeframe.

Integrated proteomic, metabolomic and physiological responses

High throughput proteomic analyses allowed the identification of 4802 and 1800 peptides and protein species, respectively, after analyzing 97851 spectra obtained from whole cell protein extracts. The number of identified proteins

represents about 12.5 % of the coding genes in the C. reinhardtii genome (Merchant et al. 2007). After data pre-processing, 1396 proteins were above the minimum abundance threshold for confident quantitation (Supplementary table S1). GC-MS allowed the unequivocal identification of 71 primary metabolites, out of these 69 were considered for quantitative purposes (Supplementary table S2). A small physiology dataset including starch, lipids (LW), carotenoids and Chlorophyll contents per cell was also considered (Supplementary table S3).

Detected proteins and metabolites were classified according to MapMan categories and 1173 proteins and 61 metabolites were assigned to functional bins along physiological measurements. These bins comprised 32 pathways for proteins and 17 for metabolites belonging to primary and secondary metabolism. Out of these, 894 proteins, 58 metabolites, and all physiological measurements except lipids could be considered quantitatively differential in at least one sampling time (ANOVA, 5% FDR). Within differentially accumulated proteins, 250 were present only in two sampling times and 386 were exclusive from one sampling time. At metabolite level only glycerol was detected for first time after 5 hours of stress (Figure 2).

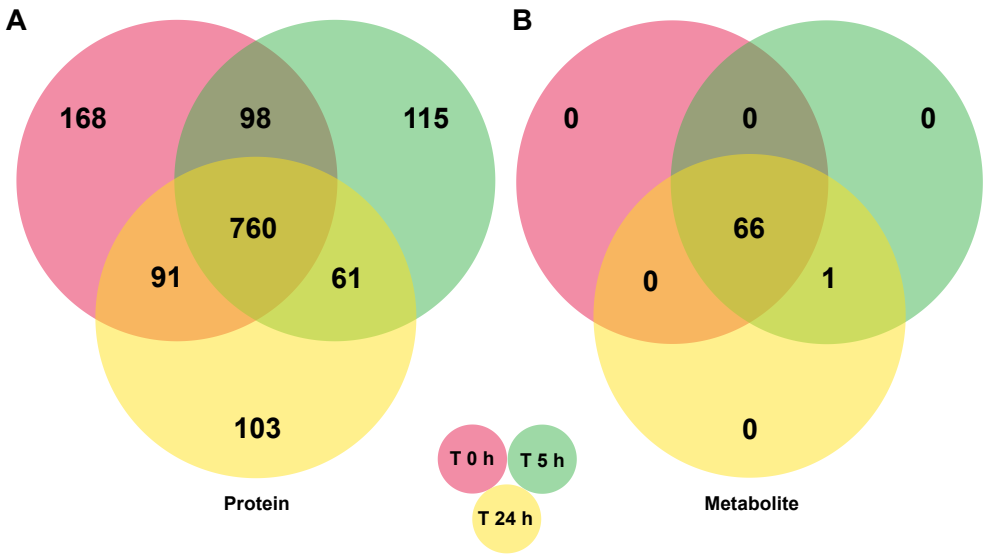


Figure 2. Venn diagram showing protein distribution between harvesting times (A). Venn diagram showing metabolite distribution between harvesting times; only glycerol showed qualitative differences (B).

The function of those proteins showing qualitative differences in their accumulation pattern give a broad idea about processes undergoing during the stress response (Supplementary table S1). Control samples exhibit characteristic proteasome elements and starch degrading isoamylases, together with many RNA and protein translation related proteins. Osmotic stress quickly induced proteins related to redox responses and photosystems, which accumulated after 5 h of exposure. Conversely, after 24 h of stress the microalgae displayed yet multiple stress response signaling elements along acclimation related ones as DNA methylation, RNA splicing, translation regulation and carbohydrate metabolism/oxidative phosphorylation related proteins, being many shared with controls. Early metabolome landscape (T5 h) was dominated by accumulating fermentation and redox related elements as lactic, 2-hydroxyglutaric and lyxonic acids. However, from the metabolic view osmolytes/osmoprotectants accumulation was this stress hallmark. Their accumulation occurred either during stress as glycerol and trehalose or exclusively on late (T24 h) acclimation like fructose, galactose, ribose, arabinose and multiple unknow sugars. These accumulated along amino acid metabolism/photorespiration related glycerate while different organic acids, amino acids and phenolic compounds fall from their initial control high levels (Supplementary table 2).

These results pointed to a differential response of Chlamydomonas reinhardtii to osmotic stress, which can be divided into differentiated and sequential stages: a quick alert/stress damage initial response can be distinguished between 0 and 5 h, ending into the activation of mechanisms leading to the cell acclimation to its new environment, 24 h after stress started. Specific individual elements and pathways abundance pattern pointed to stress signaling, ROS/redox, protein protection/turnover and carbon metabolism as key into this response as it will be depicted below.

Heatmap clustering distinguished different treatments with an adequate grouping of samples (Figure 3, Supplementary figure S1, S2). At protein level (Figure 3A) osmotic stress induced a quick reduction in transport, stress, polyamine metabolism and RNA and DNA related categories, all classified in

the same meta group. On the other hand, the abundance of sugars, lipids, electron transfer chain and related categories were increased after 5 hours of culture. Interestingly there was an increment of photosynthesis (PS) and signaling groups after 24 hours. Metabolites (Figure 3B) shared a similar distribution to those of proteins during the early stress response, but in some categories, such as fermentation and nucleotide metabolism or minor CHO metabolism, a time-shift between protein and metabolite accumulation was observed.

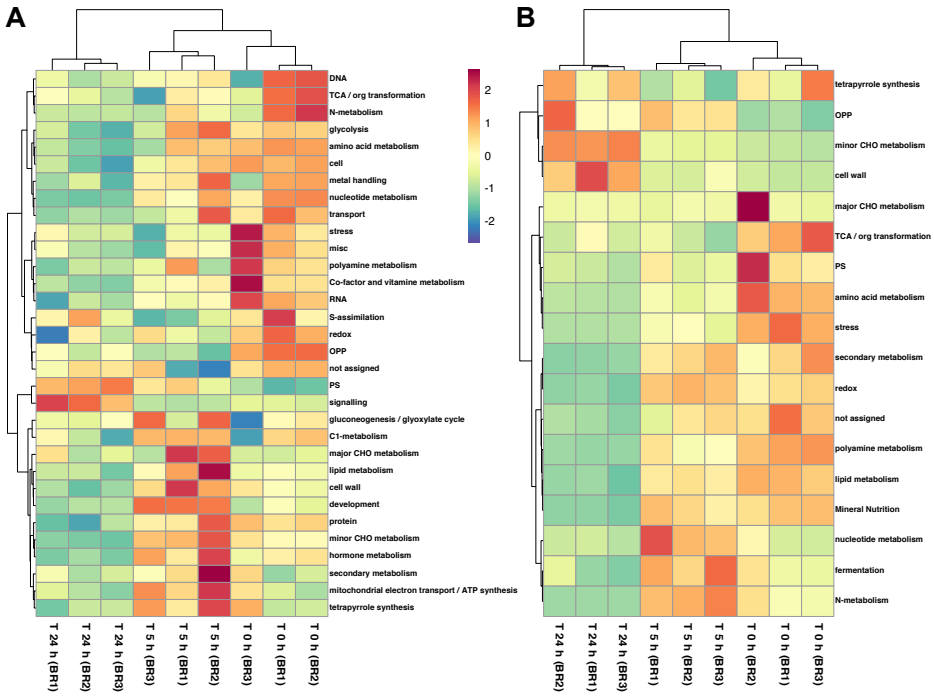


Figure 3. Heatmap biclustering plots over mapman categories protein (A) or metabolite and physiology (B) total abundance. Samples clustered according to their harvesting time and differences in abundance were observed between harvesting times for some functional categories into both plots.

Samples were also classified by PCA considering each dataset independently (Supplementary figure S3, Supplementary tables S4, S5). In proteomics dataset (Supplementary figure S3A, Supplementary table S4), principal component 1 probably gathers the variability of chloroplast stress response mechanism involving ROS CHLOROPLAST Mn SUPEROXIDE DISMUTASE (MSD3), transcription/RNA HISTONE H2A (HTA14), SUBUNIT OF EXON JUNCTION COMPLEX (UAP56), LA PROTEIN (LAL1), signaling MAGNESIUM CHELATASE SUBUNIT I (CHLI2), PROTEIN

PHOSPHATASE 1 BETA (PKL1) and sugar metabolism UDP-GLUCOSE PYROPHOSPHORYLASE (UGP1) while PC2 gathers stress acclimation, main stress nodes (as MAPK6 and several PP2s) and transcription SF3A1 SPLICING FACTOR SUBUNIT 1 (SPL1), NUCLEAR PRE-mRNA SPLICING FACTOR (PRP39) and gene regulation (DNA methyltransferase). At metabolome (Supplementary figure S3B, Supplementary table S5), PC1 was correlated to the accumulation of starch and other sugars and polyphenols, while PC2 grouped stress related metabolites.

The integration of datasets by sPLS analysis allowed an adequate classification of the samples (Figure 4A), but also the definition of an interaction network between proteins, metabolites and physiology (Figure 4B), composed of two main clusters. First cluster (Figure 4C) can be subdivided onto two smaller graphs. Lower graph gathers sugars (trehalose, fructose, and unknown mono and disaccharides) and links them with key proteins related to carbon uptake and assimilation (carbonic anhydrases) and sugar metabolism PHOSPHOFRUCTOKINASE 2 (PFK2), PHOSPHOGLUCOMUTASE (GPM2) and Cre07.g347100.t1.2, homolog to Arabidopsis GLUCOSE-6-PHOSPHATE 1-EPIMERASE. Regulatory elements such as ubiquitin ligases and AMP- or Ser/Thr-kinases and RNA related proteins are also important in this model. In the upper graph, ascorbic and citramalic acids and Leu and Ile links sugars to signal recognition proteins and specific proteases. Both graphs are linked by NADH:UBIQUINONE OXIDOREDUCTASE 16.3 kDa SUBUNIT (NUOS6) and MITOCHONDRIAL F1F0 ATP SYNTHASE SUBUNIT 6 (ATP6), involved in the production of ATP at mitochondria and a MAPK kinase. Overall this graph seems to relate initial osmotic shock and protein and starch/lipid degradation related elements with acclimation related osmolytes in form of sugars. Second cluster (Figure 4D) links proteins related to 2-hydroxyglutaric acid, an epigenetic repressor in mammal cells (Intlekofer et al., 2015). This group links epigenetic regulation (HTA14), osmotic GLYCOGEN SYNTHASE KINASE 3 (GSK3), CALMODULIN (CAM1) and chloroplast stress response PKL1, TETRAPYRROLE-BINDING PROTEIN (GUN4) proteins. Furthermore, the presence of the major regulatory kinase TOR, involved in cell cycle, protein turnover and cell survival, is also relevant.

2-hydroxyglutaric acid was also connected to chlorophyll b and to the upper half of the first cluster through regulatory (Ubiquitin ligases, GSK3), translation LAL1, RIBOSOMAL PROTEIN L7A (RPL7A) and stress-related elements (MSD3) showing altogether a clear connection between epigenetics, stress response, and proteome remodeling. Main clusters were linked by Uracil and ascorbate-related lyxonic acid through protein degradation E3 UBIQUITIN LIGASE (SKP1), 26S PROTEASOME REGULATORY SUBUNIT (RPT2), carbon metabolism Arabidopsis PANTOTHENATE KINASE (PANK) homolog Cre13.g591400.t1.1 and redox related cytochrome b5.

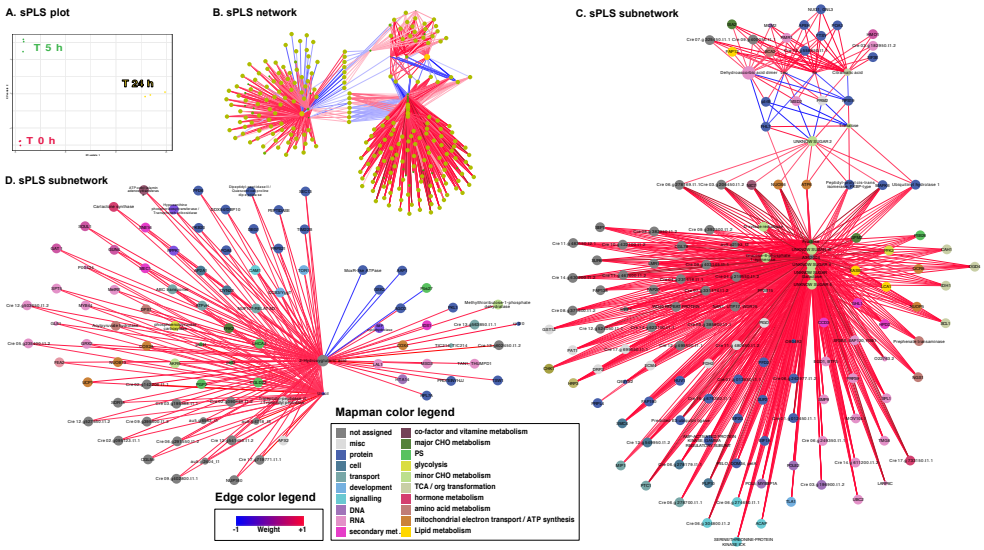


Figure 4. sPLS variable plot showing clear differentiation between harvesting times (A). sPLS based network miniature showing the links between the two graphs into which could be divided (T0-24 h and T5 h graph) (B). T0-24 h network graph relating mechanisms involved into stress alarm, signaling and acclimation (C). T5 h network graph relating early stress signaling mechanisms with emphasis on chloroplast stress signaling (D). For visualization purposes the aspect of T0-24 h and T5 h network graphs was modified from their network miniature original appearance. Network nodes were colored according to their MapMan categories linking node size and edge color to size and weight respectively. Only the edges with correlations higher or lower than ± 0.9 were visualized. Nodes without any edge above the threshold were removed from this analysis.

The overlap of a new layer considering the biological interactions (protein-protein, protein-metabolite) described in String DB to previous graph following a STITCH approach (Figure 5) supported most of the correlations

previously described. Interestingly, the graph of STITCH network corresponding to first cluster (Figure 5A) highlights (PFK2), Mn SUPEROXIDE DISMUTASE (MSD2), Cre11.g480650, PHOSPHATE ACETYLTRANSFERASE (PAT1) and CHLOROPLAST SRP RECEPTOR (FTSY) as the key proteins connecting sugars, Leu and Ile with RNA and protein translation groups with DNA replication, DNA regulation, sugar metabolism and TCA. ATP6, NUOS6 and MAPK6 which linked these groups in sPLS based networks are also present, however its importance for this model is limited except for MAPK, which links galactose to cell division and other non-identified kinases and phosphatidylinositol pathway. STITCH network corresponding to the second cluster (Figure 5B) showed PKL1, TOR, CAM1 and GSK3 as key proteins connecting protein degradation and carbon metabolism to redox homeostasis, protein synthesis, cell cycle control and epigenetic modulation.

The connection between sugars to redox (MSD2) and signaling nodes (MAPK6) and amino acid metabolism to protein synthesis/translation regulation DNA and RNA regulation were highlighted. Nodes as (PAT1), implicated into fermentative acetate production in Chlamydomonas (Magneschi et al. 2012), and FTSY were also central into this interaction. STITCH network on sPLS early response (T5 h) network define a protein synthesis/degradation, DNA and RNA regulation cluster with TOR, GSK3 and other kinases at the center, linking it to photosynthetic electron transport and redox pathways.

Stress-induced remodeling of Chlamydomonas primary metabolism

Dehydration produces a cell shrinkage and vesicularization (pictures not shown), potentially affecting membrane stability, cell osmolarity, and protein structure with an obvious impact over enzyme kinetics. In the case of energy-related pathways, this impact is high as it is depicted below.

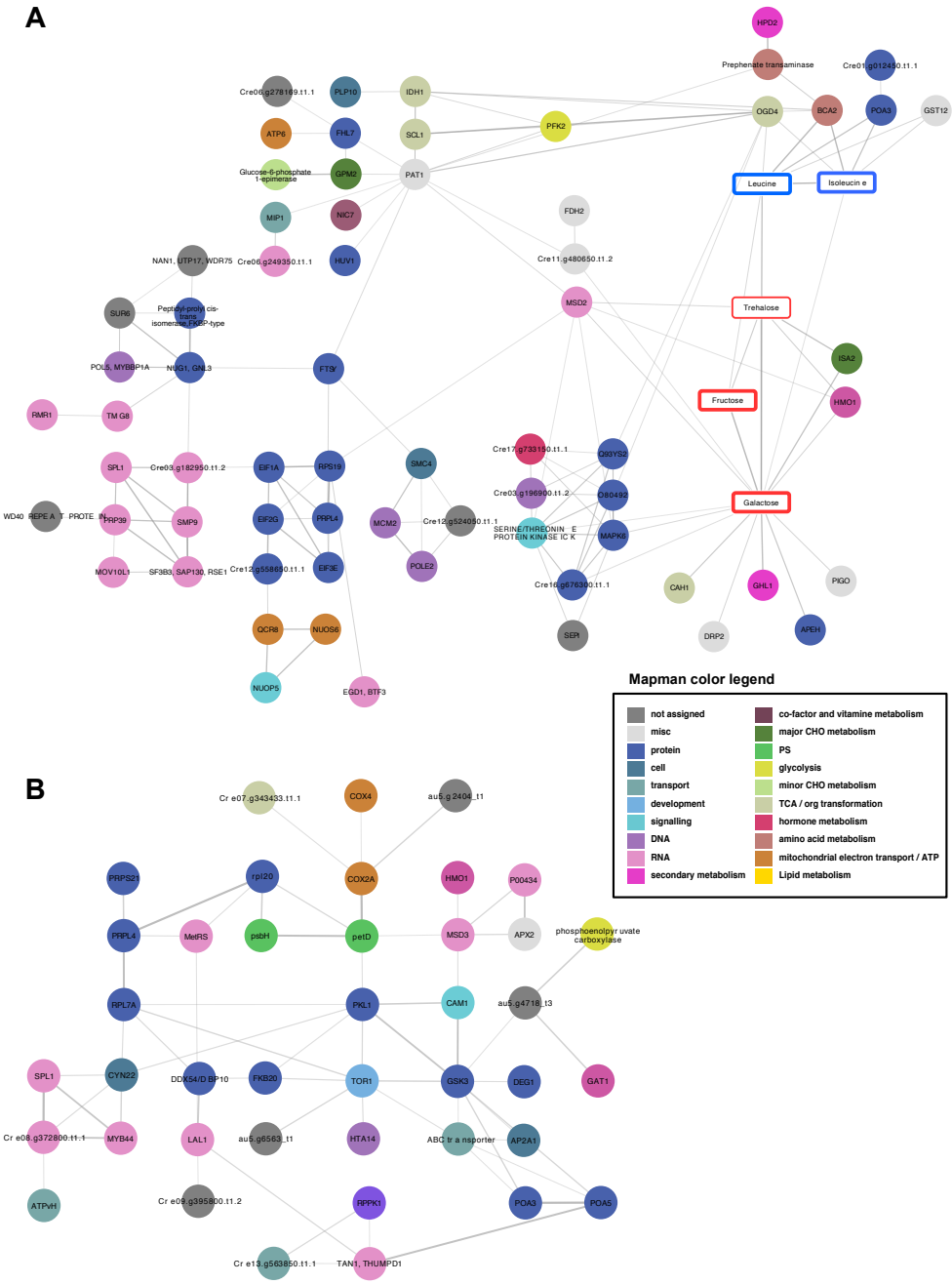


Figure 5. sPLS based STITCH networks. STITCH network nodes were colored according to their MapMan categories edge thickness was link to interaction confidence (STITCH interaction score). Confidence threshold was set at 0.4 (medium confidence). T0-24 h network relating mechanisms involved into stress alarm, signaling and acclimation. Multiple sPLS predicted interactions are confirmed highlighting multiple signaling nodes (A). T5 h network relating chloroplast mediated early stress signaling and adaptation. Multiple sPLS predicted interactions are confirmed highlighting brassinosteroids and chlorophyll precursors mediated chloroplast-nucleus retrograde signaling (B).

Regarding to photosynthetic light reactions, most of the detected proteins over accumulated with time (Supplementary table 1). The accumulation of light harvesting complex, I and II, elements correlates with an increase of chlorophyll a/b ratios (Figure 1B). Interestingly, photosystem I (PSI) core components PHOTOSYSTEM I REACTION CENTER SUBUNIT N (PSAN) and PHOTOSYSTEM I REACTION CENTER SUBUNIT V (PSAG), involved in light harvesting complexes I and II (LHCI and II) docking respectively (Takahashi, Iwai, Takahashi & Minagawa 2006), downregulated on T5 h (-0.63-, -2.05-fold), maybe indicating a change in PS complex state. Photosystem II (PSII) elements also accumulated under osmotic stress, including Mn cluster stabilizing proteins, with the exception of LUMENAL PSBP-LIKE PROTEIN (PSBP4) and CYTOCHROME b559 SUBUNIT ALPHA (PSBE), downregulated at T5 h (-1.65-, -0.78-fold). Other PSII components such as LUMEN TARGETED PROTEIN (PSBP6), PHOTOSYSTEM II REACTION CENTER PROTEIN H (PSBH), PHOTOSYSTEM II SUBUNIT 28 (PSB28) were only detectable under stress. PSBP28 controls PSII-LHCII assembly and stability (Plöchinger, Schwenkert, von Sydow, Schröder & Meurer 2016) and PSBP6 is a stress-responsive element previously described under drought stress in tomato (Tamburino et al. 2017). Cytocrome b6f elements, connecting PSII and I through linear electron flow (LEF), were also up-accumulated under stress. Despite increased photosystems and cytochrome abundance pointed to an increased LEF, nuclear encoded RIESKE IRON-SULFUR SUBUNIT OF THE CYTOCHROME b6f COMPLEX (PETC) and FERREDOXIN-NADP REDUCTASE (FNR1) proteins, down-accumulated (-1.68- and -2.97-fold at T24 and T5 h respectively) being this correlated with a reduction in LEF but not in cyclic electron flow (CEF) (Yamori et al. 2016) and to a downregulation in photosynthetic machinery while increasing antioxidants (Lintala et al. 2012), respectively. Conversely the abundance of CYTOCHROME b6f COMPLEX SUBUNIT V (PETO), an algae CEF positive regulator (Takahashi et al. 2016), was also diminished (-1.64-fold at 24h).

Carbon-fixation enzymes remain mostly unchanged, only decreasing RIBULOSE-1,5-BISPHOSPHATE CARBOXYLASE/OXYGENASE SMALL SUBUNIT 1 (RBCS1) and SEDOHEPTULOSE 1,7 BISPHOSPHATASE

(SBP1) at T5 h (-2.22-, -1.43-fold). The reduction of RBCS and the inhibition of RBCL occurs rapidly in plants under drought stress (Parry, Andralojc, Khan, Lea & Keys 2002). Interestingly photorespiration related PHOSPHOGLYCOLATE PHOSPHATASE (PGP2) accumulate at T5 along metabolic intermediate glycolate (4.1-fold at T5 h). SERINE HYDROXYMETHYLTRANSFERASE 2 and 3 (SHMT2 and 3) drop at T5 h while SERINE HYDROXYMETHYLTRANSFERASE 1 (SHMT1) isoform maintain its abundance during assay. Thermogenin-like (UCP1), probably a mitochondrial aspartate/glutamate transporter (Monné et al. 2018) accumulated, while glycine, glutamate, and serine dropped from initial high levels (-3.18-, -5.1-, -3.51-fold at T24 h) suggesting an effective sink of aminoacids by glutamate:glyoxilate aminotransferases and SHMTs but not enough activity of glycolate oxidase for supplying glycine since recycling enzyme serine:glioxylate aminotransferase abundance maintained during assay. The excess of glycolate can be excreted to the extracellular medium in Chlamydomonas (Husic & Tolbert 1986), that can be potentially reassimilated under dark period (Leboulanger, Martin-Jézéquel, Descolas-Gros, Sciandra & Jupin 1998).

Photoassimilates tend to accumulate under stress, since starch increased (0.55-fold at T24 h) (Figure 1C). Granule-bound starch synthase (1.76-fold at T5 h) and starch branching enzyme also accumulated while starch debranching enzyme ISOAMYLASE-TYPE STARCH DEBRANCHING ENZYME (ISA2) downregulated.

Besides this, the accumulation of ALPHA-AMYLASE (AMA2) (1.6-fold at T5 h) shows that starch also degrades under stress. Starch breakdown contribute to the cell glucose-1-phosphate pool, funneled under osmotic stress towards sugar synthesis, specially trehalose (7.24-fold at T24 h), by the accumulating UGP1 (2.6-fold at T5 h).

Starch accumulation rely on triose phosphate inputs from photosynthesis and acetate which are controlled by the balance between glycolytic and gluconeogenic activities. Gluconeogenic checkpoint FRUCTOSE-1,6-

BISPHOSPHATASE (FBP1) remain unchanged under stress, however, PHOSPHOENOPYRUVATE CARBOXYLASE (Cre03.g171950.t1.1), linking TCA/glyoxylate cycles to gluconeogenesis, dropped under osmotic stress (-1.74-fold T24 h). This suggest that gluconeogenesis and hence sugar/starch synthesis rely mainly on photoassimilates rather than acetate under stress. On the other hand, glycolysis-related MITOCHONDRIAL PYRUVATE DEHYDROGENASE COMPLEX, E1 COMPONENT, ALPHA SUBUNIT (PDC1) and PYRUVATE KINASE (PYK1) peaked at T5 (1.7-, 1.5-fold) along lactate (1.14-fold at T5h), while PFK2 accumulated on acclimation. These changes show how early under stress photo assimilates are driven both through gluconeogenesis to storage compounds and through glycolysis towards TCA and fermentation, as suggested by lactate accumulation. The accumulation of PFK2 on acclimation shows a glycolysis enhancement/gluconeogenesis inhibition on acclimation as glucose 2,6 bisphosphate, PFK2 product, enhances glycolytic PFK1 while inhibits gluconeogenic FBP1.

Changes into glycolysis/gluconeogenesis and growth during osmotic stress modify carbon fluxes to and from TCA with citric, malic and succinic acids dropping during acclimation (-1.96-, -2.42-, -4.35-fold at T24 h) maybe due to an increased abundance of NAD DEPENDENT ISOCITRATE DEHYDROGENASE (IDH1) and SUCCINYL-CoA LIGASE ALPHA CHAIN (SCL1). SUCCINATE DEHYDROGENASE (SDH1) dropped under stress (-3.05-fold at T24 h) and fumarate reduction (-0.75-fold at T5 h) suggest early reduced TCA oxidation due to reducing equivalents accumulation leading to the accumulation of TCA intermediaries, some as α-ketoglutarate are processed through lactate dehydrogenase to the accumulating 2-hydroxyglutarate (6.5-fold at T5 h) (Intlekofer et al. 2015).

The accumulation of reducing equivalents under osmotic stress has also consequences on mitochondrial electron transport driving to an increased QH2 pool. Although SDH1 reduced its accumulation, several subunits of the complexes I, III and IV accumulated under stress or at T5 h (Supplementary table 1) along MITOCHONDRIAL TYPE-II NADH DEHYDROGENASE (NDA1) supporting an enhancement into reducing equivalents consumption.

Increased oxidative activity ends into the acidification of intermembrane space managed by the mitochondrial ATP synthase, whose proton channel (ATP6) accumulated under stress enhancing ATP synthesis.

Initial stress response increased protein turnover and protection mechanisms

Response to stress requires a quick remodeling of the proteome in order to respond and adapt to the new environment. In this sense proteins related to protein biosynthesis and degradation peaked at T5 (Figure 3A). Two main activities were promoted after stress: protein degradation with a time-course decrement in proteasome related proteins (POAs, RTPs and RPNs) and protein synthesis with increased ribosomal (RPs and PRPs) and folding activities (FKBPs). Chaperones, T-complex, and other folding and protein stabilizing proteins showed its highest abundance also at 5 h (up to 2.5-folds change) (Supplementary table S1). T-complex and prefolding chaperones act in a coordinate way, folding cytoskeletal proteins and regulating cell cycle and transcription trough transcription factor modulation and epigenetic mechanisms (Dekker et al. 2008; Millán-Zambrano Gonzalo & Chávez Sebastián 2014). The accumulation of protein degradation and folding elements, together with the accumulation of osmolytes was previously reported in Chlamydomonas, as a consequence of heat stress (Schroda, Hemme & Mühlhaus 2015). As damaged proteins or specific isoforms are degraded, new proteins must be synthetized and multiple ribosomic proteins accumulated between 5 and 24 h. Activities not only related to protein synthesis, but also to enhanced protein secretion RIBOSOMAL PROTEIN L23a (RPL23a), COP-II COAT SUBUNIT (SEC13) (Moin, Bakshi, Madhav & Kirti 2017) and transport into nucleus, mitochondria MITOCHONDRIAL INNER MEMBRANE TRANSLOCASE (TIM22B) (Figueroa-Martínez, Funes, Franzén & González-Halphen 2008), and chloroplast CHLOROPLAST-ASSOCIATED SecA PROTEIN (SCA1), PREPROTEIN TRANSLOCASE secY SUBUNIT (SCY1) were also upaccumulated under stress (Skalitzky et al. 2011) (Supplementary table S1).

Hyperosmolarity induced alterations in water homeostasis and ROS scavenging mechanisms

High osmolarity causes a water loss that require fast and long-term control measures to protect cellular structures and maintain metabolic activity while limiting stress derived ROS damage, ultimately allowing cell acclimation to the new environment. Glycerol and trehalose accumulation (7.24-fold at T24 h for trehalose) act primarily as osmoprotectants equilibrating cytoplasmic osmolarity and protecting cellular structures but also as carbon sink together with starch. Glycerol synthesis from C3 dihydroxyacetone phosphate is a NADH consuming pathway, thus its synthesis could recycle NAD+ maintaining redox homeostasis and limiting ROS production. On the other hand, trehalose have a dual function into ROS detoxification as it is an enhancer of antioxidant gene expression (Mizunoe et al. 2018) also being a ROS scavenger (Luo, Li & Wang 2008).

The early accumulation of Arabidopsis TOCOPHEROL CYCLASE (VTE1) homolog (Cre01.g013801.t1.2), L-ASCORBATE PEROXIDASE (APX2) and lyxonate, an ascorbate metabolite, (1.8-fold at T5 h for lyxonate) suggests the early enhancement of these ROS scavenging mechanisms, which also requires the accumulation of peroxidases, GLUTATHIONE REDUCTASE (GSR1), CPYC TYPE GLUTAREDOXIN (GRX2) and MSD3. The accumulation of these elements was a consequence of a quick redox disbalance and ROS production after stress imposition.

The accumulation of GUN4, a chloroplast stress sensor, and PROTOPORPHYRINOGEN OXIDASE (PPX1) suggested the accumulation of tetrapyrrole metabolism precursors. Some of these precursors, as PPX1 product protoporphyrin IX, Mg-protoporphyrin IX and heme have a suspected role into nucleus-chloroplast stress retrograde signaling. Heme groups can modulate drought stress and chlorophyll biosynthesis related genes. Moreover, the photosensitizing nature of protoporphyrin IX can trigger chloroplastic ROS bursts enhancing chloroplast ROS detoxification response. This enhancement includes heme accumulation, which acting as a cofactor of several ROS related enzymes, improves the cell ROS detoxification response

(Nagahatenna, Langridge & Whitford 2015; Woodson 2016). GUN4 interact with protoporphyrin IX to sense the chlorophyll precursor levels and trigger retrograde responses modulating chlorophyll synthesis, being linked to the activation of a plant magnesium chelatase, an ABA modulator (Du et al. 2012), whose Chlamydomonas non-functional homolog CHLI2 (Brzezowski et al. 2016) accumulate early under stress. This proposes the early GUN4, and possibly CHLI2, involvement into chloroplast-nucleus stress retrograde signaling adjusting photosynthesis and ROS detoxification under stress.

PKL1, a brassinosteroid pathway phosphatase, accumulated early under stress along GSK3, one of its substrates. Phytohormone signaling and cytoskeleton related GSK3 (Saidi, Hearn & Coates 2012) was also linked through early accumulating 2-hydroxyglutarate to widely known stress nodes as TOR1 and CAM1 (Pérez-Pérez, Couso & Crespo 2017), into sPLS network (Figure 4D).

The interaction between early accumulated redox sensitive central cell regulator TOR, Ca^{2+}, phytohormone signaling, and epigenetic mechanisms seem to be supported on 2-hydroxyglutarate, CAM1 and GSK3. 2-hydroxyglutarate is a redox related mammal epigenetic modulator (Intlekofer et al. 2015), while calmodulin is known to integrate calcium signals under stress (Schroda et al. 2015) and GSK3 link stress to phytohormone signaling. Interestingly, HTA14 was directly linked (Figure 5B), or through 2-hydroxyglutarate (Figure 4D), to TOR1. These links confirm the epigenetic role of TOR1, previously related to histone acetylation pathways (Chen, Fan, Pfeffer & Laribee 2012), supporting novel interactions described in the networks. These interacting elements modulate different stress responses including cytoskeleton and chloroplast development (GSK3, PKL1), protein folding (CAM1) and growth regulation (TOR1), being linked both to initial cell survival strategies and latter physiological acclimation processes.

Acclimation was characterized by an accumulation of several specific sugars (unknown disaccharides), together with trehalose and glycerol. Late accumulation of trehalose and other sugars was connected to a hub of acclimation specific signaling elements including MAPK6, two protein

phosphatases 2C (Q93YS2, O80492) and a histidine kinase (Cre17.g733150.t1.1) (Figure 5A). MAP kinases and PP2Cs are known stress signalers, some specifically involved into osmotic stress response, with MAPKs as yeast HOG1 involved into osmoadaptation after a histidine kinase OSMOSENSING HISTIDINE PROTEIN KINASE SLN1 (SLN1) dependent activation. Interestingly, Chlamydomonas MAPK6 have been already linked to heat and osmotic stress signaling (Lee, Ahn & Choi 2017) and interacting histidine kinase (Cre17.g733150.t1.1), is homolog to yeast SLN1. The acclimation regulatory cluster was also linked through accumulating sugars to chloroplast stress response HEME OXYGENASE (HMO1), involved in heme synthesis, and recovering initial high levels on acclimation. HMO1 recovered its abundance when CHLOROPHYLL ANTENNA SIZE REGULATORY PROTEIN (TLA1), a nucleus/chloroplast signaler regulating Chlamydomonas antenna size (Mitra & Melis 2010) accumulated, suggesting an increase in antenna size due to a photosynthetic recovery observed into the acclimation reduction of the Chla/b ratio (Figure 1B). The distinct acclimation signaling elements were also linked to the accumulation of homologs to Arabidopsis repair DNA polymerases V and epsilon (Cre01.g012350.t1.2 and Cre10.g424200.t1.1 respectively), suggesting enhanced DNA repair mechanisms consequence of this stress.

Osmotic stressed Chlamydomonas showed a response where a sequential modulation of primary metabolism was key to first cope with the initial drawbacks associated to high extracellular osmolarity and an altered redox status and later resume growth on the new equilibrium. Quick and late responses are associated to an intensive proteometabolomic turnover driven by specific signalers related to different triggering stimulus and multiple signaling hubs homolog to those found into plant and animal response to this stress, including MAPKs and TOR.

IV. Discussion

Fast response to osmotic stress water and redox effects is based on rapid metabolome and proteome changes.

Osmotic is a particularly stressful condition for most living organisms, but especially for those adapted to live in aquatic environments. Under this stress, aquatic microalgae must react quickly in order to survive, this representing a great adaptive challenge. First strategy towards coping with this stress is increasing intracellular osmolarity through the accumulation of sugars and glycerol, aiming to avoid water loss and protect cellular structures. The accumulation of these molecules reflects a deep remodeling of primary metabolism, as it has also been reported for other stresses such as cold stress, nitrogen starvation, or salt stress (Valledor et al. 2013, 2014b; Demmig-Adams, Burch, Stewart, Savage & Adams 2017). In osmotic stress, this accumulation is probably the result of an enhanced flux of C3 metabolism directly towards glycerol or to trehalose, via gluconeogenesis rather than starch degradation. Starch degradation was respectively described as the main trehalose and glycerol source in dark grown Ostreoccocus (Hirth et al. 2017) or anoxic Chlamydomonas culture (Magneschi et al. 2012). But osmotic stress also induced a starch accumulation, so metabolism will probably shift in a similar way than reported under cold stress (Valledor et al. 2013) at least in sugars related pathways, accumulating starch and other osmolytes. The activation of these pathways, together with the reduction in the cell growth rate and respiration and the inhibition of other sinks like lipid biosynthesis, caused the accumulation of large pools of reducing equivalents (NADH and NADPH), otherwise rapidly consumed in growing cells. This drives to the downregulation NADH and NADPH producing pathways, mainly glycolysis and TCA, while increasing their recycling following fermentative pathways and glycerol production. This hypothesis was supported by the accumulation of lactic acid, glycerol and acetate related PAT1 protein, while glycolysis enhancer PFK2, SDH1 and fumarate concentration decreased. This environment generated a cell status prone to the accumulation of ROS and other toxic metabolites (Demmig-Adams et al. 2017).

The accumulation of these reactive species should be controlled for preventing further cell damage. Together with the accumulation of ROS scavenging enzymes (MSDs, APX2) the quick increase of trehalose abundance (6- or 15-fold at 5h or 24 h respectively) may be one of the main systems employed by Chlamydomonas. Trehalose accumulation, common in desiccation tolerant organisms, has been described as effective molecule to scavenge ROS and maintain protein structure, also conferring desiccation tolerance to non-tolerant species (Tapia, Young, Fox, Bertozzi & Koshland 2015). Besides this, trehalose and its phosphorylated precursor TREHALOSE-6-PHOSPHATE (T6P) have a key regulatory function in plants (Kolbe et al. 2005; Martins et al. 2013; Gazzarrini & Tsai 2014; Mizunoe et al. 2018).

The increase in starch abundance (0.5-fold after 24 h), together with the downregulation of PFK2 until 24h and the upregulation of gluconeogenesis, the accumulation of reducing equivalents and trehalose, suggest an activation of ADP-GLUCOSE PYROPHOSPHORYLASE (AGPase) induced by high NADPH, and by redox and T6P modulation (Kolbe et al. 2005; Kong et al. 2018), despite sucrose levels decreased during experiment. AGPase abundance did not change, but all its activators increased during experiment. Starch would be used to increase pyrenoid sheath thickness together with the accumulation of other carbon concentrating mechanisms (CCMs) such as carbonic anhydrases, as a strategy to reduce photorespiration.

The increase in photorespiration, consequence of osmotic stress, can be deduced after the accumulation of glycolate, PGP2, and several mitochondrial photorespiration-related enzymes. In Chlamydomonas photorespiration is not only useful for recovering 2PG after oxygenic activity of Rubisco, but also as a shuttle for moving excess carbon and reducing power from chloroplast to mitochondria (Davis, Fiehn & Durnford 2013).

The enhancement of fermentation and photorespiration was coupled to an increase in chlorophyll a/b ratio and correlating to a reduction of LEF rate as PETC and FNR1 decreased, while proteins related to antennal complexes, cytochrome and PS-LHC interaction accumulated pointing to a shift toward

cyclic electron flow. This strategy would also help cells to dissipate the excess of energy harvested by the chloroplast towards mitochondria, and in last term, extracellular medium (glycerol, glycolate excretion) while keeping the functionality of photosystem.

Most of the carbon present in the cells derives from acetate in the media under control mixotrophic conditions, which is incorporated as AcetylCoA or citrate in central metabolism through Krebs cycle, rather than photosynthesis. However, the assimilation of acetate may not be enough when all of the carbon sinks were active, as demonstrated by the accumulation of PAT1 and other related enzymes, being photosynthetic fixation probably promoted also explaining part of increased photorespiration.

Stress enhances the accumulation of chlorophyll precursors, another source of ROS, potentially driving to lipid peroxidation. To limit these activities, Chlamydomonas enhance tocopherol synthesis as was suggested by the accumulation of a VTE1 homolog. This enzyme not only catalyzes the last step of tocopherol recycling but also produces plastochromanol, another ROS scavenger from reduced plastoquinone (PQH2). Tocopherol accumulation would avoid peroxidation while VTE1 would also controls chloroplast redox status while using reduced plastoquinone as substrate (Eugeni Piller, Glauser, Kessler & Besagni 2014).

Despite cell efforts ROS will cause unavoidable damage related to extensive protein misfolding commonly associated to stress imposition (Schroda et al. 2015). Furthermore, stress imposition causes an active remodeling of the proteome, also initiated by the degradation of non-necessary proteins (Marshall & Vierstra 2018). The early importance of protein degradation elements and the continuous accumulation of synthesis, transport and protein ensemble related elements under stress support an active protein remodeling process. Enhanced protein degradation under stress induces amino acid accumulation in Arabidopsis (Usadel et al. 2008).

Thus, both remodeling and damage protein degradation activities can generate a flux of free amino acids in Chlamydomonas that can also lead to the

accumulation of glycolate and 2-hydroxyglutarate through their degradation. An enhanced amino acid degradation, also supported by the accumulation of transporters and aminotransferases, would explain the fall of free amino acid abundance under stress enhanced protein degradation.

Interestingly interaction networks demonstrated the connection between all pathways described above, being trehalose, MSDs, and PFK key nodes for explaining how Chlamydomonas regulates metabolism and control cell growth employing redox and epigenetic regulation, as it is depicted below.

Initial responses to osmotic stress are mediated by ROS, trehalose and a yeast HOG-like signaling pathway

Systemic adaption to stress involved different pathways and signaling molecules which were summarized into the sPLS network, divided in two interaction subnetworks. First subnetwork highlighted the importance of trehalose as a main interaction node bridging stress response triggers as early enhanced proteolysis and oxidative stress to an acclimation network densely interconnected with late accumulated sugars. This connection was mediated by many protein turnover related elements and redox/ROS detoxification (MSD2). Interestingly, autophagy, is enhanced both by trehalose into desiccation tolerant plants, and by ROS accumulation (Avin-Wittenberg 2019), suggesting the independent trehalose and ROS modulation of protein turnover. The analysis of biological interactions also supported the interactions between trehalose and late accumulated sugars with early enhanced proteolysis and ROS detoxification, but also highlight their link to other early processes as starch breakdown (ISA2) and HMO1 tetrapyrrole retrograde signaling. Trehalose precursor T6P can modulate the rate of starch degradation in Arabidopsis (Martins et al. 2013), moreover HMO1 has been linked to the light modulation of multiple Chlamydomonas genes including ADP GLUCOSE PYROPHOSPHORYLASE, related to accumulating starch and sugars (Duanmu et al. 2013). This points to the role of chloroplast retrograde signaling into sugar and specifically trehalose accumulation. These sugars also correlated to a cluster of stress related kinases and phosphatases with MAPK6, a homolog to Arabidopsis MAPK5, and different PP2C between them.

The involvement of MAPK kinases in osmotic stress responses has been described in yeast HOG mechanism, formed by a membrane-embedded histidine kinase osmosensor which triggers a signaling pathway which ends with the activation of HOG1 (a MAPK family protein). Once activated, its enter the nucleus regulating gene expression of stress-related genes (Babazadeh et al. 2017). SLN1-like HOG pathway histidine kinase osmosensor (Cre17.g733150.t1.1) was also present into the kinase cluster interacting with MAPK6 and a PP2C (O80492). This histidine kinase was previously identified as retinal or COP and present on the eyespot, but never related to light signaling (Fuhrmann, Stahlberg, Govorunova, Rank & Hegemann 2001). Consequently, the activation of a HOG like pathway in Chlamydomonas is feasible, and consistent with the observed accumulation of glycerol and sugars. Osmotic stress perceived by flagellar proteins and/or histidine kinase relays would trigger phosphorylation chains where several MAPK kinases and phosphatases act as central hubs driving stress response gene expression. This signaling pathway is probably redundant and/or superimposed with ROS mediated one.

Early stress signaling through known plant like stress signalers and their connection to 2-hydroxyglutarate, related to active aminoacid degradation and redox disbalance (Araújo et al. 2010), was highlighted into the second subnetwork. Biological interaction networks reinforced these signalers importance highlighting the centrality of Ca^{2+} (CAM1), phytohormone (GSK3, PKL1) and energy signaling (TOR1). Between these, TOR1 evidences along early accumulated trehalose the active tuning of proteome reshaping and the early involvement of epigenetic modulation (Avin-Wittenberg 2019). This is supported by the relation between TOR and epigenetic mechanisms (Chen et al. 2012) and its correlation to epigenetic modulators as 2-hydroxyglutarate (Intlekofer et al. 2015) and HTA14.

Long term acclimation involves specific sugar signaling, hormones, and epigenetic mechanisms

Initial responses to osmotic stress were quick, with visible changes after 5 h of induction. The drastic changes in metabolome that were quantified mostly

implied sugars and glycerol, and were the consequence of a different equilibrium in cell metabolic pathways. This equilibrium shifted from a quick growth and high respiration rates towards the accumulation of osmolytes and fermentation, and were mostly consequence of the available enzymes, rather than a wide enzymatic remodeling. However, the recovery of growth capacity after 24 h of stress exposure was the consequence of a deep proteome remodeling modulated by specific signaling mechanisms and adaptive responses.

Adaptive responses included the recovery of photosynthesis correlating with the recovery on chla/b ratio and the accumulation of PSII and Cytocrome b6f structural and stabilizing elements after 24 h. Some of these as PSBP6 related to osmotic stress in plants (Tamburino et al. 2017). This was coupled to the enhancement in protein transport between cellular compartments supporting this proteome remodeling.

The nature of osmotic stress caused a quick redox imbalance and a change in thylakoid's electron transfer chain, resulting in a pool of reduced molecules. This accumulation of reduced plastoquinone and ferredoxin probably was the initial trigger of ROS scavenging mechanisms described above, in addition to control by sugars, tetrapyrroles, or ABA (Baier & Dietz 2005). Tetrapyrrole synthesis and GUN4 abundance also increased after 5 h of stress exposure supporting the role of this system in the modulation of chloroplastic ROS production along detoxifying enzymes as MSD3 and APX2 (Brzezowski et al. 2014). This mechanism was not only required during acclimation (Foyer & Noctor 2008), but also for modulating long-term adaptive responses through regulation of available trehalose-6-phosphate (Kolbe et al. 2005) and other sugars involved in nuclear signaling through the activation of AGPase via NADPH-dependent THIOREDOXIN REDUCTASE C (NTRC) (Kong et al. 2018).

Trehalose was a key node in our interaction networks for linking all of signaling related pathways, pointing that this molecule has a regulatory role beyond osmoprotecting and ROS detoxification. In plants, trehalose-6-

phosphate (T6P), a trehalose precursor, regulates central metabolism by inhibiting SnRK1 (Gazzarrini & Tsai 2014) which is an inhibitor of TARGET OF RAPAMYCIN COMPLEX 1 KINASE (TORC1). The activity of TOR complex, TORC, is activated by sugars, hormones, and environmental stresses (Baena-González 2010). TOR showed interaction with histone acetylases and PKL1, a phosphatase related with brassinosteroid signaling, and calmodulin being this the core of one of the STITCH subnetworks. This set of proteins were correlated to a MYB transcription factor like protein (Cre07.g345350.t1.1) and to RNA splicing. MYB transcription factors participate into brassinosteroid PKL1 mediated gene regulation in Arabidopsis (Li et al. 2009) but also into maize ABA mediated drought tolerance (Wu et al. 2019).

The accumulation of a MYB transcription factor suggests the involvement of ABA into this stress response. This is also supported by the importance of the oxidative stress response, related in the microalgae to ABA signaling (Yoshida, Igarashi, Wakatsuki, Miyamoto & Hirata 2004) and by accumulating PP2Cs. These phosphatases link ABA to SnRK signaling under osmotic stress with several Chlamydomonas SnRK being sensitive to both ABA and osmotic stress (Colina et al. 2019).

ABA, together with SnRK2s, have also been related to mediate in stress adaption by regulating epigenetic mechanisms including enzymes that modify histones, DEAD-Box RNA helicases, and DNA methyltransferases. being some of them differentially accumulated during experiment (Chen, Luo, Wang & Wu 2010).

Epigenetic-related enzymes also accumulated differentially during experiment, being also present in interaction networks as middle and ending nodes, suggesting its role not only as regulators, but also as an end of other regulatory pathways. The imposition of epigenetic marks is a requirement for long-term cell adaption, since they tune the genome in order to provide the most specific response to that environment (Kronholm, Bassett, Baulcombe & Collins 2017). The inheritance of these marks would give cells an important advantage,

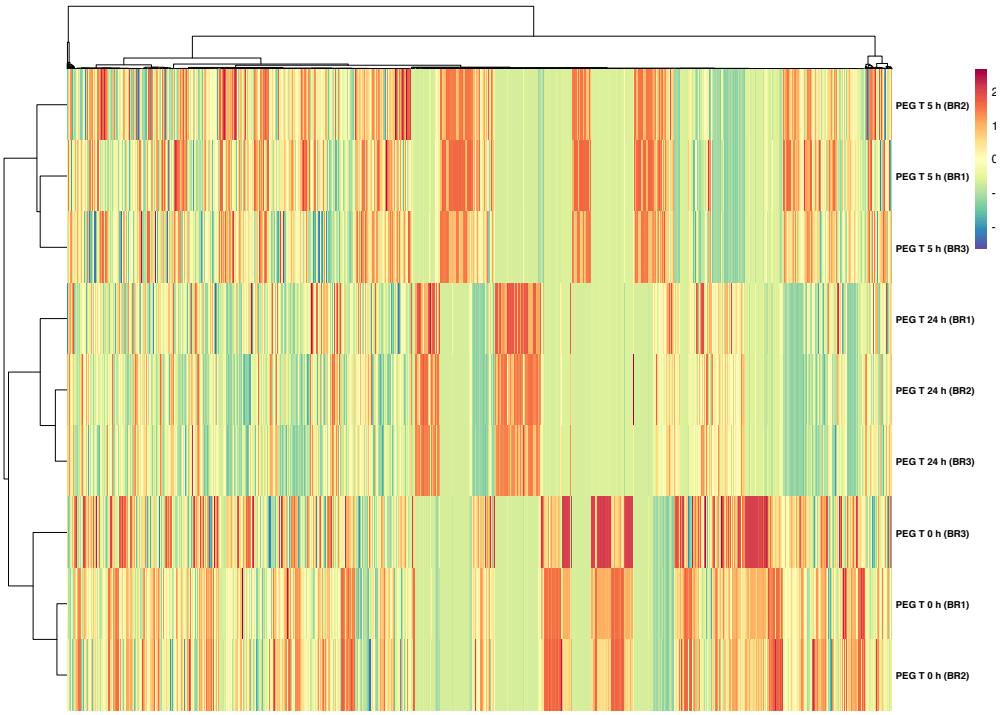
since after mitosis they will be adapted in the same way than its originally cell, allowing transgenerational culture adaption. Despite more assays will be necessary to check the extension of epigenetic memory, the accumulation of DNA methyltransferase Cre06.g249350.t1.1, an Arabidopsis METHYLTRANSFERASE 1 (MET1) homolog, after 24 h of stress and prior to cell division, supported this hypothesis. MET1 is a maintenance methyltransferase, with a clear role in keeping epigenetic patterns after cell division (Annacondia, Magerøy & Martinez 2018).

Chlamydomonas hyperosmotic response is modulated by different overlapping signaling mechanisms into which sugars and several homologs to known plant stress signalers have a key role. These systems modulate the microalgae genome not only allowing and enhancing its tolerance to stress through epigenetic mechanisms, but also modifying its biomass production with the enhancement of sugars accumulation. Thus, found osmotic stress signaling elements could be starting points into the elucidation of plant and algae signaling paths, and promising targets for the enhancement of microalgae and plant biomass yield.

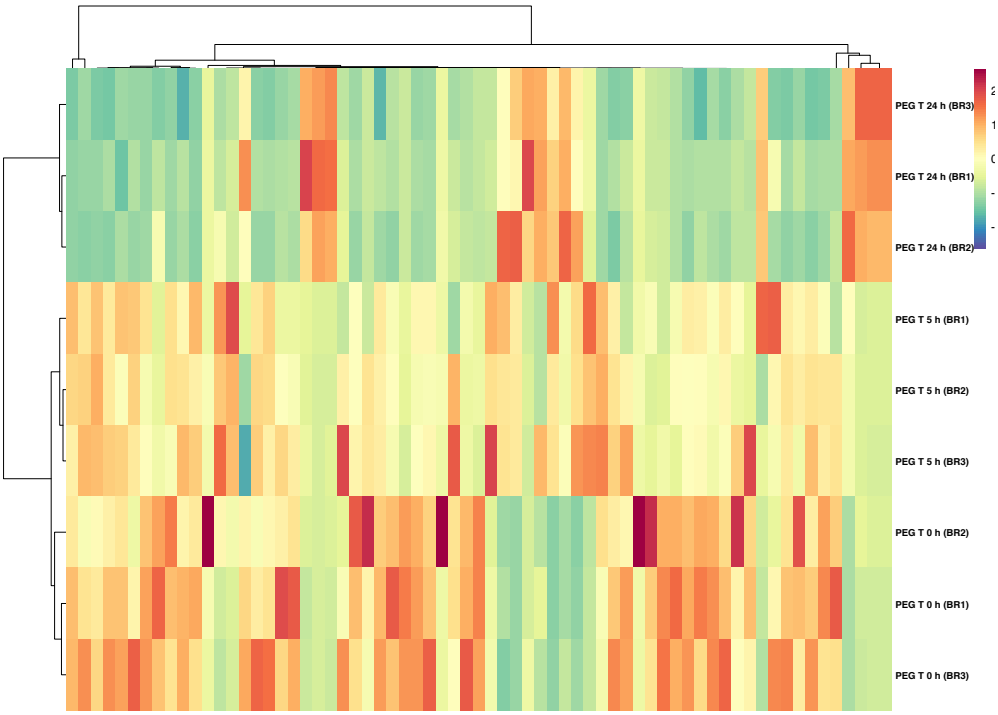
V. Conclusion

Chlamydomonas respond to osmotic stress by a quick accumulation of sugars, as a result of a profound metabolic rearrangement. Integrated proteomic and metabolomic analyses showed a response similar to land plants desiccation accumulating trehalose and glycerol as osmoprotective solutes. This accumulation is the consequence of the activation of parallel signaling pathways including a putative HOG pathway, mitochondria and chloroplast ROS signaling, organulli-nucleus communication, and potential brassinosteroid and ABA mediated pathways. Long term acclimation, was possible after a genetic reprogramming, mediated by specific epigenetic mechanisms which allow proteome remodeling and long-term cell survival. Multiple specific kinases have been identified, being MAPK6, PKL1, GUN4, MYB44, Saccharomyces SLN1 Histidine kinase osmorelay like (Cre17.g733150.t1.1) promising targets for further osmotic response characterization or exploitation towards engineering more productive strains.

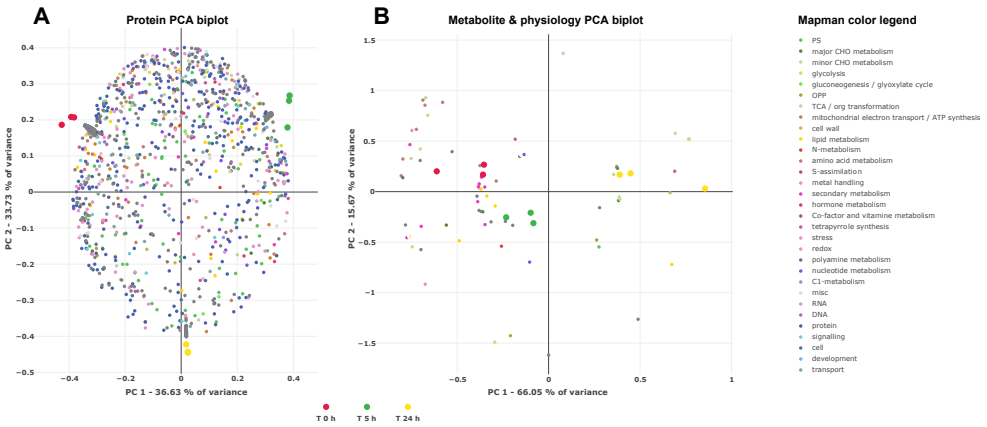
VI. Supplementary figures and tables



Supplementary figure S1. Protein heatmap biclustering plot over the individual protein variables abundance. Samples cluster according to their harvesting time and block differences into the accumulated proteins are observed between harvesting time.



Supplementary figure S2. Metabolite and physiology heatmap biclustering plot over the individual variables abundance. Samples cluster according to their harvesting time and block differences into the variable abundance are observed between harvesting time.



Supplementary figure S3. Independent PCA biplots over z-score transformed data from protein (A) and metabolite and physiology (B) showing divergence between different harvesting time samples and the individual variables correlation to each component. Bigger dots represent individual samples and were colored according to their harvesting time (0, 5, 24 h), while small dots represent individual variables and were colored according to their MapMan category. A, protein variables PCA biplot B, metabolite and physiology PCA biplot.

VII. Supplementary material available only on the USB drive

excel file including the large supplementary tables S1 to S6 whose headings are listed below:

Supplementary table S1: List of the 1396 quantified proteins in whole cell fractions with their abundances estimated following a NSAF approach. Protein are designated with their respective Chlamydomonas JGI v5.5, Viridiplantae-UniProt or Augustus database accession. Percent of coverage, number of unique peptides used for identification and score are indicated. Abundance data displayed underwent filtering, imputation and sample abundance-based balancing. The mean abundance ± SD for each sampling time is indicated as well as their ANOVA p- and q-values (BH), and the post-hoc Tukey HSD test p-values. Deflines, symbols and MapMan bins were manually curated.

Supplementary table S2: List of the 67 quantified metabolites. Metabolite names were included along their Golm metabolome database identifiers used as uncharacterized compounds names. Retention time (RT) was included along the mass/charge ratios (m/z 1 and m/z 2) of the two most characteristic fragmentation ions for each compound. Metabolite abundance was estimated from the peak areas of the indicated characteristic ions. Abundance data underwent filtering, imputation and sample abundance-based balancing. The mean abundance respect to control ± SD for each sampling time are indicated as well as ANOVA p- and q-values (BH) and post-hoc Tukey (HSD) test p-values. MapMan bins and deflines were manually curated.

Supplementary table S3: Physiological parameters relative to cellular density included into the integrative analysis. Abundance data underwent filtering, imputation and sample abundance-based balancing. Between included measures were total lipids and starch weight, along with chlorophyll a, b and carotenoids concentration. For each parameter is represented the harvesting time mean and SD. Significative differences were detected trough a one-way ANOVA (α=0.05) over log10 transformed data. Tukey HSD post-hoc test (α=0.05) was performed in order define differences between the different harvesting times.

Supplementary table S4: Loadings relating each protein variable contribution to each of the nine generated components into the protein PCA analysis. Proteins were identified by their respective Phytozome v5.5, Viridiplantae-UniProt or Augustus identifier.

Supplementary table S5: Loadings relating each metabolite and physiological variable contribution to each generated component into the metabolite PCA analysis. Metabolites were identified by their respective name or for uncharacterized compounds their respective Golm database identifier.

Supplementary table S6: sPLS loadings relating the contribution of each of the z-score transformed variables from X (protein) or Y (metabolite and physiology) dimensions to each two generated components. Proteins were identified by their respective Phytozome v5.5, UNIPROT-Viridiplantae or augustus identifier. Metabolites were identified by their respective name or for uncharacterized compounds their Golm database identifier. The number of variables to keep into each of the sPLS model dimensions was tuned, maintaining 125 X and 9 Y variables.

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Systemic UV-B/C stress adaptation in Chlamydomonas reinhardtii.

CHAPTER CONTENTS

I. Introduction

Organisms are well adapted to surface and underwater solar UV-A/B irradiation, with specific sensing systems allowing the deployment of acclimation and photomorphogenic responses. Conversely, responses to UV-C have been poorly characterized due to the atmospheric blockage of this

stressor. Besides this, the characterization of UV-C sensing, signaling and response mechanisms have gained new interest on its effects over biomass production, stress resistance and development (Takeno, 2016) (Ahmed and Schenk, 2017; Xu et al., 2017, Yanqun Xu 2019). Since UV-C sensing and response mechanisms are mostly unknown, current research focuses on the study of UV-A/B, oxidative and pathogen stresses response mechanisms as potential responsive systems.

While UV-A/B, oxidative and pathogen stressors effects converge in Reactive Oxygen Species (ROS) generation and macromolecule damage, UV-B/C can directly damage DNA and proteins (Stingele, Habermann and Jentsch, 2015; Mullenders, 2018). These similar but different stresses involve specific signalers as damaged DNA (Mullenders, 2018), ROS (Urban et al., 2016), salicylic acid (SA), and jasmonates (JA) (Xu et al., 2018; Takeno, 2016). These signalers are shared between UV-B and UV-C responses (Besteiro et al., 2011; Dakup and Gaddameedhi, 2017; Zhang et al., 2017), modulating UV stress responses including the accumulation of UV absorbing and ROS scavenging compounds, and the enhancement of DNA repair and protein turnover.

Besides damage-based UV sensing and response mechanisms, plants can directly sense UV-A/B through specific receptors as UV-B specific ULTRAVIOLET-B RECEPTOR (UVR8), and blue/UVA specific photoreceptors identified in Arabidopsis thaliana. The excitation of UVR8 by UV-B can directly trigger UV-B acclimation responses through specific signaling pathways (Liang, Yang and Liu, 2018). Photoacclimation to UV-B in Chlamydomonas rely on an enhancement of photosynthetic protein turnover mediated by UVR8 (Tilbrook et al., 2016). Conversely, UV-C effect on UVR8 is unclear (Christie et al., 2012; Jiang et al., 2012) although signaling elements related to UVR8 have been associated to UV-C signaling in Arabidopsis thaliana (Xie et al., 2012).

UVR8 functions overcome the activation of UV stress response as has been proposed as the input for UVB signals into plant circadian oscillators (Oakenfull and Davis, 2017). UV tuning of circadian oscillators, dependent on

different photoreceptors, is key for the adjustment of the intensity of UV responses as DNA repair to day length UV intensity variation in plant and animal systems (Horak and Farré, 2015; Dakup and Gaddameedhi, 2017). Recently, in Arabidopsis thaliana also was revealed that epigenetic mechanisms play an important role into the control of UV responses with UVR8 also involved through the induction of HISTONE H3 Lys 9/Lys 14 (H3K9/K14) acetylation over its regulated gene loci (Velanis, Herzyk and Jenkins, 2016).

UV involvement into circadian and epigenetic mechanisms shows how UV has been engaged during plant evolution into the control of complex growth/development and cycle regulation processes. These photomorphogenic effects are unclear for UV-C, although this radiation does induce specific changes into plant metabolome and pathogenicity related genes expression. These changes enhance pathogen tolerance in plants and induce the accumulation of valuable compounds as sterols and flavonoids into a wide phylogenetic range from microalgae to plants (Ahmed and Schenk, 2017; Xu et al., 2017, Yanqun Xu 2019). Storage compounds as triacylglycerols (TAG) and UV shielding compounds also accumulate in microalgae under different UV-A/B treatments (Tian and Yu, 2009; Forján et al., 2011; Srinivas and Ochs, 2012; Kumar et al., 2018). Thus, response to the differential UV-A/B and UV-C stressors shares a metabolic rearrangement response after the accumulation of different protective, structural and storage compounds. Differences come at the signaling level with the involvement of JA and SA into UV-C signaling both for these rearrangements and for enhanced pathogen tolerance (Lee et al., 2016; Chen et al., 2018; Xu et al., 2019). Besides this, other candidates might be mediating the metabolic modulation under both UV-B and UV-C highlighting SUCROSE NON-FERMENTING RELATED KINASES/ CHLAMYDOMONAS SNRK (SnRK/CKIN) and related DUAL SPECIFICITY TYROSINE REGULATED KINASES (DYRK) kinases. Both have been related to carbon fluxes control under stress and SnRK/CKIN have been found sensitive to UV-B/C in Chlamydomonas (Schulz-Raffelt et al., 2016; Colina et al., 2019).

The different UV responses rely on radiation properties with UV-A/B inducing promising photomorphogenic effects while UV-C enhance biotic stress resistance. All wavelengths also induce specific metabolic modulation. These involve promising UV applications on the modulation of biomass production in microalgae, the control of plant development or the reduction of pesticide usage. The chlorophyte Chlamydomonas reinhardtii has been selected for the characterization of this response due to its available genomic/proteomic resources, closeness to industry relevant species, and plant-like UV signaling pathways. Moreover, response to UV-B (Tilbrook et al., 2016) and to the effect of JA and SA treatments (Xu et al., 2018; Lee et al., 2016) have already been characterized in the microalgae from an omic perspective. All this makes the system ideal and convenient for the characterization of the effect of UV-B/C induced metabolic rearrangement and developmental effects. Therefore, the aim of this study is to render an image of the Chlamydomonas response system under low intensity UV-B/C radiation through the integration of the microalgae proteome and metabolome levels changes under stress. This integration, involving both novel data processing strategies and interaction knowledge, would allow the identification of key factors related to UV-B and UV-C signaling mechanisms and photomorphogenetic/metabolic responses.

II. Material and methods

Strains and Cultures

Chlamydomonas reinhardtii CC-503 cw92 cultures were grown on a culture chamber (25 °C, 120 rpm, continuous light and 85 μE m^2 s^{-1} light intensity provided by Sylvania GroLux lamps) in HEPES Acetate Phosohate (HAP) culture media (L. Valledor *et al.*, 2013). HAP was formulated from Tris Acetate Phosphate (TAP) (Harris, 2009), substituting Tris for HEPES as a Mass Spectrometry (MS) compatible buffer. To induce UV stress cultures were continuously irradiated with low UV-B/C doses (kW/mxxxxxx) from XXX lamps. Initial culture was prepared 48 h before experiment start diluting twentyfold a seed culture originated from a single colony. After 48 h, cells were harvested and diluted to $1 \cdot 10^5$ cell/mL splitting the resulting culture volume between three flasks. The three biological replicates were sampled at

0, 5 and 24 h by centrifuging 50 mL of media at 4000 x g (Figure 1). Pellets were immediately frozen in liquid nitrogen and employed for obtaining proteins and metabolites according to Valledor et al. (Valledor, Escandón, *et al.*, 2014).

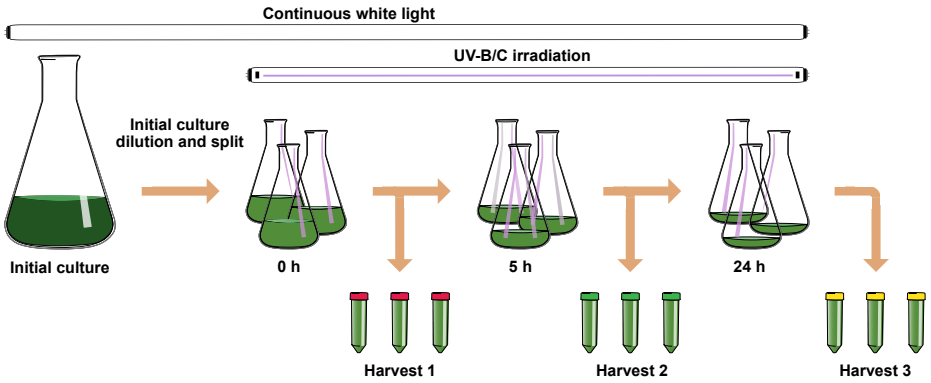

Figure 1. Graphic schema of the experimental system showing the setting and progress of the experiment. The initial Chlamydomonas culture grown under control conditions was diluted and split between three flasks just before experiment start. These three cultures (biological replicates) were continuously irradiated with UV-B/C light during the 24 h time course experiment, taking one sample from each one at 0 h (red cap tubes), 5 h (green cap tubes) and 24 h (yellow cap tubes) after experiment start.

Quantitative Proteome Analysis (GeLC-LTQ-Orbitrap MS)

The preparation of protein samples for MS/MS analysis was performed following Valledor, *et al.* (2014) recommendations. Ten micrograms of tripsin digested peptides were loaded per injection into a one-dimensional nano-flow LC-Orbitrap/MS and resolved in a 90-min gradient from 5 to 40% (v/v) acetonitrile/0.1% (v/v) formic acid using a monolithic C18 column (Chromolith RP-18r, Merck, Darmstadt, Germany). MS analysis was performed on an Orbitrap LTQ XL mass spectrometer (Valledor *et al.*, 2013).

Raw data coming from Orbitrap were searched with Proteome Discoverer version 2.1 (Thermo) SEQUEST algorithm as previously described (Valledor *et al.*, 2012), employing a label-free quantification based on precursor's areas.

Chlamydomonas 5.5 protein (18750 accessions), Chlamydomonas chloroplast & mitochondria (84 accessions) and Swissprot-viridiplantae (36097 accessions) databases were employed for protein identification. Only high confidence proteins (at least one significant peptide, XCorr > 1.8, FDR 5%) present in all the biological samples of at least one treatment were considered for this analysis.

Metabolite GC-MS Analysis

Polar fraction analysis were carried out following (Valledor, Furuhashi, *et al.*, 2014) protocol with some minor changes on a triple quad instrument (TSQ Quantum GC; Thermo). In brief, 1 μL of sample was injected, and GC separated into a HP-5MS capillary column (30 m 9 0.25 mm 9 0.25 mm) (Agilent Technologies). Oven temperature was increased from 80 °C to 200 °C at 3 °C per min and then reduced to 25 °C at 10 °C per min and maintained at 25 °C for 3 min, maintaining post run conditions at 30 °C for 4 min. Mass spectrometer was operated in electron impact (EI) mode at 70 eV in a scan range of m/z 40–600. The identification of metabolites was based on the spectral characteristics and GC retention times of each individual metabolite through its comparison with the retention times and spectral characteristics of standards available in our in-house library and in Golm Metabolome Database (Fernie *et al.*, 2004).

Biostatistical analyses

R v3.3 (R Core Team, 2019) software core functions and pRocessomics package (available at https://github.com/Valledor/pRocessomics) were used for all performed statistical procedures.

Proteomics and metabolomics datasets missing values were imputed through a sequential K-Nearest Neighbor algorithm. Imputation was performed only when one value per sampling point was missing. Protein and metabolite abundances were re-estimated afterwards following a sample-centric approach

(individual peak areas divided by total peak area per sample). Data was then subjected to univariate (one-way ANOVA; 5% FDR followed by a Tukey HSD post-hoc) and multivariate analyses (principal component analysis, heatmap clustering and sparse partial least squares regression analysis).

sPLS-based multivariate models (Le Cao *et al.*, 2008) were employed to build correlation networks employing the R package mixOmics (Lê Cao *et al.*, 2012) using proteins as predictors and metabolites as response. Correlation network nodes were used as imput to build known protein-protein/protein-metabolite interaction networks into the STITCH platform (Szklarczyk *et al.*, 2016). Resulting networks were visualized and processed in Cytoscape V3.6.1 (Shannon *et al.*, 2003) ans Cytoscape plug in StringApp (Doncheva *et al.*, 2019) was used for STICH network import, setting interaction confidence to 0.4 (medium confidence).

III. Results

Integrated proteomic and metabolomic responses on Chlamydomonas exposed to UV-B/C stress

Proteomic analyses allowed the identification of NNNN peptides and 1441 protein species, after analyzing NNNN spectra obtained from whole cell protein extracts. After data pre-processing, 885 proteins were above the abundance threshold for confident quantitation (Supplementary table S1). GC-MS allowed the unequivocal identification of 69 primary metabolites, out of these 68 were considered for quantitative purposes (Supplementary table S2). Detected proteins and metabolites were classified according to MapMan V4 categories (Schwacke *et al.*, 2019). 703 proteins and 54 metabolites were assigned to functional bins. These bins comprised 27 pathways for proteins and 8 for metabolites covering different cellular processes. Out of these, 398 proteins and 5 metabolites could be considered quantitatively differential in at least one sampling time (ANOVA, 5% FDR)(Supplementary table S1, S2; Figure 2).

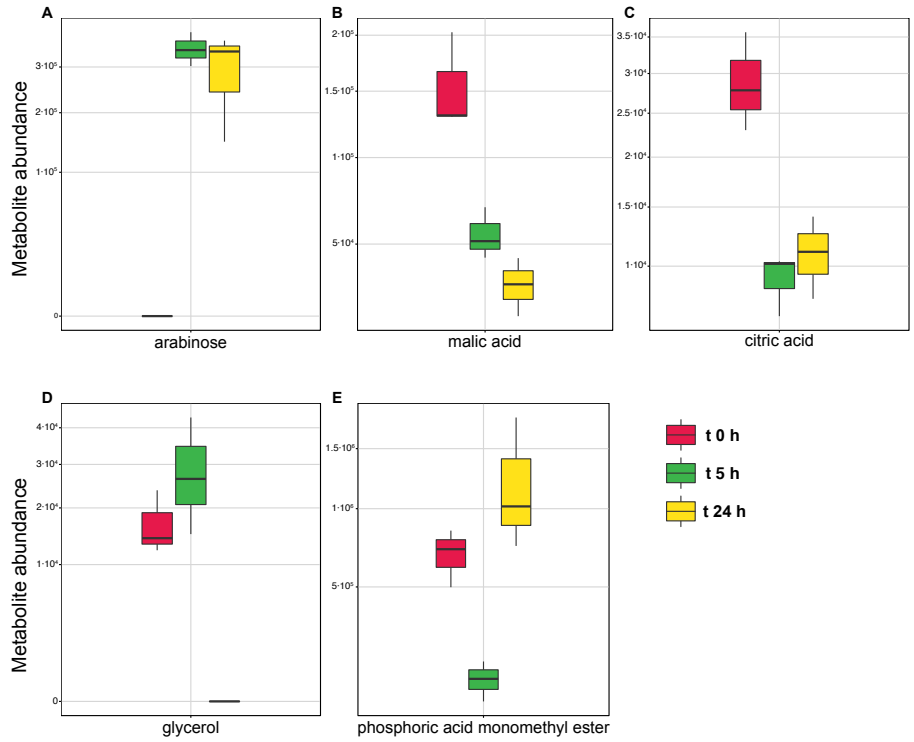

Figure 2. Time-course accumulation of differentially accumulated metabolites (ANOVA, 5% FDR) under UV-B/C stress in Chlamydomonas involved in sugar metabolism (arabinose) (A), central carbon metabolism (malic acid, citric acid) (B, C), REDOX and carbon fixation (glycerol) (D), and mineral nutrition (phosphoric acid monomethyl ester) (E). Box plot representation of the filtered and imputated untransformed metabolite data (Supplementary Table S2).

Heatmap clustering based on mapman-categories distinguished the different treatments with an adequate grouping of samples (Figure 3). At protein level (Figure 3A) UV-B/C stress induced a quick reduction on multi process regulation, RNA processing, Protein biosynthesis and Cell cycle related categories, all classified in the same meta group. On the other hand, the abundance of Cell wall, Protein degradation, External stimuli response, Carbohydrate metabolism and Cellular respiration categories, within the same cluster, increased after 5 h. Interestingly, Redox homeostasis increased 24 h after stress start when Photosynthesis bin reached its maximum abundance. Metabolites (Figure 3B) showed a divergent distribution to those of proteins, highlighting Amino acid and Carbohydrate metabolism categories time shifts with their protein counterparts. Interestingly, within individual metabolites,

redox-related glycerol and different amino acids accumulated after 5 h along photorespiration and amino acid metabolism related glycolic acid. On the other hand, the increase of several unknown sugars abundance after 24 h characterized acclimation (Supplementary table S1, S2).

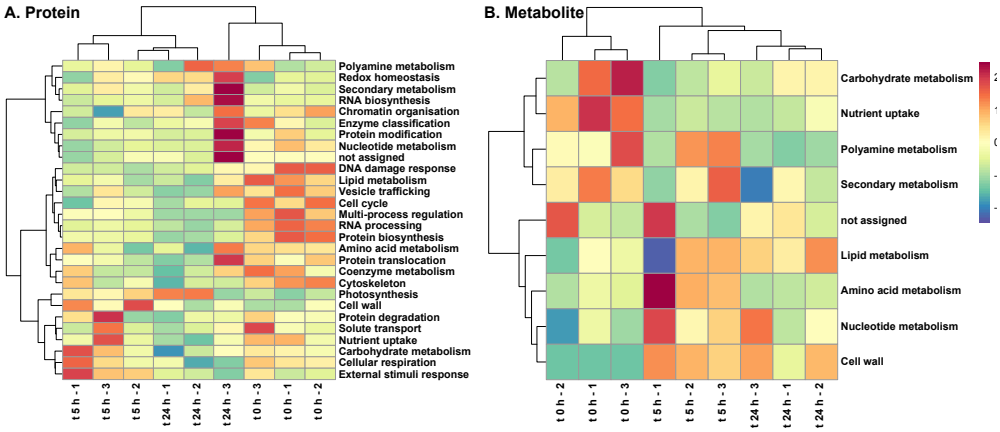

Figure 3. Heatmap biclustering plots over mapman categories protein (A) or metabolite (B) total abundance. Samples clustered according to their harvesting time and differences in abundance were observed between harvesting times for some functional categories into both plots.

Principal Component Analysis (PCA) allowed an adequate classification of samples considering each omic level independently (Figure 4; Supplementary table S3, S4). In proteomics dataset (Figure 4A; Supplementary table S3) loadings of the components showed that principal component 1 (PC1) potentially gathers variability related to the photoacclimation to UV-B/C involving photosynthesis PHOTOSYSTEM II D2 PROTEIN (psbD), PHOTOSYSTEM I REACTION CENTER SUBUNIT V (psaG), and CHLOROPHYLL A-B BINDING PROTEIN (LHCB4); C/N metabolism, plastid protein assemblage PEPTIDYLPROLYL ISOMERASE (FKB16-3), CYCLOPHILLIN TYPE PEPTIDYL-PROLYL CIS-TRANS ISOMERASE (CYN38), and GrpE PROTEIN HOMOLOG (MGE1); and catechin metabolism CHALCONE ISOMERASE (Cre12.g517100.t1.1). Principal component 2 (PC2) gathers early response elements with signaling WD40 REPEAT PROTEIN (Cre12.g495650.t1.2); splicing FORKHEAD-ASSOCIATED DOMAIN-CONTAINING PROTEIN (CGL86); carbon fixation, lipid degradation and C3 transport RIBULOSE-1,5-BISPHOSPHATE

CARBOXYLASE/OXYGENASE LARGE SUBUNIT (RBCL), NADP MALIC ENZYME 5, 6 (MME5, MME6), ACYL-CoA OXIDASE/DEHYDROGENASE (ACX), and 3-HYDROXYACYL-CoA DEHYDROGENASE (HCD1); nucleotide metabolism ADENYLATE/GUANYLATE CYCLASE (CYG29), and ADENYLATE KINASE (ADK3); and mitochondrial/chloroplastic oxidative stress/protein damage HEAT SHOCK PROTEIN 70C, E (HSP70C, HSP70E), and TYPE II NADH DEHYDROGENASE (NDA5). At metabolome level (Figure 4B, Supplementary table S4) PC1 explain early response with photorespiration enhancement (glycolate), phenolic and nucleotide metabolism (protocatechuic acid, 4-hydroxybenzoic acid and uracil), while PC2 joins late accumulated sugars and UV shielding catechin, both related to acclimation.

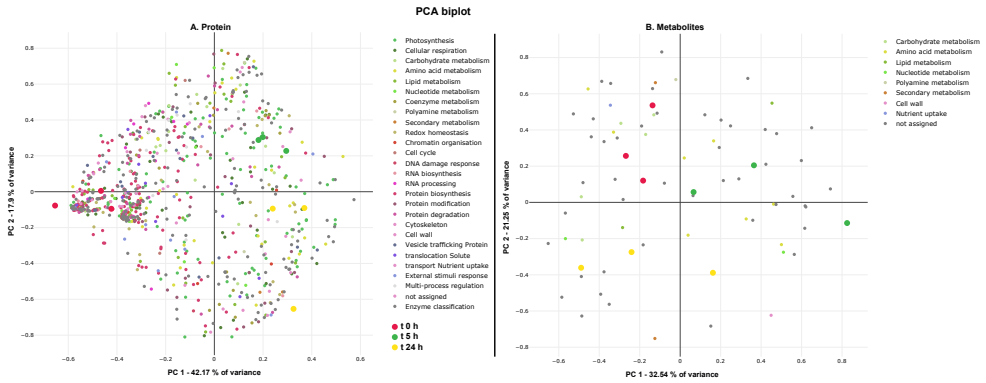

Figure 4. Independent PCA biplots over z-score transformed data from protein (A) and metabolite (B) showing divergence between samples from different harvesting times and the correlation of each individual variables to each displayed component. Bigger dots represent individual samples and were colored according to their harvesting time (0, 5, 24 h), while small dots represent individual variables and were colored according to their mapman category. A, protein variables PCA biplot B, metabolite and physiology PCA biplot.

Besides the adequate classification of protein and metabolite databases obtained by PCA, a protein-metabolite correlation network was defined (Figure 5B) through the integration of both datasets employing sPLS regression, also allowing a good sample classification (Figure 5A, Supplementary table S5). Within the resulting network (Figure 5B) the cluster centered on early depleting metabolites (citrate, malate, glutamate,

dehydroascorbate) and early accumulated arabinose gathers multiple translation (ribosomal subunits), development MINA53 MYC INDUCED NUCLEAR ANTIGEN (MINA53) homolog (Cre07.g356600.t1.2), stress PHOSPHATASE 2A (PP2A) homolog (Cre03.g199983.t1.1), C/N metabolism PYRUVATE KINASE (PYK2), and ISOCITRATE LYASE (ICL2) related proteins also depleting upon stress start. Remaining clusters were centered into early accumulated glycerol and late accumulated UNKNOW SUGAR 5 gathering t 5 and t 24 h response elements respectively. Glycerol cluster joined redox and carbon fixation RIBOFLAVIN KINASE (RFK2), GLUTATHIONE S-TRANSFERASE homolog (CPLD58), AQUAPORIN (MIP1), NDA5, and MME5; translation modulation S-ADENOSYL-L-METHIONINE-DEPENDENT METHYTRANSFERASE (SAM MTase), RIBOSOMAL PROTEIN S19 (RPS19), EUCARIOTIC TRANSLATION INITIATION FACTOR 1A (EIF1a), LEUCYL-tRNA SYNTHETASE (TSL1), LYSYL-tRNA SYNTHETASE (SYK1), and METHIONYL tRNA SYNTHETASE (TSM2); and signaling (WD40 REPEAT PROTEIN) elements. Early accumulated glycerol correlated negatively to a cluster with elements accumulated after 24 h centered in UNKNOW SUGAR 5. Fatty acid (FA) synthesis B-KETOACYL-[ACYL-CARRIER-PROTEIN] SYNTHASE III (KASIII) (Cre04.g216950.t1.2) and a DYRK like kinase (Cre01.g008550.t1.1 or au5.g1142_t1) positively correlated to the late accumulated sugar. A novel FILAMENTOUS TEMPERATURE SENSITIVE H like (FTSH) like protease connected the cluster centered into early depleted elements with the clusters gathering elements accumulated after 5 and 24 h of UV-B/C irradiation.

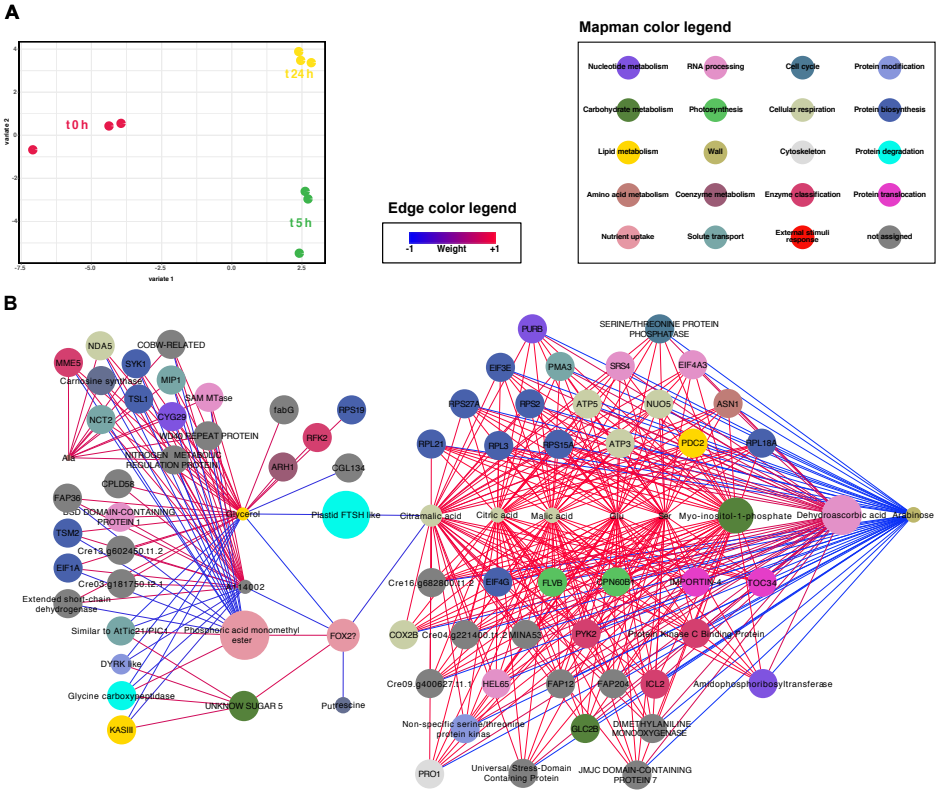

Figure 5. sPLS variable plot showing clear differentiation between harvesting times (A). sPLS based network showing the response differentiation between Chlamydomonas early and acclimation responses (B). Network nodes were colored according to their MapMan categories linking node size and edge color intensity to size and weight respectively. Only the edges with correlations higher or lower than ± 0.6 were visualized. Nodes without any edge above the threshold were removed from this analysis.

Since these networks were only based on mathematical models, STITCH analysis was applied to improve these interactions based on biological models (Figure 6). STITCH network highlighted early accumulated glycerol connections to protein biosynthesis, respiration, lipid metabolism and oxidative stress response. Same network connected translation related EIF1a, PP2A like (Cre03.g199983.t1.1) and FLAGELLAR ASSOCIATED PROTEIN 204 (FAP204) to C/N metabolism and translation modulation. Moreover, at the selected interaction confidence level translation regulation related MINA53 with the unknown WD40 repeat protein were paired but isolated from remaining STITCH network elements.

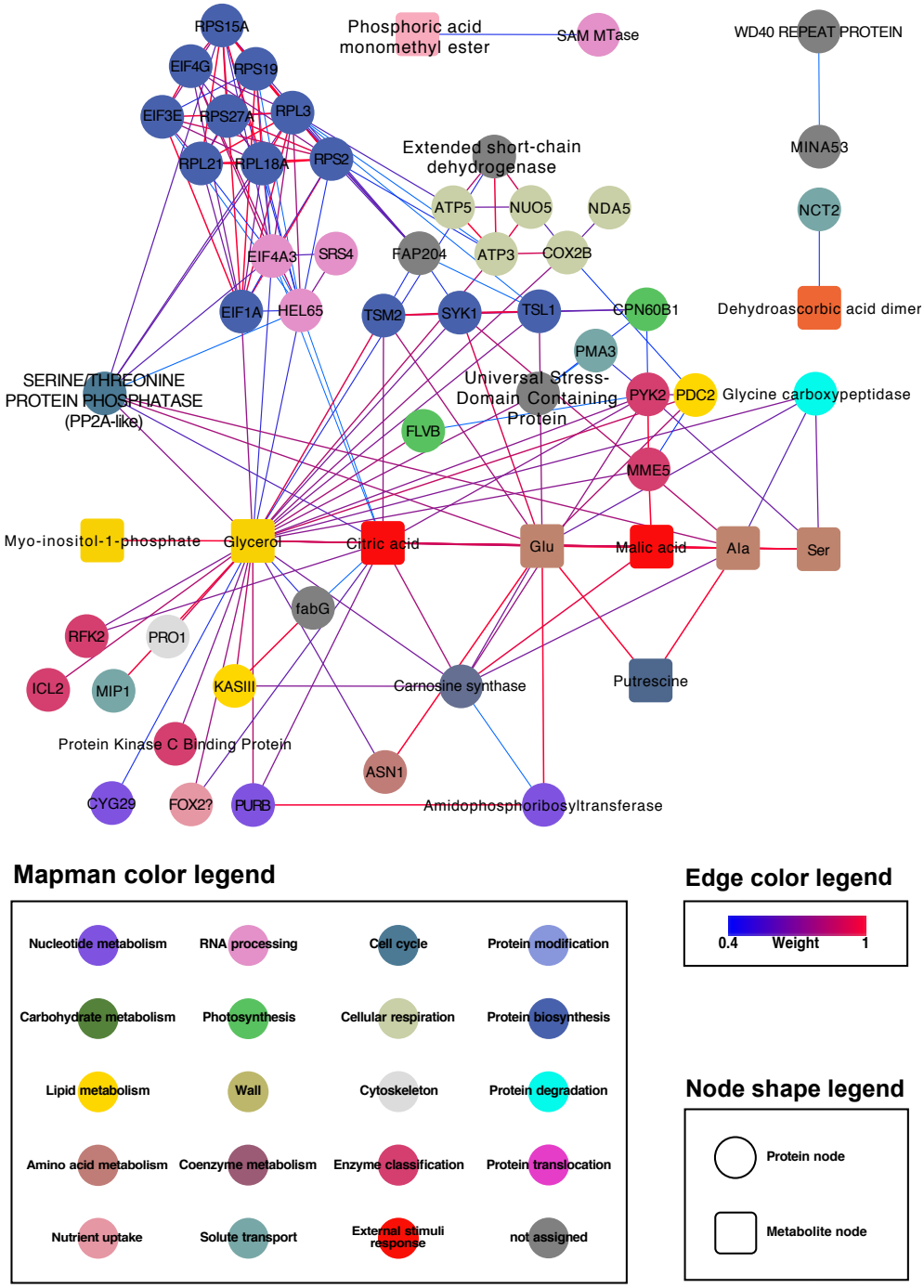

Figure 6. sPLS based STITCH network. STITCH network nodes were colored according to their MapMan categories edge color was link to interaction confidence (STITCH interaction score). Confidence threshold was set at 0.4 (medium confidence). Network highlighted C3 derived glycerol redox valve central role under UV-C, between protein biosynthesis, respiration modulation, and lipid metabolism, oxidative stress response. Same network connected translation related EIF1a, PP2A like (Cre03.g199983.t1.1) and FAP204 to C/N metabolism and translation modulation. Translation/development related MINA53 was also connected into this network to unknow WD40 repeat protein.

UV-B/C irradiation hit mitochondrial electron transport enhancing ROS production and inhibiting oxidative phosphorylation

Mitochondrial respiration downregulated under UV-B/C stress with the depletion of multiple subunits of respiratory complexes, excepting the early accumulated IV subunit (COX4) (Supplementary table S1). Interestingly, UV-B/C treatment increased the abundance of respiratory complexes chaperones. NUOAF4, chaperone of the large complex I, was exclusively detected on early stress response and HSP70C, a subunit of the IV associated HSP70 complex, peaked at this stage (1-fold at t 5 h) (Supplementary table S1). HSP70 can complex with MGE1 and COX4, also up-accumulated under stress, allowing the incorporation of COX4 into the IV complex (Bottinger *et al.*, 2013). Moreover, the accumulation of HSP70C has been described in response to heat stress induced protein misfolding in Chlamydomonas (Schroda, Hemme and Mühlhaus, 2015), suggesting a UV-B/C induced protein damage. UV irradiation affects mitochondrial proteins and triggers ROS bursts (Urban *et al.*, 2016), probably inducing the observed accumulation of ROS scavenging catechin, catechin biosynthesis related CHALCONE ISOMERASE (Cre12.g517100.t1.1) and redox related TCA ISOCITRATE DEHYDROGENASE (IDH2) under UV-B/C (0.8-, 1.5-, 2.2-fold at t 24 h) (Supplementary table S1, S2). Moreover, also redox related RIBOFLAVIN KINASE (RFK2) was exclusively detected under early stress (Supplementary table S1). RFK overexpression protect cells from oxidative stress enhancing GSH metabolism (Hirano *et al.*, 2011). Besides not a mitochondrial protein, HLA8 nuclease accumulated under stress (1.9 max. fold at t 24 h) (Supplementary table S1). This nuclease, is induced by high light in Chlamydomonas (Soon Im and Grossman, 2002) and its homolog (AtCaN2) in Arabidopsis overexpression enhances oxidative stress and cell death (Sui *et al.*, 2018).

Low intensity UV-B/C irradiation modulates thylakoid electron transport and fixed carbon allocation under inhibited respiration

Under UV-B/C stress, central photosystem II (PSII) subunits PsbD (0.9-fold at t 5 h) and PsbF peaked and was exclusively detected at t 5 h, respectively, while antennal LHCB4 and STRESS-RELATED CHLOROPHYLL A/B

BINDING PROTEIN 1 (LHCSR1) were up-accumulated on acclimation (1-, 3- max. fold at t 24 h) (Supplementary table S1). PSII oxygen evolving complex (OEC) subunits are specially sensible to UV-B, and their damage reduce linear electron flow (LEF) explaining the reduced photosystem I (PSI) damage under UV stress (Zhang *et al.*, 2016). Although none of the OEC subunits abundance levels changed, the abundance of LEF related CYTOCHROME b6f RIESKE IRON-SULFUR CENTER SUBUNIT (PETC) was reduced from previously high levels (-9.6-fold at t 24 h) (Supplementary table S1) suggesting a LEF downregulation. On the other hand, the accumulation of PSI proteins P700 CHLOROPHYLL A APOPROTEIN A2 (psaB) and psaG (2.4- and 2.7-fold at t 5 and 24 h respectively), PLASTOCYANIN (PCY1) and FERREDOXIN NADP REDUCTASE (FNR1) (1.6-,1-fold at t 24 h) (Supplementary table S1) related both to LEF but also cyclic electron flow (CEF) suggested an enhancement of the latter under UV-B/C. CEF promotion, characteristic of several stress situations as high light or chilling and linked to PSII inhibition (Huang *et al.*, 2018), was also evidenced by the accumulation of PSII central subunit PsbD, and antennal LHCB4 and LHCSR1(Supplementary table S1) working as a photosynthetic protein protection and turnover mechanism (de Bianchi *et al.*, 2011; Kiss *et al.*, 2012; Rusaczonek *et al.*, 2015).

The evidences of an enhanced CEF and PSII photoinhibition are linked to the progressive increase of LHCSR1 under stress (Supplementary table S1). This protein participates into a photoprotective mechanism transferring excitation energy from microalgae PSII associated LHCII to PSI and free LHCII (Kosuge *et al.*, 2018), and its accumulation is UVR8 dependent in Chlamydomonas (Allorent *et al.*, 2016). The enhancement of PSII quenching and PSII subunit synthesis under UV-B/C stress was coupled to the accumulation of photosynthetic complexes repair and assembly proteins as THYLAKOID LUMINAL FACTOR (TEF14), CYN38 and CHLOROPLASTIC DNAJ-LIKE PROTEIN (CDJ1) (2.7 and 2.4, 1.2-fold at t 24 h) (Supplementary table S1). Arabidopsis mutants on TEF14 and CYN38 orthologs fail to assembly and repair PSII (Fu *et al.*, 2007; Liu and Last, 2017). Thus, the accumulation of TEF14 and CYN38 along PSII subunits, all supporting an enhanced PSII turnover, and the evidences of enhanced PSII quenching through LHCSR1

suggest the tested UV-B/C radiation focus on PSII and support the photoinhibition of PSII and the enhancement of CEF. Chlamydomonas CDJ1 organizes chloroplastic HSPs under heat stress (Willmund *et al.*, 2008) suggesting its accumulation under UV-B/C an enhanced protection of photosynthetic proteins and complexes. Besides the enhanced photosystem protection, UV damages photosystems elements requiring the removal of damaged subunits and the ensemble of newly synthesized ones. The accumulation of a FTSH-like Chlamydomonas chloroplastic protease (Q32065) along PSII psbD and photosystem ensemble/repair proteins (Supplementary table S1) supported the protein removal, synthesis and ensemble steps associated to photosystems damage.

The up-accumulation under UV-B/C of redox related NDA5 oxidoreductase, whose Arabidopsis homolog (NDA1) is a chloroplastic/mitochondrial NADPH dependent oxidoreductase (Carrie *et al.*, 2008), and tocopherol biosynthesis PHYTOL KINASE (CGL134) (Supplementary table S1) support the radiation effect on redox homeostasis. Other redox regulators as THIOREDOXIN M (TRXm) accumulated under stress (2.5-fold at t 24 h) or as GLUTATHIONE-S-TRANSFERASE (CPLD58) was detected exclusively after 5 h of stress. On the other hand, redox regulated chloroplastic proteins NADP MALATE DEHYDROGENASE (MME5), accumulated early under stress (5-fold at t 5 h) (Supplementary table S1). This enzyme is part of the malate shuttle transferring not only carbon but excess reducing power from chloroplast to cytoplasm and other organules. The early accumulation of MME5 concurred with the accumulation of chloroplastic glycolate/glycerate (TEF24), triose phosphate:Pi (APE2) antiporters (2.9-, 4-fold at t 5 h) and glycerol transporter (MIP1) (Supplementary table S1). Moreover, triose phosphate related glycerol, photorespiratory glycolate and alanine accumulated early under stress (0.7-, 1.7-, 1.1-fold at t 5 h). This was coupled to the early accumulation of photorespiration related enzymes as SERINE and ALANINE GLYOXILATE TRANSAMINASES (SGA1, AGT2) (1.5-, 1.8-fold at t 5 h) (Supplementary table S1, S2).

The early accumulation of glycerol, glycolate and chloroplastic C3 transporters was synchronized with the accumulation of RuBisCO large subunit (RBCL) and OPP 6-PHOSPHOGLUCONATE DEHYDROGENASE (1-, 1.3-fold at t 5 h) (Supplementary table S1). Starch synthesis was also early enhanced and GRANULE BOUND STARCH SYNTHASE (STA2) (2.1-fold at t 5 h) and STARCH BRANCHING ENZYME (SBE3) accumulated at t 5 h (Supplementary table S1). Low intensity UV-C or UV-B treatments increase RuBisCO content and activity in cyanobacteria and microalgae (Zhang *et al.*, 2015; Peng, Yang and Gao, 2017; Phukan, Rai and Syiem, 2018). Besides this, RBCL methyltransferase (RBCMT1) and RuBisCO small subunit isoform 1 (RBCS1) depleted under stress. Interestingly, RuBisCO small subunit isoform 2 (RBCS2) abundance did not change under stress (Supplementary table S1).

Observed inhibition of mitochondrial respiration and early cytoplasm flooding with photosynthates and amino acids could be explained through down-accumulating ISOCITRATE LYASE (ICL1, 2, 3) (-6.1-fold at t 24 h for ICL1) (Supplementary table S1). Chlamydomonas ICL mutants have a downregulated acetate assimilation, respiration, glyoxilate cycle and gluconeogenesis and their TCA is shifted towards aminoacid synthesis and accumulation (Plancke *et al.*, 2014). Alanine accumulated at t 5 h along SERINE ACETYLTRANSFERASE (SAT3) (2.3-fold at t 5 h), aminoacid/GSH metabolism OXOPROLINASE (Cre07.g325748.t1.1), CYSTATIONINE BETA LYASE (METC) and aromatic aminoacid, quinones and phenolic compounds related CHORISMATE SYNTHASE. Leucine biosynthesis ISOPROPYLMALATE DEHYDROGENASE (LEU3) (3.6-fold t 24 h) and METHYLCROTONYL CoA CARBOXYLASE alpha subunit accumulated under stress (Supplementary table S1, S2).

UVR8 related and alternative signalers modulate development and metabolism under UV-B/C.

Although Chlamydomonas UV-B response involves UVR8 and many of its described associated factors (Tilbrook *et al.*, 2016) the effect of UV-C on the sensory protein is still unclear (Christie *et al.*, 2012; Jiang *et al.*, 2012; Xie *et al.*, 2012). UV-B/C treatment induced LHCSR1 and CHALCONE

ISOMERASE (Cre12.g517100.t1.1) (Supplementary table S1), both UVR8 effectors (MÃ¼ller-Xing, Xing and Goodrich, 2014; Allorent *et al.*, 2016), suggesting the involvement of UVR8 into this response. Moreover, the involvement of SA signaling is also probable as SA synthesis related BENZOATE-4-MONOOXIGENASE (Cre01.g000350.t1.1) accumulated on t 24 h (4-fold) (Supplementary table S1). This monooxigenase turns 4-hydroxybenzoic acid, which reduced its accumulation at t 24 h (-0.4-fold) (Supplementary table S2), into benzoic acid, a SA precursor. This change is compatible with an enhanced SA synthesis, although SA and enzymes directly related into SA synthesis were not detected. Moreover, the observed metabolite pattern under UV-B/C resembled the described metabolite changes into SA treated Chlamydomonas (Lee *et al.*, 2016). SA induces sugars (glucose, fructose, galactose), glycerol derived glyceric acid and nitrogen metabolism related elements (glutamate, alanine, uracil, adenine) accumulation in Chlamydomonas (Lee *et al.*, 2016). In this assay glycerol, uracil (1.1-fold at t 5 h) and alanine accumulated early in Chlamydomonas, while glucose and unknown sugars as UNKNOW SUGAR, UNKNOW SUGAR 4 and UNKNOW SUGAR 5 accumulated 24 h after UV-B/C irradiation start (1-, 1.2-, 1.2-, 1-fold at t 24 h) (Supplementary table S2).

Besides UVR8 and SA related elements, different unknown, signaling like, development and translation modulation elements accumulating at t 5 h were clustered into the sPLS network (Figure 5, Supplementary figure S1). Within these elements, STITCH network (Figure 6) linked WD40 REPEAT PROTEIN to development related MINA53, down-accumulating under stress (Supplementary table S1). The mutation of the human homolog of MINA53, MINA53/RIOX2, inhibit DNA replication/repair mechanisms and cell proliferation into human cell lines (Xuan *et al.*, 2018). MINA53 was also linked into sPLS network (Figure 5) through C/N metabolism to JmjC protein JMJC DOMAIN CONTAINING PROTEIN 7 and HISTONE-ARGININE N-METHYLTRANSFERASE (PRMT2). Interestingly, Arabidopsis homolog to JMJC DOMAIN CONTAINING PROTEIN 7, JMJ32, is a HISTONE H3 lysine 27 (H3K27) demethylase regulating flowering time upon temperature change through FLOWERING LOCUS C (FLC) modulation (Gan *et al.*,

2014), and PRMT2 Arabidopsis homolog, PROTEIN-ARGININE N-METHYLTRANSFERASE (PRMT10) a HISTONE H4 arginine 3 (H4R3) methyltransferase, also modulate FLC expression (Niu *et al.*, 2007). Although the FLC gene is absent from Chlamydomonas, these proteins suggest the involvement of the applied radiation into the epigenetic modulation of different developmental processes into the microalgae. sPLS and STICH networks also highlighted the link between C/N metabolism and protein synthesis to early accumulated translation modulation related EIF1a and aminoacyl tRNA synthases and to signaling PP2A like (Cre03.g199983.t1.1), exclusively detected into control samples (Figure 5, 6) (Supplementary table S1). This novel PP2A like phosphatase is homolog to several Arabidopsis PP2A which are key nodes into plant immunity integrating pathogen perception at membrane level with pathogen response at multiple levels including SA, ABA and TOR signaling and C/N metabolism modulation (Durian *et al.*, 2016; Punzo *et al.*, 2018). More directly related to the modulation of carbon metabolism under stress, sPLS network (Figure 5) highlighted a correlation between DYRK kinase (Cre01.g008550.t1.1 or au5.g1142_t1) and the production of sugars and glycerol. This DYRK kinase, classified as CMGC_DYRK-PRP4 into the iTAK database (Zheng *et al.*, 2016), accumulated 24 h after UV-B/C irradiation start (Supplementary table S1). Other Chlamydomonas DYRK as TAR1 and STD1 are known for their roles into carbon storage under nutrient stress (Kajikawa *et al.*, 2015; Schulz-Raffelt *et al.*, 2016).

UVB/C regulation of cell proliferation

While modulating the expression of stress related genes and enhancing protein turnover, the early depletion of PP2A like SERINE/THREONINE PROTEIN PHOSPHATASE and MINA53 under UV-B/C (Supplementary table S1) also suggest an early Chlamydomonas proliferation stop. PP2As are connected to different signaling pathways including SA and ABA, and through TOR modulation can regulate cell growth in response to the environment (Durian *et al.*, 2016; Punzo *et al.*, 2018; Tang *et al.*, 2018). ROS is described to enhance PP2A gene demethylation and subsequent mTORC inhibition driving to a halt

in cell proliferation (Tang *et al.*, 2018). The connection of PP2A to protein synthesis and C/N metabolism into the STITCH network (Figure 6) is suggestive on the PP2A like protein mediated tuning of TOR pathway into UVB/C stressed Chlamydomonas. Thus, PP2A down-regulation would lead to a proliferation enhancement, although it is important to consider that PP2A regulation is complex and Arabidopsis PP2A have been found both positive and negative regulators of ABA response in different tissues (Punzo *et al.*, 2018). Conversely, the down-regulation of MINA53 point to a cell proliferation stop under UV stress. The mutation of the human homolog of MINA53, MINA53/RIOX2, renders cell lines with inhibited DNA replication/repair mechanisms, and cell proliferation (Xuan *et al.*, 2018).

Mixed UV-B/C irradiation affected Chlamydomonas causing redox unbalance and ROS production through its damaging effects over photosystem and mitochondrial respiratory complexes. Chlamydomonas early response to UV-B/C effects is focused on the avoidance of ROS and direct UV damage to cellular structures trough, redox modulating, ROS scavenging, and protein protection/turnover responses. On the other hand, adaption is related to the avoidance of PSII damage through the CEF, quenching and UV shielding mechanisms, to cell proliferation and development modulation, and to the modulation of carbon metabolism through kinases as DYRK (Cre01.g008550.t1.1) accumulating along different sugars. The proteins involved into these differentiated UV-B/C responses suggest the overlapping involvement of UVR8 SA and redox based signaling mechanisms. Moreover, these responses are linked to protein expression/epigenetic modulation elements as JMJ32, MINA53 and PRMT2 that might be driving the proteogenomic changes after UV adaptation and even photomorphogenic effects in Chlamydomonas. All these elements would help for further UV-B/C stress characterization or exploitation towards the generation of enhanced strains.

IV. Discussion

Chlamydomonas response to UV-B/C stress is based on redox modulation

Plants and also algae are exposed to ever changing light environments and continuously forced to adapt. For UV stress, adaption is focused on damage avoidance and repair mechanisms mainly centered on photosynthesis but also globally on protein and redox modulation. Tested UV-B/C radiation photoinhibited Chlamydomonas PSII as the evidences of an enhanced PSII protein turnover, repair and energy quenching are between the most relevant of the observed UV-B/C response features. Besides this, although PSII was the main UV-B/C target, tested stress also induced an extensive protein damage and the alteration of the cell redox status. All these UV-B/C effects explain an early response based in redox homeostasis, protein turnover/repair and photoprotection. In Arabidopsis, it was reveal that UV and light stresses photoinhibit PSII and enhance the production of ROS, triggering a response based on the accumulation of ROX quenchers as tocopherol and the maintenance of PSII functionality (Eugeni Piller, 2014). The response to the production of ROS and the breakage of redox homeostasis under UV-B/C is based on the synthesis of UV-shielding phenolics and antioxidant tocopherol, and the modulation of reduced equivalents (NADPH) and tyllakoidal reduced plastoquinone (PQH_2) pools to avoid overreduction. Tested UV-B/C stress induced the accumulation of diverse phenolic compounds in Chlamydomonas (protocatechuic acid, 4-hydroxybenzoic acid, catechin) and phenolic metabolism enzymes as BENZOATE-4-MONOOXIGENASE (Cre01.g000350.t1.1) and CHALCONE ISOMERASE, but also enhanced tocopherols synthesis as suggested the accumulation of a predicted PHYTOL KINASE (CGL134). Phenolic compounds and tocopherol are accumulated in UV irradiated plants (Pascual *et al.*, 2017). Arabidopsis PHYTOL KINASE 1 (VTE5) participates into tocopherol synthesis through the recycling of phytol from degraded chlorophyll (Valentin *et al.*, 2006), thus the presence of a PHYTOL KINASE under UV-B/C also suggest UV/ROS damage to the antenna pigments.

The shielding and scavenging/stabilizing function of phenolics and tocopherol is complemented by the activity of also UV-B/C induced oxidoreductase NDA5. The Arabidopsis homolog to this protein, NDC1, reduce oxidized plastoglobuli tocopherol and plastoquinone on accumulating NADPH. The reduced plastoquinone (PQH_2) into plastoglobuli can easily diffuse back to thylakoidal membranes (Eugeni Piller *et al.*, 2011; Eugeni Piller, 2014) participating into redox buffering process which can also contribute to ATP synthesis trough the cycling of electrons around PSI.

The enhancement of NDA5 redox exhaust under UV-B/C is complemented by an increase in carbon fixation, a NADPH consuming process, co-occurring with an enhanced photorespiration and photosynthetic/photorespiratory carbon efflux from chloroplast. The increase in carbon fixation was supported by the accumulation on RBCL and the maintainment of RuBisCO ACTIVASE (RCA1) and RuBisCO SMALL SUBUNIT 2 (RBCS2) levels sugessiting an increased RuBisCO activity. Interestingly, the differential behavior of the RBCS1 subunit suggest a possible holoenzyme configuration change under stress. The increase in RuBisCO activity and the early accumulation of glycerol, sugars, starch synthesis enzymes support the enhancement on carbon fixation, coupled to its export to the cytoplasm as suggested by the early accumulation of NADPH dependent malate shuttle (MME5) and triose phosphate:Pi (APE2) transporter. On the other hand, same RuBisCO activity along the early accumulation of glycolate, glycolate/glicerate (TEF24) transporter and mitochondrial SERINE/ALANINE GLYOXILATE TRANSAMINASES (SGA1, AGT2) supports a photorespiratory increase. While NDA5 and carbon fixation can directly avoid the overreduction of the photosynthetic electron chain photorespiration and carbon export would act as a shuttle moving excess reducing power in the form of malate, glycolate and glycerol to other cellular compartments or the extracellular medium (Davis, Fiehn and Durnford, 2013; Demmig-Adams *et al.*, 2017). This carbon efflux would supply mitochondria with ATP under the evidences of a inhibited electron transport.

Proteome reorganization key process in UV-B/C stress response of Chlamydomonas

Proteins are the main target of UV damage, thus, standing under UV require the enhancement of protein turnover mechanisms involving the degradation of damaged proteins, the synthesis of new ones and its fast and coordinated folding and integration into different complexes. PSII is one of the most affected protein complexes under UV with the radiation damaging its central subunits and activated specific responses based on its turnover. The early accumulation of mitochondrial stress chaperone HSP70C subunit, linked to increased mitochondrial protein damage in Chlamydomonas (Schroda, Hemme and Mühlhaus, 2015), and chloroplastic HSP organizer CDJ1 support a global increase protein damage under UV-B/C stress. Moreover, the also early accumulation of free amino acids supports an enhanced degradation. The activation of the specific PSII turnover response under UV-B/C was supported by the accumulation of FTSH like protease, FTSHs are associated to damaged PSII under UV stress (Pascual *et al.*, 2017), central PSII subunits (psbD) and the accumulation of elements related to the correct subunit folding and ensemble as TEF14 and CYN38.

Either the synthesis of new spare proteins to substitute for damaged ones or the modulation of the proteome to acclimate to the new conditions require a controlled enhancement of protein synthesis modulated at translation transcription and epigenetic levels. This enhancement on UV-B/C stressed Chlamydomonas was supported at translation level by the up-accumulation of translation initiation complex recruiter EIF1a and different aminoacyl tRNA synthetases, and the down-accumulation of FAP204 whose human homolog, OLA1, inhibits translation initiation complex formation (Chen *et al.*, 2015). Protein interaction network supports this linking EIF1a to ribosomal subunits and connecting those to tRNA synthetases through FAP204. On the same way but into a different level early transcriptional and epigenetic changes also support the reshape of the proteome under early UV-B/C stress. sPLS network linked epigenetic related JmjC protein JMJC DOMAIN CONTAINING PROTEIN 7 and PRMT2 demethylase and methyltransferase depletion to depleting mitochondrial metabolism and sugar accumulation, while links early

accumulated redox related glycerol to multiple RNA processing elements. Arabidopsis homologs to the last two JMJ32 and PRMT10 are linked environment based FLC modulation (Niu *et al.*, 2007; Gan *et al.*, 2014). Its depletion would be related to the UVB/C mediated control of stress response genes.

The late acclimation to UV-B/C stress was coupled to photosynthetic changes centered into the downregulation of LEF and photorespiration and to the enhancement of CEF into a process dependent on the thylakoidal lumen pH. The exposition of Chlamydomonas to high light intensities generates a reduction of the lumenal pH due to a disbalance between the ATP production and consumption rates, limiting ATP synthase access to ADP and Pi and thus the release of H^+. Moreover, a low lumenal pH complicates the export of protons by the cytochrome b6f to the lumen, limiting PQH_2 reduction and LEF (Erickson, Wakao and Niyogi, 2015). This high light responses supports the early redox alteration observed under UV-B/C but also shows how described early measures against UV-B/C as the enhancement of C3:Pi exchange and ATP consuming carbon fixation also could be contributing to dissipate the ΔpH through the enhancement of ATP synthase access to ADP and Pi. Moreover, low luminal pHs could be activating quenching in Chlamydomonas as suggested the accumulation of quenching related LHCSR1, a low stromal pH activated protein (Kosuge *et al.*, 2018), under UV-B/C. LHCSR1 channels the excitation energy from PSII associated LHCII to PSI further protecting PSII. The accumulation of PSII uncoupler LHCSR1 along CEF related elements suggest the late enhancement of cyclic electron flow under late UV-B/C acclimation. Thus the accumulation of LHCSR1 and the enhancement of CEF would be part of a larger PSII protection/repair mechanism through its uncoupling and the production of needed ATP for this repair. These UV-B/C acclimation changes matched the accumulation of multiple sugars and the phenolic catechin coupled to the fall on early enhanced C3 transport and photorespiration indicating the recovery of redox balance. The accumulation of soluble sugars is observed under different plant abiotic stresses associated to a ROS scavenging function (Sami *et al.*, 2016; Escandón et al., 2016). The late accumulation of sugars could be mediated by a DYRK

kinase as sPLS network connected late accumulated sugar (UNKNOW SUGAR 5) to a novel DYRK kinase. Interestingly, DYRK kinases modulate starch and oil accumulation into stressed Chlamydomonas (Schulz-Raffelt *et al.*, 2016)

UV-B/C modulation of Chlamydomonas metabolism and development.

UVR8 is involved into the deployment of responses to UV-B in Chlamydomonas and land plants as Arabidopsis (Allorent *et al.*, 2016; Liang, Yang and Liu, 2018). The accumulation of UVR8 proxies, LHCSR1, CHALCONE ISOMERASE and catechin, a CHALCONE ISOMERASE derivative, under tested UV-B/C stress suggested the involvement of UVR8 into the microalgae UVB/C stress response. Moreover, protein damage related novel FTSH protease (Q32065) and PSII subunit psbD, also accumulating under tested stress could as well be beacons of the UVR8 involvement into Chlamydomonas UVB/C response. UVR8 induces the accumulation of photoprotective LHCRS1 and phenolic metabolism CHALCONE ISOMERASE in Chlamydomonas under UVB stress (Tilbrook *et al.*, 2016). The link between detected FTSH and UVR8 is less clear as it was not detected under UVB in Chlamydomonas (Tilbrook *et al.*, 2016), however, Arabidopsis FTSH protease FTSH8 is a known UVR8 effector and several FTSH proteases accumulate in *Pinus radiata* under UV (Brown *et al.*, 2005; Pascual *et al.*, 2017). UVR8 has also been proposed to modulate psbD mRNA stability (Tilbrook *et al.*, 2016). Besides this, LHCSR1 is also sensible to high light, PSII inhibition, and linked to Ca^{2+} signaling (Maruyama, Tokutsu and Minagawa, 2014). Thus, LHCSR1 accumulation would also be the response to other stimulus (i.e ROS), also relevant under UVB and UVC stresses.

The evidences of an early ROS burst under UVB/C stress are compatible with the involvement of SA signaling into Chlamydomonas UVB/C response. This was supported by the accumulation of SA close enzyme BENZOATE-4-MONOOXIGENASE and the observed metabolic changes under UV-B/C, including the late sugars accumulation, close to those observed on SA treated Chlamydomonas (Lee *et al.*, 2016). Besides this, directly SA related enzymes

or SA itself were not detected. Moreover, some of the found phenolic metabolism elements as CHALCONE ISOMERASE (Cre12.g517100.t1.1) would also be related to enhanced catechin, tocopherol and plastoquinone synthesis under stress. Furthermore, Chlamydomonas SA signaling is barely described and the microalgae lacks homologs to its most important plant receptor (NDR1), suggesting an alternative signaling.

V. Conclusions

Chlamydomonas response to UV-B/C stress is based on the avoidance of UV/ROS protein damage requiring globally from the enhancement of UV shielding, protein protection/turnover, redox modulation mechanisms, and specifically from the fine tuning of UV sensitive photosystems. Microalgae early response to the UV irradiation is mainly based on protein turnover/protection, keeping different processes functionality, and metabolic/redox modulation, limiting further UV damage. This fast response is coupled to the activation of parallel UV-B and UV-C signaling pathways including UVR8 and ROS/SA signaling that would be driving a fine UV-tuned control of cell proliferation, gene expression and protein translation before Chlamydomonas late and more complex acclimation responses. Acclimation was related to specific proteome changes focused on the photosystems modulation, uncoupling PSII and enhancing CEF, and on the accumulation of specific UV shielding and ROS scavenging metabolites. Some of the found early response signalers as translation modulator FAP204, cell proliferation related PP2A like protein and MINA53, and late acclimation metabolic modulator DYRK have been identified as promising targets for the further characterization of the UV-B/C response or the exploitation of the UV metabolic modulation mechanisms towards the engineering of more productive strains.

VI. Supplementary materials available only on the USB drive

Table S1. List of the 885 quantified proteins in whole cell fractions with their abundances estimated following a NSAF approach. Protein are designated with their respective Chlamydomonas JGI v5.5, Viridiplantae-UniProt or Augustus database accession. Percent of coverage, number of unique peptides used for identification, residues and protein molecular weight are indicated. Displayed data underwent filtering, imputation and sample abundance-based balancing. The mean abudance ± SD for each sampling time are indicated as well as their ANOVA p- and q-values (BH), and the post-hoc Tukey HSD test p-values calculated over Log10 transformed data. Deflines, symbols and Mapman bins were manually curated.

Table S2. List of the 68 quantified metabolites. Metabolite names were included along their Golm metabolome database identifiers used as uncharacterized compounds names. Retention time (RT) was included along the mass/charge ratios (M/Z 1 and MZ 2) of the two most characteristic fragmentation ions for each compound. Metabolite abundance was estimated from the peak areas of the indicated characteristic ions. Abundance data underwent filtering, imputation and sample abundance-based balancing. The mean abudance respect to control ± SD for each sampling time are indicated as well as ANOVA p- and q-values (BH) and post-hoc Tukey (HSD) test p-values calculated over Log10 transformed data. Mapman bins and deflines were manually curated.

Table S3. Summary of z-score transformed protein abundance PCA. Scores of the nine generated components for each sample are showed along proportion of the total sample variance explained by each component and loadings relating each protein variable contribution to each generated component. Proteins were identified by their respective Phytozome v5.5, Viridiplantae-UniProt or Augustus identifier.

Table S4. Summary of z-score transformed protein abundance PCA. Scores of the nine generated components for each sample are showed along proportion of the total sample variance explained by each component and loadings relating each protein variable contribution to each generated component. Proteins were identified by their respective Phytozome v5.5, Viridiplantae-UniProt or Augustus identifier.

Table S5. Summary of z-score transformed protein, metabolite and physiology abundance sPLS. Model was tuned keeping 125 protein (X) and 9 metabolite/physiology (Y) variables. Loadings relating each keep protein (X) or metabolite and physiological variables (Y) contribution to each two generated components. Proteins were identified by their respective Phytozome v5.5, UNIPROT-Viridiplantae or Augustus identifier. Metabolites were identified by their respective name or for uncharacterized compounds their Golm database identifier.

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CHAPTER 4

Genome-wide identification and characterization of CKIN/SnRK gene family in *Chlamydomonas reinhardtii*

CHAPTER CONTENTS

I. Introduction

Algae are promising organisms for biotechnological applications, ranging from the production of biofuels or feedstock to the accumulation of high value-added molecules. This, together with its ease of cultivation, simple life cycle, high growth rate, phenotypic plasticity, and capability to accumulate biofuel-related molecules (lipids, starch) or secondary metabolites such as ß-carotene, astaxanthin or Omega-3(Bajhaiya, Ziehe Moreira & Pittman 2017) make this group of organisms one of the most studied biological systems.

However, several studies report that the accumulation of these molecules requires the imposition of an external stress. For instance the accumulation of TAG (triacylglycerides), essential for biodiesel production, is mainly triggered by nutrient limitation (Merchant, Kropat, Liu, Shaw & Warakanont 2011; Cakmak *et al.* 2012; Valledor, Furuhashi, Recuenco-Muñoz, Wienkoop & Weckwerth 2014b; Iwai, Ikeda, Shimojima & Ohta 2014). The accumulation of other valuable molecules such as carotenes (Skjånes, Rebours & Lindblad 2012) or hydrogen (Gonzalez-Ballester, Jurado-Oller & Fernandez 2015) is also dependent on nutrient or environmental stresses. This need for stressing algae cultures forces a two-phase cultivation strategy in which cells are first grown under optimal conditions until enough biomass is produced and then an abiotic stress is imposed to trigger the accumulation of specific molecules (Cheng & He 2014; Minhas, Hodgson, Barrow & Adholeya 2016). This approach requires longer cultivation times and/or the expense of energy to apply a certain stress (e.g. removing a nutrient from the media), making this production method economically unsustainable (Radakovits, Jinkerson, Darzins & Posewitz 2010).

Studying metabolic and regulatory networks involved in stress response is thus a mandatory first step to identify targets with potential to improve microalgae strains and culture practices (Guarnieri & Pienkos 2015). Among the possible cellular mechanisms proposed as targets for microalgae production enhancement, there is evidence that the cAMP-dependant SnRK1 protein kinase family [Sucrose non-fermenting-1 (Snf1)-related protein kinase; known as Snf1 in yeast/mammals and AKIN in *Arabidopsis thaliana*] mediates the connexion between central metabolism, gene regulation, and stress response, together with hexokinases (HXK) and Sucrose-Phosphate Phosphatase (SPP) (Baena-González & Sheen 2008; Halford & Hey 2009). This protein family is also interconnected with TOR (Robaglia, Thomas & Meyer 2012), epigenetic pathways (Gendrel, Lippman, Yordan, Colot & Martienssen 2002) and with the activation/repression of entire metabolic branches in other organisms (Shaw 2009), making it a good candidate for further studies in microalgae.

The first described SnRK family member was the yeast Snf1, being well-known by its role in lipid accumulation (Kamisaka, Tomita, Kimura, Kainou & Uemura 2007) and glucose repression, regulating carbon metabolism (Baena-González & Sheen 2008). Globally, AKIN/SnRK1/Snf1/AMPK kinases concentrate divergent stress signals by activating specific enzymes and transcription factors, related to metabolic regulation, protein biosynthesis and cell organization, as part of a complex system aimed to increase cell survival under unfavourable energetic balance (Halford & Hey 2009)(Nukarinen *et al.* 2016). AMPK regulates through direct phosphorylation or associated γ subunits ADP-AMP levels sensing(Oakhill, Scott & Kemp 2012), with also associated β subunits regulating substrate specificity and cell location(Polge, Jossier, Crozet, Gissot & Thomas 2008). These kinase-regulatory subunits protein complexes are remarkably well conserved across Eukarya and deeply rooted into the life tree. Orthologs to AMPK and γ subunits have been found into Bacteria and Archaea kingdoms, being β subunits exclusive from eukaryotes(Roustan, Jain, Teige, Ebersberger & Weckwerth 2016). Plants are an exception of this family conservation showing unique γ functionally equivalent βγ proteins and γ-like subunits without direct SnRK1 interaction(Lumbreras, Albà, Kleinow, Koncz & Pagès 2001). This exceptionality comes also at regulation level, with a sugar phosphate mediated regulation of the energy sensing kinase(Yadav *et al.* 2014). Moreover, although AMPK duplication events are common in plants and animals(Roustan *et al.* 2016), plants SnRK family underwent an extensive duplication and diversification event, giving origin to three subfamilies: SnRK1/AKIN (the closest to Snf1), SnRK2 and SnRK3(Halford, Bouly & Thomas 2000). All these plant SnRK subfamilies share a common Ser/Thr kinase domain, followed by a UBA and a KA1 domain in SnRK1. In SnRK2 an osmotic stress activation domain I is present after the kinase domain, while SnRK3 contains a NAF/FISL domain (Coello, Hey & Halford 2011). There is strong evidence that subfamilies SnRK2 and 3 evolved after gene duplication of SnRK1 in order to enable plants to develop networks capable of linking stress, ABA, and calcium signalling with metabolic and epigenetic responses (Halford & Hey 2009). In plants, some SnRK2 are also key transductors in ABA-mediated

responses to salt and other abiotic stresses (Coello *et al.* 2011), presenting specific ABA interaction acidic motifs known as domain II (Yoshida *et al.* 2006) and placed after the characteristic osmotic stress activation domain I.

In *Arabidopsis thaliana*, thereafter Arabidopsis, the SnRK family is composed by 38 members and 3 subfamilies: SnRK1 (3 genes), SnRK2 (10 genes), and SnRK3 (25 genes) (Hrabak *et al.* 2003). SnRK1 has been linked to increased tolerance to nitrogen stress (Wang, Peng, Li, Yang & Li 2012) and to energy sensing and gene regulation (Robaglia *et al.* 2012). It showed to be implied in plant response to starvation and energy deficit by coordinating ATP/cAMP, redox status and C/N ratios to regulate broad metabolic branches, either directly by phosphorylation of enzymes or indirectly by phosphorylation of transcription factors (Polge & Thomas 2007; Halford & Hey 2009; Bernhard *et al.* 2017). SnRK1 has been considered a potential target to improve plant performance under unfavourable conditions (Coello *et al.* 2011). SnRK2 and SnRK3 showed to have also a key role in signalling pathways that regulate plant response to nutrient limitation, drought, cold, salt, and osmotic stress (Coello *et al.* 2011). The SnRK2 subfamily has an essential role in gene expression regulation through the activation of bZIP transcription factors and SWI/SNF/helicase complexes (Baena-González & Sheen 2008; Fujii, Verslues & Zhu 2011) tightly connected to epigenetic mechanisms that perfectly control gene activation or repression. However, little is known about the structure or the role of this protein kinase family in microalgae stress response mechanisms and its relation with biotechnological processes, such as the accumulation of high value-added molecules like energetic molecules (sugars and lipids) (Sato, Matsumura, Hoshino, Tsuzuki & Sato 2014) or pigments (astaxanthin, lutein, and β-carotene).

The Chlorophyceae *Chlamydomonas reinhardtii,* thereafter Chlamydomonas, shares common ancestry with vascular plants (Hannon, Gimpel, Tran, Rasala & Mayfield 2010). Therefore, it is expected that many of its responses to limiting conditions would be similar (Grossman 2000). Few studies suggest the involvement of SnRK family, named CKIN in Chlamydomonas(Valledor, Furuhashi, Hanak & Weckwerth 2013), in stress response, namely under sulphur (Gonzalez-Ballester, Pollock, Pootakham &

Grossman 2008; González-Ballester *et al.* 2010) and nitrogen deprivation (Valledor *et al.* 2014b), and cold stress (Valledor *et al.* 2013). Gonzalez-Ballester et al. (Gonzalez-Ballester *et al.* 2008) reported the existence of eight putative SnRK2-like genes in Chlamydomonas, while Valledor et al. (Valledor *et al.* 2013) found three cold stress-responsive proteins showing sequence homology with Arabidopsis AKIN10/11 family (CKIN1, CKIN2, and CKIN3). Both authors suggested that, like in plants, Chlamydomonas abiotic stress response is mediated by CKINs. Notably, it has been recently reported by Sato et al. (Sato *et al.* 2014) that *SAC1* and *SnRK2.2* act as positive regulators of DGTT1, enhancing TAG synthesis under Sulphur starvation in Chlamydomonas. Chlamydomonas SnRKs similarity to land plants is conceivably not only limited to direct stress SnRK induction, but also ABA-SnRK interaction. ABA showed to be involved in Chlamydomonas cell signalling during osmotic stress response (Yoshida, Igarashi, Mukai, Hirata & Miyamoto 2003). However, microalgae ABA-mediated responses seem to be less complex than in land plants as little or no homology was found between most land plants ABA receptors/effectors and the Chlamydomonas proteome (Hauser, Waadt & Schroeder 2011; Lu & Xu 2015). Considering that SnRKs control entire branches of the metabolism in Arabidopsis and other studied models, the identification of CKIN stress-specific dynamics, will potentially reveal new targets for further bioengineering research aiming to accumulate economically relevant biomolecules.

Therefore, in the present study, we aim to fully describe the entire set of genes belonging to the CKIN family in Chlamydomonas and its potential implication in specific-stress response mechanisms and in ABA-mediated responses. The combination of Chlamydomonas and other microalgae genome mining, plant protein-protein interaction databases, and quantitative reverse transcription PCR (RT-qPCR) allowed not only the definition of this family and its evolutive history, but also defining its interacting networks and testing its expression levels under exogenous ABA addition, ABA synthesis inhibition, and a wide-range of stressful conditions. The results herein presented represent a great advance in microalgae and stress biology research, defining a new set of potential targets for biotechnological improvement. Although SnRK are a key

group of protein kinases for biotechnology, this family was never fully characterized in microalgae.

II. Material and methods

CKIN sequence identification and classification in Chlamydomonas

Chlamydomonas CKIN family genes were initially defined by BLASTP comparison against the Chlamydomonas proteome v5.5 (Merchant *et al.* 2007) available at Phytozome (Goodstein *et al.* 2012) employing Chlamydomonas (Gonzalez-Ballester *et al.* 2008; Valledor *et al.* 2013) and Arabidopsis (Coello *et al.* 2011) previously identified CKINs and SnRKs as query (Supplementary Table S1). Homology was considered for e-values lower than 10^{-25} generating a first uncurated sequence list (Supplementary Table S2).

On a second step, Inter Pro Scan (Zdobnov & Apweiler 2001) was used to define the domain structure of all candidate sequences, filtering out those proteins with no SnRK domain structure (e.g. CDPKs). Furthermore, BLASP query sequences domains (Supplementary Table S3) were used as reference to search for potential CKINs into the Chlamydomonas genome using BIOMART (Smedley *et al.* 2015). Protein sequences with characteristic SnRK/CKIN domains, were aligned with M-Coffee (Wallace, O'Sullivan, Higgins & Notredame 2006).

Maximum likelihood (ML) sequence trees were built into PhyML(Guindon, Lethiec, Duroux & Gascuel 2005) platform employing M-Coffee general alignment distances after a TCS (Transitive Consistency Score)(Chang, Di Tommaso, Lefort, Gascuel & Notredame 2015) alignment filtration. One hundred bootstrap replicates were done into the same PhyML platform over the filtered alignment data to assess tree consistency. Different tree topologies were evaluated including a different set of ortholog SnRK proteins sequences (Supplementary Table S6) and Chlamydomonas CDPK sequences. Same tree-group sequences were aligned together using M-Coffee to validate sequence adhesion to their group through distinctive sequence motif conservation. These block alignments were curated using g-blocks (Castresana 2000). COILS

(Lupas, Van Dyke & Stock 1991) and different phosphoproteomic datasets were used for alignment enrichment with coiled-coil predicted regions and phosphosites.

Gene duplication was inferred from tree topologies and intra SnRK sequence comparison by BLASTP (Pérez-Bercoff, Makino & McLysaght 2010). Intra tree group comparisons of BLASTP e-values and identity % were used as parameters for duplicity consideration. Three confidence thresholds for high, medium and low duplication origin probability were respectively defined at 10^{-50}, 10^{-45}, and 10^{-40} for e-values, and 50, 45, and 40 for identity %.

Volvox, Dunaliella, Coccomyxa, Chlorella and Ostreococcus sequences homologous to Chlamydomonas and Arabidopsis CDPKs and SnRKs were obtained from Phytozome (Palenik *et al.* 2007; Prochnik *et al.* 2010; Blanc *et al.* 2010; Polle *et al.* 2017) employing the methods previously applied to Chlamydomonas genome mining as described above. SnRK/CDPK sequence groups found in Chlamydomonas, Volvox, Dunaliella *and* Ostreococcus were curated both manually and through iTAK application(Zheng *et al.* 2016). Identified SnRK/CKIN sequences in microalgae species and Arabidopsis along with Chlamydomonas CDPK as an outgroup, were aligned using M-Coffee. A ML sequence tree was built into PhyML platform from the TCS filtered M-Coffee global alignment distances. One hundred bootstrap replicates were done into the same PhyML platform over the filtered alignment data to assess tree consistency. Transfer bootstrap expectation (TBE) bootstrapping method(Lemoine *et al.* 2018) with 100 replicates was used along conventional Felsenstein's method for improving deep branches bootstrap support.

Chlamydomonas culture and stress response characterization

Chlamydomonas strain CC-503 cells were grown on a closed incubator (25 °C, 120 rpm, 16 h light:8 h dark photoperiod and 190-200 μE m^2 s^{-1} light intensity provided by warm white LEDs) in liquid HAP culture media (Harris, Stern & Witman 2009) supplemented with 10 mM sodium acetate at an initial cell density of 3-5 x 10^5 cells mL^{-1}. Basal media composition and growth conditions were changed according to Table 2 to test different abiotic stresses.

Fifty mL culture samples were collected at the beginning of the experiment (0 h) and 48 h after the start of the assay. Samples were centrifuged (4000 rpm, 6 min) and resulting cell pellet masses were estimated gravimetrically and frozen using liquid nitrogen. Four biological replicates of each stressful scenario were performed.

Expression analysis of CKIN genes under a variety of environmental stresses

A precise estimation of SnRK abundance based on RT-qPCR was performed over 9 experimental situations (Table 2). RNA was extracted from the previously frozen pellets using the method described by Valledor et al. (Valledor *et al.* 2014a). 1.7 μg of DNA free RNA was used for cDNA synthesis.

RT-qPCR analysis was performed using the CFX96 Touch Real-Time PCR detection system (Bio-Rad). The individual reactions contained 1x Maxima SYBR Green qPCR Master Mix (Thermo Scientific), 0.5 μM of each primer, 2 % DMSO, and 0.7-0.8 μg of cDNA. The amplification protocol consisted in 1x [95 °C, 10 min], 50 x [95 °C, 15 s; 61 °C, 30 s; Fluorescence reading] and a final melting curve. Relative expression levels were determined in 22 independent experiments for each primer pair (Supplementary Table S7). Each individual experiment was performed with two analytical replicates for each biological replicate from each condition tested. *UBQ* and *RCK1* were selected as endogenous controls after testing the expression stability of *IDA5*, *UBQ*, *TUB*, and *RCK1* with the geNorm software (Vandesompele *et al.* 2002). Gene expression of each SnRK was evaluated by calculating ΔΔCq values (Supplementary Table S5b) according to the recommendations proposed by Hellemans et al. (Hellemans, Mortier, De Paepe, Speleman & Vandesompele 2007).

NGS-based transcriptomic datasets of *Chlamydomonas reinhardtii* available at the AlgaePath repository (Zheng *et al.* 2014) were mined to perform a complementary characterization of the CKIN family expression under stressful conditions. Five different datasets were analysed: sulphur depletion (González-Ballester *et al.* 2010), nitrogen deprivation (Miller *et al.* 2010), low CO_2

content (Fang *et al.* 2012), oxidative stress and iron deprivation (Urzica *et al.* 2012). Each CKIN gene was searched using their gene accession, and its fold change variation under stress was obtained by comparison to non-stressed abundance values. In all cases, abundances within each dataset were normalized against its corresponding abundance in controls (Supplementary Table S5a). The integration of NGS and RT-qPCR abundances was done based on the z-score of the fold change variation between control and stress situation.

ABA-induced regulation of CKINs

HAP medium was supplemented either with 500 μM ABA or 50 μM fluridone (carotenoid and ABA synthesis inhibitor through Phytoene desaturase inhibition) following previous works(Yoshida *et al.* 2003; Née *et al.* 2017). CC503 cell cultures were grown under the previously described basal conditions for 48 h, and compared to its corresponding controls. RNA extraction and RT-qPCRs were performed over 3 biological replicates each treatment. The effect of ABA and fluridone was monitored by quantifying the expression of the ABA-inducible genes Beta-Carotene Hydroxylase (*CHX*) and Ascorbate peroxidase (*APX*) (Du *et al.* 2010).

Bioinformatic and statistical analyses

All the procedures for the identification and classification of Chlamydomonas SnRK were performed locally employing the bioinformatics suite Geneious v7 (Biomatters Inc.), with the exception of Inter Pro Scan (Zdobnov & Apweiler 2001) and BIOMART (Smedley *et al.* 2015) searches that were performed at the European Bioinformatics Institute (ebi.ac.uk) and Phytozome (phytozome.jgi.doe.gov) websites, respectively.

Protein-protein functional interactions were identified by using STRING v10 (Szklarczyk *et al.* 2015) and ChlamyNet (Romero-Campero *et al.* 2016) databases. CKINs protein sequences were uploaded to STRING application and database was queried considering Chlamydomonas as a reference organism. Two related networks were made, one showing highest confidence (over 0.85 STRING interaction score) known interactions. The other network

including also high confidence (over 0.7 STRING interaction score) known and predicted interactions. CKINs protein sequences were also uploaded to ChlamyNet application, containing a Chlamydomonas transcript-based correlation network. A smaller network was made out of the original containing only first level CKIN interactions. STRING and ChlamyNET resulting networks were represented using Cytoscape v3.4 (Cytoscape Consortium 2016).

R v.2.4 software (R Core Team 2016) core functions and the gplots2 and pheatmap packages were used under the R Studio Environment (RStudio Team 2016) to perform the statistical analyses and heatmap plotting of transcriptomic data. ANOVA and t-test (α=0.05 in both cases) were respectively employed for comparing the expression values ($\Delta\Delta$Cq) of CKINs under different stresses, values previously processed using EasyqPCR package for R(S 2012). When mining available datasets in Algaepath, the abundance of each CKIN under the different stress situations was compared to its corresponding non-stressed controls considering fold change.

Data availability

Sequence data analysed in this work, with their IDs listed at Supplementary Table S4 and Supplementary Table S6 are available at phytozome, JGI Chlorella NC64A genome portal and NCBI nucleotide repositories; https://phytozome.jgi.doe.gov, https://genome.jgi.doe.gov/ChlNC64A_1 and https://www.ncbi.nlm.nih.gov respectively. RT-qPCR datasets generated and analysed during the current study are available from the corresponding author on reasonable request. Phosphoproteomic and transcriptomic datasets analysed during this study are included in Wang, H. et al. 2014, Werth, E.G. et al. 2017 and de la Fuente Van Bentem, S. et al 2008 works (de la Fuente van Bentem *et al.* 2008; Wang *et al.* 2014; Werth *et al.* 2017) (and its respective Supplementary Information files) as well as into the Algaepath and ChlamyNet databases at http://algaepath.itps.ncku.edu.tw and http://viridiplantae.ibvf.csic.es respectively.

III. Results

Identification of SnRK protein orthologs in Chlamydomonas

Initial BLAST searches against Chlamydomonas genome employing Arabidopsis SnRKs and identified Chlamydomonas CKIN sequences (Supplementary Table S1) as queries determined 110 proteins that showed significant homology to this family (e-value < 10^{-25}; Supplementary Table S2). Calcium Dependent Protein Kinases (CDPKs), CKINs, and other protein were present in this initial set due to the conserved Ser/Thr kinase domain. Further analyses of protein domains allowed the unequivocal distinction between CKINs and other proteins attending to other domains specifically present in this family (UBA, KA1/αCTD, CBS, Immunoglobulin E-set/CBM and βCTD/ASC/AMPKβI). The combination of BLAST and protein domain validation resulted in the identification of 19 putative CKIN sequences (Table 1). Furthermore, manual analysis of genome employing domain family annotations present in BIOMART database allowed the determination of three new sequences, making a total of 22 sequences. Out of these, 10 genes were previously described by Gonzalez-Ballester et al. (Gonzalez-Ballester *et al.* 2008) and Valledor et al. (Valledor *et al.* 2013), while 12 were found in Chlamydomonas for the first time. Protein alignment, phosphorylation site identification, functional prediction of unannotated domains, expression and protein interaction analyses were performed for the curation of the identified sequences as described below.

Table 1. Chlamydomonas reinhardtii (Cre) BLAST (e-value < 10^{-25}) and BIOMART SnRK/CKIN sequence search hits. Cre and Arabidopsis thaliana (Ath) ortologs sequence accessions, homology search e-values (not for exclusive BIOMART hits), Cre sequence names, and domain identifiers (ID) provided. CKIN were grouped according to sequence similarity and protein domains layout into different clusters (CKIN1: containing the Serin/Threonin Kinase PTHR24343:SF183, IPR015940 and IPR001772 domains; S1 R: regulatory subunits of CKIN1 with PTHR10343, PTHR13780:SF35, IPR032640, IPR000644, IPR006828 and IPR013785 domains; CKIN2: containing the Serin/Threonin Kinase PTHR24343, PTHR24343:SF169, PTHR24343:SF207, PTHR24343:SF167 or PTHR24343:SF200 domains, with CKIN1L exception,

containing the MAP/microtubule affinity-regulating kinase PTHR24346:SF5 and IPR015940 domains). *Chlamydomonas sequences previously referred to as CKIN family by [1] Valledor et al. (2013) and CKIN2 subfamily [2] by Gonzalez-Ballester et al. (2008).

Cre **Accession**	***Ath*** **Accession**	**e-value**	**Name**	**Cluster**	**Domain ID**
Cre04.g211600.t1.1	AT3G01090.1	0	CKIN1*[1]	CKIN1	PTHR24343:SF183 IPR015940 IPR001772
Cre13.g570250.t1.1	AT1G78290.2	$5.96e^{-48}$	CKIN1L	CKIN1	PTHR24346:SF5 IPR015940
Cre10.g457500.t1.1	AT4G16360.1	$1.86e^{-69}$	CKIN β	S1 R (β)	PTHR10343 IPR032640 IPR006828
Cre12.g484350.t1.3	AT1G09020.1	$2.88e^{-52}$	CKIN βγ	S1 R (βγ)	PTHR13780:SF35 IPR032640 IPR000644
Cre12.g528000.t1.2		Domain	CKIN γ*[1]	S1 R (γ)	PTHR13780:SF49 IPR000644 IPR013785
Cre02.g075850.t1.1	AT4G33950.1	$1.33e^{-57}$	CKIN2.1*[2]	CKIN2	PTHR24343
Cre12.g499500.t1.1	AT1G78290.2	$4.02e^{-117}$	CKIN2.2*[2]	CKIN2	PTHR24343:SF207
Cre02.g075900.t1.1	AT5G66880.1	$6.60e^{-73}$	CKIN2.3*[2]	CKIN2	PTHR24343
Cre11.g477000.t1.2	AT5G08590.1	$7.14e^{-29}$	CKIN2.4*[2]	CKIN2	PTHR24343
Cre03.g209505.t1.1	AT1G78290.2	$2.60e^{-63}$	CKIN2.5*[2]	CKIN2	PTHR24343
Cre11.g481000.t1.2	AT4G33950.1	$1.91e^{-88}$	CKIN2.6*[2]	CKIN2	PTHR24343
Cre06.g292700.t1.2	AT4G33950.1	$8.68e^{-103}$	CKIN2.7*[2]	CKIN2	PTHR24343
Cre10.g466350.t1.1	AT4G33950.1	$2.96e^{-152}$	CKIN2.8*[2]	CKIN2	PTHR24343:SF169
Cre16.g657350.t1.2	AT1G78290.2	$5.77e^{-59}$	CKIN2.9	CKIN2	PTHR24343
Cre17.g707800.t1.2	AT1G78290.2	$9.81e^{-46}$	CKIN2.10	CKIN2	PTHR24343:SF167
Cre02.g076000.t1.2	AT5G63650.1	$2.10e^{-40}$	CKIN2.11	CKIN2	PTHR24343
Cre17.g707650.t1.1		Domain	CKIN2.12	CKIN2	PTHR24343:SF167
Cre12.g485600.t1.2	AT1G78290.2	$5.64e^{-47}$	CKIN2.13	CKIN2	PTHR24343:SF167
Cre13.g568050.t1.3	AT4G33950.1	$4.77e^{-58}$	CKIN2.14	CKIN2	PTHR24343
Cre08.g384250.t1.2		Domain	CKIN2.15	CKIN2	PTHR24343
Cre07.g329850.t1.1	AT5G63650.1	$4.69e^{-37}$	CKIN2.16	CKIN2	PTHR24343
Cre16.g685389.t1.1	AT1G78290.2	$6.54e^{-33}$	CKINL*[1]	CKIN2	PTHR24343:SF200

M-Coffee alignment of 4 Chlamydomonas CDPK along the 19 identified Chlamydomonas catalytic CKIN sequences allowed the definition of three putative catalytic functional clusters (Figure 1a). A fourth cluster was conformed with the 3 identified CKIN1 regulatory sequences aligned with their Arabidopsis, *Homo sapiens* and *Saccharomyces cerevisiae* orthologs (Figure 1b). Although closely related to CKIN2, CKINL was excluded from the kinase group during alignments curation. The first identified catalytic cluster involved the SnRK1/AKIN complex, including Chlamydomonas

catalytic subunit α, CKIN1, (Serin/Threonin Kinase (PTHR24343), UBA, and KA1/αCTD domains) and CKIN1L (Serin/Threonin Kinase (PTHR24343) and UBA domains). CKIN1L displayed also unique features as a long N-terminal unconserved sequence and lacked conserved Thr189, key into CKIN1 activation(Stein, Woods, Jones, Davison & Carling 2000), and the regulatory KA1/αCTD domain (Supplementary Figure S1). The second cluster, SnRK1 regulatory subunits, included those non-catalytic subunits of the SnRK1 complex: CKIN β (Immunoglobulin E-set/CBM and βCTD/ASC/AMKβI domains), CKIN βγ (Immunoglobulin E-set/CBM and CBS domains) and related CKIN γ (CBS domains). CKIN γ showed more identity to plant γ subunits and *Saccharomyces cerevisiae* γ-like SDS23(Deng, Lee, Schutt & Moseley 2017) than to true γ-acting proteins as plant βγ and human γ subunits (Figure 1b). The sequences belonging to these two clusters were conserved across evolution as shown by its curated alignments using M-Coffee (Figure 1c).

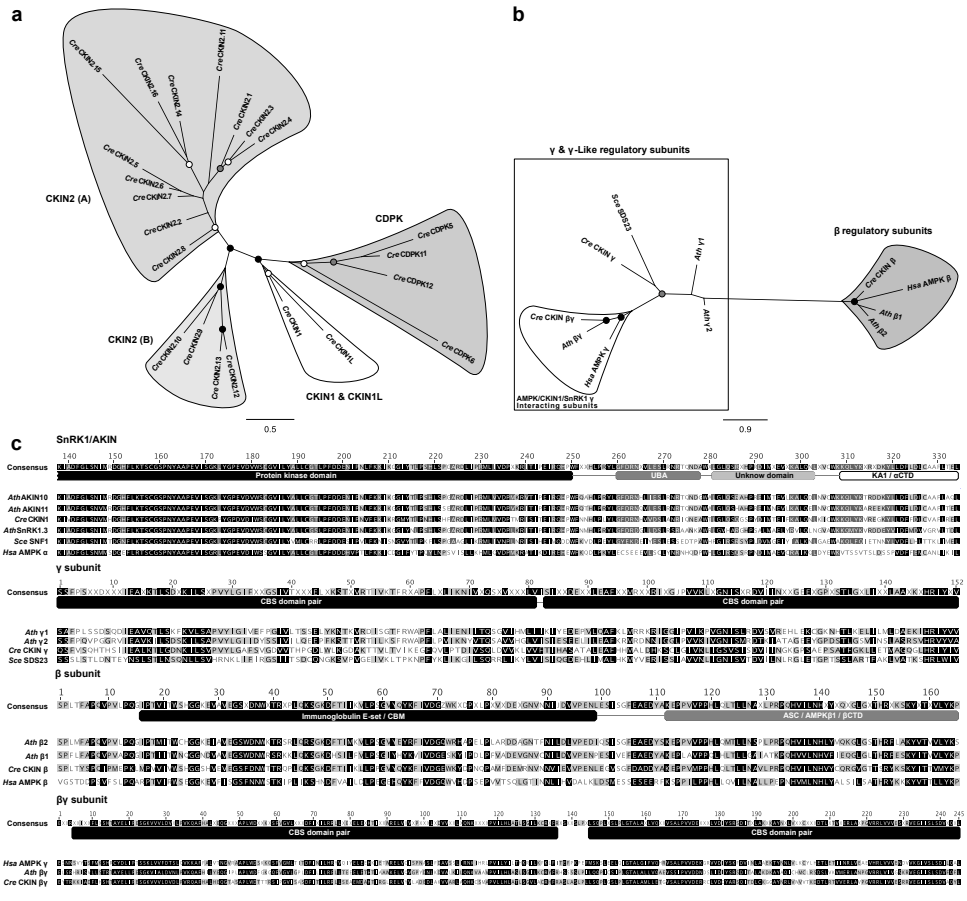


Figure 1: M-Coffee based sequence clustering and structural analysis of *Chlamydomonas reinhardtii* (*Cre*) CKIN sequences. Tree a arranged catalytic CKIN sequences into 3 clusters, namely CKIN2 (A), CKIN2 (B) and CKIN1 & CKIN1L, including a fourth CDPK cluster. Tree b included all *Cre* SnRK1/CKIN1 regulatory interacting and related sequences along with *Arabidopsis thaliana* (*Ath*), *Homo sapiens* (*Hsa*) and *Saccharomyces cerevisiae* (*Sce*) orthologues. Regulatory sequences group into two clusters, namely β regulatory subunits and γ and γ-Like regulatory subunits. This second cluster differentiates between CKIN1/SnRK1/AKIN/AMPK interacting subunits and γ-Like non-interacting subunits. FBP bootstrap support for both trees are indicated through color circles over nodes (black: >90, grey: >80, white: >70). c, M-Coffee curated alignments of CKIN1/SnRK1/AKIN and associated regulatory protein clusters. *Cre* sequences show same domain structure as seen on *Ath*, *Sce* and *Hsa* SnRK1/SNF1/αAMPK being *Hsa*AMPK the more dissimilar one.

Sequences belonging to the Chlamydomonas CKIN2 subfamily were characterized by a SRK2 Serin/Threonin Kinase domain, which is shared with CKIN1. M-Coffee alignment tree arranged CKIN2 sequences into two clusters, CKIN2 (A) and CKIN2 (B) (Figure 1a). The Serin/Threonin Kinase domain (PTHR24343) was present in all Chlamydomonas CKIN2 sequences and its Arabidopsis orthologs (Figure 1c; Supplementary Figure S2; Supplementary Figure S3; Supplementary Table S3). Conversely, SRK variants SRK2C (PTHR24343:SF207), SRK2D (PTHR24343:SF169), SRK2E (PTHR24343:SF167), and SRK2F (PTHR24343:SF200) Serin/Threonin Kinase domains seem to be unique to Chlamydomonas CKIN2 subfamily. These domains are characteristic of the newly found elements (Supplementary Table S3). In plants, these kinases have two conserved serine or threonine phosphorylable residues into the activation loop required for gaining kinase activity (Vlad *et al.* 2010). Although Serin/Threonin Kinase domain showed slight variations across Chlamydomonas CKINs, it can be considered that activation region is conserved and functional since this site has been revealed as differentially phosphorylated in various environments (Supplementary Figure S2, S3) after reanalyzing available phosphoproteomic datasets (de la Fuente van Bentem *et al.* 2008; Wang *et al.* 2014; Werth *et al.* 2017).

Chlamydomonas CKIN2 (A) has regions homologous to Arabidopsis osmotic stress-dependent activation domain I after its kinase domains, where CKIN2 (B) sequences showed a conserved region after kinase domain but less similar to the plant domain I (Supplementary Figure S2, S3). In CKIN2.2 and 2.6-2.8 ABA-dependent activation domains II-like sequences also followed domain I, but were smaller and less rich in acidic residues than its Arabidopsis counterparts (Gonzalez-Ballester *et al.* 2008) (Supplementary Figure S2).

Most Chlamydomonas CKINs showed sequence variations not present in Arabidopsis SnRKs. Similarly to the extra loops previously described in the kinase domains of CKIN2.1, 2.3 and 2.4 (Gonzalez-Ballester *et al.* 2008) (Supplementary Figure S2, S3), extra sequences were found not only restricted to kinase domain. Some domains I and II, and N- and C-terminal regions showed length and sequence variation (Supplementary Figure S2, S3). These extra sequences harbored phosphorylation sites, glycine rich patches (into C-terminal tails) or in the case of CKIN2.4 and 2.14, coiled-coil predicted regions (Supplementary Figure S2, S3).

CKIN2 subfamily was further analyzed along CKIN1 and its regulatory subunits to determine gene duplicities and evolutive relations between the members of this family (Figure 2). CKIN genes mapped to 12 chromosomes, with *CKIN2.10* and *2.12*, and *CKIN2.1*, *2.3* and *2.11* were close in their respective chromosomes. Homology results showed that *CKIN2.2* and *CKIN2.5-2.7* evolved by duplication of *CKIN2.8*, the sequence exhibiting higher homology to higher plants and closer to *CKIN1*. On the other hand, *CKIN2.1*, *2.3* and *2.4*, *CKIN2.14* and *2.10*, and *CKIN2.12* and *2.13* evolved from three different ancestors no longer conserved in Chlamydomonas.

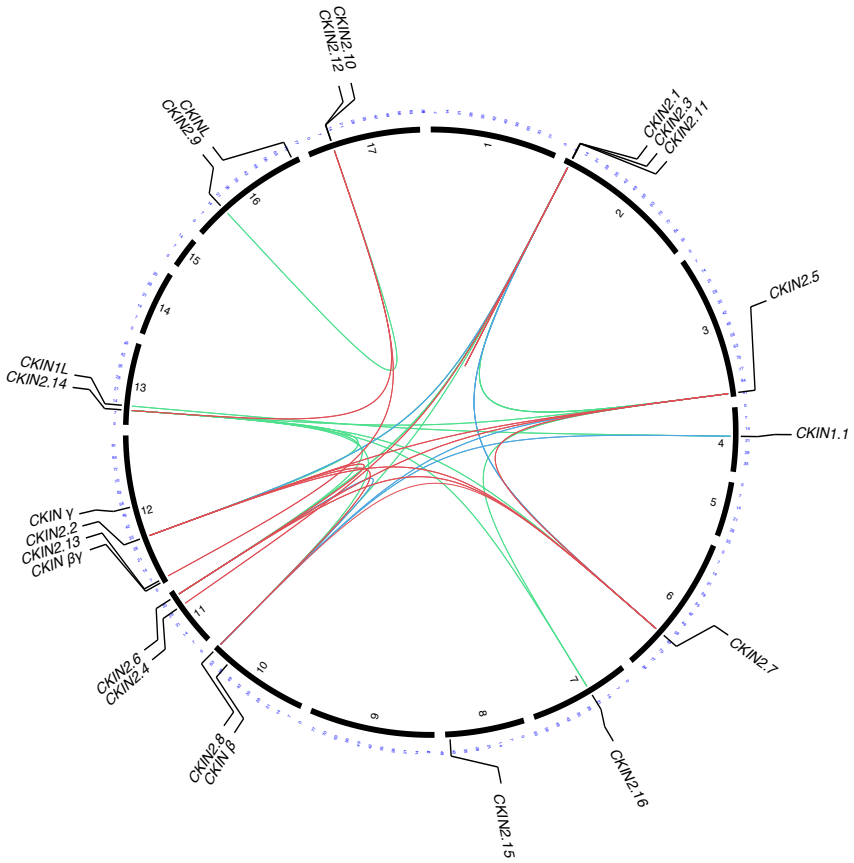


Figure 2: CKIN family evolution in Chlamydomonas. Chlamydomonas CKIN family genes were represented along chromosomes and gene duplications showed as links between duplicated elements. Link colour and thickness show BLASTP e-value and % identity based duplication confidence. Red thicker links joins genes coming from highly probable duplication events with e-values lower than 10^{-50} and more than 50 % identity, blue links joins genes coming from mid probable duplication events with e-values lower than 10^{-45} and more than 45 % identity and green links joins genes coming from low probable duplication events or ancient duplication events with e-values lower than 10^{-40} and more than 40 % identity.

Sequences belonging to Arabidopsis SnRK3 subfamily, characterized by the presence of a Ser/Thr kinase (PTHR24343), Ca-dependent protein kinase (PTHR24347), and NAF/FISL (IPR018451) domains, were not found in the Chlamydomonas genome (Supplementary Table S3).

The employment of the strategy described above over *Ostreococcus lucimarinus*, *Chlorella variabilis*, *Coccomyxa subellipsoidea*, *Volvox carteri*, and *Dunaliella salina*, showed divergent SnRK/CKIN family structures between land plants and microalgae (Supplementary Table S4). All studied species had one SnRK1 catalytic subunit, but had different elements belonging to SnRK2 and 3 subfamilies. Chlamydomonadaceae species lacked SnRK3 subfamily orthologs but had large and diverse SnRK2/CKIN2 (A) groups composed by CKIN2 proteins with uncharacterized insertions and long C-terminal extensions, and others similar to plant SnRK2s as it was observed in Chlamydomonas. Chlorella and Coccomyxa shared with Chlamydomonadaceae a SnRK2/CKIN2 (B) group and a reduced SnRK/CKIN2 (A) group making a SnRK2 subfamily more similar to Arabidopsis compared to Chlamydomonoceae. In turn, Chlorella and Coccomyxa had SnRK3 elements. Interestingly, Ostreococcus, the closer genome to land plants among the analyzed, contains only a small number of Arabidopsis-like sequences not including SnRK3, making the smallest SnRK families among the studied ones (Figure 3, Supplementary Table S4).

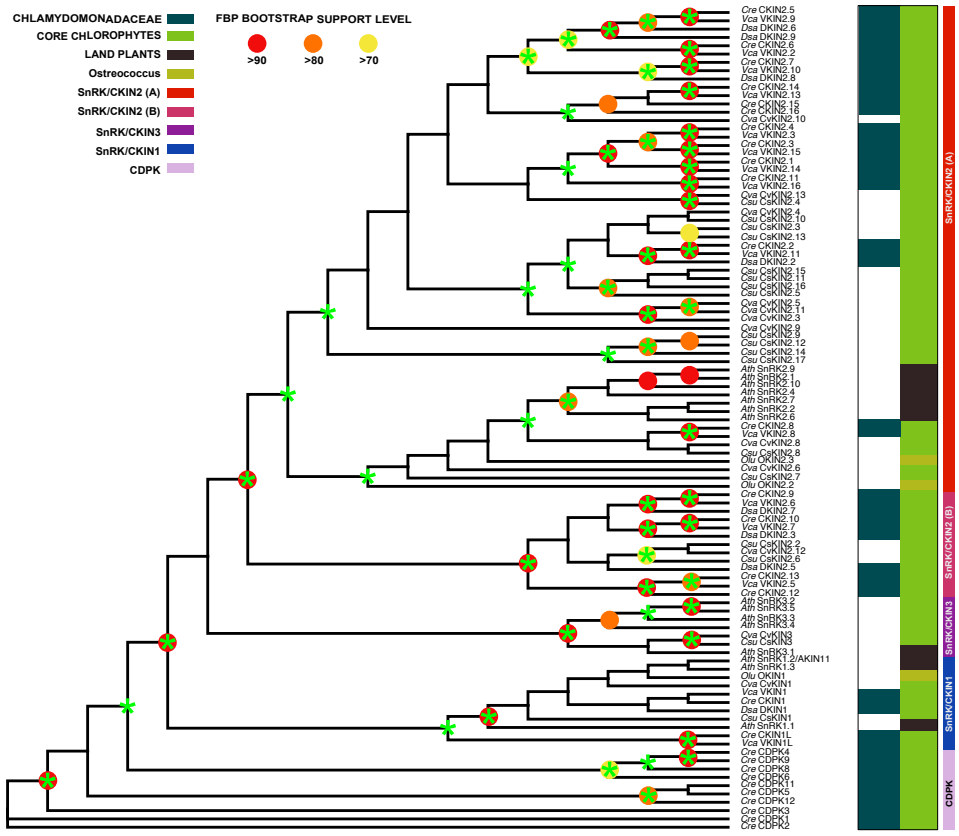


Figure 3: SnRK/CKIN family evolution between Chlorophyta and higher plants. TCS curated M-Coffee alignment based MS sequence tree. TBE bootstrap confidences over 80 % are marked with a green asterisk over nodes. *Chlamydomonas reinhardtii* (*Cre*) SnRK sequences were included along with *Arabidopsis thaliana* (*Ath*), *Chlorella variabilis* (*Cva*), *Coccomyxa subellipsoidea* (*Csu*), *Dunaliella salina* (*Dsa*), *Ostreococcus lucimarinus* (*Olu*) and *Volvox carteri* (Vca) orthologues. Tree arranged SnRK/CKIN sequences into 4 clusters, namely SnRK/CKIN3, SnRK/CKIN1, SnRK/CKIN2 (A) and SnRK/CKIN2 (B). Subfamily distribution between species shows SnRK3 restricted to land plants and basal core Chlorophytes. SnRK/CKIN2 subfamily resulted highly variable between species, from the reduced and close to land plants Ostreococcus subfamily to the diverse and large Chlamydomonadaceae subfamilies.

Moreover, the use of iTAK database(Zheng *et al.* 2016) and kinase classification(Lehti-Shiu & Shiu 2012) over all Chlamydomonas, Volvox, Dunaliella and Ostreococcus genomes showed similar grouping than proposed following our approach. SnRK1 sequences were classified as CAMK_AMPK and almost all found SnRK2 sequences fell into SNF like CAMK_OST1L kinase group. In Chlamydomonas, the divergent CKINL and CKIN1L sequences made an exception being classified respectively into CAMK_CAMKL-PASK-PIM and CAMK_Cr-1, close to SNF microalgae exclusive groups. Ortologs to these sequences were also found in Volvox, Dunaliella, and Chlorella.

Expression profiles of Chlamydomonas CKINs under abiotic stress

RNA was isolated from CC-503 strain after 48 h of exposure to different stressful situations described in Table 2. The application of these stresses significantly affected cell growth, reducing multiplication rate in all cases, except for phosphorous limitation and UV irradiation (Supplementary Figure S4). These results were complemented with a targeted analysis of available RNAseq datasets studying the response of this organisms to nitrogen (Miller *et al.* 2010; Boyle *et al.* 2012), sulphur (González-Ballester *et al.* 2010), iron and carbon dioxide (Fang *et al.* 2012) deficiencies and hydrogen peroxide-induced oxidative damages (Urzica *et al.* 2012; Fischer *et al.* 2012).

Table 2: Variations of the basal HAP culture media to test different abiotic stress conditions *in Chlamydomonas* CKINs.

Assay	Modification of basal media
Nitrogen deprivation (-N)	Substitution of ammonium chloride by potassium chloride.
Carbon deprivation (-C)	Removal of carbon sources from the media.
Sulphur deprivation (-S)	Substitution of $MgSO_4$, $ZnSO_4$, $FeSO_4$, and $CuSO_4$ by $MgCl_2$, $ZnCl_2$, $FeCl_2$, and $CuCl_2$.
Phosphorous limitation (5 % P)	Reduced phosphorous (5 % of the TAP medium).
Heat Stress (40 °C)	Increase incubator temperature to 40 °C.
Cold Stress (4 °C)	Decrease incubator temperature to 4 °C.
UV radiation Stress (UV)	30 minute UV irradiation each 24 h.
Salt stress (0.25 M NaCl)	Addition of 0.25 M sodium chloride to basal media.
Osmotic stress (20 % PEG)	Addition of 20 % (w/v) PEG 4000 to basal media.

The expression of Chlamydomonas CKIN genes greatly varied under the abiotic stresses tested, with different ranges of overexpression/repression in function of the analysed gene and stress (Figure 4, Supplementary Table S5). PEG-induced osmotic and UV stresses triggered the strongest responses of this family, with an average CKIN abundance increase of 3.9- and 4.5-fold respectively and compared to control. Contrarily, the transition from mixotrophic to autotrophic conditions, H_2O_2-induced oxidative damage, and nitrogen limitation did not induce overall abundance change of the CKIN family genes. Although this reflects the dynamics of the entire CKIN family under several stresses, it is more interesting to analyse the dynamics of specific CKIN genes to look for possible targets for future bioengineering studies.

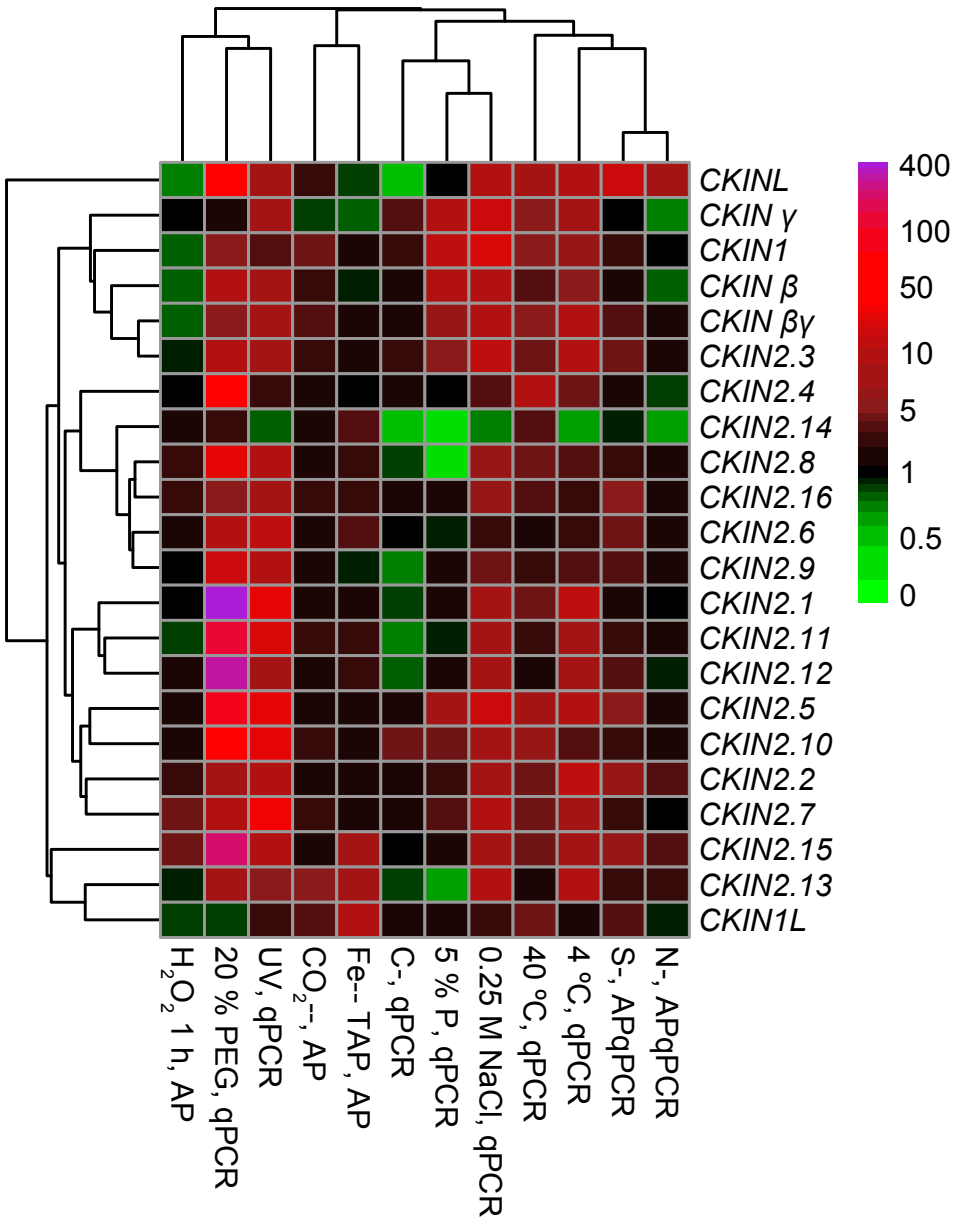


Figure 4: Heatmap and cluster representation of the changes in the abundance of CKIN family members under tested stress situations compared to control growth. Tree was built based on Euclidean distances and WPGMA aggregation method. Normalized fold-change values (z-scores) were used to avoid artefacts related to the different expression magnitudes of the members of this family. Legend: nitrogen (N-, APqPCR), carbon (C-, qPCR), and sulphur (S-, APqPCR) deprivation, phosphorous limitation (5 % P, qPCR) deprivations, heat and cold stress (40 °C, qPCR and 4 °C, qPCR, respectively), UV radiation stress (UV, qPCR), salt (0.25 M NaCl, qPCR) and osmotic (20 % PEG, qPCR). AP: expression data obtained from AlgaePath Database, qPCR: RT-qPCR dataset obtained in our laboratory.

CKIN genes also showed different ranges of response within each treatment, from the small abundance variations of *CKIN2.14* and *CKIN1L* (2.28- to -10-fold and 2.94- to -2.43-fold change; maximum and minimum change considering all stresses, respectively) to the strongest response of *CKIN2.1* and *CKIN2.12* (440- to -1.47-fold and 354- to -1.28-fold change respectively). Salt, temperature, UV and PEG-induced osmotic stress caused the overexpression of all genes of these family at different levels, except for *CKIN2.14*, downregulated under salt, low-temperature and UV stress and *CKIN1L*, downregulated by PEG. On the other hand, carbon limitation (both the reduction of available CO_2 and the transition from mixotrophic to autotrophic growth) led to the overexpression of genes belonging to the CKIN1 complex, *CKIN2.3*, *CKIN2.10* and *CKIN1L*, while nutrient deficiencies (S, N, P, Fe) induced gene-specific responses. Although Arabidopsis γ and AKIN10/11 or SnRK1.3 interaction have not been identified, Chlamydomonas CKIN γ (close to plant γ and Saccharomyces SDS23) showed a close expression pattern to CKIN1 and its regulatory subunits clustering in the same group. Moreover, *CKINL* differential expression pattern under tested stresses supported its exclusion from the CKIN2 subfamily.

Changes in CKIN expression in response to ABA

Due to the importance of ABA in SnRK signalling in other biological systems we tested the expression levels of all CKIN genes after 48 h of culture in basal media supplemented with 500 μM ABA following Yoshida et al. (Yoshida *et al.* 2003) or 50 μM fluridone. The expression of 6 out of 22 CKIN genes were affected under one or both culture conditions (Figure 5). *CKIN2.14* was upregulated under ABA treatment but downregulated with fluridone. *β subunit*, *CKIN2.1*, *2.2*, and *2.10* were upregulated with fluridone, whereas only ABA addition upregulated *CKIN2.5*.

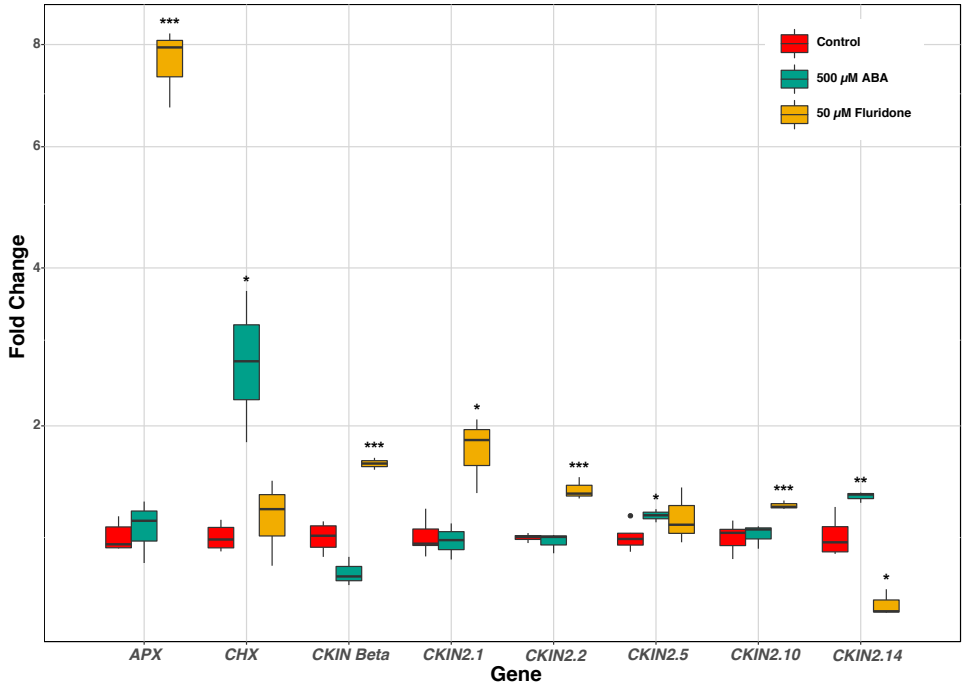


Figure 5: Expression change of CKINs after 48 h of culture in 500 µM ABA or 50 µM fluridone. Carotene hydroxylase (*CHX*), an ABA inducible gene, and Ascorbate peroxidase (*APX*), an oxidative stress inducible gene, were included in the analysis to test the effectiveness of the treatment. This plot only shows the 6 CKINs that were responsive to either ABA or fluridone treatment (ANOVA, *p<0.05, **p<0.01, ***p<0.001, n=6). RT-qPCR data was processed with EasyqPCR package according to Hellemans et al (Hellemans et al., 2004).

STRING and ChlamyNet based CKINs interaction and co-expression networks

CKINs showed to be a part of a complex interaction network, as shown into the STRING(Szklarczyk *et al.* 2015) and ChlamyNet(Romero-Campero, Perez-Hurtado, Lucas-Reina, Romero & Valverde 2016) databases. STRING encompasses a collection of predicted and experimentally proven protein-protein interactions in Chlamydomonas and other species. The Chlamydomonas CKINs present in the STRING database turned out to be associated between them and with several biochemical and regulatory pathways (Figure 6; Supplementary Figure S5). Three main CKIN clusters were defined based on the interactions observed. The first one, the hub of this network, includes CKIN α, β, and βγ subunits of the CKIN1 complex. This cluster is directly linked to CKIN2, carbon and nitrogen metabolism, and

mRNA splicing. In addition, it was indirectly linked through Ca-dependent mechanisms to autophagy and DNA remodelling and maintenance mechanisms. No interactions were found for the non-interacting CKIN γ subunit. Second and third CKIN clusters comprise CKIN2 subfamily, being linked to different Protein Phosphatase 2 C (PP2C) family phosphatases. CKIN2.4, 2.9, 2.13, 2.16, and CKINL only showed interaction with PP2CF, PP2C3, and A8IIX1 while the other set of CKIN2 and CKIN1L was also interacting with 9 other PP2C and related phosphatases. ChlamyNet database comprises a Chlamydomonas transcript correlation network. Eight CKINs were identified in this database being part of several clusters (Supplementary Figure S6). CKIN β, CKIN2.12, and CKIN1L were into the same group, directly connected to carbon (macromolecule and hexose metabolism, photosynthesis) and nitrogen metabolism (amino acid metabolism and protein turnover). CKIN2.6 formed a second cluster directly related with autophagy elements. CKIN γ conformed the third major cluster interacting with transcriptional regulation elements, autophagy and protein folding/assemblage. CKIN 2.3 and 2.16 were also related to transcriptional regulation and CKIN 2.7 to autophagy, heat response and protein folding.

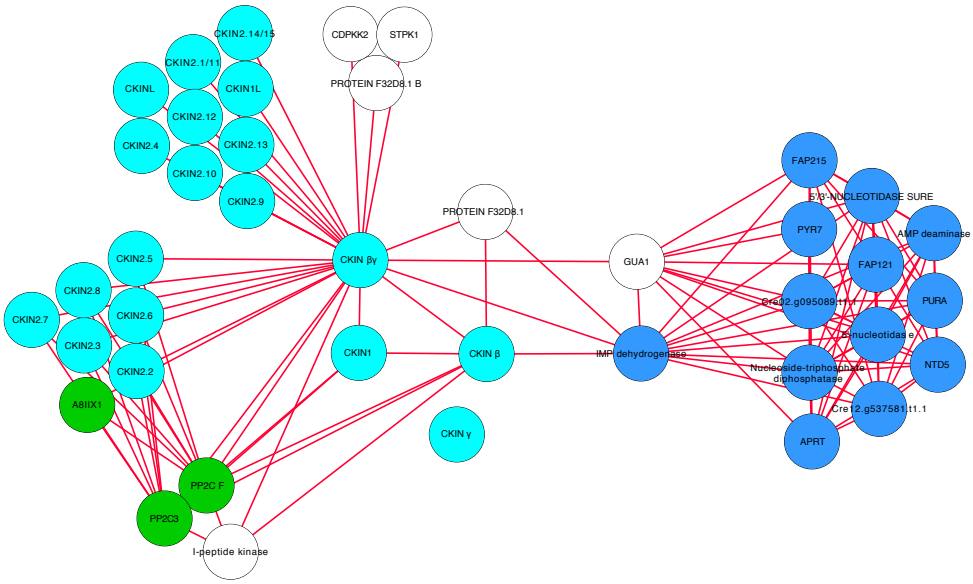


Figure 6: High confidence interaction network of CKIN proteins. CKINs (light blue) interact between them and with major regulatory groups according to KEGG classification: hormone-signalling response (green), nitrogen acquisition and derived pathways (dark blue). White nodes represent bridge proteins.

Other proteomic and metabolomic interaction networks, as the Chlamydomonas nitrogen starvation and recovery response STRING based network presented by Roustan et al.,(Roustan, Bakhtiari, Roustan & Weckwerth 2017) and the nitrogen starvation response sPLS correlation network published by Valledor et al.(Valledor *et al.* 2014b), were surveyed for CKIN or CKIN related elements. Although no CKINs were identified into the first network, interactants as the flagellar associated protein (FAP121) and the protein kinase CDPKK2 were shared between CKIN and nitrogen stress response network. The second network only included CKIN γ.

IV. Discussion

The SnRK family is conserved in all eukaryotes, as SnRK1/SNF1/AMPK/CKIN1, functioning as a sensor of cell energetic status (Halford *et al.* 2004; Li, Peng, Zhang, Shi & Wang 2010; Nukarinen *et al.* 2016). In plants, this family evolved into two more subfamilies, 2 and 3, which are key players in ABA-dependent and -independent stress response mechanisms (Zhang, Mao, Wang & Jing 2010; Fujii *et al.* 2011; Coello *et al.* 2011). In Chlamydomonas, SnRK/CKINs were initially described under sulphur deprivation (Davies & Grossman 1999), and later related to a wide range of stress responses (Gonzalez-Ballester *et al.* 2008; González-Ballester *et al.* 2010; Valledor *et al.* 2013, 2014b) and indicated as potential targets for increasing the production of industry-demanding biomolecules (Sato *et al.* 2014). However, these studies focused on the functional characterization of some CKINs rather than defining the whole family, explaining why only 10 CKINs were described in Chlamydomonas so far, when in land plants like Arabidopsis this family comprises 38 members (Hrabak *et al.* 2003).

Mining Chlamydomonas genome using homology searches allowed the definition of a large number of candidate genes, most of them only homologous to the Ser/Thr kinase domain. Furthermore, CKIN and CDPK protein families showed great similarity, being the absence of a Ca-binding EF-hands domain the key to distinguish these families (Hrabak *et al.* 2003). In

consequence, all BLAST hits were later curated by protein domain analyses. This strategy was later expanded to a domain-based genome mining looking for sequences containing all the domains required for a protein to be classified as CKIN. This proved to be useful allowing the incorporation of two novel CKIN sequences (*CKIN2.12*, and *CKIN2.15*) otherwise lost during data mining.

Overall, in silico genomic analyses resulted in the characterization of 21 proteins belonging to the CKIN family. As this family has not been deeply studied in other algae, comparisons are difficult to perform but recently, one of these analyses described only 2 elements belonging to SnRK1/CKIN1 subfamily in *Volvox carteri* (Wu, Wang, Duan, Li & Hou 2017). Out of these, only one sequence can be undoubtedly classified as SnRK1, since its kinase, UBA, and KA1/αCTD domains are highly conserved across evolution (Hardie 2007). On the other the other sequence cannot be assigned to this group since regulatory residues were not present, being more similar to CKIN1L. Conversely, any SnRK2/CKIN2 elements were described in this species. The application of the described above workflow to the analysis of Volvox, Dunaliella, Coccomyxa, Chlorella and Ostreococcus genomes allowed the definition of progressively larger and more complex SnRK2s subfamilies as the species were closer to Chlamydomonas. Identified SnRK kinase members in Chlamydomonas, Volvox, Dunaliella and Ostreococcus were identified as such into the iTAK database (Zheng *et al.* 2016) supporting Chlamydomonas CKINL exclusion. These analyses validated the method capabilities to discover new SnRK/CKINs, highlighting the need of specific methodologies beyond homology searches, such as protein domain analyses, for the fully characterization of a protein family when dealing with families showing atypical evolution such as CKIN2 subfamily in algae (Kulik, Wawer, Krzywińska, Bucholc & Dobrowolska 2011). Interestingly, from the genomes analysed, only land plants and humans had more than one SnRK1. SnRK1/AMPK duplication is common between plants and vertebrates whereas no more than one Snf/CKIN1 subfamily member has been identified in fungi and invertebrates(Roustan *et al.* 2016). This suggests a probable complexity related feature. Vertebrates and plants needed to diversify this regulatory

mechanism to cope with their more complex life cycles and somatic structures, or more stringent environments(Buitink, Thomas, Gissot & Leprince 2004; Fragoso *et al.* 2009). This increasing somatic/environmental complexity fits the functional diversification found in some land plants and vertebrates SnRK1/AMPKs. Supporting this is the Arabidopsis SnRK1.3 reproductive tissues focused expression(Schmid *et al.* 2005), same species AKIN10/11 differentiated response under stress(Fragoso *et al.* 2009) and muscle/liver focused expression of human AMPK α2 which is related to osteogenesis and adipogenesis(Wang *et al.* 2016).

On the other hand, and despite the conservation of SRK2 Serin/Threonin kinase domain of the CKIN2 subfamily, more efforts are needed to be paid to unequivocally classify these elements. If CKIN2 subfamily originated from duplication and divergence of CKIN1, a common ancestor or "founder like" to all SnRK2/CKIN2s would diverse this subfamily in different groups ranging from CKIN1 to land plants SnRK2s. Both CKIN2 subgroups (A/B) were conserved within core Chlorophytes, while those closer to SnRK1, CKIN2 (B), were lost or changed in land plants. SnRK2/CKIN2 (A), including plant SnRKs, was larger and more diverse in core Chlorophytes, with almost all this sequence diversity being Chlamydomonadaceae exclusive. In Chlamydomonas, CKIN2.2 and 2.8 can be considered the common ancestors of most of its CKIN2 (A) sequences, despite some ancestor of this group long CKIN2s may be lost along CKIN2 (B) group ancestor. Furthermore, despite being present in Chlorella and other Chlorophytes, this ancestor CKIN2 (B) group has been lost in Ostreococcus, exhibiting only plant-like CKIN2s, this may also be happening due to its reduced genome size. Although atypical and different from land plants, algal CKINs are supposed to be engaged in most of the functions land plants SnRKs are involved. This is sustained by the gene balance hypothesis, which explains the conservation observed, predicting that transcription factors and proteins belonging to signalling networks are more likely to be retained (Birchler & Veitia 2010).

In plants, SnRK1-based sugar signalling acts as a master regulator of carbon/energy ratios and is strongly linked to hormonal regulation and

different signal transduction pathways (Kleinow *et al.* 2000; Jossier *et al.* 2009). In Chlamydomonas this complex resulted to be very sensitive to most of the imposed stresses. However, the suppression of carbon in culture media did not caused the highest overexpression rates of this complex, being more responsive to salt and extreme temperatures. The damages that these stresses represent to algae causing an energy shock are probably greater than those from removing acetate from media (that will still interchange CO_2 with air). The metabolic remodeling required to survive under extreme conditions mediated by SnRKs has been reported in different plant systems (Valledor *et al.* 2013; Baena-Gonzalez & Hanson 2017), being the interaction of the SnRK1 complex with specific transcription factors and kinases a basic mechanism to trigger proteome and metabolome remodelling. CDPKK2, a Chlamydomonas ortholog to Arabidopsis GRIK1, known for activating the land plant SnRK1(Shen, Reyes & Hanley-Bowdoin 2009) is one of the kinase intermediaries highlighted into presented STRING based SnRK network. The kinase was also present as central node into Chlamydomonas nitrogen depletion and recovery stress network(Roustan *et al.* 2017) supporting the complex activity under abiotic stresses other than carbon depletion.

Conversely to CKIN1, CKIN2 proteins were more difficult to classify due to its partial divergence to its land plant counterparts. Previously described CKIN2 elements, falling all into the CKIN2 (A) group, exhibited a high similarity to its Arabidopsis SnRK2s orthologs. On the contrary, newly discovered elements, all CKIN2 (B) sequences and the Chlamydomonadaceae exclusive SnRK2 (A) sequences, were more dissimilar. However, all Chlamydomonas CKINs exhibited a Ser/Thr kinase domain and, in the case of CKIN2 (A) sequences a land plant-like regulatory domain I which is less conserved in Chlamydomonadaceae sequences CKIN2.11 and CKIN2.15-16. This domain is related to osmotic and salt stress responses in plants (Yoshida *et al.* 2006; Kulik *et al.* 2011). The domain I function seems to be mostly conserved in Chlamydomonas, as all elements containing it, except CKIN2.14, were overexpressed under hyperosmotic and saline stresses. Interestingly CKIN2 (B) group sequences exhibited a conserved region after kinase domain which is different than land plants domain I. CKIN2 (B) sequences and CKIN2

(A) CKIN2.11 and CKIN2.15 were also overexpressed under hyperosmotic and saline stresses probably due to the existence of an algae specific domain and other sensing/signalling mechanisms respectively.

The presence of different regulatory sequences, some of them elongated, may explain the differential classification of CKIN2 after M-Coffee analysis, which focused on most conserved regions. CKIN2 (A) grouped the closest sequences to the common land plant-algae ancestor and all Chlamydomonadaceae exclusive ones. Some of these sequences were almost identical in length and sequence to Arabidopsis while most, including the closest Chlamydomonas CKIN2 to Arabidopsis SnRK2s (CKIN2.8), had long C-terminal ends and/or extra sequence loops. These features are probably related to a multiple environmental stress response capacity. CKIN2 (A) sequences have a heterogeneous response pattern under different stresses. Besides this, their expression levels under sulphur or nitrogen starvation, phosphorous limitation and oxidative stress were higher than CKIN2 (B) sequences

CKIN2 (B) contained core Chlorophyte exclusive sequences. Sequences of this smaller group are more homogeneous, with a conserved region after kinase domain in place of regulatory domain I and long C-terminal ends (excepting CKIN2.9). Within the CKIN2 (B) cluster, CKIN2.13 and 2.10 were the most overexpressed CKIN2 under carbon starvation and autotrophic growth respectively, surpassing CKIN1 complex. This correlates this CKIN2 (B) "ancestor" group sequences with an energy stress related function commonly associated to CKIN1 complex.

Besides differences, CKIN2 (A/B) sequences share the osmotic/UV responsiveness, excepting CKIN2.14 and 2.4, CKIN1L, and CKIN γ. CKIN2s as *CKIN2.1*, *2.12*, and *2.15* were strikingly upregulated (c.a. 400-fold) under osmotic stress.

Response heterogeneity and group size makes difficult to propose a common regulatory mechanism for CKIN2 (A). Moreover, despite they share with CKIN2 (B) and land plants a well conserved kinase-dependent activation loop (Boudsocq, Droillard, Barbier-Brygoo & Laurière 2007; Vlad *et al.* 2010), most

of its sequences also display extra sequence loops/elongations whose function is probably diverse. Furthermore, the presence of phosphorylation sites, coiled-coil protein interaction motifs, glycine and glutamine rich regions in CKIN2 (A) sequences points to a probable CKIN2 regulatory function. Plant SnRK2 protein ends usually have an important role in their regulation trough phosphorylation (Belin *et al.* 2006). Moreover, coiled-coil structures, know by their function in protein-protein interactions (Burkhard, Stetefeld & Strelkov 2001), hold different kind of protein interactions in plant SnRKs, such as the interaction with PP2Cs (Nolan, Saeed & Rose 2006). In addition to the activation loop and these C-terminal potential regulatory regions, the regulation of SnRK2 in Arabidopsis requires the presence of two regulatory domains. All CKIN2 (A) had a domain I with different degrees of degeneration, but domain II like regulatory sequences could only be recognized into CKIN2.2 and CKIN2.6-2.8, the closest CKIN2s to land plants SnRK2 subfamily.Although regulatory domain II is characteristic of some CKIN2 (B), these sequences are shorter that those present in land plants. In land plants this domain, with its characteristic acidic patch, is present in highly ABA responsive elements, being named ABA box, despite some SnRK2s can respond to ABA without this structure (Kobayashi, Yamamoto, Minami, Kagaya & Hattori 2004; Yoshida *et al.* 2006; Kulik *et al.* 2011).

Interestingly, *CKIN2.14* and *CKIN2.5* were the only responsive elements to exogenous ABA, regardless of not having acidic patches in their sequences. ABA regulatory mechanisms are well described in land plants, where the acidic domain II mediates the inactivation of the kinase through a SnRK2-PP2C interaction (Belin *et al.* 2006). ABA, interacting with PYR /PYL/RCAR ABA receptors inhibits PP2C, allowing the kinase activation (Santiago *et al.* 2009; Yin *et al.* 2009). Although all of these elements are not present in microalgae or have a very low similarity to higher plants (Lu & Xu 2015) (Hauser *et al.* 2011), string-based protein-protein analyses demonstrated the interaction between SnRK2s and PP2C, protein phosphatase $Mg2+/Mn2+$ dependent (PPM) and Protein phosphatase type 1 isoform K (PP1K) phosphatases, and CAB and CDP kinases, all core players in ABA signalling (Minkoff, Stecker & Sussman 2015). Therefore, *CKIN2.14* and *2.5* might be elements in an ABA

regulatory pathways in Chlamydomonas, highlighting the need of novel SnRK-PP2C interaction sequences and alternative ways in which ABA breaks this interaction. This hypothesis is also supported by the effect of exogenous application of fluridone, which increased the expression of *CKIN2.1*, *2.2*, *2.5* and *2.10* despite it can also be considered as an oxidative stress induction since CKIN β subunit was also induced.

Results clearly showed low ABA signalling dependence of CKIN2s in Chlamydomonas. In lesser extent, ABA-independent regulation of SnRKs occurs in land plants, as in the case of Arabidopsis *SnRK2.1*, *2.4*, *2.5*, *2.9* and *2.10,* induced by osmotic stress following an ABA-independent pathway (Kulik *et al.* 2011). Arabidopsis ABA-independent SnRKs regulate transcripts of stress related genes under hyperosmotic conditions, thus complementing ABA-dependent SnRK2s function (Soma *et al.* 2017). In Chlamydomonas, ABA-independent CKIN2s were also responsive to osmotic stress, as in land plants. But contrary to plants, specific responses to low nitrogen (Valledor *et al.* 2014b) or sulfur deprivation (Gonzalez-Ballester *et al.* 2008) seem to be triggered in an ABA-independent manner. These results support the idea that the plant-specific SnRK2/CKIN2 subfamily plays a crucial role in stress response signaling both in Arabidopsis and Chlamydomonas.

These mechanisms are not entirely related to energy-saving decisions, but lead to a complex remodelling of cell metabolism, as demonstrated by the interactions with DNA repair and maintenance pathways and TOR in Arabidopsis (Shen *et al.* 2007), and in Chlamydomonas to a similar and complex interaction network as it was proposed by STRING and ChlamyNet analyses. The fact that the expression of a large number of CKIN2s is induced by a single stress suggests a great compensatory effect or pleiotropy within this family in Chlamydomonas. It is well-known that the consequence of most stresses is oxidative damage and/or low energy syndrome (Kobayashi *et al.* 2005; Shin, Alvarez, Burch, Jez & Schachtman 2007) but it is striking the high number of elements triggered in Chlamydomonas under most of the studied stresses given their low intensity. On the other hand, the high responsiveness to osmotic, salt, and UV stresses of this family is crucial for a freshwater alga

in natural environments, since they cannot control neither water quality nor environmental UV irradiation. Thus, a fast, flexible, and efficient mechanism is required to ensure algae survival under unfavourable environmental conditions.

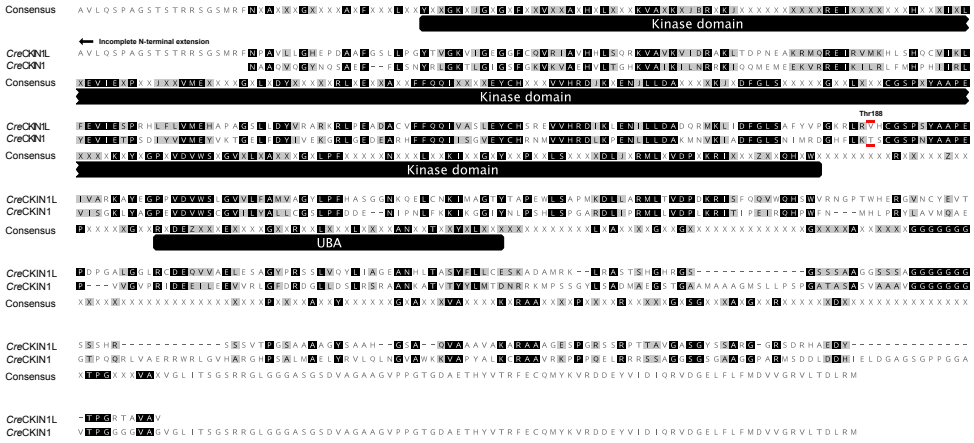
The apparent lack of specificity of CKIN confronts to other plant systems, with elements with very specific functions and stress responsiveness. Land plants also had a third SnRK subfamily, comprising proteins kinases interacting with calcineurin B-like calcium binding domains (Harmon 2003; Kulik *et al.* 2011), mostly involved in drought and salt resistance, being the SOS (salt overly sensitive) the best-known mechanism (Ji *et al.* 2013). The characteristic NAF/FISL (Hrabak *et al.* 2003) domain of SnRK3 is not present in Chlamydomonas, but Chlorella and Coccomyxa have one element. This suggests that SnRK3 is not only characteristic of land plants but is also present in the last common ancestor and lost in some core Chlorophytes. Homologs of this sequence were not found in Ostreococcus (the closest microalgae to land plants) but this would be easily explained by its reduced genome. Moreover the reduced SnRK3 subfamily size in microalgae makes easy their lost through mutation, being probably compensated in their function by SnRK2s, specific CDPK proteins (Hamel, Sheen & Séguin 2013) or by SNF/SKP1/Ubiquitin ligase complexes, already identified as key elements of hormone, sugar, and stress responses (Farrás *et al.* 2001). In line with this, Chlamydomonas, Volvox, and Dunaliella have larger and more diverse SnRK2/CKIN2 subfamilies than Chlorella and Coccomyxa, both containing one SnRK3 element.

V. Conclusion

The genome-wide approach used in this work over Chlamydomonas for the identification of its CKIN family have completed previous work in this species while extending it to other microalgae. This kinase family description has shed light to its unique structure and sequence features in Chlamydomonadaceae, highlighting the conserved abiotic stress sensitivity of the Chlamydomonas members. Thus, paving the way for the description of novel CKIN and

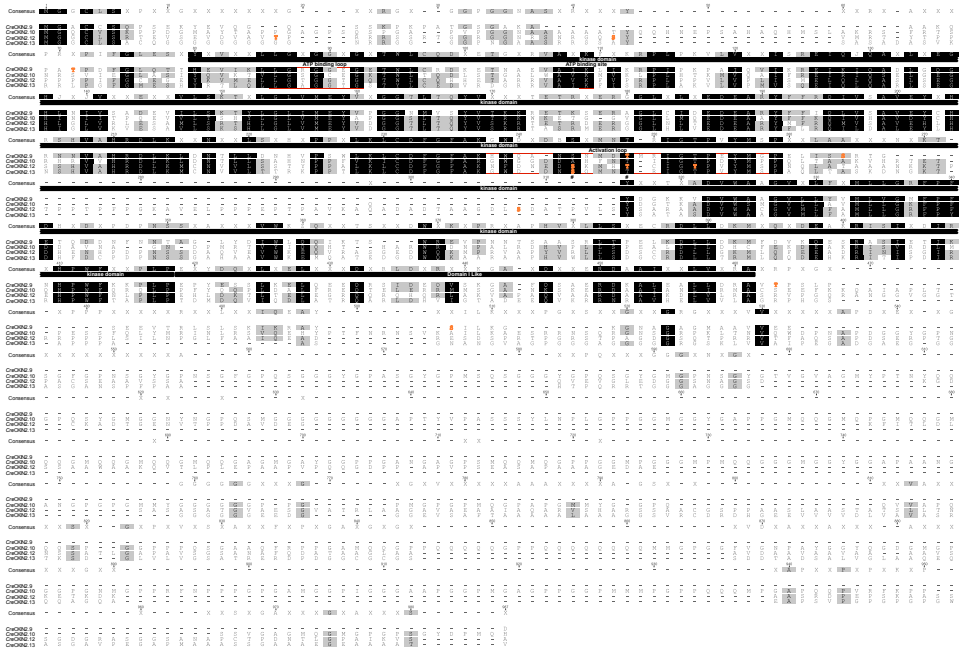
probably ABA mediated stress responsive pathways in microalgae. The shared and unique core Chlorophytes SnRK/CKIN family structure make Chlamydomonas a suitable system for novel stress response mechanisms identification and a better fitted model than land plants for the identification of algae specific targets for biofuels and secondary metabolites production enhancement in these species.

VI. Supplementary figures and tables

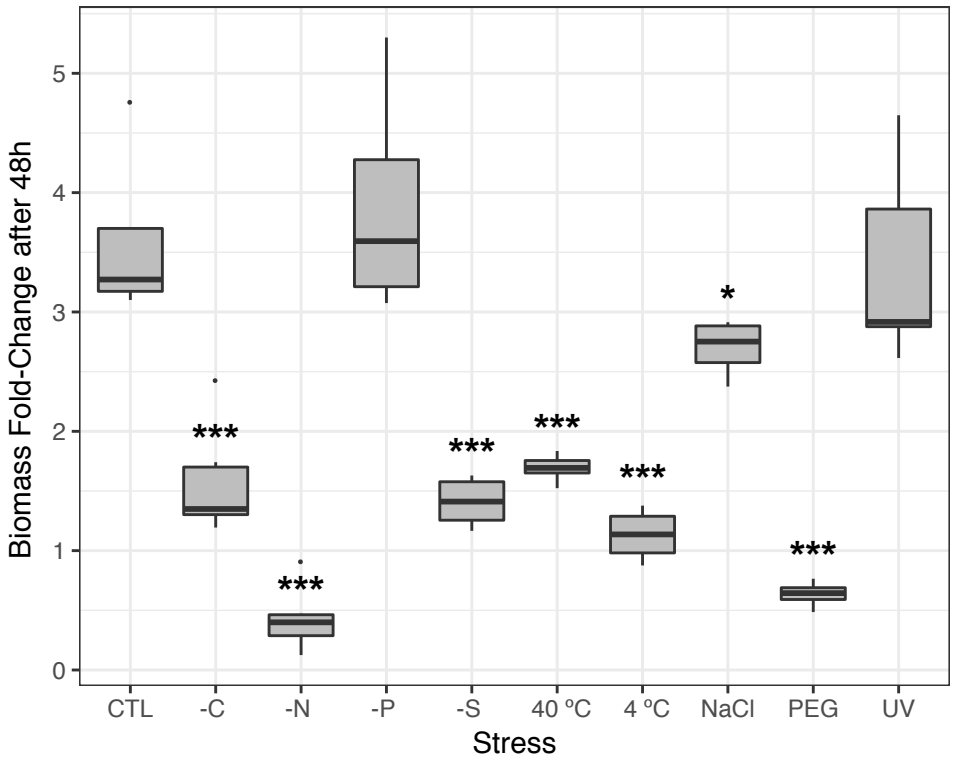


Supplementary Figure S1: Muscle based sequences structural analysis of *Chlamydomonas reinhardtii* (*Cre*) CKIN1 and CKIN1L. Both sequences share a highly analogous kinase domain followed by a shared UBA domain. CKIN1L has a long uncharacterized N- terminal region (not shown) that does not align with SnRK1' (¬).

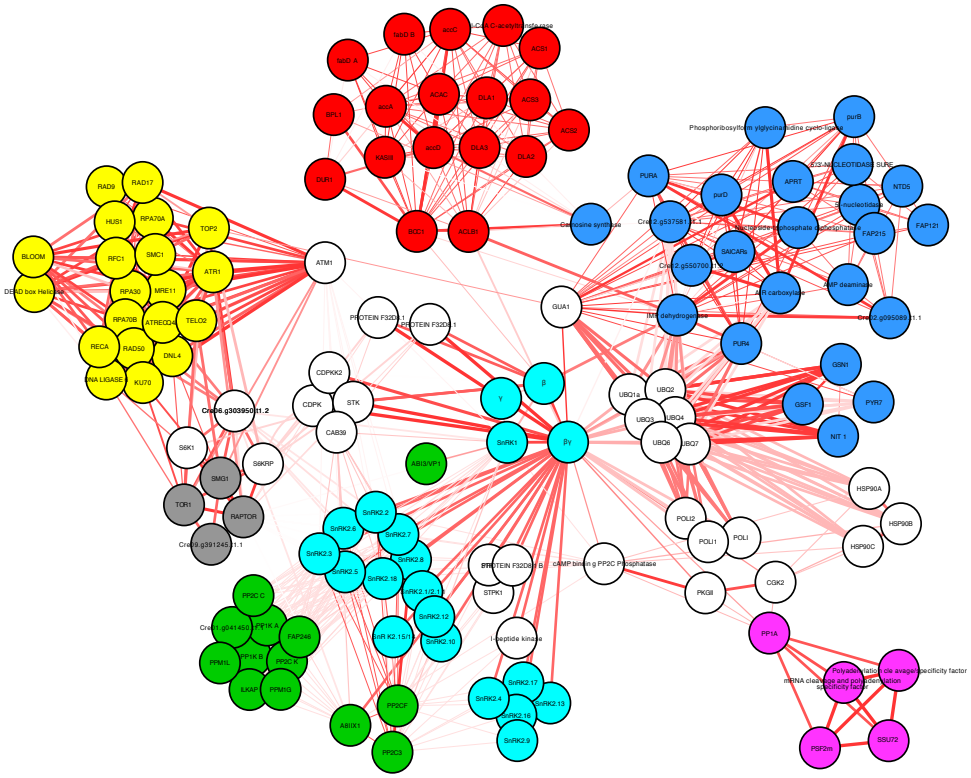
Supplementary Figure S2: M-Coffee based sequence structural analysis of Chlamydomonas (*Cre*) CKIN2 (A) group. CKIN2.4 and 2.11 show extra sequence loops and along CKIN2.16 long C-terminal ends. CKIN2.15 have a short N-terminal region. CKIN2.4 and 2.14 share predicted coiled-coil regions (rectangled) placed in CKIN2.4 into kinase domain inclusions and in CKIN2.14 in their C- terminal tail. Conserved ATP binding loop/site, and activation loop are marked. Within activation loop, conserved Serin/Threonin activation residues are highlighted (#). Phosphorylated S or T sites are marked in bold orange characters. Domain II like acidic patches into CKIN2.2 and CKIN2.6-8 are highlighted in pink.



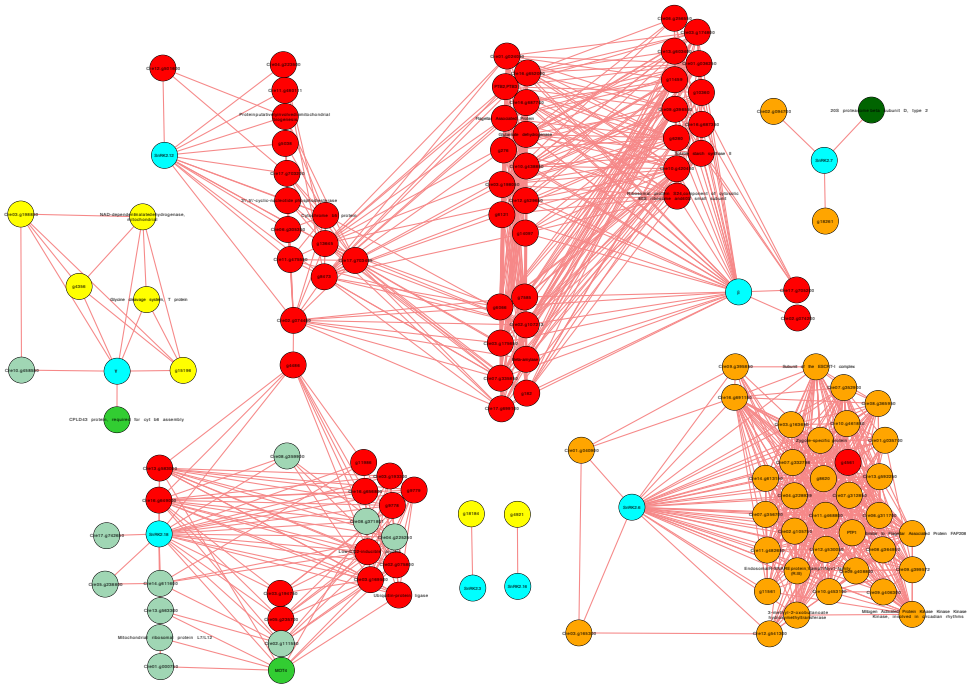
Supplementary Figure S3: M-Coffee based sequence structural analysis of Chlamydomonas CKIN2 (B) group. All sequences shared kinase domain sequence and a conserved structure (Domain I like) after the catalytic domain. Conserved ATP binding loop/site, and activation loop are marked. Within activation loop, conserved Serin/Threonin activation residues are highlighted (#). Phosphorylated S or T sites are marked in bold orange characters. These terminal regions were also rich in glycine residues in most sequences.



Supplementary Figure S4: Boxplot representation of the changes in Chlamydomonas biomass after stress assays. Legend: Control (CTL), Nitrogen (-N), Carbon (-C), and Sulphur (-S) deprivation, Phosphorous limitation (5 % P) deprivations, heat and cold stress (40 °C and 4 °C, respectively), UV radiation stress (UV), salt (0.25 M NaCl) and osmotic (20 % PEG). Gravimetrical measures of pellet weight at the stress phase (48 h) were normalized by its corresponding controls (0 h). Significant changes induced by stress were marked as * (t-test, $p<0.05$) or *** (t-test, $p<0.001$).



Supplementary Figure S5: STRING based Interaction networks of CKIN proteins. CKINs (**cyan**) interact with major regulatory groups according to KEGG classification: hormone-signalling response (**green**), autophagy (**grey**), DNA repair/transcription (**yellow**), nitrogen acquisition and derived pathways (**dark blue**), carbon homeostasis (**red**), mRNA splicing (**purple**). White nodes represent bridge proteins, mainly Ca-dependent signalling-related, heat shock, transport, or ubiquitin proteins. Edge thickness represent the combined interaction score provided by STRING-db (confidence range 0.7-0.999) while edge color intensity shows the experimental evidence of the interactions (confidence range 0.131-0.989).



Supplementary Figure S6: ChlamyNet based interaction network of CKIN proteins. CKINs (**cyan**) interact with major regulatory groups according to GO classification: Protein complex assembly and response to misfolded protein (**green**), response to heat and misfolded protein (**dark green**), protein phosphorilation, ribosome biogenesis and macromolecule biosynthesis (**red**), autophagy, GTPase activity and vesicle mediated transport (**orange**), chromatin organization and postranscriptional organization (**yellow**), photosynthesis and hexose metabolic process (**turquoise**).

Supplementary Table S1. *Arabidopsis thaliana* (*Ath*) and *Chlamydomonas reinhardtii* (*Cre*) query sequences used for BLAST homology search to identify putative CKIN sequences into the *C. reinhardtii* genome. Names and accessions (ID) were indicated below. *Chlamydomonas sequences previously referred to as CKIN family by [1] Valledor et al. (2013) and SnRK2 subfamily by [2] Gonzalez-Ballester et al. (2008).

Name	*Ath/Cre* ID	Name	*Ath/Cre* ID	Name	*Ath/Cre* ID
AKIN10	AT3g01090	SnRK3.3	AT4g14580	SnRK3.23	AT1g30270
AKIN11	AT3g29160	SnRK3.4	AT5g57630	SnRK3.24	AT5g10930
β1	AT5g21170	SnRK3.5	AT5g45810	SnRK3.25	AT5g25110
β2	AT4g16360	SnRK3.6	AT5g45820	CKIN1*[1]	Cre04.g211600.t1.1
β3	AT2g28060	SnRK3.7	AT2g34180	CKIN γ*[1]	Cre12.g528000.t1.2
βγ	AT1g09020	SnRK3.8	AT5g58380	CKIN2.1*[2]	Cre02.g075850.t1.1
γ1	AT3g48530	SnRK3.9	AT4g18700	CKIN2.2*[2]	Cre12.g499500.t1.1
γ2	AT1g69800	SnRK3.10	AT3g23000	CKIN2.3*[2]	Cre02.g075900.t1.1
SnRK2.1	AT5g08590	SnRK3.11	AT5g35410	CKIN2.4*[2]	Cre11.g477000.t1.2
SnRK2.2	AT3g50500	SnRK3.12	AT1g01140	CKIN2.5*[2]	Cre03.g209505.t1.1
SnRK2.3	AT5g66880	SnRK3.13	AT4g24400	CKIN2.6*[2]	Cre11.g481000.t1.2
SnRK2.4	AT1g10940	SnRK3.14	AT4g30960	CKIN2.7*[2]	Cre06.g292700.t1.2
SnRK2.5	AT5g63650	SnRK3.15	AT5g01820	CKIN2.8*[2]	Cre10.g466350.t1.1
SnRK2.6	AT4g33950	SnRK3.16	AT3g17510		
SnRK2.7	AT4g40010	SnRK3.17	AT2g26980		
SnRK2.8	AT1g78290	SnRK3.18	AT2g25090		
SnRK2.9	AT2g23030	SnRK3.19	AT2g38490		
SnRK2.10	AT1g60940	SnRK3.20	AT1g29230		
SnRK3.1	AT5g01810	SnRK3.21	AT1g48260		
SnRK3.2	AT5g07070	SnRK3.22	AT2g30360		

Supplementary Table S2. Uncurated BLAST results for *Chlamydomonas* protein. Sequences were considered homologous for e-values lower than 10^{-25}.

Cre Accession	e-value	*Ath* Accession	*Cre* Accession	e-value	*Ath* Accession
Cre10.g457500.t1.1	1.86e-69	AT4G16360.1	Cre09.g400330.t1.1	1.91e-37	AT1G30270.1
Cre01.g001800.t1.1	3.71e-32	AT2G34180.1	Cre09.g400330.t2.1	1.96e-37	AT1G30270.1
Cre01.g003524.t1.1	7.85e-37	AT4G24400.2	Cre09.g412750.t1.2	1.91e-43	AT2G30360.1
Cre01.g009500.t1.2	1.90e-46	AT5G35410.1	Cre10.g428650.t1.2	1.16e-45	AT5G25110.1
Cre01.g010000.t1.2	1.31e-29	AT5G39440.1	Cre10.g443300.t1.2	5.71e-29	AT3G01090.1
Cre01.g016570.t1.1	1.49e-29	AT2G25090.1	Cre10.g457700.t1.2	6.47e-66	AT3G01090.1
Cre01.g029500.t1.2	1.47e-27	AT2G26980.3	Cre10.g463500.t1.1	3.60e-27	AT5G07070.1
Cre01.g029850.t1.2	3.03e-37	AT4G24400.2	Cre10.g464100.t1.2	6.14e-33	AT5G25110.1
Cre01.g034900.t1.2	2.16e-30	AT3G29160.1	Cre10.g465900.t1.2	6.17e-31	AT4G18700.1
Cre01.g036650.t1.1	3.25e-32	AT3G01090.1	Cre10.g466350.t1.1	2.96e-152	AT4G33950.1
Cre01.g036700.t1.2	3.30e-30	AT3G01090.1	Cre11.g467568.t1.1	8.50e-36	AT1G29230.1
Cre01.g037100.t1.1	6.68e-34	AT1G30270.1	Cre11.g477000.t1.2	2.35e-29	AT5G63650.1
Cre01.g048650.t1.1	9.37e-35	AT3G01090.1	Cre11.g481000.t1.2	1.91e-88	AT4G33950.1
Cre02.g074370.t1.2	2.51e-41	AT4G18700.1	Cre12.g484350.t1.1	2.88e-52	AT1G09020.1
Cre02.g075350.t1.2	3.38e-33	AT3G29160.1	Cre12.g485600.t1.2	5.64e-47	AT1G78290.2
Cre02.g075350.t2.1	2.12e-33	AT3G29160.1	Cre12.g486350.t1.1	9.55e-38	AT3G01090.1
Cre02.g075350.t3.1	3.38e-33	AT3G29160.1	Cre12.g493250.t1.2	8.45e-34	AT2G30360.1
Cre02.g075850.t1.1	1.33e-57	AT4G33950.1	Cre12.g498650.t1.2	4.16e-28	AT3G01090.1
Cre02.g075850.t2.1	1.06e-53	AT4G33950.1	Cre12.g499500.t1.1	4.02e-117	AT1G78290.2
Cre02.g075900.t1.1	6.60e-73	AT5G66880.1	Cre12.g499850.t1.1	1.48e-37	AT5G25110.1
Cre02.g076000.t1.2	1.07e-40	AT5G08590.1	Cre12.g508900.t1.2	4.55e-31	AT3G01090.1
Cre02.g076900.t1.1	5.58e-45	AT3G23000.1	Cre12.g509000.t1.1	4.89e-32	AT3G01090.1
Cre02.g106650.t1.1	3.39e-44	AT2G26980.3	Cre12.g514650.t1.2	6.81e-35	AT1G30270.1
Cre02.g107800.t1.1	2.45e-32	AT3G01090.1	Cre12.g527000.t1.2	4.34e-39	AT5G25110.1
Cre02.g112333.t1.1	1.51e-30	AT2G30360.1	Cre12.g529550.t1.2	1.63e-36	AT1G30270.1
Cre02.g113600.t1.2	1.12e-30	AT2G25090.1	Cre12.g537400.t1.1	1.90e-40	AT2G30360.1
Cre02.g114750.t1.2	2.61e-48	AT1G01140.2	Cre12.g538300.t1.1	4.36e-28	AT5G35410.1
Cre03.g144484.t1.1	5.09e-43	AT3G01090.1	Cre12.g560350.t1.1	9.83e-34	AT5G45820.1
Cre03.g153150.t1.2	4.07e-37	AT2G38490.1	Cre12.g560350.t2.1	9.60e-34	AT5G45820.1
Cre03.g164900.t1.1	3.35e-27	AT2G34180.1	Cre13.g564500.t1.1	2.17e-41	AT1G01140.2
Cre03.g169500.t1.2	4.41e-27	AT5G45810.1	Cre13.g567550.t1.1	2.85e-32	AT1G29230.1
Cre03.g190050.t1.1	6.79e-35	AT3G01090.1	Cre13.g567550.t2.1	3.31e-32	AT1G29230.1
Cre03.g199050.t1.2	2.14e-49	AT1G30270.1	Cre13.g568050.t1.1	2.59e-58	AT5G66880.1
Cre03.g209505.t1.1	3.81e-63	AT1G78290.2	Cre13.g570250.t1.1	1.56e-76	AT3G01090.1
Cre04.g211600.t1.1	0	AT3G01090.1	Cre13.g571700.t1.1	8.10e-52	AT3G01090.1
Cre04.g220700.t1.2	4.92e-52	AT5G10930.1	Cre13.g579200.t1.2	2.46e-36	AT2G25090.1
Cre04.g228800.t1.1	4.76e-29	AT1G30270.1	Cre13.g592000.t1.2	4.10e-37	AT3G01090.1
Cre05.g232750.t1.2	9.50e-33	AT3G01090.1	Cre14.g612000.t1.2	1.76e-35	AT1G30270.1
Cre06.g251050.t1.1	1.44e-62	AT3G29160.1	Cre16.g657350.t1.2	5.77e-59	AT1G78290.2
Cre06.g265550.t1.2	2.99e-43	AT5G10930.1	Cre16.g659400.t1.1	2.12e-28	AT2G34180.1
Cre06.g268150.t1.1	2.08e-27	AT5G39440.1	Cre16.g659400.t2.1	3.09e-29	AT1G30270.1
Cre06.g292700.t1.2	8.68e-103	AT4G33950.1	Cre16.g663200.t1.1	1.43e-38	AT3G29160.1
Cre06.g296200.t1.2	9.41e-46	AT3G29160.1	Cre16.g665364.t1.1	1.16e-53	AT3G01090.1
Cre06.g306950.t1.1	1.10e-31	AT5G01810.1	Cre16.g669800.t1.2	1.14e-50	AT5G25110.1
Cre07.g317300.t1.1	2.83e-28	AT5G01820.1	Cre16.g672602.t1.1	2.58e-26	AT5G35410.1
Cre07.g328850.t1.2	1.45e-28	AT1G29230.1	Cre16.g676309.t1.1	1.32e-27	AT5G01810.1
Cre07.g328900.t1.2	3.33e-50	AT2G26980.3	Cre16.g685389.t1.1	4.18e-37	AT4G18700.1
Cre07.g329850.t1.1	3.09e-37	AT5G08590.1	Cre17.g705000.t1.2	7.65e-55	AT5G25110.1
Cre07.g348450.t1.1	2.51e-30	AT5G01810.1	Cre17.g705350.t1.1	4.72e-39	AT5G01810.1
Cre07.g351150.t1.2	1.10e-27	AT5G45820.1	Cre17.g707800.t1.2	9.81e-46	AT1G78290.2
Cre08.g359900.t1.1	1.71e-35	AT2G30360.1	Cre17.g719450.t1.1	4.92e-34	AT5G10930.1
Cre08.g382800.t1.2	2.80e-35	AT5G10930.1	Cre17.g728700.t1.1	2.71e-35	AT2G30360.1
Cre08.g385050.t1.1	2.98e-28	AT3G01090.1	Cre17.g733300.t1.1	6.38e-33	AT4G24400.2
Cre09.g388000.t1.2	4.15e-36	AT2G25090.1	Cre17.g747397.t1.1	5.09e-28	AT4G30960.1
Cre09.g391245.t1.1	3.93e-47	AT1G01140.2	Cre19.g750597.t1.1	6.08e-52	AT3G01090.1

Supplementary Table S3. SnRK/CKIN clusters, protein domain names, and domain identifiers (ID) both in *Arabidopsis thaliana* (*Ath*) and *Chlamydomonas reinhardtii* (*Cre*), and corresponding *Cre* sequence names. SnRK clusters according to sequence similarity and protein domain layout (CKIN1/SnRK1/AKIN: containing the Serin/Threonin Kinase PTHR24343:SF183, IPR015940 and IPR001772 domains; S1R: regulatory subunits of SnRK1 with PTHR10343, PTHR13780:SF35, IPR032640, IPR000644, IPR006828 and IPR013785 domains; CKIN2/SnRK2: containing the Serin/Threonin Kinase PTHR24343, PTHR24343:SF169, PTHR24343:SF207, PTHR24343:SF167 or PTHR24343:SF200 domains, with CKIN1L exception, containing the MAP/microtubule affinity-regulating kinase PTHR24346:SF5 or the Serin/Threonin protein kinase PTHR24343:SF302 and IPR015940 domains).

SnRK Cluster	Domain name	*Ath* Domain ID	*Cre* Domain ID	*Cre* Name
CKIN1/SnRK1/AKIN	KA1 domain	IPR001772	IPR001772	CKIN1
	Serin/Threonin Kinase	PTHR24343	PTHR24343	CKIN1
	Serin/Threonin Kinase	PTHR24343:SF183	PTHR24343:SF183	CKIN1
	Serin/Threonin Kinase	PTHR24343:SF156		
	UBA domain	IPR015940	IPR015940	CKIN1
	MAP/microtubule affinity-regulating kinase		PTHR24346 PTHR24346:SF5	CKIN1L
	Serin/Threonin protein kinase		PTHR24343:SF302	
	UBA domain			CKIN1L
			IPR015940	CKIN1L
S1 R (β)	AMPK, β subunit	PTHR10343	PTHR10343	CKIN β
	SnRK 1, β-1 regulatory subunit	PTHR10343:SF51		CKIN β
	Immunoglobulin E-set	IPR014756	IPR014756	CKIN β
	AMPK, glycogen-binding domain	IPR032640	IPR032640	CKIN β
	Association with the SNF1 complex domain (ASC)	IPR006828	IPR006828	CKIN β
S1 R (βγ)	AMPK, γ regulatory subunit	PTHR13780	PTHR13780	CKIN βγ
	AMPK, γ regulatory subunit		PTHR13780:SF35	CKIN βγ
	AMP-activated protein kinase, glycogen-binding domain	IPR032640	IPR032640	CKIN βγ
	CBS domain	IPR000644	IPR000644	CKIN βγ
	Immunoglobulin E-set	IPR014756	IPR014756	CKIN βγ
S1 R (γ)	AMPK, γ regulatory subunit	PTHR13780	PTHR13780	CKIN γ
	AMPK, γ regulatory subunit	PTHR13780:SF47		
	SnRK 1, γ regulatory subunit	PTHR13780:SF36		
	AMPK, γ regulatory subunit		PTHR13780:SF49	CKIN γ
	CBS domain	IPR000644	IPR000644	CKIN γ
CKIN2/SnRK2	Serin/Threonin kinase	PTHR24343	PTHR24343	CKIN2
	Serin/Threonin Protein Kinase SRK2D-Related		PTHR24343:SF169	CKIN2.8
	Serin/Threonin Protein Kinase SRK2C		PTHR24343:SF207	CKIN2.2
	Serin/Threonin Protein Kinase SRK2E		PTHR24343:SF167	CKIN2.12 CKIN2.19 CKIN2.10 CKIN2.13
CKINL	Serin/Threonin Protein Kinase SRK2F		PTHR24343:SF200	CKINL
SnRK3	Serin/Threonin Kinase	PTHR24343	PTHR24343	
	Calcium/calmodulin-dependent/calcium-dependent protein kinase	PTHR24347	PTHR24347	
	NAF	IPR004041		
	NAF/FISL	IPR018451		

Supplementary Table S4. *Arabidopsis thaliana* (*Ath*), *Chlamydomonas reinhardtii* (*Cre*), *Chlorella variabilis* (*Cva*), *Coccomyxa subellipsoidea* (*Csu*), *Dunaliella salina* (*Dsa*) and *Volvox carteri* (*Vca*) sequences used for the design of the figure 3 sequence tree: sequence identifier (Seq ID) followed by sequence name (Name).

Seq ID	Name	Seq ID	Name
AT3g01090	*Ath* AKIN10	jgi|ChlNC64A_1|140361|IGS.gm_3_00100	*Cva* CvKIN2.10
AT3g29160	*Ath* AKIN11	jgi|ChlNC64A_1|142950|IGS.gm_5_00279	*Cva* CvKIN2.11
AT5g39440	*Ath* SnRK1.3	jgi|ChlNC64A_1|143933|IGS.gm_6_00507	*Cva* CvKIN2.12
AT5g08590	*Ath* SnRK2.1	jgi|ChlNC64A_1|145069|IGS.gm_8_00397	*Cva* CvKIN2.13
AT3g50500	*Ath* SnRK2.2	jgi|ChlNC64A_1|140646|IGS.gm_3_00385	*Cva* CvKIN2.14
AT5g66880	*Ath* SnRK2.3	e_gw1.10.111.1|16775	*Csu* CsKIN1
AT4g33950	*Ath* SnRK2.6	e_gw1.3.241.1|12786	*Csu* CsKIN2.2
AT4g40010	*Ath* SnRK2.7	estExt_fgenesh1_pg.C_100215|47867	*Csu* CsKIN2.3
AT2g23030	*Ath* SnRK2.9	estExt_fgenesh1_pg.C_240007|49286	*Csu* CsKIN2.4
AT1g60940	*Ath* SnRK2.10	estExt_fgenesh1_pm.C_150088|54361	*Csu* CsKIN2.5
AT5g01810	*Ath* SnRK3.1	estExt_Genemark1.C_30088|64800	*Csu* CsKIN2.6
AT5g07070	*Ath* SnRK3.2	estExt_Genemark1.C_30287|64904	*Csu* CsKIN2.7
AT4g14580	*Ath* SnRK3.3	estExt_Genemark1.C_90162|66347	*Csu* CsKIN2.8
AT5g57630	*Ath* SnRK3.4	estExt_Genemark1.C_240049|68309	*Csu* CsKIN2.9
AT5g45810	*Ath* SnRK3.5	estExt_Genewise1.C_100280|24306	*Csu* CsKIN2.10
Cre04.g211600.t1.1	*Cre* CKIN1	estExt_Genewise1.C_190274|25912	*Csu* CsKIN2.11
Cre13.g570250.t1.1	*Cre* CKIN1L	estExt_Genewise1Plus.C_30441|27599	*Csu* CsKIN2.12
Cre02.g075850.t1.1	*Cre* CKIN2.1	estExt_Genewise1Plus.C_30509|27636	*Csu* CsKIN2.13
Cre12.g499500.t1.1	*Cre* CKIN2.2	estExt_Genewise1Plus.C_30548|27662	*Csu* CsKIN2.14
Cre02.g075900.t1.1	*Cre* CKIN2.3	estExt_Genewise1Plus.C_100351|29722	*Csu* CsKIN2.15
Cre11.g477000.t1.2	*Cre* CKIN2.4	estExt_Genewise1Plus.C_100354|29725	*Csu* CsKIN2.16
Cre03.g209505.t1.1	*Cre* CKIN2.5	fgenesh1_pg.20_#_52|44853	*Csu* CsKIN2.17
Cre11.g481000.t1.2	*Cre* CKIN2.6	e_gw1.5.212.1|14400	*Csu* CsKIN2.18
Cre06.g292700.t1.2	*Cre* CKIN2.7	Dusal.0281s00020|Dusal.0281s00020.1	*Dsa* DKIN1
Cre10.g466350.t1.1	*Cre* CKIN2.8	Dusal.0029s00005|Dusal.0029s00005.1	*Dsa* DKIN2.2
Cre13.g568050.t1.3	*Cre* CKIN2.14	Dusal.0056s00014|Dusal.0056s00014.1	*Dsa* DKIN2.3
Cre16.g657350.t1.2	*Cre* CKIN2.9	Dusal.0106s00018|Dusal.0106s00018.1	*Dsa* DKIN2.5
Cre17.g707800.t1.2	*Cre* CKIN2.10	Dusal.0181s00032|Dusal.0181s00032.1	*Dsa* DKIN2.6
Cre12.g485600.t1.2	*Cre* CKIN2.13	Dusal.0186s00019|Dusal.0186s00019.1	*Dsa* DKIN2.7
Cre02.g076000.t1.2	*Cre* CKIN2.11	Dusal.0617s00007|Dusal.0617s00007.1	*Dsa* DKIN2.8
Cre08.g384250.t1.2	*Cre* CKIN2.15	Dusal.0826s00006|Dusal.0826s00006.1	*Dsa* DKIN2.9
Cre17.g707650.t1.1	*Cre* CKIN2.12	fgenesh1_pm.C_Chr_6000070|12650	*Olu* OKIN1
Cre07.g329850.t1.1	*Cre* CKIN2.16	eugene.0100010317|28923	*Olu* OKIN2.2
Cre02.g074370.t1.2	*Cre* CDPK1	ost_03_006_039|86646	*Olu* OKIN2.3
Cre02.g106650.t1.3	*Cre* CDPK2	Vocar.0025s0127.1	*Vca* VKIN1
Cre06.g265550.t1.2	*Cre* CDPK3	Vocar.0048s0006|Vocar.0048s0006.1	*Vca* VKIN2.2
Cre06.g296200.t1.2	*Cre* CDPK4	Vocar.0027s0049|Vocar.0027s0049.1	*Vca* VKIN2.3
Cre07.g328900.t1.2	*Cre* CDPK5	Vocar.0031s0053|Vocar.0031s0053.1	*Vca* VKIN2.5
Cre01.g048650.t1.1	*Cre* CDPK6	Vocar.0004s0065|Vocar.0004s0065.1	*Vca* VKIN2.6
Cre13.g564500.t1.3	*Cre* CDPK8	Vocar.0021s0079|Vocar.0021s0079.1	*Vca* VKIN2.7
Cre13.g571700.t1.1	*Cre* CDPK9	Vocar.0029s0126|Vocar.0029s0126.1	*Vca* VKIN2.8
Cre19.g750597.t1.1	*Cre* CDPK11	Vocar.0013s0156|Vocar.0013s0156.1	*Vca* VKIN2.9
Cre01.g003524.t1.1	*Cre* CDPK12	Vocar.0024s0254|Vocar.0024s0254.1	*Vca* VKIN2.10
jgi|ChlNC64A_1|57611|estExt_fgenesh3_pg.C_80094	*Cva* CvKIN2.1	Vocar.0006s0284|Vocar.0006s0284.1	*Vca* VKIN2.11
jgi|ChlNC64A_1|33664|estExt_Genewise1Plus.C_10631	*Cva* CvKIN2.3	Vocar.0001s0337.1	*Vca* VKIN2.12
jgi|ChlNC64A_1|34706|estExt_Genewise1Plus.C_50310	*Cva* CvKIN2.4	Vocar.0001s1516|Vocar.0001s1516.1	*Vca* VKIN2.13
jgi|ChlNC64A_1|57249|estExt_fgenesh3_pg.C_50159	*Cva* CvKIN2.5	Vocar.0001s1602|Vocar.0001s1602.1	*Vca* VKIN2.14
jgi|ChlNC64A_1|135543|IGS.gm_14_00076	*Cva* CvKIN2.6	Vocar.0001s1603|Vocar.0001s1603.1	*Vca* VKIN2.15
jgi|ChlNC64A_1|138144|IGS.gm_2_00611	*Cva* CvKIN2.8	Vocar.0001s1604|Vocar.0001s1604.1	*Vca* VKIN2.16
jgi|ChlNC64A_1|138887|IGS.gm_22_00128	*Cva* CvKIN2.9		

Supplementary Table S5: Fold-change variations in the abundance of the different members of the CKIN family on data taken from Algaepath (a), and under the different stress situations that were tested by qPCR (control vs 48 h of stress).

a) Algaepath

Name	S-	N-	CO_2--	H_2O_2, 1.0h	Fe--
CKIN1	1.77	0.89	2.69	0.57	1.32
CKIN1L	2.18	0.79	2.10	0.64	9.49
CKIN β	1.30	0.54	1.78	0.59	0.72
CKIN βγ	2.33	1.40	2.03	0.51	1.17
CKIN γ	0.93	0.40	0.60	0.96	0.58
CKIN2.1	1.23	0.96	1.39	0.91	1.05
CKIN2.2	4.11	2.41	1.24	1.58	1.31
CKIN2.3	2.60	1.39	1.94	0.76	1.15
CKIN2.4	1.44	0.60	1.04	0.82	0.81
CKIN2.5	3.52	1.38	1.23	1.06	1.49
CKIN2.6	2.56	1.47	1.29	1.26	2.08
CKIN2.7	1.59	0.87	1.69	2.55	1.20
CKIN2.8	1.60	1.11	1.39	1.51	1.68
CKIN2.9	2.13	1.28	1.01	0.98	0.77
CKIN2.10	1.77	1.24	1.62	1.02	1.01
CKIN2.11	2.00	1.18	1.84	0.63	1.66
CKIN2.12	2.32	0.77	1.26	1.25	1.75
CKIN2.13	1.60	1.58	3.45	0.77	7.82
CKIN2.14	0.75	0.33	1.42	1.27	2.28
CKIN2.15	4.48	2.40	1.01	2.87	8.00
CKIN2.16	3.03	1.40	1.86	1.52	1.82
CKINL	16.04	5.61	1.82	0.48	0.69

b) qPCR assays

Name	S-	N-	C-	5 % P	20 % PEG	0.25M NaCl	40 °C	4 °C	UV
CKIN1	2.08	0.75	1.72	11.98	3.38	18.61	3.28	4.54	2.08
CKIN1L	2.94	0.41	1.35	1.04	0.64	1.87	2.59	1.03	1.52
CKIN β	1.42	0.33	1.37	8.03	8.33	8.70	2.15	3.21	7.53
CKIN βγ	5.23	1.72	1.12	4.05	3.35	10.18	3.57	8.97	6.32
CKIN γ	1.37	0.51	2.11	9.31	1.28	16.03	3.75	6.77	5.40
CKIN2.1	1.00	0.76	0.67	1.33	440.00	6.72	2.75	13.54	21.95
CKIN2.2	11.62	2.72	1.09	1.98	5.15	6.59	2.83	13.64	9.07
CKIN2.3	4.73	1.34	1.51	3.03	8.74	12.74	2.77	9.95	5.79
CKIN2.4	1.64	0.42	1.22	0.94	29.80	2.21	9.16	2.91	1.91
CKIN2.5	10.38	1.50	1.17	5.16	105.61	14.33	6.72	8.30	21.22
CKIN2.6	4.44	1.12	0.95	0.80	8.97	1.60	1.22	1.86	13.63
CKIN2.7	2.15	0.65	1.05	2.09	8.05	9.10	2.69	6.26	25.96
CKIN2.8	1.74	0.62	0.69	0.19	21.20	4.09	2.83	2.47	10.69
CKIN2.9	4.99	1.13	0.46	1.27	16.89	2.78	1.74	2.12	9.12
CKIN2.10	1.98	1.16	2.61	2.60	71.84	7.35	4.82	2.20	22.04
CKIN2.11	2.45	1.17	0.49	0.72	134.08	7.97	1.78	5.87	19.37
CKIN2.12	4.47	1.16	0.58	1.38	354.10	5.94	1.25	6.46	7.76
CKIN2.13	1.87	0.44	0.67	0.37	6.71	9.19	1.47	9.78	3.37
CKIN2.14	0.28	0.10	0.25	0.16	1.64	0.48	2.16	0.38	0.52
CKIN2.15	13.77	2.21	0.91	1.39	230.70	5.73	2.65	6.67	10.71
CKIN2.16	6.87	1.88	1.02	1.20	3.52	4.24	2.22	1.78	6.99
CKINL	82.94	16.79	0.27	0.94	31.78	10.65	7.31	10.78	5.90

Supplementary Table S6. Sequences of *Arabidopsis thaliana* (*Ath*), *Saccharomyces cerevisiae* (*Sce*), *Homo sapiens* (*Hsa*), and *Chlamydomonas reinhardtii* (*Cre*) employed for building sequence trees. Organism identification followed by sequence name (Name) and sequence identifier (Seq ID).

Name	Seq ID	Name	Seq ID
Ath AKIN10	AT3g01090	*Cre* CKIN2.6	Cre11.g481000.t1.2
Ath AKIN11	AT3g29160	*Cre* CKIN2.7	Cre06.g292700.t1.2
Ath SnRK1.3	AT5g39440	*Cre* CKIN2.8	Cre10.g466350.t1.1
Hsa AMPK α1	NP_006242.5	*Cre* CKIN2.9	Cre16.g657350.t1.2
Cre CKIN1	Cre04.g211600.t1.1	*Cre* CKIN2.10	Cre17.g707800.t1.2
Ath β1	AT5g21170	*Cre* CKIN2.11	Cre02.g076000.t1.2
Hsa AMPK β2	NP_005390.1	*Cre* CKIN2.12	Cre17.g707650.t1.1
Cre CKIN1L	Cre13.g570250.t1.1	*Cre* CKIN2.13	Cre12.g485600.t1.2
Sce SNF1	NP_010765.3	*Cre* CKIN2.14	Cre13.g568050.t1.3
Ath β2	AT4g16360	*Cre* CKIN2.15	Cre08.g384250.t1.2
Cre CKIN β	Cre10.g457500.t1.1	*Cre* CKIN2.16	Cre07.g329850.t1.1
Ath βγ	AT1g09020	*Cre* CKINL	Cre16.g685389.t1.1
Hsa AMPK γ1	NP_002724.1	*Ath* SnRK3.1	AT5g01810
Cre CKIN βγ	Cre12.g484350.t1.3	*Ath* SnRK3.2	AT5G07070
Ath γ1	AT3g48530	*Ath* SnRK3.3	AT4g14580
Ath γ2	AT1g69800	*Ath* SnRK3.4	AT5g57630
Cre CKIN γ	Cre12.g528000.t1.2	*Ath* SnRK3.5	AT5g45810
Ath SnRK2.2	AT3g50500	*Cre* CDPK1	Cre02.g074370.t1.2
Ath SnRK2.6	AT4g33950	*Cre* CDPK2	Cre02.g106650.t1.3
Ath SnRK2.7	AT4g40010	*Cre* CDPK3	Cre06.g265550.t1.2
Ath SnRK2.9	AT2g23030	*Cre* CDPK4	Cre06.g296200.t1.2
Ath SnRK2.10	AT1g60940	*Cre* CDPK5	Cre07.g328900.t1.2
Cre CKIN2.1.	Cre02.g075850.t1.1	*Cre* CDPK6	Cre01.g048650.t1.1
Cre CKIN2.2	Cre12.g499500.t1.1	*Cre* CDPK8	Cre13.g564500.t1.3
Cre CKIN2.3	Cre02.g075900.t1.1	*Cre* CDPK9	Cre13.g571700.t1.1
Cre CKIN2.4	Cre11.g477000.t1.2	*Cre* CDPK11	Cre19.g750597.t1.1
Cre CKIN2.5	Cre03.g209505.t1.1	*Cre* CDPK12	Cre01.g003524.t1.1

Supplementary Table S7. Primer pairs used for RT-qPCR analyses. All primers were designed with Tm greater than 60°C and PCR were all conducted at an annealing temperature of 61°C.

Name	ID	Forward primer sequence	Reverse primer sequence
ACT	Cre13.g603700.t1.2	GCGGCTACTCGTTCACCACCAC	TCTCCTGCTCGAAGTCCAGGGC
UBI	Cre03.g159200.t1.1	CTACCGCCGTTCCTGTTCCTGC	CTGGCGGCAGTAGCACCACATC
TUB	Cre04.g216850.t1.2	GTCCAAGCTGGGCTTCACCGTC	GGCGGCAGATGTCGTAGATGGC
RCK1	Cre06.g278222.t1.1	CGACAAGAGCGTGCTGGTCTGG	GTCAGGCAGAACTGGCCATCGG
CKIN1	Cre04.g211600.t1.1	TCATGCACCCGCACATCATCCG	CAGCCGCCCCTTCTCCACAATG
CKIN1L	Cre13.g570250.t1.1	CGTGGCCAGCCTCGAGTACTGC	GGGCACGTAGAAGGCGGACAGG
CKIN β	Cre10.g457500.t1.1	GATGAGCTGACAGCCGCCAACC	CACCGTGGCTCCACACGATGAC
CKIN βγ	Cre12.g484350.t1.3	ACGGCTCTCCTGGGTTGTTTGC	TTGACGTTGCCAAGCGGGTCTG
CKIN γ	Cre12.g528000.t1.2	CGTGCTGATGCAGGAGCTGGAG	TCACGGTGTGCACCTTCTTGGC
CKIN2.1	Cre02.g075850.t1.1	GGTCAAGCGTGAAGTGCGAACC	GCAGTCCGCGTACTCCATCACC
CKIN2.2	Cre12.g499500.t1.1	CATCTGGAGCTGCGGCGTGATG	GCGGCGGGATGTGGTAGTCAAC
CKIN2.3	Cre02.g075900.t1.1	CGTGTTCGGCCTCGACTACTGC	GCAGGTTGAAGGGGTGCTCCAG
CKIN2.4	Cre11.g477000.t1.2	GCGCGAAATTCAGTCCCATCGC	CCCTGGTCAGCGAACTCCATGC
CKIN2.5	Cre03.g209505.t1.1	CGGTGGACTACTGCCACAAGCG	GGACCTGAAGTCGGCCTTGCTG
CKIN2.6	Cre11.g481000.t1.2	GGCGGGAGCTTGTTCCACTACG	TCCAGCTTGATGTCGCGGTTGG
CKIN2.7	Cre06.g292700.t1.2	GCTGCCGCTGCTCAAAATCTGC	GTACTGGTCCGTGGCGCGAATC
CKIN2.8	Cre10.g466350.t1.1	GTGTGCCACCGGGATCTGAAGC	TCTTGGGCTGGCTGTCAAACGC
CKIN2.9	Cre16.g657350.t1.2	GGACGAGGCGCGGTACTTCTTC	GTCGCACAGCTTCAGCCAGGAG
CKIN2.10	Cre17.g707800.t1.2	GGCCTGCAGCGAGGAGTTCAA	GCGGTTGAAGGTGGGGTAGTGC
CKIN2.11	Cre02.g076000.t1.2	ACCTGAAGCGCGAGGTGGTGTG	GCTCCGCCAGCCACTGCTTGAG
CKIN2.12	Cre17.g707650.t1.1	GGTGATGGAGCTGCTGGGCACAG	GCTTGCAGCTGCAGCTCCATGC
CKIN2.13	Cre12.g485600.t1.2	AGGACGAGCACAAGCGCATCA	ACGGGTGGCCAGGTCCTGTATG
CKIN2.14	Cre13.g568050.t1.3	CTGGAGCCGCGGGTTCTACAAG	TCCATCACCACCGCCAGGTAGG
CKIN2.15	Cre08.g384250.t1.2	CATCTGGTCCTGCGGCGTGGTG	TCCTCGCGCGGCACCATGATG
CKIN2.16	Cre07.g329850.t1.1	CATGTCGGCCGCCTCCCTTGAC	CGCGTGCGGCAGATGGTGTAGG
CKINL	Cre16.g685389.t1.1	GACGTCGCCCATGGGTCAGCAC	GGAGGTGCTACTGCCGGCATCC
CHX	Cre04.g215050.t1.2	ATGATGCTGGCTTCGCGTCCTG	CACAATGGGGTCCGCGACCTTC

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CHAPTER 5

Genetic engineering of *Chlamydomonas reinhardtii* omic derived candidates

CHAPTER CONTENTS

I. Introduction

The use of mutant organisms has contributed alone to the characterization of many genes, especially those related to simple traits. However, genome edition by itself has a reduced power when it comes to complex traits as stress

resistance or biomass production since these traits are consequence of the interaction of a large number of loci. This complexity has motivated the use of complementary strategies based on high-throughput omic techniques coupled to systems biology allowing the high-resolution modelling of biological systems. These system models can help to identify candidates which are commonly unrelated to the previous knowledge. However, the gap between the math-based assumptions about a candidate function and the actual function of the candidate needs to be filled through validation. Currently, different genome edition techniques drive this validation step between other techniques through the modulation and/or blockage of the candidate genes expression, and the description of the interactants and location of the proteins that they encode. Omics and systems biology characterization of the candidate function and interactants through the detailed description and modelling of the effects of their modulation and their interactants (Nukarinen *et al.* 2016).

Multiple strategies have been used for the engineering of *C. reinhardtii* genome with different levels of success (Jinkerson & Jonikas 2015). The isolation of knockout lines from random insertional mutagenesis is one of the most successfully applied techniques into the microalgae (Gonzalez-Ballester *et al.* 2011; Zhang *et al.* 2014; Li *et al.* 2016, 2019) along the generation of miRNA based silenced lines (Molnar *et al.* 2009). On the other hand, site directed techniques as homologous recombination and CRISPR/Cas have a low efficiency in the microalgae. Moreover, the low efficiency of recombinant protein expression in Chlamydomonas has motivated the development of different strategies centered in the use of strong constitutive promoters as HSP70-RBCS2, introns and autocatalytic sequences enhancing transcription, transcript processing and translation efficiency (Jinkerson & Jonikas 2015; Baier, Wichmann, Kruse & Lauersen 2018).

All these strategies rely in vector constructions and different transformation strategies for the delivery of genetic modules into the Chlamydomonas genome, involving different resistance genes that allow the recovery of the transformed colonies. Although vectors are convenient supports for genetic

transformation, many studies challenge their capabilities requiring the synchronous insertion of multiple elements or their association to different elements from purification tags and fluorescent proteins to inducible promoters in the same assay. The need of multiple gene transformations is common into the characterization of gene families with high functional redundancy members such as the Arabidopsis SnRK2 subfamily. The recovery of a defective SnRK2 phenotype required the generation of a decuple mutant coupling a multiple transformation with a mating strategy in order to increase the number of altered genes using a limited diversity of constructions (Fujii, Verslues & Zhu 2011). On the other hand, the use of inducible promoters is especially important when characterizing the function of lethal genes as Arabidopsis SnRK1 (Nukarinen *et al.* 2016) and Chlamydomonas centrin (Koblenz & Lechtreck 2005).

Genome editing strategies commonly require the mix of different gene and regulatory sequences which are dispersed between different backbones and not ready for their ensemble into a new construction. Moreover, many of these sequences are not optimized for the Chlamydomonas codon usage. Thus, the edition of Chlamydomonas genome is a time-consuming task, mostly dedicated to the edition and ensemble of the different pieces involved. In order to skip these initial steps, multiple works have initiated a standardization labor producing vector sets with Chlamydomonas optimized and easily exchangeable modules including fluorescent proteins, signal peptides, promoters, intron sequences and different antibiotic selection markers (Lauersen, Kruse & Mussgnug 2015; Crozet *et al.* 2018).

Between the different candidates found into this thesis the SnRK kinases and the mitochondrial ATP synthase proton channel MITOCHONDRIAL ATP SYNTHASE SUBUNIT A (ATP6) were considered the best options for characterization. The SnRKs conserved role into stress response and their link to biomass production makes the microalgae orthologs to these kinases (CKINs) promising for the modulation of their biomass content. However, the lethality derived from the complete disruption of the Arabidopsis SnRK1 subfamily and the functional redundancy observed into the same species

SnRK2s have required from complex characterization strategies and foreseen the need of similar approaches for the microalgae CKIN/SnRK family. On the other hand, ATP6 was also found to have a key role into osmotic and oxidative stress response, linked to the maintenance of mitochondrial electron flow. However, the characterization of ATP6 will probably be easier due to the structural role and specificity of this protein. CKINs and ATP6 features would allow the use of different strategies based on single/multiple transformation and constitutive/inducible expression for their characterization. Accounting on these diverse needs and on commented vector ensemble issues we propose the design and ensemble of a modular genetic construction capable to drive multiple mutant strategies thanks to an exchangeable resistance and to control the inducible expression of lethal genes.

II. Material and methods

Strains and culture conditions

Chlamydomonas reinhardtii strain CC1690 $NIT1^+$ $NIT2^+$ (Chlamydomonas resource center, University of Minnesota) was used into all described transformation procedures. Liquid cell cultures were grown into TAP medium within Erlenmeyer flasks and keep into an incubator at 25 ºC, 150 rpm shaking and 100 μmol photons $\cdot m^2 \cdot s^{-1}$ white light provided by LEDs. Semisolid cultures also into TAP medium were keep under same growing conditions without shaking. Certain tap components were changed during transformation and induction procedures (Table 1).

Table 1. Variations of the basal TAP culture media directed to transformation and expression modulation procedures and to the test of candidate gene expression under stressing conditions.

Assay/procedure	Modification of basal media
NIT1 promoter induction	Substitution of ammonium chloride for potassium nitrate as nitrogen source.
Paromomycin based selection	Addition of 20 mg/mL Paromomycin to basal media.

Oxyfluorfen based selection	Addition of 3 μM Oxyfluorfen to basal media.
Salt stress	Addition of 0.25 M sodium chloride to basal media.
Singlet oxygen stress	Addition of 6 mM Bengal Rose to basal media.

Extraction of the *NIT1* promoter

NIT1 promoter sequence (NIT1p) as described by (Ohresser, Matagne & Loppes 1997) was amplified from CC1690 gDNA using specific primers targeting the promoter ends (Table 2) including restriction tags for SpeI and StuI. PCRs were performed using Phusion high fidelity polymerase (ThermoFisher Scientific) following the polymerase recommendations for high GC amplicons and the following program 1x [98 ºC 30s], 45x [98 ºC 10 s, 60 ºC 30 s, 72 ºC 27 s], 1x [72 ºC 5 min].

Resulting fragment was ligated with a pJET1.2/blunt vector (ThermoFisher Scientific) following manufacturer's recommendations for blunt end products. Ligation product was used to transform *E. coli* and transformed colonies were grown in liquid LB medium before the purification of the resulting plasmid constructions through miniprep. NIT1p containing purified construction was validated through sanger sequencing (STAB VIDA).

Amplification of *ATP6*, *CKIN2.12* and *PPX* coding sequences and insertion of *ATP6* and *CKIN2.12* into the pSI108 vector.

The isolation of the *ATP6*, *CKIN2.12* and PROTOPORPHYRINOGEN IX OXIDASE (*PPX*) coding sequences (CDS) was started with the extraction of Chlamydomonas RNA following Valledor *et al.* 2014 protocol. *PPX* DNA complementary strand was synthesized out of 1 μg of RNA using a specific *PPX* primer (Table 2) and a RevertAid reverse transcriptase (RT) (ThermoFisher Scientific) into a 10 μL reaction following the RT specifications for long GC rich amplicons and gene specific primers. For the remaining genes

standard RT reaction conditions were used over same RNA amount using random hexamers.

ATP6 and *CKIN2.12* coding sequences were amplified from cDNA samples using specific primers targeting the sequences ends including restriction tags for EcoRV and EcoRI. Tagged *ATP6* and *CKIN2.12* sequences were then inserted into the pSI180 vector using the EcoRV/RI restriction sites into the vector MCS (Figure 3A). Resulting pSI108 based HSP70-RBCS2::*ATP6* and HSP70-RBCS2::*CKIN2.12* constructions were validated through sanger sequencing (STAB VIDA)

Production of an oxyfluorfen resistance gene through the single point mutation of *PPX*

PPX CDS ends were tagged with specific restriction sequences for NdeI and BglII out of the previously generated cDNA using a specific primer pair (Table 2, Figure 1A).

The introduction of the *rs-3* mutation, a nonsynonymous G to A transition changing Valine 389 for Methionine, into the tagged *PPX* sequence involved three different PCR reactions. First one amplified a 1178 bp fragment from the gene start to the mutation site while second one produced a 545 bp fragment from the mutation site to the gene end. These reactions used two different primer pairs including the *rs-3* mutation site (Table 2, Figure 1). A third PCR reaction was carried out over the partially complementary products of the first two ones allowing the ensemble of the mutated PPX sequence (Figure 1, Table 2).

All PCRs were carried out using Phusion high fidelity DNA polymerase (ThermoFischer Scientific) following the polymerase specifications for high GC content amplicons. Mutated *PPX* sequence (*PPX rs-3)* was ligated with a pJET1.2/blunt vector (ThermoFisher Scientific) following recommendations for blunt end products. Ligation product was used to transform *E. coli* and transformed colonies were grown in liquid LB medium before the purification of the plasmidic construction containing the *PPX rs-3* sequence through

miniprep. *PPX rs-3* sequence into purified construction was validated through sanger sequencing (STAB VIDA).

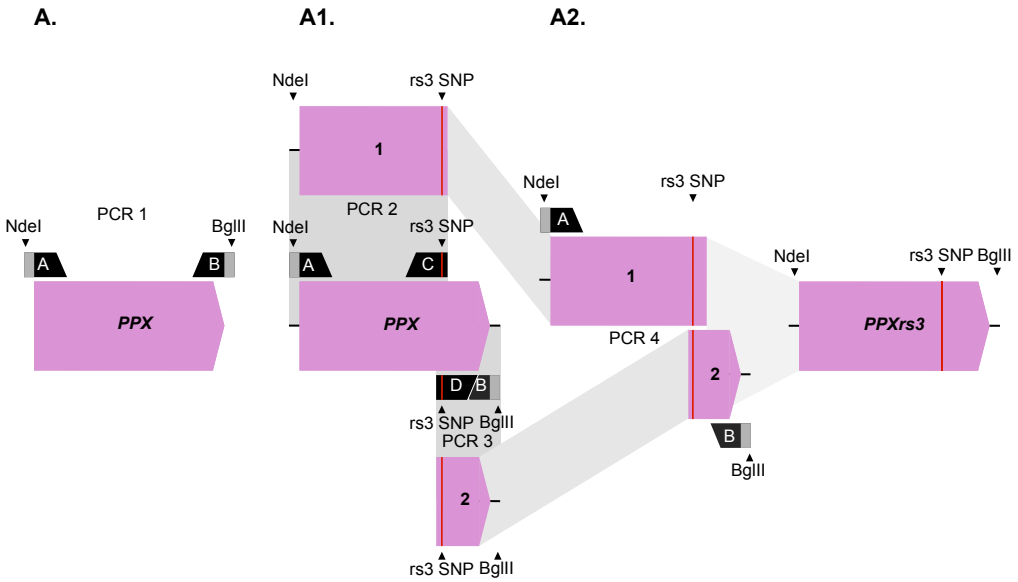

Figure 1. Mutation of the PPX gene through PCR. A, PCR based tagging of wild type *PPX* sequence ends with primers A and B including restriction sites for NdeI and BglII (PCR1). A1, a second PCR (PCR2) using the tagged PPX sequence as template and primers A and C amplificated the fragment 1 while a third PCR (PCR3) using primers D and B and the same template produced the fragment 2. Primers C and D were complementary and contained the mutated *rs-3* site. A2, partially complementary fragments 1 and 2 containing the *rs-3* mutation were hybridized into PCR4 using A and B primers to produce the full length mutated *PPX rs-3* sequence.

Ensemble of the pSticky vector

The ensemble of the pSticky vector started from the pSI108 backbone (Figure 2). Resistance marker cassette was emptied and restriction tags for NdeI and BglII added between the cassette 3' and 5' UTRs (Figure 1A-A7). Resulting construction and *PPX rs-3* sequences were digested with NdeI and BglII and then ligated (Figure 1B-B2).

New restriction tags for StuI and SpeI were added to the sides of the second expression cassette promotor into the edited pSI108 construction carrying the *PPX rs-3* resistance marker. A fragment containing the promoter, the 5'UTR

sequence and part of the multiple cloning site of the second expression cassette of the edited pSI108 vector was excised with SmaI. Parallelly tags for StuI and SpeI were added to NIT1 promoter while the region between 5' and the second SmaI restriction site into the original MCS was tagged with SpeI and SmaI restriction sites. Both modules were inserted into pJET1.2/blunt vectors and ensembled through their digestion and religation (Figure 2C-C6). Ligated module containing the NIT1p was amplified and digested to allow its insertion into the *PPX rs-3* containing pSI108 (Figure 1C8).

All these steps involved different PCR amplifications, restriction and ligation steps detailed into figure 1. PCR reactions were performed using Phusion high fidelity polymerase (ThermoFisher Scientific) using different primer pairs, melting temperatures and extension times (Figure 1, Table 2). Ligations were performed with ligase T4 (ThermoFisher Scientific).

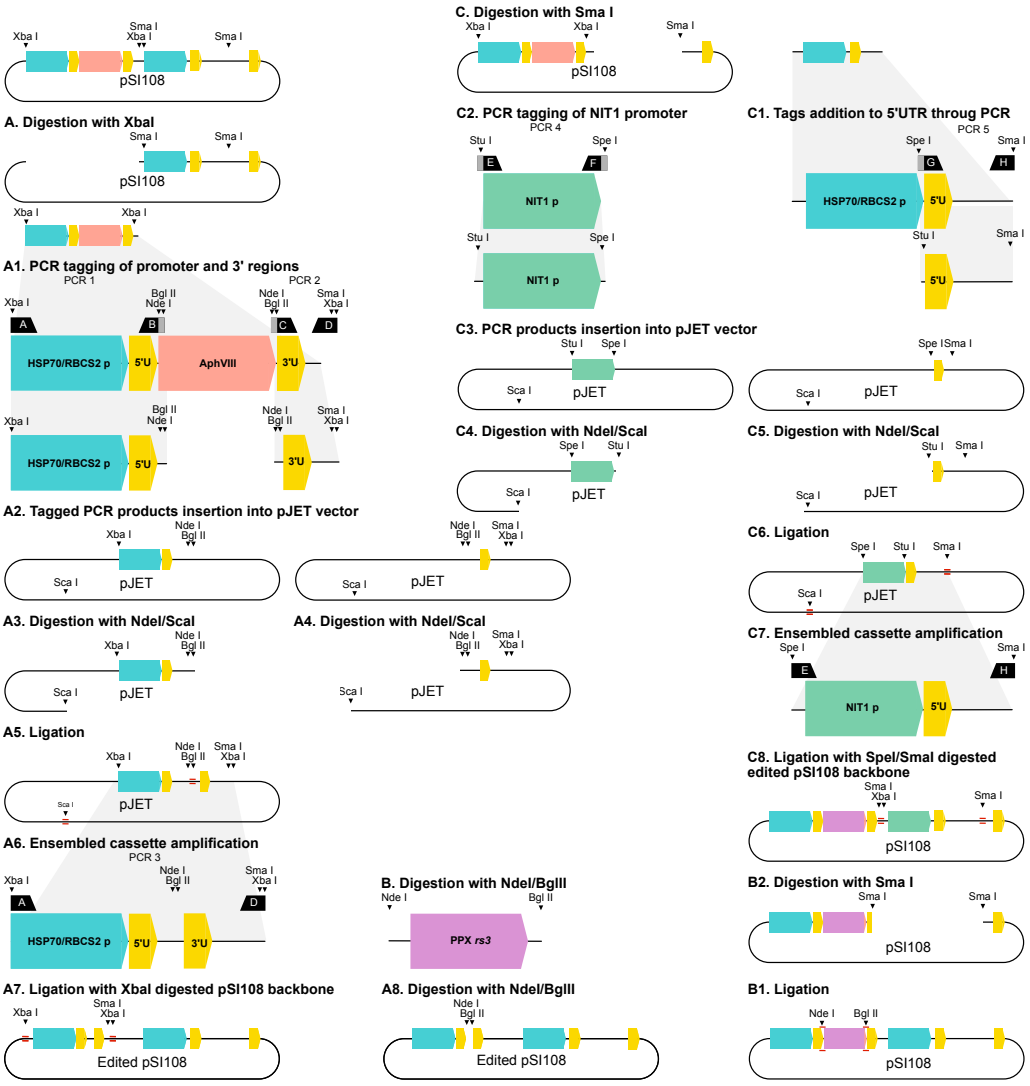

Figure 2. Ensemble of the pSticky vector. Digestion of the original pSI108 vector allowing the excision of the resistance cassette (A). Promoter and 3' UTR regions surrounding the AphVIII resistance gene were tagged through PCR with specific restriction sequences (A1). Resulting PCR products were integrated into pJET vectors (A2) and through the digestion (A3, A4) and religation (A5) of these both tagged modules were fused. New cassette was amplified and inserted into XbaI digested pSI108 backbone (A7) digesting afterwards the ligation product to allow the insertion of the PPX *rs3* resistance gene (A8, B, B1). Parallelly original pSI108 was digested with SmaI (C). Restriction tags were added to the NIT1 promoter sequence (C2) and to the region between 5'UTR and second SmaI restriction site (C1) into the gene of interest cassette. Both fragments were separately inserted into pJET vectors and through digestion and relegation of resulting pJET

constructions fused (C4-6). NIT1 cassette was amplified (C7) and after digestion with xxx and sss ligated with the digested PPX rs3 containing pSI108 (C8).

Insertion of the *CKIN2.12*, *CFP* and *AphVIII* coding sequences into the pSticky vector

CKIN2.12 sequence into pSI108 based HSP70-RBCS2:: *CKIN2.12* construction was excised with EcoRI and EcoRV and inserted into pSticky previously digested with the same enzymes to create the pSticky based NIT1p::*CKIN2.12* construction.

CYAN FLUORESCENT PROTEIN (CFP) coding sequence was amplified from a previously existing vector pC19 with specific primers adding EcoRI and EcoRV tags to the sequence ends (Table 2). PCR product was digested with *Eco*RI and *Eco*RV and ligated with a pSticky digested with the same enzymes to create the pSticky based NITp::*CFP* construction.

AphVIII was amplified from pSI108 vector using an specific primer pair adding EcoRI and EcoRV tags to the sequence ends (Table 2). PCR product was digested with EcoRI and EcoRV and ligated with a pSticky digested with the same enzymes to create the NITp::*AphVIII* construction.

Resulting pSticky based constructions NITp::*CKIN2.12*, NITp::*CFP* and NITp::*AphVIII* were validated through sanger sequencing (STAB VIDA).

Chlamydomonas transformation

Chlamydomonas was transformed by electroporation, briefly Chlamydomonas liquid cultures below $3 \cdot 10^6$ cell/mL were centrifuged and the resulting pellet washed twice into max efficiency transformation reagent for algae (ThermoFisher Scientific) before its resuspension in the same buffer at a final concentration of $1 \cdot 10^8$ cel/mL. 250 uL aliquots of this high-density suspensions were mixed with xxx of the corresponding construction and electroporated into 0.4 cm cold electroporation cells at 500 V, 50 µF and 800 Ω. Electroporated cell suspensions were incubated in10 mL TAP medium

without antibiotic and under low light and room temperature, being plated afterwards into TAP media containing the respective selective antibiotic.

NIT1 promoter induction

Cultures transformed with NIT1p containing constructions were induced by substituting nitrogen source from ammonia to nitrate. Liquid cultures were centrifuged at 2000 x g, supernatant discarded and resulting pellets washed with nitrate containing TAP. Suspensions were centrifuged again and resulting pellets resuspended to final assay density into TAP nitrate medium.

Cells transformed with NIT1p::*AphVIII* construction were plated into paromomycin containing NIT1p inductive medium and basal TAP medium (table 1). NIT1p induction of the *AphVIII* gene expression under inductive conditions was screened by resistant colony count. Moreover, cells carrying the pSticky based NIT1p::*CFP* were placed under NIT1p inductive conditions for 24 h harvesting samples at the experiment start, and at t 15 min, 30 min, 1 h, 2 h, 4 h, 8 h and 24 h after the induction start. Promotor induction time was tested on the evolution of CFP fluorescence, observed under a confocal microscope (leicaxxxxx)

III. Results

The pSticky vector allow the easy exchange of genetic modules, including both promoters and selectable markers

Generated pSticky vector included an editable resistance gene cassette thanks to the insertion of NdeI and BglII restriction tags between RBCS2 3' and 5'UTR regions into the original pSI108 resistance gene cassette, allowing the exchange of the resistance gene sequence (Figure 3). pSticky vector included a second cassette whose modularity was not only restricted to the gene of interest as the promotor sequence was also exchangeable through the added SpeI and StuI restriction sites (Figure 3). Different modules were adapted to the construction including the resistance markers *AphVIII* and *PPX rs-3*, the promoter sequences HSP70-RBCS2 and NIT1p and reporter protein sequences as *CFP*.

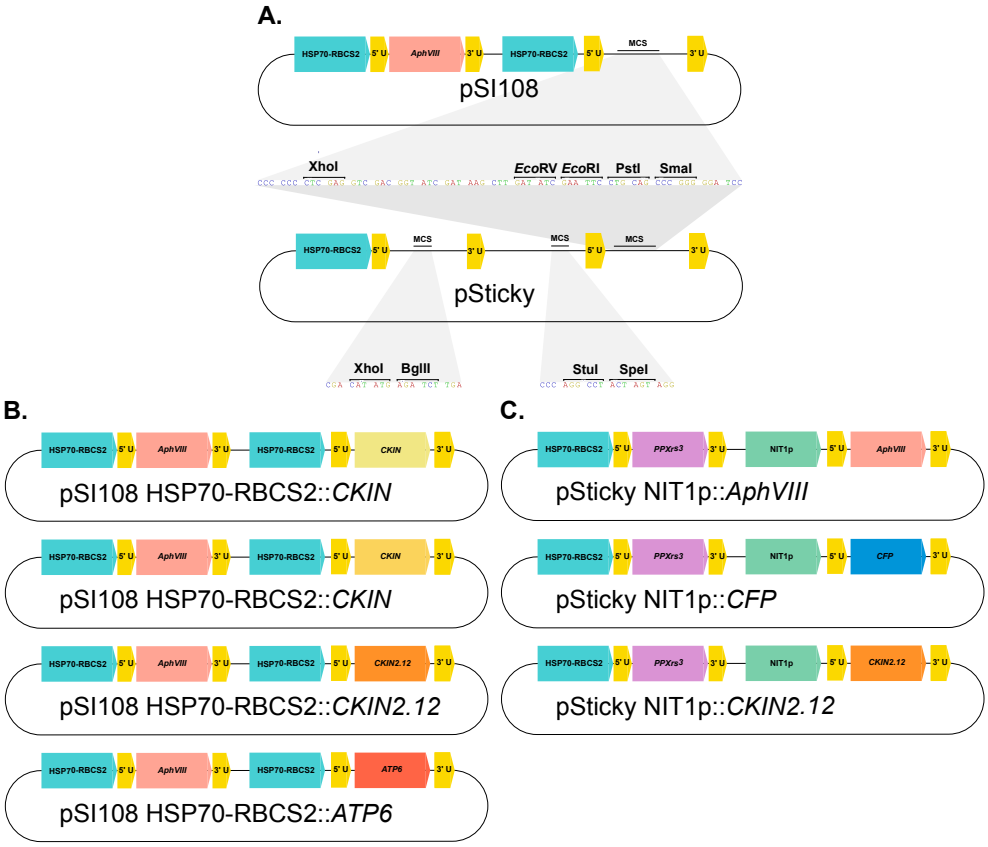

Figure 3. A. Maps of the pSI108 (upper) and pSticky (lower) constructions detailing the sequences and restriction sites for the multiple cloning sites (MCS) into the gene of interest cassette (shared between both constructions) and the pSticky exclusive ones allowing the exchange of the resistance gene and the gene of interest cassette promotor. B, pSI108 vector based constructions C, pSticky based constructions.

The transformation of Chlamydomonas with the mutated PPX confers the microalgae a stable resistance to oxyfluorfen

PPX was successfully mutated as confirmed sanger sequencing and inserted into the resistance gene cassette of the pSticky backbone. Resulting construction was used to transform Chlamydomonas through electroporation, allowing the recovery of 234 oxyfluorfen resistant colonies containing the resistance gene cassette as confirmed by colony PCR. This not only validated the capability of the mutated *PPX rs-3* sequence to confer Chlamydomonas resistance to the *PPX* inhibitor oxyfluorfen but also confirms the functionality of resistance gene cassette into the new pSticky construction.

NIT1 promoter drives the nitrate inducible expression of *AphVIII* resistance marker, confering a modulable sensitivity to paromomycin, and *CFP*

AphVIII resistance gene sequence was successfully placed under the control of NIT1 promotor into the pSticky based NIT1p::*AphVIII* construction. The transformation with this construction rendered paromomycin resistant colonies exclusively when the transformation product was plated into paramomycin containing inductive medium (452 paromomycin resistant colonies vs. 0 into the paromomycin containing basal TAP medium without nitrate). This result supported the functionality of the inducible NIT1 promoter into the generated pSticky vector (Figure 4A). Moreover the speed of the NIT1 promoter to induce the expression of a certain gene was tested through the transformation of Chlamydomonas with the pSticky based NIT1p*::CFP* construction. Between the 245 obtained colonies one was selected for the experiments. The transformed colony showed blue fluorescency exclusively under inductive conditions, and fluorescence started to be observed 30 min after induction start. This result support not only the promoter functionality but its fast gene induction (Figure 4B).

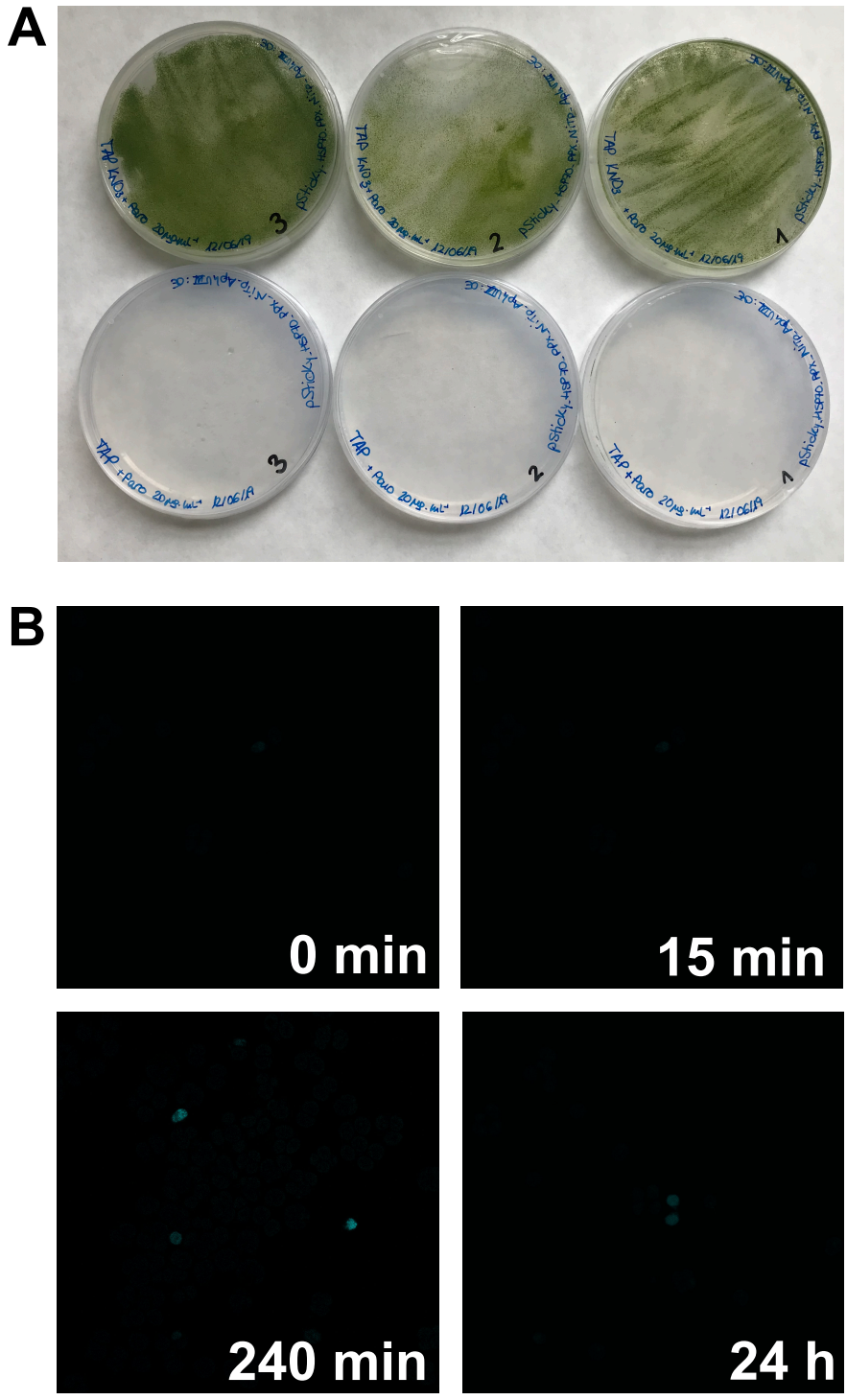

Figure 4. A, result of the plating of NIT1p::*AphVIII* transformed cells into paromomycin containing inductive (upper plates) and basal medium (lower). B, confocal microscopy images of the CFP blue fluorescence evolution at different time points upon induction start.

The overexpression of *CKIN2.12*, *CKINβγ*, *ATP6* and *CKINγ*

The coding sequences from *CKIN2.12*, *CKINβγ*, *ATP6* and *CKINγ* were successfully amplified from Chlamydomonas cDNA and inserted into the pSI108 vector under the control of the constitutive promotor HSP70-RBCS2. The transformation of Chlamydomonas with these constitutive expression constructions rendered 504, 356 and 427 paromomycin resistant colonies for HSP70-RBCS2::*CKINβγ*, HSP70-RBCS2::*ATP6* and HSP70-RBCS2::*CKINγ* respectively while no resistant colonies were recovered when transforming with the HSP70-RBCS2::*CKIN2.12* constitutive expression construction.

Twenty transformed colonies were selected from HSP70-RBCS2::*CKINβγ*, HSP70-RBCS2::*ATP6* and HSP70-RBCS2::*CKINγ* plates and genotyped to confirm the integration of the respective genes. Selected *CKINβγ* and *CKINγ* colonies did not have phenotypic differences in growth, pigments and starch/lipid content with empty pSI108 transformed colonies when growth under control conditions or under salt stress.

Five of the twenty genotyped HSP70-RBCS2::*ATP6* colonies were assayed for their resistance to salt stress against empty pSI108transformed strains. The qPCR over salt exposed control and transformed cells showed the ATP6 expression levels being higher into two of the five overexpressing lines than into the empty pSI108 control lines (data not shown). These two *ATP6* overexpressing strains showed a higher growth rate than control ones under tested salt stress (Figure 5).

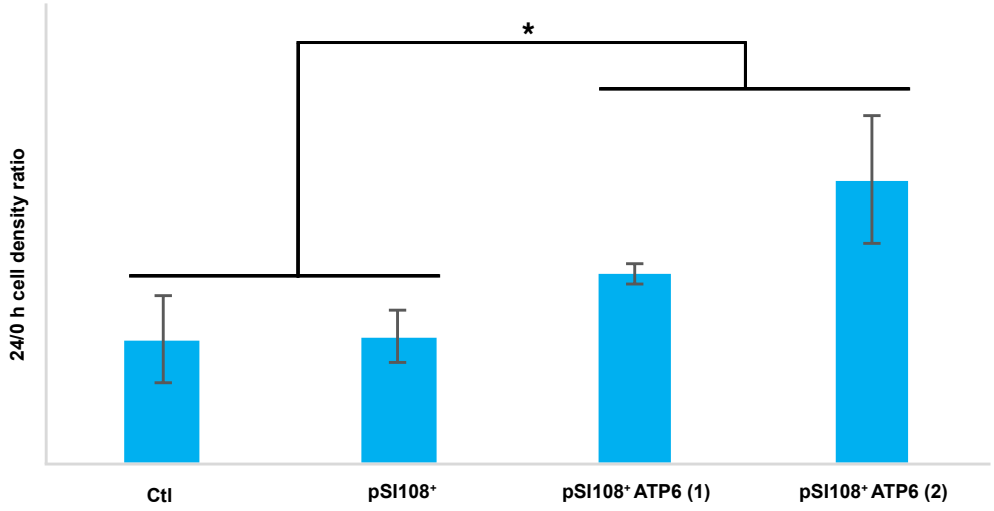

Figure 5. Cell density ratio after 24 h growth into liquid TAP medium with salt (25 mM NaCl) of control cell lines (Ctl), empty vector transformed lines (pSI108$^+$) and two different strains transformed with the ATP6 overexpression constructions (pSI108$^+$ ATP6 1 and 2).

The inducible expression of *CKIN2.12.*

To avoid the possible lethal effect derived from the constitutive expression of *CKIN2.12*, the gene coding sequence was inserted after the NIT1 inducible promoter into the pSticky based NIT1p::*CKIN2.12* construction. The transformation of Chlamydomonas with this construction rendered 245 oxyfluorfen resistant colonies after their plating into the oxyfluorfen containing medium under non-inducible conditions. Twenty oxyfluorfen resistant colonies were genotyped and three of these inoculated into inductive conditions. NIT1p::*CKIN2.12* induced cells were viable and showed no difference in growth with empty pSI108 transformed strains when growth under control conditions.

IV. Discussion

The use of different selection markers is needed when inserting different constructions into the same cell. Multiple resistance genes have been successfully tested into *C. reinhardtii*. However, some resistance genes provide a broad spectrum of resistances and, on the other side, some antibiotic combinations have cross effects. Thus, we chose a combination of the antibiotic resistance marker *AphVIII* and the herbicide resistance marker *PPX*

rs-3. these genes give Chlamydomonas an stable resistant phenotype without described cross resistances and cross effects between their selection agents (Bruggeman, Kuehler & Weeks 2014).

The *AphVIII* resistance marker was easily recovered from pSI108 construction, but *PPX rs-3* was produced from the original Chlamydomonas *PPX* coding sequence through a PCR based mutation strategy. The *rs-3* mutation gave *PPX rs-3* transformed Chlamydomonas a stable resistance to oxyfluorfen allowing the use of the mutated gene as an efficient selection marker. Interestingly, the resistance derived from the overexpression of *PPX rs-3* might not be exclusively related to the *rs-3* mutation (Randolph-anderson *et al.* 1998). The overexpression of the wild type Arabidopsis PPOX (the Arabidopsis ortholog to PPX) in *Nicotiana tabacum* gave the transformed plants resistance to the oxyfluorfen-like herbicide acylfluorfen (Lermontova & Grimm 2000).

The use of both *AphVIII* and *PPX rs-3* resistance genes have also allowed the validation of the function of the resistance and gene of interest cassettes into the pSticky vector. Moreover, the insertion of *AphVIII* under the control of NIT1 promoter into the pSticky based NIT1p::*AphVIII* construction has also allowed to test the functionality of the inducible promotor with the recovery of paromomycin resistant colonies exclusively under NIT1p inductive conditions. NIT1 promotor have been successfully used in other works to selectively induce the expression of metabolic selection markers and silencing RNA constructs in Chlamydomonas (Ohresser *et al.* 1997; Koblenz & Lechtreck 2005).

Other reports have characterized the promotor speed to drive the expression of a certain gene upon the imposition of inductive conditions (Ohresser *et al.* 1997). The transformation with the pSticky based NITp::*CFP* construction, containing the fluorescent protein gene CFP, allowed the evaluation of the induction speed of the NIT1 promoter. The observed induction time, based in the accumulation of CFP fluorescence, was coherent to the time described into other works. Moreover, the use of a fluorescence gene rather than a selection marker as *AphVIII* might have avoided skews into the observed

cassette expression efficiency due to the dependence of the cells to the resistance gene.

The original pSI108 constitutive construction has allowed the overexpression of different omic derived candidates discovered into the system biology-based models of the microalgae response to osmotic stress including *CKIN2.12*, *CKINβγ*, CKINγ and ATP6. Strikingly, the constitutive overexpression of *CKIN1* associated regulatory subunits *CKINβγ* and *CKINγ* did not induce phenotypic changes under tested conditions. This is interesting due to the central role of the highly conserved *SnRK1/CKIN1* subfamily into stress response and the centrality of *CKINβγ* into the Chlamydomonas STRING protein-protein interaction network (Colina *et al.* 2019). This points to the need to perform alternative stress assays to effectively test these genes. Moreover, available insertional mutant libraries don't have insertional mutants for CKIN1 and its associated regulatory sequences. This absence could be pointing to the lethality of CKIN1 subfamily. This problem has been circumvented in plants through the use of silencing constructs coupled to inducible promoters (Nukarinen *et al.* 2016) that would also work for the Chlamydomonas CKIN1 subfamily.

On the other hand, the overexpression of *ATP6* did induce phenotypic changes, increasing the microalgae resistance to salt stress. This nuclearly encoded mitochondrial protein, part of the proton channel of the mitochondrial ATP synthase, was found to be accumulated under osmotic stress. Moreover, the systemic models generated out of Chlamydomonas osmotic exposition protein and metabolite data correlated the protein to the multiple sugars accumulating under osmostress. These sugars were the most significative outcome of the osmostress response related to the maintainment of the osmotic equilibrium, the protection of cell structures and also the avoidance of ROS damage. The fact that is a nuclear encoded mitochondrial subunit made it an interesting target as it would allow the mitochondrial modulation from nuclei avoiding transforming the mitochondrial genome. Moreover, in plants, the enhancement of subunit a (plant ATP6 structural analog) expression under stress is a common response along other respiration

related mitochondrial subunits, allowing an enhanced resistance to stress. This enhancement is probably related to an increase in mitochondrial respiration, which is related to a reduction into ROS accumulation.

Along subunit a, other subunits enhance their expression under stress highlighting MtATP6. Multiple stresses induce the accumulation of this protein and its overexpression enhance Arabidopsis and yeast resistance to salt and oxidative stress. Interestingly, MtATP6 is not a Chlamydomonas ATP6 ortholog and it have an regulatory/stabilizing role into the plant and yeast mitochondrial ATP synthases rather than an structural one. Besides this, the maintenance of ATP synthase integrity under stressing conditions by the enhancement of these different subunits may contribute to the maintenance of ATP synthesis under stress and the control of proton gradients across mitochondrial inner membrane limiting oxidative damage.

The most surprising outcome between the chosen gene group was the lethality of CKIN2.12 constitutive overexpression. This effect points to the key function of the protein or at least to the toxic effect of its overexpression breaking the system dosage balance or abnormally interacting with other routes (Prelich 2012). This effect is greatly unexpected for a protein predicted to be an ortholog to plant SnRK2s as there are no reports of the overexpression or the blockage of these kinases having lethal effects. Besides this, this kinase is part of the CKIN2B group within the Chlamydomonas CKIN2 subamily, gathering secuences more divergent to plant SnRK2 than those into CKIN2A.

The overexpression lethal effect was not observed when inducing *CKIN2.12* expression into the context of an inducible promoter as NIT1p.This difference might be pointing to a developmental or a cell density dependent effect where the overexpression of the gene was only lethal under certain culture densities or during specific developmental stages.

The discovery of this kinase effects and its uniqueness opens the door to the characterization of novel stress and metabolic signaling pathways that could be unique to the microalgae.

V. Conclusion

Overexpression of osmotic stress candidate effector gene *ATP6* and microalgae specific CKIN/SnRK *CKIN2.12* changed the response of *C. reinhardtii* response to osmotic stress and was lethal for the microorganism respectively. On the other hand, the overexpression of plant-like Chlamydomonas *CKIN1* related *CKINβγ* and *CKINγ*, and *CKIN2.2* did not produce any phenotypic change in the microalgae under the tested conditions. The absence of phenotype has been related to the presence of functional redundancy between these elements, while the lethal effect of *CKIN2.12* overexpression suggests a key role for the microalgae specific protein probably into microalgae specific routes. These problems motivated the development of a new vector frame (pSticky) allowing to overcome them through multiple mutation strategies (functional overlapping) and inducible expression strategies (lethality) which has been successfully tested. Despite the unexpected results coming from CKIN candidates, the effect of the modulation of ATP6 expression points to the central role of the mitochondrial electron transport (*ATP6* is a key structural elements into this system) and validates the omics-systems biology used approach which originally placed this gene as a key piece into the microalgae response to osmotic stress.

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CHAPTER 6

General discussion

The environmental and socioeconomical issues derived from the extensive use of plants as biomolecule providers has motivated the search for alternatives, highlighting the use of microalgae. Besides this, the induction of biomolecule production into microalgae is unavoidably linked to stress, reducing their bulk biomass production and the profitability of their derived products (Khan, Shin & Kim 2018).

Making microalgae reliable and profitable biomolecule providers needs, thus, the characterization and modulation of their stress response systems in order to avoid or at least limit the stress associated costs and/or increase their biomass yields. However, stress response is a highly complex system where thousands of different level variables are involved. The omics and systems biology-based approaches are probably the best available ones to understand and modulate these complex traits on the modelling capabilities of systems biology and the omics power to feed modelling algorithms with high resolution multilayer data of the system.

Different omics/systems biology works have already described and modelled the stress response of the microalgae model *Chlamydomonas reinhardtii* under different stressing stimuli, discovering a system with features between those observed into land plants and yeasts (González-Ballester *et al.* 2010; Valledor, Furuhashi, Hanak & Weckwerth 2013; Valledor, Furuhashi, Recuenco-Munoz, Wienkoop & Weckwerth 2014). Besides this, these works have focused on classical biomass inductive stresses as Nitrogen deprivation, Sulphur deprivation and salt stress, while other promising ones as UV irradiation and dehydration have been less covered.

All the characterized response systems converge into central signaling nodes modulating, among other processes, the biomass modulation responses. The SnRK kinases, a highly diverse kinase family into the Plantae kingdom, are one of these central nodes linking different stress perception/signaling pathways to multiple stress effector genes (Coello, Hey & Halford 2011). Besides this, these kinases are still poorly characterized in Chlorophytes. Although these species SnRKs have shown similarities to land plants ones, the long divergence with plants would have originated differences that might be related to microalgae exclusive stress response mechanisms.

The omics and systems biology-based characterization of UV-B/C and dehydration stress responses performed into this thesis have identified the central role of these kinases in the microalgae stress response as previously did the works characterizing the nitrogen (Valledor *et al.* 2014) and sulphur depletion (Gonzalez-Ballester, Pollock, Pootakham & Grossman 2008; González-Ballester *et al.* 2010), and cold stress responses (Valledor *et al.* 2013) in *C. reinhardtii*. The involvement of these kinases into the microalgae stress response and the probable presence of microalgae specific SnRK related mechanisms have motivated the use of different methodologies for the description and characterization of the microalgae SnRK family aiming to uncover their origin and function, and evaluate their potential as biomass modulators.

C. reinhardtii and related microalgae species have centered multiple works characterizing their response to salt stress (Wang *et al.* 2018), but little characterization have been done on the simpler dehydration response. This focus can be explained on the effective and efficient modulation of the microalgae biomass when exposed to salt stress. However, salt stress is not a common one for freshwater Chlamydomonas and the mix of osmotic and ionic primary stresses into salt stress would complicate the description of response pathways and their linkage to specific stress stimulus. Thus, a mild dehydrating osmotic stress was chosen as resembles more accurately the stresses received by the microalgae into its natural environment, exclusively

involves osmotic stress as primary and stress and modulates the microorganism biomass content.

As into other stresses, the microalgae resilience under dehydration relies on controlled proteome and metabolome rearrangements which led between other systemic changes to the accumulation of valuable compounds. The great deal of complex multilevel rearrangements behind the observed systemic changes into other stresses, motivated the high-throughput screening of the osmostress induced changes into multiple omic layers, including the microalgae proteome, metabolome and physiology. The large multilevel data recovered after the exposition of the microalgae to dehydration allowed the rendering of a stress response model which was closer to land plants and microalgae desiccation than to salt stress responses. The observed metabolic changes matched those previously described into osmotically stressed *C. reinhardtii* and other chlorophytes, highlighting the accumulation of glycerol and soluble sugars (Husic & Tolbert 1986; Gustavs, Eggert, Michalik & Karsten 2010). These compounds played an osmolyte/osmoprotector role, but glycerol also worked as fast modulator of the cell redox status. Moreover, the integration of trehalose, between accumulated sugars, into the response model suggest a signaling role for the dissacharide into the microalgae osmostress response that could resemble its SnRK1 related role in plants (Kolbe *et al.* 2005; Tsai & Gazzarrini 2014).

The accumulation of this plant signaling sugar matched the parallel activation of different osmostress signaling pathways into the microalgae. These included a putative HOG pathway represented by an homolog to Saccharomyces SLN1 Histidine kinase osmorelay like (Cre17.g733150.t1.1), mitochondria and chloroplast ROS signaling MAPK6 and ATP6, organulli-nucleus communication through GUN4, and potential brassinosteroid and ABA mediated pathways represented by GUN4, MYB44 and different PP2C phosphatases. SnRKs could be involved into the observed sugar signalling role under osmostress (Kolbe *et al.* 2005; Tsai & Gazzarrini 2014) but also be part of the ABA/ROS signalling interacting with the identified PP2C (Coello *et al.* 2011). These signalers could be explaining the observed metabolic

rearrangement, and as into other stresses driving a genetic reprogramming, mediated by specific epigenetic mechanisms behind the observed proteome and metabolome remodeling allowing long-term cell survival.

These elements also modulate plant and yeast osmostress response and are promising points for further osmotic response characterization or exploitation towards engineering more productive strains.

Same multi-level omics and systems biology strategy was used into the characterization of UV-B/C response. Against dehydration, UV-B/C irradiation is a less common stress for microalgae due to the atmospheric filtering of UV-C and the water shield on UV-B. However, this radiation also drives to interesting changes into the biomass composition of plant and microalgae, centered on the accumulation of pigments and antioxidant compounds (Srinivas & Ochs 2012; Ahmed & Schenk 2017; Xu *et al.* 2017, 2019).

The accumulation of these compounds summarizes a response based on the avoidance of UV and UV derived ROS damage to macromolecules, involving a timeline of events including the enhancement of UV shielding, protein protection/turnover, redox modulation mechanisms, and specifically from the fine tuning of UV sensitive photosystems.

Microalgae early response to the UV irradiation is mainly based on the maintenance of the different cellular processes functionality and the avoidance of further damage through enhanced protein turnover/protection and metabolic/redox modulation. This fast response was coupled to the activation of parallel UV-B and UV-C signaling pathways including UVR8 and probably ROS/SA signaling that would be driving a fine UV-directed tuning of cell proliferation, gene expression and protein translation before Chlamydomonas late and more complex acclimation responses. Some found early response elements would be after the early changes as redox modulator NDE5, translation modulator FAP204, and cell proliferation related PP2A like protein and MINA53.

Acclimation was related to specific proteome changes focused on the modulation of photosystems, highlighting the uncoupling of PSII and the enhancing of CEF, and on the accumulation of specific UV shielding and ROS scavenging metabolites. The late metabolic modulation could be related to a novel DYRK kinase accumulating on acclimation along a described UVR8 target (LHCSR1), contributing to acclimation photosynthetic changes.

The identified targets are promising for the further characterization of the UV-B/C effect on metabolism and photosynthesis or towards the exploitation of the UV metabolic modulation mechanisms into the engineering of more productive strains.

Many evidences from different stress responses support the conservation of the plant SnRK role in energy sensing and stress-adaptive responses into microalgae systems. However, differences between both systems SnRKs and stress response systems could difficult the use of the plant SnRK knowledge into the improvement of microalgae biomass production through the modulation of these kinases. Thus, the centrality of this kinases into the stress response, its transversality along the plantae kingdom and the identification of their particularities in plant and microalgae systems has motivated the definition of the Chlamydomonas CKIN family (SnRK in Arabidopsis) and those of other microalgae species.

Twenty-two sequences were defined as plant SnRK orthologs in Chlamydomonas after a genome-wide analysis and classified into two subfamilies: CKIN1 and CKIN2. While CKIN1 subfamily is reduced to one conserved member and a close protein (CKIN1L), a large CKIN2 subfamily clusters both plant-like and algae specific CKIN2s.

The responsiveness of these genes to abiotic stress situations was tested by RT-qPCR. Results showed that almost all elements were sensitive to osmotic and UV stresses while showing different degrees of sensibility to other abiotic stresses, as occurs in land plants, revealing their specialization and the family pleiotropy for some elements.

The regulatory pathway of this family may differ from land plants since these sequences shows unique regulatory features and some of them are sensitive to ABA, despite conserved ABA receptors (PYR/PYL/RCAR) and regulatory domains are not present in this species.

Core Chlorophytes and land plant showed divergent stress signaling, but SnRKs/CKINs share the same role in cell survival and stress response and adaption including the accumulation of specific biomolecules. This fact places the CKIN family as well-suited target for bioengineering-based studies in microalgae (accumulation of sugars, lipids, secondary metabolites), while promising new findings in stress biology and specially in the evolution of ABA-signaling mechanisms.

The extension of the family characterization to other chlorophyte species revealed the heterogeneity of the family between microalgae species from the reduced plant like group found on Ostreococcus to the Chlamydomonadales with a Chlamydomonas like arrangement.

Besides this, although most of the microalgae CKINs have shown a high sensitivity to stress, the link between stress and CKINS is still undisclosed. Stress/ABA sensitive CKINs and other Chlamydomonas omic derived stress response candidates still need to be characterized in order to identify their actual function into the stress response. This step commonly relies on genome edition techniques, allowing the elucidation of the gene function through the description of the effects of their modulation, the identification of their interactants and/or the discovery of their location. The characterization of the Chlamydomonas CKIN family has been started with the identification of the *CKIN2.1* and *CKIN2.2* role into Sulphur deprivation through the description of the response to Sulphur stress into *CKIN2.1* and *CKIN2.2* KO and recovered cell lines (Gonzalez-Ballester *et al.* 2008).

The plant family function has been much more described with different works covering the subfamilies SnRK1, SnRK2 and SnRK3 (Coello *et al.* 2011). The characterization of the first two plant subfamilies involved many problems whose solutions have been valuable into the approaches to characterize the

microalgae family. Arabidopsis and rice needed multiple gene mutants for the characterization of the link between their SnRK2 subfamilies and the response to osmotic stress due to the functional overlapping of their members (Fujii, Verslues & Zhu 2011). On the other hand, the edition of the highly conserved SnRK1 kinases has shown to be lethal into Arabidopsis requiring from the use of inducible repression strategies (Nukarinen *et al.* 2016).

Thus, the conservation observed between Chlamydomonas and Arabidopsis SnRK subfamilies 1 and 2 suggested that same lethality and functional overlapping problems could arise when characterizing the microalgae subfamilies. Indeed, the constitutive expression of Chlamydomonas *CKIN2.2*, close to Arabidopsis SnRK2s did not modify the microalgae phenotype. Conversely, the overexpression of the Chlamydomonadal specific *CKIN2.12* was lethal. Strikingly, the overexpression of *CKIN1/SnRK1* associated regulatory sequences *CKINβγ* and *CKINγ* wasn't lethal and did not produce phenotype changes in the microalgae.

In order to overcome the lethality and functional overlapping issues probably associated to CKINS a modular vector, pSticky, was constructed allowing the easy exchangeability of resistance genes and promotors. This construction would allow the generation of Chlamydomonas lines with multiple mutated CKINs and the inducible expression of lethal genes. Two resistance genes, *AphVIII* and *PPX rs-3*, will drive the multiple transformation strategies in the microalgae allowing the selection of the multiple transformant strains. In the case of *PPX rs*-3, the gene was successfully mutated from the wild type *PPX* to produce the resistance to the herbicide oxyfluorfen. Moreover, the inclusion of NIT1 nitrate inducible promoter into the construction have already allowed the inducible expression of target genes. The inducible expression of *CFP* and *AphVIII* allowed the testing of the system with easily screenable genes. *CKIN2.12* was inserted afterwards after the NIT1 promoter, however, the inducible expression of the sequence has no lethal effects and did not produce phenotypic changes in the microalgae.

On the other hand, the constitutive expression of the osmotic stress candidate *ATP6* enhanced the microalgae resistance to salt stress. The involvement of this protein into the mitochondrial transport chain and oxidative phosphorylation evidences the importance of these processes, and more specifically of oxidative stress response, into the response to osmotic stress. Osmostress induced oxidative stress in microalgae and the overexpression of this subunit could be enhancing the oxidative stress response. The overexpression of similar subunit in plants enhance their competence.

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CHAPTER 7

Conclusions

I. Chlamydomonas response to osmotic stress is characterized by a quick accumulation of sugars and glycerol as a consequence of the activation of parallel signaling pathways related to osmosensing, ROS, plastid-nucleus retrograde signaling, and potentially ABA and brassinosteroids. Ultimately, all these were linked to the deployment of long-term acclimation mechanisms through their control of a genetic reprogramming, which mediated by epigenetic mechanisms, drove the observed remodeling of the proteome, allowing the cell survival to the hyperosmotic environment.

II. Integrated proteomic and metabolomic approach over the osmotic stress response allowed the identification of key sensing and signaling targets as MAPK6, PKL1, ATP6, GUN4, MYB44, Histidine kinase osmorelay like (Cre17.g733150.t1.1) and different PP2Cs. The plant and yeast orthologs to these proteins are also related to osmostress response, showing a Chlamydomonas osmostress response system similar to those already described into land plants and yeasts.

III. Chlamydomonas response to UV-B/C stress is characterized by the fast activation of proteome repair/protection and redox modulation mechanisms. These give way to acclimation mechanisms focused on photosynthesis as the uncoupling of PSII and the enhancement of CEF, and the accumulation of ROS scavenging/UV shielding compounds, needing from a more slow proteome turnover which modulated by different epigenetic mechanisms was driven by different early triggered UV-B/C dependent signaling pathways.

IV. The same integrative approach over proteome and metabolome data of UV-B/C stressed cells pointed to the synchronous involvement of UV-C related ROS/SA and UV-B related UVR8 signaling pathways into the described response. This approach also allowed the identification of key factors as FAP204, PP2A like protein, MINA53, DYRK and LHCSR1 related to translation, redox, cell proliferation, biomass and photosynthetic electron transport modulation under stress.

V. Osmotic and UV stress related elements are promising targets for the further characterization of the microalgae osmotic stress response and the exploitation of these microorganisms towards the engineering more productive strains. Moreover, the multiple parallelisms between the plant and microalgae response systems places the microalgae as interesting models for plant stress research.

VI. The characterization of the microalgae response systems to osmotic and UV stresses showed complex and overlapping signaling pathways. Both systems displayed many specific elements but also shared evidences of the involvement of central stress signalers as the plant and microalgae conserved SnRK kinase family, which had been previously found to be involved into the microalgae response to cold, sulphur and nitrogen deprivation stresses.

VII. The chosen sequence homology and domain-based strategy used into the identification of the microalgae SnRKs allowed the completion of the description of the Chlamydomonas SnRK gene family and the description of those of related microalgae species. This strategy identified plant like CKIN/SnRK, along microalgae specific sequences which had been previously unnoticed and are probably after novel signaling mechanisms; and a reduced to absent SnRK3 subfamily in microalgae.

VIII. Microalgae CKIN/SnRKs are monophyletic with plant SnRKs although they have followed a divergent evolutive path with the development of more heterogeneous SnRK2 subfamilies, specially between Chlamydomonadales, and their small to absent SnRK3 subfamilies.

IX. The microalgae CKIN sequences are responsive to different stresses, highlighting their high sensitivity to UV and osmotic stresses and the sensitivity that some of them displayed under the modulation of ABA.

X. The overexpression of Chlamydomonas *CKIN1* related *CKINβγ* and *CKINγ*, and *CKIN2.2* did not produce any phenotypic change under the tested conditions probably due to the functional redundancy of these elements while the overexpression of *CKIN2.12* was lethal, suggesting a possible key role for the microalgae protein. These problems motivated the development of a new vector frame (pSticky) allowing to overcome these problems through multiple mutation strategies (functional overlapping) and inducible expression strategies (lethality).

XI. Overexpression of osmotic stress candidate effector gene *ATP6* changed *C. reinhardtii* response to osmotic stress, pointing to the central role of the mitochondrial electron transport (*ATP6* is a key structural elements into this system) while validates the omics-systems biology used approach which placed this gene as a key piece into the response to osmotic stress.

Description of the *P. pinaster* PpiKIN/SnRK family and their links to Arabidopsis and microalgae SnRKs

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I. SnRKs, promising targets for getting forestry species ready for climate change

Stress stimuli can effectively modulate biomolecule production into microalgae and plants, but at the cost of reducing their biomass yields. This yield losses vexes plant producers globally as the incidence and intensity of environmental stresses is rising under the current context of global warming. These changes have already produced variations into crop and forestry yields and they are predicted to worsen into the near future (Bussotti, Pollastrini, Holland & Brüggemann 2015; Parent *et al.* 2018; Tigchelaar, Battisti, Naylor & Ray 2018; Ladwig, Chandler, Guiden & Henn 2019).

climate change effects would be especially negative for the slow growing forestry species as *Pinus pinaster*, an autochthonous species from the Mediterranean basin with a key importance for this region forestry sector. Thus, multiple efforts have been started directed to the use of better management strategies and the generation/selection of more resistant and productive tree varieties (Bussotti *et al.* 2015). Besides this, the complexity of

the plant productivity and stress resistance traits and the long generative time of tree species complicates these approaches.

Omics and systems biology are the best suited techniques to face the biological complexity problem; however, the long generation times of tree species still complicates the required functional validation of omics candidates. Model species as Arabidopsis have already been used for the validation of candidate genes from tree species due to its closeness and short generation times (Valledor *et al.* 2018). Moreover, the shorter generative times (days vs months in Arabidopsis) and simpler but plant-like response systems of the microalgae model *C. reinhardtii* would further ease forestry species research.

The CKIN/SnRK kinases, with a central role into stress response and a specific involvement in the modulation of biomass production, are promising candidates for the enhancement of plant/microalgae productivity and stress resistance (Coello, Hey & Halford 2010; Colina *et al.* 2019). These kinases would also be valuable targets into enhancement strategies in tree species as *P. pinaster*; besides this, they have not been described in the gymnosperm and the long generative times of this species will complicate their characterization. The description of the *P. pinaster* SnRKs would bypass the generation time problem through the identification of their orthologs into fast-growing species as *Arabidopsis thaliana* and *Chlamydomonas reinhardtii*. The modulation of these orthologs and/or their substitution for their *P. pinaster* counterparts would allow the characterization of the *P. pinaster* sequences avoiding the time-consuming transformation and/or selection strategies in the tree species. Moreover, the identification of the orthology links with microalgae and plant families would trace down the evolution of the complex plant SnRK families and evidence the specific features into the poorly characterized gymnosperms families.

II. Identification of the P. pinaster SnRK/PpiKIN family

The description of the *P. pinaster* SnRK/PpiKIN family faced the lack of a sequenced genome into this non model species; thus, the search was based on

the species available transcriptome (Canales *et al.* 2014) and derived protein models available at Plaza (Proost *et al.* 2014). Arabidopsis SnRKs sequences (Coello *et al.* 2010) were used as query into BLAST and BLASTP based searches into the bioinformatic suite Geneious (Biomatters Inc.), considering for further analyses the sequences with e-values lower than e^{-25}. Homology based searches resulted into the identification of a large sequence group containing SnRKs and other sequences. Non-SnRKs were filtered out from homology results through the analysis of their domain composition using InterProScan (Mitchell *et al.* 2019), keeping those sequences with the Arabidopsis SnRKs canonical domain composition as specified by Colina *et al.* (2019).

This search and filter strategy resulted into the confidently identification of 39 *P. pinaster* SnRK/PpiKIN sequences. Between the identified PpiKINs, 31 were classified as SnRK3 (PpiKIN3), 6 as SnRK2 (PpiKIN2) and the two remaining ones as SnRK1 and SnRK1 like (PpiKIN1 and PpiKIN1-L respectively) relying on their domain composition (Table 1). As expected, *P. pinaster* and Arabidopsis SnRK/PpiKIN families have similar size and structure without algae specific SnRK2(B) sequences and a large SnRK3 subfamily. Interestingly, the Pinus family have a SnRK1-L sequence as into Chlamydomonas and *V. carteri* families (Table 1).

Table 1. Number of sequences from each SnRK subgroup, namely SnRK1, SnRK1-L, SnRK2(A), SnRK2(B) and SnRK3 into the SnRK families of *A. thaliana*, *P. pinaster*, *O. lucimarinus*, *C. subellipsoidea*, *C. variabilis*, *D. salina*, *C. reinhardtii* and *V. carteri*.

Species	SnRK1	SnRK1-L	SnRK2(A)	SnRK2(B)	SnRK3
Arabidopsis thaliana	3	-	8	-	28
Pinus pinaster	1	1	6	-	31
Ostreococcus lucimarinus	1	-	2	-	-
Coccomyxa subellipsoidea	1	-	15	2	1
Chlorella variabilis	1	-	9	1	1
Dunaliella salina	1	-	4	3	-
Chlamydomonas reinhardtii	1	1	14	2	-
Volvox carteri	1	1	10	3	-

III. Description of the P. pinaster PpiKIN family structure and evolution

PpiKIN sequences were aligned with other SnRK sequences belonging to *A. thaliana* and different microalgae species as *C. reinhardtii*, *Dunaliella salina*, *Volvox carteri*, *Chlorella variabilis*, *Coccomyxa subellipsoidea* and *Ostreococcus lucimarinus* using the M-Coffee consensus alignment method (Wallace, O'Sullivan, Higgins & Notredame 2006). Inconsistent columns were removed from the resulting alignment through the Transitive Consistency Score algorithm (TCS) (Chang, Di Tommaso, Lefort, Gascuel & Notredame 2015). Curated alignment distances were used into the generation of maximum likelihood (ML) trees whose consistency was evaluated through Transfer Bootstrap Expectation score (TBE, 500 replicates) into the Booster platform (Lemoine *et al.* 2018).

The Pinus sequences clustered into three main groups containing all SnRK1, SnRK2 and SnRK3 orthologs of the different included species. All the SnRK1 orthologs clustered together along the SnRK1-L sequences. Within the SnRK1 subgroup the closest sequence to *P. pinaster PpiKIN1* was the Arabidopsis *SnRK1/AKIN10* and the *O. lucimarinus OKIN1* was the basal branching sequence (Figure 1). The larger Arabidopsis SnRK1 subfamily has been related to the more complex life cycle of the model angiosperm with one of its SnRK1 (*SnRK1.3*) being exclusively expressed into the plant reproductive tissues. Moreover, the *OKIN1* basal position points to this species SnRK1 as the closest to the common algae-plant ancestor. Moreover, the clustering of *C. reinhardtii*, *V. carteri* and *P. pinaster* SnRK1 like sequences along SnRK1 (Figure 1) support their relation to the SnRK1 subgroup and suggest the conservation of these sequences across Plantae kingdom. Despite this, *PpiKIN1-L* lacked the long (1 Kb) N-terminal extension present into the microalgae *CKIN1-L* and *VKIN1-L* (data not shown).

SnRK3 was the biggest sequence group, mostly represented by the Arabidopsis and Pinus elements. Microalgae SnRK3 cluster (cluster 1), with *C. subellipsoidea CsKIN3* and *C. variabilis CvKIN3*, was at the base of this group

followed by four more clusters containing exclusively Arabidopsis and *P. pinaster* sequences (clusters 2-5) (Figure 1). Interestingly, the closest plant SnRK3 cluster to the microalgae specific cluster 1, the cluster 2, contained the Arabidopsis sequence *SnRK3.11*. The derived protein, SOS2, is involved into the plant salt stress signaling pathway SOS. The proximity of *SnRK3.11* to the microalgal SnRK3s is suggestive of the ancient origin of this pathway and the possible involvement of the microalgae SnRK3s into salt stress response. *P. pinaster PpiKIN3.3* and *PpiKIN3.4* were the closest sequences to *SnRK3.11*/SOS2 (Figure 1) and thus the most probable gymnosperm orthologs of the salt stress related kinase. Clusters 3 to 5 contain most of the plant sequences. Interestingly, many of the Arabidopsis sequences into these clusters are involved into ABA stress signaling and expressed under certain developmental times and into specific tissues (Zhou *et al.* 2015). Thus, the diversification of the plant SnRK3 subfamily may have been related to the increase in structure and life cycle of land plants and to their adaption to the more stressing land environment.

SnRK2 sequences clustered into SnRK2(A) and (B) as previously described (Colina *et al.* 2019) with all the *P. pinaster* sequences falling into the SnRK2(A) group. The gymnosperm SnRK2s clustered into this group along Arabidopsis ones and a small group of microalgae SnRK2s, plant like SnRK2, conforming the subgroup 2 (Figure 1). These plant like microalgae sequences were the most interesting SnRK2 orthologs towards the use of microalgae as models for the characterization of the PpiKIN2 subfamily function.

APPENDIX 1: Description of the *P. pinaster* PpiKIN/SnRK family and their links to Arabidopsis and microalgae SnRKs.

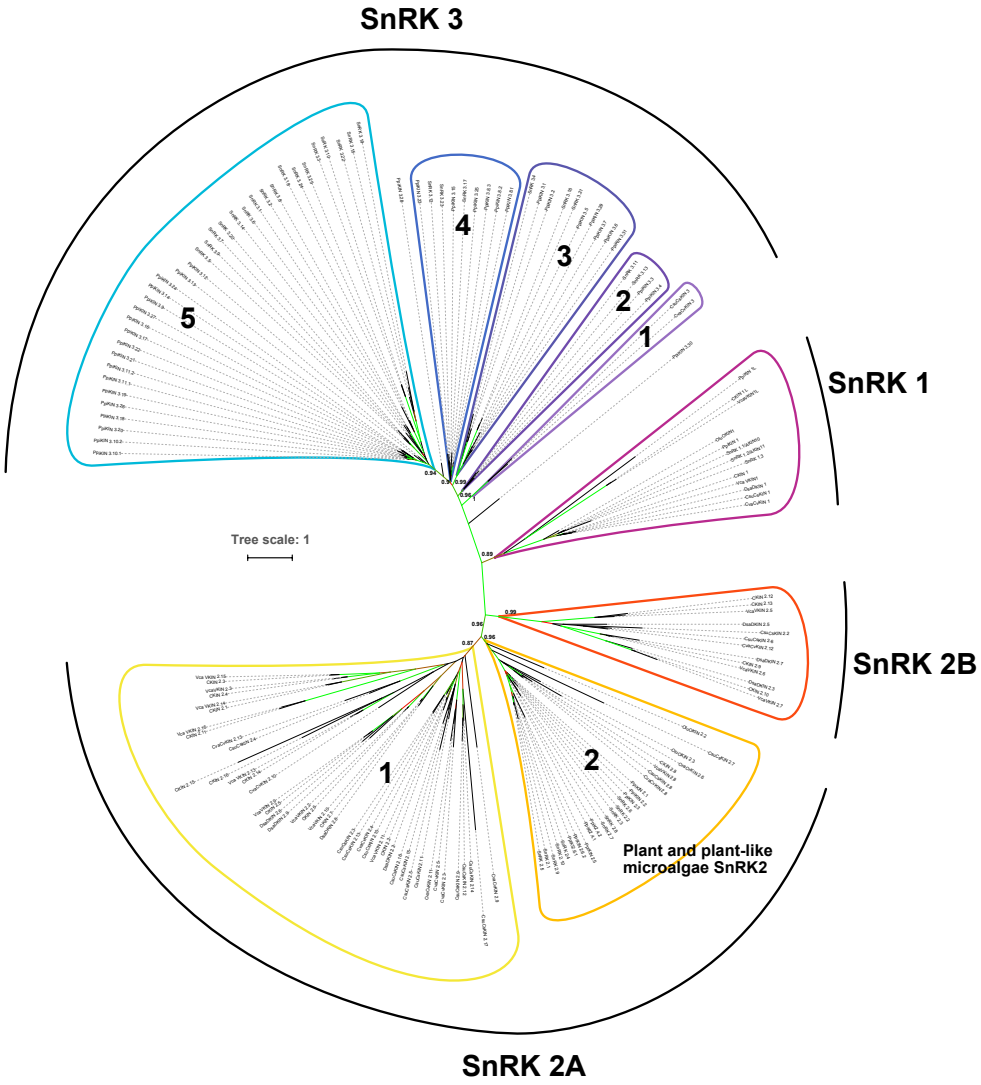

Figure 1. Unrooted ML tree of *Pinus pinaster* (pPIKIN), *Arabidopsis thaliana* (SnRK), *Chlamydomonas reinhardtii* (CKIN), *Volvox carteri* (VcaVKIN), *Dunaliella salina* (DsaDKIN), *Chlorella variabilis* (CvaCvKIN), *Coccomyxa subellipsoidea* (CsuCsKIN) and *Ostreococcus lucimarinus* (OluOKIN) SnRK sequences. Tree branches are colored according to their TBE bootstrap value, thus, branches with TBE values below 0.8 are black while these above 0.8 TBE are colored from red (0.8) to light green (1). Tree divided the sequences into the tree SnRK families (SnRK1, 2, 3). SnRK1 subfamily was divided into SnRK1 like and SnRK1 sequences, SnRK3 sequences was divided into five different subgroups and SnRK2 sequences were divided between SnRK2A and SnRK2B. *P. pinaster* PpiKIN2 sequences were all included into the SnRK2(A) group along Arabidopsis SnRK2s.

IV. Description of the SnRK2 subfamily structure and evolution

All the plant and plant-like SnRK2 into the cluster 2 within SnRK2(A) were aligned using the consensus aligner M-Coffee (Wallace *et al.* 2006). The resulting alignment was curated trough TCS (Chang *et al.* 2015) and the distances of the curated alignment used to build a rooted ML tree whose consistency was evaluated through Felsestein's bootstrap (FBP, 500 replicates) into the booster platform (Lemoine *et al.* 2018).

Resulting tree confidently separated plant and microalgae SnRK2 placing *O. lucimarinus OKIN2.2* as the closest microalgae SnRK2 to plant SnRK2s. Arabidopsis SnRK2s were grouped into three groups, namely I (ABA insensitive), II (Low ABA sensitivity) and III (high ABA sensitivity) as previously described (Kulik, Wawer, Krzywińska, Bucholc & Dobrowolska 2011). Most *P. pinaster* sequences clustered with the Arabidopsis sequences into I and III groups, however no sequences clustered with Arabidopsis group II SnRK2s (Figure 2).

This tree also showed that the ABA sensitive sequences were closer to the ancestral SnRK2 than the insensitive ones being suggestive of the early involvement of ABA into SnRK2 based signaling pathways. Despite this, the expression of Chlamydomonas *CKIN 2.8*, a plant like SnRK2, is not sensitive to exogenous ABA treatments (Colina *et al.* 2019).

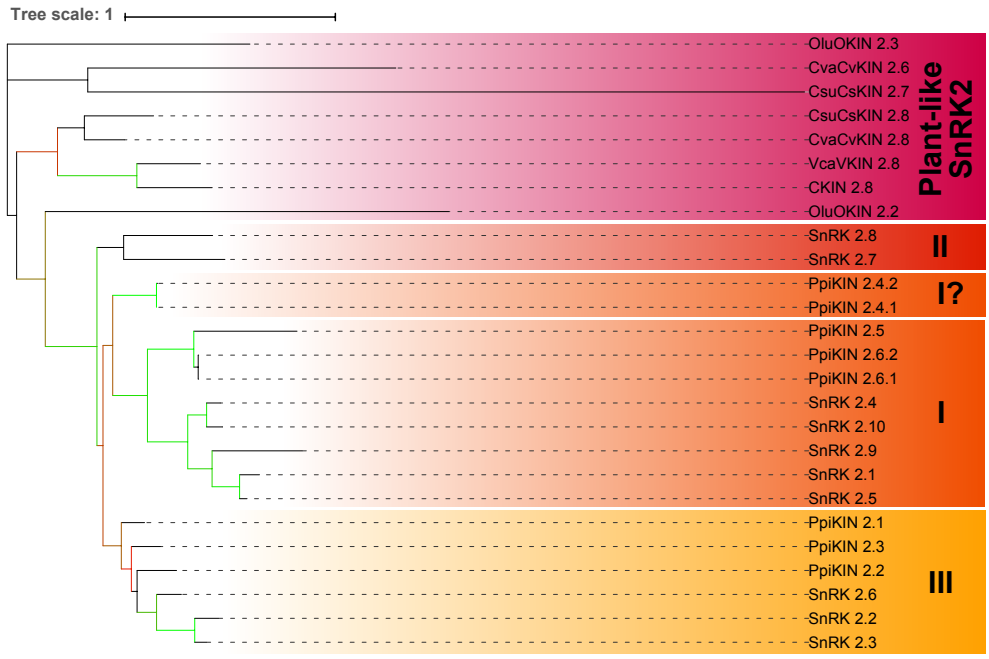

Figure 2. Rooted ML tree over *Pinus pinaster* (pPIKIN 2) and *Arabidopsis thaliana* (SnRK 2) SnRK 2 sequences along *Chlamydomonas reinhardtii* (CKIN 2), *Volvox carteri* (VcaVKIN 2), *Dunaliella salina* (DsaDKIN 2), *Chlorella variabilis* (CvaCvKIN 2), *Coccomyxa subellipsoidea* (CsuCsKIN 2) and *Ostreococcus lucimarinus* (OluOKIN 2) plant-like SnRK2s. Tree branches are colored according to their TBE bootstrap value, thus, branches with TBE values below 0.8 are black while these above 0.8 TBE are colored from red (0.8) to light green (1). Tree confidently separated microalgae and plant sequences, plant SnRK2 group was separated into groups I (ABA insensitive), II (low ABA sensitivity) and III (ABA sensitive).

Group III SnRK2 sequences were highly similar between Arabidopsis and *P. pinaster*, with highly conserved regulatory domains I and II (ABA box) after the ser/thr kinase domain supporting their involvement into the gymnosperm ABA signaling (alignment not shown). On the other hand, although the kinase domain and domain I was similar between the Arabidopsis group I sequences and *P. pinaster PpiKIN2.1*, *2.4*, *2.5*, *2.9* and *2.10*, the Pinus and Arabidopsis sequences diverged after domain I end (alignment not shown). Interestingly, *PpiKIN2.4* shared C-terminal motifs with the ABA sensitive group III sequences, and *PpiKIN2.6* had long insertions into these regions not found into Arabidopsis SnRK2 sequences but resembling those found into microalgae sequences as Chlamydomonas *CKIN2.8*.

V. Conclusion

Unsurprisingly *P. pinaster* and Arabidopsis SnRK family resemblance is high, supporting the use of Arabidopsis for the functional characterization of the Gymnosperm SnRKs. Moreover, the SnRK families of microalgae species as Chlamydomonas, *O. lucimarinus*, *C. subellipsoidea* and *C. variabilis* could also be valuable. Microalgae species have plant like SnRK1 and 2 sequences, and *C. subellipsoidea* and *C. variabilis* also plant like SnRK3 sequences within simpler response systems. Despite the utility of this models, the comparison of their SnRK families also evidences important differences as the absence of clear orthologs to the Arabidopsis I and II SnRK2 subgroups, the simpler *P. pinaster* SnRK1 subfamily, the large groups of microalgae specific SnRK2s and the absence of SnRK3 into most microalgae species. Besides these differences could complicate the use of Arabidopsis and microalgae species for the validation of the *P. pinaster* SnRKs, they also show the evolution of the SnRK family pointing to the early origin of SnRK3-based salt stress signaling, the conservation of SnRK1-L sequences into plant species and the early origin of the ABA sensitive SnRK2s.

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Resumen

TABLA DE CONTENIDOS

I. Introducción

Las consecuencias negativas sobre el medioambiente y la seguridad alimentaria que se derivan del uso de las plantas como principales proveedores de biomasa, han motivado la búsqueda de alternativas entre las que destaca el uso de las microalgas. Estos microorganismos, comparten muchas de las características de las plantas sin la necesidad de grandes superficies para su cultivo, sin embargo, la acumulación de biomoléculas en estos organismos esta ligada a un proceso de inducción por estrés, que también reduce su rendimiento en biomasa y la rentabilidad de los productos derivados de estas.

Conseguir que las microalgas sean una fuente de biomasa rentable y eficiente, necesita atajar el problema de la inducción por estrés, eliminando o al menos reduciendo los costes que este proceso introduce. La mayoría de las biomoléculas de interés industrial se acumulan bajo estrés porque forman parte de la respuesta al propio estrés, por ello, convertir las microalgas en los productores de biomasa del futuro pasa necesariamente por la caracterización de su sistema de respuesta a estrés y sus vínculos con la producción y

modulación de la composición de biomasa. A pesar de la claridad del objetivo, la respuesta a estrés es un proceso de gran complejidad en el que se implican miles de variables de diferentes niveles. Este problema ha motivado el uso de estrategias basadas en las ómicas y la biología de sistemas, que son las mejores disponibles para la resolución de problemas biológicos que, como este, implican una gran dimensionalidad frente a la que las alternativas reduccionistas tradicionales han fallado.

Múltiples trabajos ya se han basado en estas técnicas para describir el sistema de respuesta a estrés de la microalga modelo *Chlamydomonas reinhardtii* ante diferentes estreses abióticos, identificando un sistema con características intermedias entre las descritas en otros de plantas y levaduras. Estos trabajos también coinciden en la identificación de nodos señalizadores centrales que modulan, entre otras, las respuestas a estrés basadas en la modulación de la composición de la biomasa. Entre los diferentes nodos identificados, las kinasas de la familia *Sucrose non Fermenting Related Kinases* (SnRK), muy diversa en el reino Plantae y conocida en plantas por su rol en la conexión de distintas vías de percepción y señalización, con múltiples genes efectores, son uno de los mas destacados. A pesar de esto, estas quinasas todavía están poco caracterizadas en las clorófitas, donde a pesar de las similitudes identificadas con la familia de plantas, la gran divergencia evolutiva con respecto a ellas puede haber originado diferencias importantes que pueden estar tras mecanismos exclusivos de respuesta.

Por ello, el planteamiento de esta tesis parte de la importancia de la caracterización del sistema de respuesta a estrés en la microalga modelo *C. reinhardtii*. Se centra en su respuesta a estreses aún poco caracterizados en la microalga, pero con efecto sobre su perfil de biomasa como son el estrés osmótico y ultravioleta. Así pues el objetivo principal es identificar nodos centrales en el sistema de respuesta a estos estreses, centrándose preferentemente en aquellos relacionados con la modulación de la composición de biomasa.

II. Resultados y discusión

Caracterización de la respuesta a estrés osmótico

Las microalgas son capaces de sobrevivir a cambios en el contenido de agua de los medios que habitan gracias a diversos mecanismos que permiten el mantenimiento del equilibrio osmótico y el crecimiento bajo estas condiciones. Como en otras respuestas a estrés, los mecanismos de respuesta a estrés osmótico implican cambios profundos en el proteoma y el metaboloma que se encuentran tras la adaptación final al estrés y, más específicamente, tras respuestas típicas al mismo como es la acumulación de biomoléculas de interés con función osmoprotectora (azúcares y glicerol). La inducción de la acumulación de biomoléculas junto con la falta de conocimiento sobre los efectos específicos del estrés osmótico -múltiples trabajos ya han descrito los efectos de su combinación con el estrés iónico bajo estrés salino- han motivado la caracterización de la respuesta a dicho estrés en la microalga modelo *C. reinhardtii*, mediante su exposición a un estrés osmótico suave durante un periodo de 24 h, similar al que la microalga de agua dulce/salobre puede encontrarse en su medio natural.

La medida del cambio de miles de proteínas y decenas de metabolitos, combinada con la toma de diferentes medidas fisiológicas durante la exposición a este estrés permitió la reconstrucción de una imagen dinámica de este sistema de respuesta a través de una aproximación de biología de sistemas. Esta ha mostrado, como en trabajos anteriores, la similitud de la respuesta de la microalga con las de plantas y levaduras, y la importancia que tienen la acumulación de osmoprotectores como el glicerol y de azúcares (entre los que destacó la trehalosa tanto por su rápida acumulación como por su posible rol en la señalización del estrés).

El desarrollo de esta respuesta fue el resultado de la activación de distintas vías de señalización incluyendo una posible vía HOG, vías basadas en la producción de especies reactivas de oxígeno, comunicación retrógrada núcleo-orgánulo, y potencialmente mecanismos basados en ABA y brasinosteroides. Estas vías estaban íntimamente ligadas a la aclimatación a largo plazo a través

de su participación en la activación de diversos mecanismos de respuesta a través de un proceso de modulación génica mediado por diferentes mecanismos epigenéticos.

Dentro de estas vías de señalización se han identificado diferentes quinasas como MITOGEN ACTIVATED PROTEIN KINASE 6 (MAPK6), PROTEIN KINASE LIKE 1 (PKL1) y una proteína similar al osmorelé SLN1 de *Saccharomyces cerevisiae* (Cre17.g733150.t1.1), así como otros elementos señalizadores como GUN4 y MYB44. Junto a estos, también destaca la identificación de efectores como la subunidad de la ATP sintasa mitocondrial ATP6, que junto con los elementos señalizadores son prometedores en la continuación de la investigación de la respuesta a este estrés y la generación de nuevas variedades mas productivas.

Caracterización de la respuesta al estrés ultravioleta

Las microalgas también se encuentran expuestas al estrés ultravioleta, que penetra en la capa superficial de las masas de agua que habitan. *C. reinhardtii* cuenta con mecanismos de percepción, señalización y respuesta a radiación ultravioleta B que son similares a los ya descritos en plantas, sin embargo, la respuesta a ultravioleta C aún esta poco descrita en ambos grupos de organismos. Por otra parte, la irradiación con ultravioleta B y/o C tiene efectos muy prometedores desde un punto de vista industrial, dado a que induce cambios en la composición de biomasa tanto de las microalgas como de las plantas sin implicar su aplicación grandes costes energéticos.

El estrés ultravioleta es más simple que el osmótico en cuanto a los estreses primarios que lo componen. La radiación ultravioleta daña las estructuras celulares tanto directamente como a través de las especies reactivas de oxígeno cuya generación fomenta. A pesar de esto, los mecanismos de respuesta a este estrés ya descritos en plantas y en la propia microalga, muestran, como en el estrés osmótico, un sistema de respuesta complejo donde la radiación ultravioleta modula la expresión de muchos genes tanto efectores como implicados en procesos de desarrollo. Estos factores han motivado la caracterización de la respuesta de la microalga ante una dosis de radiación

ultravioleta B y C no letal que permita la adaptación de la microalga, enfrentándose a la complejidad esperada de esta respuesta mediante una aproximación basada en el análisis proteometabolómico y la integración de ambos niveles ómicos mediante un enfoque de biología de sistemas con el objetivo de identificar nodos centrales de este sistema de respuesta.

La respuesta a la radiación ultravioleta B y C en *C. reinhardtii* esta basada en la evitación tanto del daño infligido por la radiación, como indicó el fomento de la acumulación de compuestos que absorben la radiación ultravioleta, la promoción de los mecanismos de protección y recambio del proteoma, la modulación de la homeostasis redox y el control de los fotosistemas, especialmente el fotosistema dos, por su alta sensibilidad a este estrés.

Entre estos mecanismos la protección y el recambio de las proteínas, y la rápida modulación redox centraron la respuesta temprana de la microalga, basada mayormente en la prevención y reparación del daño causado por la radiación incidente. Esta respuesta rápida estuvo probablemente acoplada a la activación de distintas vías de señalización dirigidas a ultravioleta B (UVR8) y ultravioleta C (especies reactivas de oxígeno/ácido salicílico) que pueden estar conduciendo la modulación de la proliferación celular y la expresión génica tras las respuestas tempranas y el proceso final de aclimatación.

La aclimatación a este estrés se pudo relacionar con cambios proteometabolómicos específicos centrados en la modulación de la actividad de los fotosistemas mediante el desacoplamiento del PSII y el fomento del flujo cíclico de electrones, y en la acumulación de distintos compuestos protectores frente al ultravioleta y los radicales libres. Estos procesos, fueron asociados a distintos señalizadores tempranos como FAP204, un posible modulador de la traducción, las proteínas PP2A like y MINA53, relacionadas ambas con el control de la proliferación celular, y una nueva DYRK quinasa, que acumulándose durante la aclimatación pudo ser responsable de los cambios metábolicos observados bajo este estrés. Las características de estos elementos los convierten en objetivos prometedores de cara a la continuación de la caracterización de este estrés,r así como para la explotación del efecto

modulador de la radiación ultravioleta sobre la composición de biomasa, permitiendo el desarrollo de cepas más productivas.

Descripción de la familia CKIN/SnRK en *C. reinhardtii*

La implicación de múltiples fostatasas 2C (PP2Cs) bajo estrés osmótico, la importancia de la señalización por especies reactivas de oxígeno, y posiblemente ABA, bajo estrés osmótico y ultravioleta, y la modulación de la composición de biomasa de la microalga observada bajo los estreses descritos apuntan hacia la implicación de la familia de quinasas Sucrose non Fermenting Related Kinases (SnRK) en la coordinación de la respuesta a estrés en *C. reinhardtii*. Esta familia ya se conoce por su papel en la percepción del estado energético de la célula y la modulación de las respuestas a estrés abiótico en plantas, y también ha sido relacionada con la respuesta a distintos estreses como el estrés por deficiencia de azufre y el estrés por bajas temperaturas en la microalga.

La probable implicación central de estas quinasas en las respuestas descritas en Chlamydomonas motivó la identificación y descripción de los miembros de esta familia que fue designada como CKIN en *C. reinhardtii,* así como el trazado del proceso evolutivo que siguió esta familia de quinasas. Para ello, se realizó un minado de la información de los genomas de *C. reinhardtii* y otras especies próximas de algas clorófitas y mamiellales. Este análisis identificó 22 secuencias en *C. reinhardtii* como ortólogas a las SnRK de la planta modelo *Arabidopsis thaliana* dividiéndolas en dos subfamilias, CKIN1 y CKIN2, correspondientes con las subfamilias SnRK1 y 2 de plantas, sin identificar ortólogos a las quinasas de la subfamilia SnRK3. Mientras que la subfamilia CKIN1 estaba formada por un único miembro y una proteína similar (CKIN1L), la subfamilia CKIN2 de la microalga es grande y diversa con miembros similares a los de plantas y otros exclusivos de microalgas.

La descripción de las quinasas de esta familia se complementó con la descripción de su sensibilidad a diferentes estreses abióticos y mediadores de la respuesta a estrés como el ácido abscísico mediante PCR cuantitativa. La mayor parte de estas secuencias mostraron sensibilidad a estrés osmótico y

ultravioleta, y en menor medida a otros estreses revelando la sensibilidad de la familia al estrés abiótico y la posible redundancia funcional ante la sensibilidad de distintas CKIN2 a los mismos estreses.

Tanto la presencia de secuencias CKIN2 exclusivas de microalgas, como la de motivos reguladores únicos, o la sensibilidad de algunas de las CKIN2 a ácido abscísico hace probable la implicación de estas secuencias en vías únicas de microalgas. Esto es especialmente probable para las secuencias sensibles a ácido abscísico dado a que Chlamydomonas no cuenta con los receptores de esta molécula en plantas (PYR/PYL/RCAR) ni sus CKIN presentan los elementos regulatorios asociados a la regulación de las SnRK2 por esta fitohormona.

Tanto las clorófitas como las plantas muestran sistemas de señalización del estrés divergentes, sin embargo, las quinasas SnRK/CKIN comparten una función similar en estos sistemas de respuesta incluyendo la acumulación de biomoléculas. Esto sitúa a las CKIN como buenos objetivos dirigidos a la mejora de la producción de biomasa en microalgas. Por otra parte, sus elementos diferenciales prometen nuevos descubrimientos sobre los mecanismos únicos de su respuesta a estrés.

Caracterización funcional de candidatos ómicos resultantes de la descripción de los sistemas de respuesta a estrés ultravioleta y osmótico en *C. reinhardtii* mediante técnicas de ingeniería genética.

La caracterización ómica de la respuesta a los estreses osmótico y ultravioleta en *C. reinhardtii* incluida en esta tesis ha permitido la identificación de múltiples candidatos implicados en los sistemas de respuesta a sendos estreses. Entre ellos destacan componentes estructurales de la F_1F_0 ATP sintasa mitocondrial, con una estructura diferente y aún poco caracterizada en microalgas, como su subunidad seis (ATP6), y elementos señalizadores centrales como los de la familia CKIN que también cuentan con muchos miembros exclusivos de microalgas. La relevancia de estas proteínas en la respuesta a estrés, y la exclusividad de muchas de ellas, motivaron la

caracterización de su implicación y función específica en la respuesta a estrés de la microalga.

La modulación individual y constitutiva de la expresión de distintas CKIN2 y subunidades reguladoras asociadas a CKIN1 no produjo cambios fenotípicos en las cepas mutantes a excepción de *CKIN2.12* cuya sobreexpresión es probablemente letal al no haberse recuperado colonias transformantes para este gen. La caracterización de los ortólogos de las CKIN en plantas, como las SnRK de *A. thaliana*, también fue complicada debido tanto a la redundancia funcional de muchos de sus genes como a la letalidad causada por la modulación de la expresión de otros. Estos problemas requirieron en su día estrategias de mutación múltiple o de expresión inducible para permitir la recuperación de mutantes con fenotipos distinguibles. Los resultados observados en *C. reinhardtii* también se asociaron a las mismas causas, la redundancia funcional y la letalidad, motivando el inicio de una estrategia que permita la obtención de mutantes múltiples y la expresión inducible de distintas CKIN. Para llevar a cabo esta estrategia se desarrolló un nuevo vector de estructura modular en el que ya se ha probado el intercambio rápido de distintos genes y secuencias reguladoras, y que facilitará el desarrollo de las estrategias de mutación y modulación de la expresión necesarias para la caracterización de este grupo de candidatos.

A diferencia de los resultados obtenidos con los candidatos de la familia CKIN, la modulación del candidato efector, ATP6, mediante su sobreexpresión incrementó la resistencia de las cepas transformantes al estrés osmótico. Los efectos de la sobreexpresión de esta proteína, parte de la cadena de transporte electrónico mitocondrial, indican claramente la importancia del mantenimiento de la homeostasis de este proceso, y también señala a la relevancia de la modulación de la respuesta a estrés oxidativo bajo estas condiciones.

Los datos de caracterización existentes hasta el momento validan la estrategia de caracterización del sistema de respuesta a estrés de la microalga basada en la descripción de diferentes niveles ómicos y su integración mediante técnicas

de biología de sistemas al haber conseguido que la modulación de la expresión de un elemento predicho como principal en el sistema de respuesta (ATP6) modulase la respuesta a estrés. Por otra parte, aunque los resultados sobre la caracterización de las CKIN aún son limitados la falta de fenotipos distinguibles entre sus mutantes apunta ya a paralelismos con las SnRK2s de plantas como la redundancia funcional, mientras que la letalidad asociada a *CKIN2.12*, una quinasa exclusiva de microalgas, sugiere que cumple una función clave en la regulación celular pero dentro de vías probablemente exclusivas de microalgas.

III. Conclusiones

Esta tesis, centrada en estreses abióticos aún poco caracterizados en la microalga modelo *Chlamydomonas reinhardtii* como son el estrés ultravioleta y el estrés osmótico, ha confirmado como previamente han hecho otros trabajos la complejidad de las respuestas a estrés de este organismo unicelular, sus similitudes con las de plantas y levaduras, y su implicación en la producción/modulación de biomasa. Para ello, se ha empleado una estrategía basada en distintas ómicas (proteómica y metabolomica) y en su integración a través de algoritmos incluidos dentro de la biología de sistemas, que no solo han permitido evidenciar la complejidad de estos sistemas sino también realzar nodos clave en estos. Aunque muchos de estos nodos, incluyendo la familia de quinasas CKIN/SnRK son comunes entre plantas y microalgas, otros han resultado ser específicos de microalgas, y la implicación de alguno de ellos en la respuesta a estrés ha sido validada, confirmando la capacidad de la estrategia empleada para los objetivos inicialmente previstos. De una forma más específica las conclusiones de esta tesis son las siguientes:

I. La respuesta a estrés osmótico en *C. reinhardtii* se caracterizó por la rápida acumulación de azúcares y glicerol como consecuencia de la activación de vías de señalización solapantes relacionadas con la percepción de un medio hiperosmótico, especies reactivas de oxígeno, la comunicación retrógrada entre el núcleo y el cloroplasto, y posiblemente señalizadores como el ácido abscísico

y los brasinoesteroides. Todas ellas han sido relacionadas con la activación de distintos mecanismos implicados en la aclimatación a través de su dirección de un proceso de reprogramación genómica, que mediado por distintos elementos epigenéticos fue reponsable de la remodelación observada en el proteoma y permitió la supervivencia de la célula al medio hiperosmótico.

II. La estrategia de integración de los datos procedentes del proteoma y el metaboloma de *C. reinhardtii* expuesta a estrés osmótico permitió la identificación de nodos principales en el sistema de percepción, señalización y respuesta de la microalga como MAPK6, PKL1, ATP6, GUN4, MYB44, una proteína homóloga al osmorelé asociado a histidina quinasa que dirige la percepción del estrés osmótico en levaduras (Cre17.g733150.t1.1), y diferentes fosfatasas 2C (PP2Cs). Los ortólogos de estas proteínas en plantas y levaduras también están relacionados en la respuesta a estrés osmótico, mostrando un sistema de respuesta similar al de plantas y levaduras.

III. La respuesta a ultravioleta B y C en Chlamydomonas se caracteriza por la rápida activación del la reparación y protección de las proteínas, así como en diversos mecanismos que permiten el mantenimiento de la homeostasis redox. Estos mecanismos dan paso a otros más relacionados con la aclimatación centrados en la fotosíntesis, como el desacoplamiento del fotosistema dos, el fomento del flujo cíclico de electrones y la acumulación de compuestos protectores frente a la radiación ultravioleta y las especies reactivas de oxígeno derivadas de esta; y un complejo remodelado del proteoma, que a través de distintos mecanismos epigenéticos es modulado por distintas vías sensibles a ultravioleta.

IV. La misma aproximación integrativa sobre los distintos niveles ómicos descritos (proteóma y metaboloma) en las células estresadas por ultravioleta apuntó al solapamiento de la señalización dependiente de ácido salicílico y especies reactivas de oxígeno, sensible a ultravioleta C, y la señalización de ultravioleta B mediada por UVR8. Esta estrategia también permitió la

identificación de distintos elementos clave como FAP204, proteína similar a PP2A, MINA53, DYRK y LHCSR1, relacionadas con el control de la traducción, la modulación redox, la proliferación celular, la regulación de la composición de biomasa y la modulación de la fotosíntesis bajo estrés.

V. Los elementos identificados como centrales en la respuesta a los estreses ultravioleta y/o osmótico son de gran interés tanto para la continuación de la caracterización de la respuesta de la microalga a estos estreses como para el desarrollo de cepas más productivas. Además, los paralelismos identificados entre los sistemas de respuesta de plantas y microalgas sitúan a la microalga como un modelo de gran interés en la investigación vegetal tanto dirigida a microalgas como a plantas.

VI. La caracterización de las respuestas a estrés osmótico y ultravioleta mostró sistemas complejos y solapantes que a pesar de presentar elementos señalizadores y efectores específicos también mostraron evidencias de la implicación de señalizadores comunes como las quinasas de la familia SnRK, cuya implicación en la respuesta a otros estreses como el estrés por frio o por deficiencia de azufre y nitrógeno ya ha sido observada.

VII. La estrategia basada en homología y búsqueda de dominios conservados para la identificación de los miembros de la familia CKIN/SnRK en *C. reinhardtii* y otras microalgas permitió completar la descripción de la familia de la microalga, identificando elementos similares a las SnRK de plantas, pero también secuencias exclusivas de microalgas, que probablemente están implicadas en mecanismos específicos de las mismas, y una reducida subfamilia SnRK3 sin representantes en Chlamydomonadales.

VIII. A pesar de que la presencia de CKIN específicas de microalgas, especialmente en las Chlamydomonadales, sugirió que la familia de quinasas de la microalga ha seguido un camino evolutivo diferente, los análisis filogenéticos realizados indicaron que las familias identificadas en microalgas eran monofiléticas con las de plantas.

IX. Las CKIN de *C. reinhardtii* resultaron ser sensibles a los distintos estreses abióticos ensayados destacando su sensibilidad a los estreses osmótico y por radiación ultravioleta y la respuesta de alguna de ellas a la modulación del ácido abscísico.

X. La sobreexpresión de *CKIN2.2* y las secuencias reguladoras asociadas a *CKIN1* como *CKINβγ* y *CKINγ* no tuvo efectos distinguibles en el fenotipo de la microalga, probablemente debido a la existencia de redundancia funcional entre estos elementos, mientras que la sobreexpresión de *CKIN2.12* tuvo un efecto letal que sugirió su papel central en la señalización celular. Estos problemas motivaron el desarrollo de un nuevo vector (pSticky) que permita superarlos mediante el uso de estrategias de mutación múltiple (solapamiento funcional) y expresión inducible (letalidad).

XI. La sobreexpresión del gen efector *ATP6* produjo cambios en la respuesta de *C. reinhardtii* a estrés osmótico, lo cual apunta al papel central de la cadena de transporte electrónico mitocondrial, en la que esta proteína está implicada, en la respuesta al estrés y al mismo tiempo valida la estrategia inicialmente empleada al situar a este gen como elemento implicado en la respuesta a este estrés.

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