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UNIVERSITE DE GENEVE FACULTE DES SCIENCES
Departement de Botanique et Biologie Végétale Professor M. Goldschmidt-Clermont

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New Steps Toward Understanding The Regulation Of Photosystem I Biogenesis In *Chlamydomonas reinhardtii*

THESE

presentée à la Faculté des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences, mention biologie

par
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de
Dijon (France)

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RESUME

La photosynthèse est un mécanisme primordial à la vie telle que nous la connaissons. Sur terre la quasi-totalité de la biomasse est produite par la photosynthèse ou en est dérivée, comme bon nombre de nos matières premières telles que le bois, le charbon ou le pétrole. Comme son nom l'indique, il s'agit d'une réaction de synthèse qui tire son énergie de la lumière émise par le soleil. La collecte de l'énergie lumineuse se fait en deux phases, la première directement dépendante de la lumière qui génère de l'ATP et du pouvoir réducteur (NADPH) et la seconde phase qui utilise ces dernières molécules pour l'incorporation du CO_2 pour former des molécules organiques.

Malgré sa grande diversité évolutive, la photosynthèse se déroule quasiment toujours dans des systèmes membranaires complexes appelés thylakoïdes présents notamment chez les cyanobactéries, organismes procaryotes. Chez les eucaryotes photosynthétiques, ces thylakoïdes sont retrouvés dans une organelle particulière, le chloroplaste, originaire de l'incorporation endosymbiotique d'une cyanobactérie dans une cellule eucaryote primitive. Les mécanismes responsables de la phase lumineuse de la photosynthèse utilisent quatre complexes majeurs dont les activités sont couplées par une chaine de transport d'électrons et une accumulation de ions H+ dans la lumière des thylakoïdes. Ces complexes sont nommés photosystème II, cytochrome b_6f, photosystème I, et ATPsynthase. Ils sont composés d'une dizaine a une trentaine de sous unités chacun. L'évolution endosymbiotique du chloroplaste a eu pour effet la translocation d'une grande majorité des gènes d'origine cyanobactérienne vers le noyau, mis à part quelques gènes dont la structure et la fonction rendraient difficile leur translocation. Ce déplacement massif de gènes chloroplastiques vers le noyau a été accompagné d'une complexification du génome nucléaire, du protéome du chloroplaste, et des relations entre les deux systèmes génétiques. De nouvelles formes de signalisation et de régulation ont évolué pour coordonner la synthèse de différentes protéines codées par les deux compartiments, dont certaines s'assemblent stoechiométriquement dans les complexes du chloroplaste tout en adaptant leur quantité aux conditions environnementales.

Chlamydomonas est un organisme unicellulaire modèle avec un seul chloroplaste, qui possède la capacité de se multiplier aussi bien en hétérotrophie qu'en autotrophie. Il est donc possible d'étudier des mutations affectant la photosynthèse sans perdre la viabilité de l'organisme. Ainsi, il a été identifié et caractérisé un grand nombre de gènes nucléaires

impliqués dans la biogénèse des complexes photosynthétiques. Des composants encodés par le noyau de la machinerie d'expression chloroplastique, des enzymes de certaines voies métaboliques et des sous-unités des photosystèmes ont été découverts. La stabilisation, la maturation ou la traduction des ARN messagers du chloroplaste sont des activités trouvées régulièrement pour des protéines encodées dans le noyau. Par exemple il a été mis en évidence que pas moins de 14 locis nucléaires indépendants sont nécessaires au trans-splicing de l'ARN de *psaA*, une sous-unité majeure du photosystème I encodée dans le chloroplaste.

Cette thèse décrit l'identification de MAC1, une protéine nouvellement identifiée encodée par le génome nucléaire, impliquée dans la stabilisation de l'ARN messager de *psaC*, sous-unité du photosystème I. L'identification de ce gène a été possible suite à l'isolation d'un mutant d'insertion dont le génotypage par PCR inverse qui a caractérisé le gène affecté. Ce gène code pour MAC1 qui est une protéine de 100 kDa environ contenant des répétitions de type « Tetratricopeptide repeats », TPR ou HAT (demi TPR) notamment présentes dans de nombreuses protéines qui lient l'ARN dans les chloroplastes des algues et des plantes. Ainsi le mutant *mac1* s'est révélé transcrire *psaC* normalement mais présenter une déstabilisation de l'ARN de *psaC* induisant une perte complète de la protéine PsaC et une perte du phototropisme. La protéine MAC1 se localise dans la fraction soluble du chloroplaste lors d'un fractionnement cellulaire ou lors de sa visualisation par immunofluorescence. MAC1 affecte le 5'UTR de *psaC* comme il a été montré dans l'analyse de transformants chloroplastiques exprimant un rapporteur luciférase chimérique. L'insertion dans le 5'UTR d'une structure polyG, dont il a été démontré dans des études antérieures qu'elle bloque la progression des exonucleases, n'a pas réussi à stabiliser l'ARN messager mature de *psaC* mais a rétablit partiellement l'accumulation du précurseur dicistronique *psaC-petL*. Cela suggère soit un possible rôle de MAC1 sur des zones de l'ARNm autres que le 5'UTR, soit l'existence de différente variantes du 5'UTR, la forme mature étant exempte de la partie contenant le polyG.
Le rôle de MAC1 dans la régulation a été démontré à plusieurs niveaux. Tout d'abord, l'expression de MAC1 en différentes quantités dans une série de transformants est suivie par une accumulation proportionnelle de l'ARN de *psaC*, ce qui suggère que MAC1 joue un rôle limitant. Alors qu'il est connu que le photosystème I est dégradé en absence de fer dans le milieu, en conditions mixotrophiques l'ARN de *psaC* et la protéine MAC1 sont tous deux réduits de deux fois tandis que les protéines PsaC et PsaA sont fortement déstabilisées. Enfin, MAC1 s'avère être phosphorylée dans des conditions de croissance mixotrophiques (les autres

conditions de croissance n'ont pas été testées) alors que la carence en fer et des conditions favorisant l'oxydation ou la réduction de la chaine de transport d'électrons (état 1 ou état 2) modifient sensiblement cette phosphorylation. Cette observation soulève la question du rôle physiologique de cette phosphorylation sur l'activité de MAC1.

Les organismes sont capables de s'acclimater en modifiant leur population protéique en fonction de leurs conditions de croissance. Chez les organismes photoautotrophes, cette acclimatation met en jeu la régulation de la capture de la lumière par la machinerie photosynthétique. L'efficacité de cette dernière dépend de la disponibilité des substrats NADP et ADP, accepteurs de l'énergie capturée, leur disponibilité étant dépendante de nombreux facteurs environnementaux comme la température et la présence en quantité suffisante des nutriments. Ainsi une capture trop importante de l'énergie lumineuse induirait la production d'espèces réactives de l'oxygène (ROS) nocives. Les résultats obtenus suggèrent que cette limitation par les accepteurs déclenche une acclimatation à long terme de la machinerie photosynthétique. Cela implique une forte réduction de l'accumulation du photosystème I en haute lumière, mais des changements modérés des taux de synthèse et de dégradation. L'effet observé découlerait alors d'un changement subtil du ratio biogénèse/protéolyse.

Les mécanismes qui gouvernent l'accumulation des protéines chloroplastiques sont encore incomplètement compris et ne présentent que quelques exemples de régulation. Des technologies de protéomique et d'intéractomique devront être employées pour les comprendre et les domestiquer, notions prenant tout leur sens dans la perspective du développement des biotechnologies.

SUMMARY

Photosynthesis is a mechanism essential to life. On earth almost all biomass is produced by photosynthesis directly or indirectly, as are many of our raw materials such as wood, coal or oil. As its name suggests, this is a biosynthesis pathway which takes its energy from light emitted by the sun. The conversion of light energy by photosynthesis involves two phases, the first one is directly dependent on light and generates chemical energy in the form of NADPH and ATP, the second one uses these molecules for the fixation of inorganic CO_2 to form organic molecules.

Despite its evolutionary diversity, the first phase of photosynthesis generally takes place in complex membrane systems called thylakoids. These are present cyanobacteria, which are prokaryotic phototrophs, and in photosynthetic eukaryotes, where the thylakoids are located in the chloroplast. This organelle originated from the endosymbiotic incorporation of a cyanobacterium in a primitive eukaryotic cell. The light phase of photosynthesis involves four major complexes whose activities are coupled by a redox electron transfer chain and the accumulation of protons in the lumen of the thylakoids. These complexes are named photosystem II , cytochrome b_6f, photosystem I and ATPsynthase. Each complex is composed of roughly a dozen of subunits, binding many pigments and cofactors. The endosymbiotic evolution of the chloroplast has resulted in translocation to the nucleus of a great majority of the original cyanobacterial genes. This massive displacement of chloroplast genes to the nucleus led to increased complexity. New forms of signaling and regulation evolved to coordinate the synthesis of different proteins encoded by the two compartments, to allow their stoichiometric assembly in the chloroplast complexes while adapting to environmental conditions.

Chlamydomonas is a model unicellular green alga with a single chloroplast, which has the ability to grow using phototrophy or heterotrophy. It is therefore possible to study mutations that disrupt photosynthesis without affecting the viability of the organism. This allowed the identification and characterization of a large number of nuclear genes involved in the biogenesis and function of photosynthetic complexes, as well as in some metabolic pathways. Many post-transcriptional events of chloroplast gene expression, such as stabilization, maturation or translation of mRNA, are directed by proteins encoded in the nucleus. For example as many as 14 independent nuclear loci are necessary for trans-splicing of *psaA*

mRNA , which encodes a major subunit of photosystem I, in the chloroplast.

This thesis reports the identification of MAC1 (Maturation of *psaC*), a newly-discovered protein encoded by the nuclear genome, involved in stabilizing the mRNA of *psaC*, which encodes a subunit of photosystem I. The identification of the *MAC1* gene was based on the isolation and mapping of an insertional mutant. MAC 1 is a protein of about 100 kDa containing TPR / HAT (Tetratricopeptide / Half a Tetratricopeptide) repeats. These repeats are also present in other chloroplast RNA-binding proteins of algae and plants. In the *mac1* mutant *psaC* is transcribed at normal rates, but the *psaC* mRNA is unstable, inducing a complete loss of the PsaC protein and therefore a loss of photoautoprophy. Cell fractionation and immunofluorescence indicated that the MAC1 protein is localized in the soluble fraction of the chloroplast. It acts through the 5'UTR of *psaC*, as shown by the analysis of chloroplast transformants harboring a chimeric luciferase reporter. Small sRNAs that map to the 5'end of the *psaC* mRNA are absent in the *mac1* mutant and may thus represent footprints of MAC1 and of its putative protein partners. Insertion in the 5'UTR of a tract of polyG, which is known from previous studies to block the progression of exonucleases, failed to stabilize the *psaC* mRNA but did partially restore the accumulation of the dicistronic *psaC-petL*-precursor. This suggests an additional role for MAC1 either on parts of the mRNA other than the 5'UTR, or the existence of different 5'UTR, the mature form being exempt of the part containing the polyG. A role for MAC1 in the regulation of *psaC* expression was demonstrated at different stages. First, the expression of different amounts of MAC1 in an allelic series of transformants was accompanied by parallel changes in the accumulation of *psaC* mRNA, suggesting a limiting role of MAC1 for *psaC* accumulation. Second, iron limitation in conditions of mixotrophic growth (other growth conditions were not tested) causes the degradation of photosystem I. Concomitantly, MAC1 protein and *psaC* mRNA are both reduced approximately two-fold in amount, while the PsaC and PsaA proteins are strongly destabilized. Finally, MAC1 is phosphorylated, and its phosphorylation is significantly modulated in response to the availability of iron or to conditions that favor the oxidation or the reduction of the electron transfer chain (state1 or state2). This raises interesting questions on the physiological role of phosphorylation in regulating the activity of MAC1.

Living organisms are able to acclimate to their environment by changing their protein content. In photoautotrophic organisms, this is particularly apparent in the acclimation responses that regulate light absorption by the photosynthetic machinery. The effectiveness of photosynthesis depends on the availability of the acceptors for the collected energy, ADP

and NADP, which is affected by many environmental factors such as temperature and the presence of sufficient quantities of nutrients. Thus, absorption of excess light energy induces the production of harmful reactive oxygen species (ROS). Acclimation to changes in light intensity and CO2 availability was investigated in cultures of Chlamydomonas. The results suggest that limitation by the acceptors triggers long-term acclimation of the photosynthetic machinery. This involves a pronounced reduction of the amount of photosystem I in high light, but only moderate changes in its rates of synthesis and degradation were observed. The acclimation to high light could result from subtle changes in the ratio of biogenesis to proteolysis.

The mechanisms that determine the accumulation of chloroplast proteins are still poorly understood and there are only few examples of regulation. Proteomics and interactomic technologies will be used to better understand and tame the regulation mechanisms in order to facilitate advances in biotechnologies

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PREAMBLE

Photosynthesis research in its context

A major motivation for my interest in doing research on photosynthesis is the promise that it holds to address global problems of energy supply, sustainable development and technologies. The potential contribution of photosynthesis is briefly outlined in this Preamble and further detailed in Appendix I.

Photosynthesis in the production of energy

Today, human beings face shortages of the resources that would be needed to perpetuate or improve their lifestyle. The development of our culture as well as population growth imply growing needs in terms of energy. The amount of usable energy on earth is limited, making our future development dependent on the way we harness it. The resources we can dispose of derived in their vast majority from hydrogen fusion reactions in the sun that transform mass to energy. A very small fraction of what is produced in the sun is received on earth as light and is transformed to heat (at the origin of wind energy, and hydraulic energy for example) and to biomass through photosynthesis. Part of the latter was converted to fossil fuels such a as oil, gas or coal. Those byproducts are now transformed or burned to produce mechanical energy or heat, sometimes with additional intermediary steps. While nuclear energy from uranium, as produced today, is a finite resource, many other technologies are in development to harness other fissile atoms or to control hydrogen fusion. It is estimated that deuterium fusion could provide 60 billion years of energy as currently consumed by mankind (Cowley, 2015). However, it will still take several decades before this technology is ready.

On the other hand, photosynthesis is a direct sun-energy collecting mechanism and is thought to offer powerful alternatives to fossil fuels. Artificial photosynthesis consists in mimicking the chemical reactions that generate reducing power in photoautotrophic organisms to produce electricity. In some technologies, photosynthesis inspired the engineering of chlorophyll-like structures with metal-coordinated double-bounds within complex molecules linked to electrodes. This involves the coordination of metals to electrode-bound resonance structures (Frischmann et al., 2013). It is also feasible to extract the light-harvesting apparatus

from crude plant material and to link it to electrodes to produce electricity (Mershin et al., 2012). Photosynthetic energy in algae can also be redirected to produce hydrogen gas (Melis and Happe, 2001) that can drive electricity generation in fuel cells. Last but not least, it is also possible, and already commercially applied, to produce energy from plant or algae biomass. This biomass is treated chemically or enzymatically to produce « biodiesel » or is fermented to produce methane « biogas » or ethanol « bio-ethanol ». This sector is quickly developing as it was estimated, for example, that the global market for enzymes for biofuel production is growing by 10% a year (Brindle, 2015).

Photosynthesis as a tool for biotechnology.

Photosynthesis seems to be a short-term solution to partly sustain energy needs (while nuclear energy is predicted to offers more promising yields and upscalability), and also appears quite promising from a biotechnological point of view. Many complex molecules produced by the chemical industry can be produced by photosynthetic organisms . Secondary metabolites from plants have been extracted and used as drugs. It is estimated that around 80% of the 30,000 natural pharmaceutical products are of plant origin (Rao and Ravishankar, 2002; Rischer et al., 2013). Their production through engineered photosynthetic organisms would decrease their cost on the long term and would make their price independent from the fluctuating rates of petroleum. In unicellular algae, hemagglutinin, vitamins, poly unsaturated fatty acids, antioxidants, glycerol, transgenic proteins (Mayfield et al., 2007; Rasala et al., 2010), toxic fatty acids used to improve algae monoculture (Skjanes et al., 2013), poly-3-hydroxybutyrate (PHB) (Chaogang et al., 2010) have been successfully produced. As a vaccine against Plasmodium, an antigen with a fused cholera-toxin adjuvant has been expressed in the chloroplast (Gregory et al., 2013). In cyanobacteria, ethylene and isobutyraldehyde, both major precursors in chemical industries, were produced by genetic modifications (Takahama et al., 2003; Atsumi et al., 2009).

INTRODUCTION

Photosynthesis

Photosynthesis can be defined as a mechanism that transforms light energy into chemical energy. In living organisms, it allows phototrophy, which is the ability to complete a reproductive cycle with light as the only energy source.

The chloroplast and the thylakoid membrane

In eukaryotic phototrophs, the photosynthetic machinery is compartmentalized in a subcellular organelle, called the chloroplast, which is surrounded by several membrane layers. At the beginning of the 20th century, the Russian botanist Konstantin Mereschkowsky discovered chloroplasts while studying lichens, and subsequently published a book in 1910 entitled "*The theory of two plasms as the basis of symbio*gene*sis, a new study of the origins of organisms*," in which he formulated a theory about the possible endosymbiotic origin of chloroplasts. There is now a body of evidence that supports the cyanobacterial origin of chloroplasts (Alda et al., 2014). There is evidence that endosymbiosis may not be a unique event. The nuclear genome content in ancestral archeal genes (host genome) proteobacterium genes (mitochondrial genome), and cyanobacterial genes (chloroplastic genome) does not explain the supernumerary and multiphyletic bacterial genes present in a eukaryote genome. Single gene phylogenies reveal genome chimerism with a large subset of genes not inherited from the three known ancestors. This suggests the occurrence of either multiple events of ephemeral endosymbiosis, acquisition of genes from phagocytized organisms, or any other horizontal gene transfer (e.g., through viruses) that could have chimerized the three ancestral genomes (Ku et al., 2015). Multiple endosymbiosis events are also at the origin of different photosynthetic lineages, which are spread widely across the phylogenetic tree. A secondary endosymbiosis event is believed to account for the origin of the multiple chloroplast layers in euglenophytes and some dinoflagellates, and a tertiary endosymbiosis event could explain the four membranes present around the chloroplast in haptophytes, heterokonts, cryptophytes, and chlorarachniophytes (Cavalier-Smith, 2000).

Chloroplasts have their own genome and many features that resemble those of

cyanobacteria such as outer and inner membranes, nucleoids, PEP (Plastid Encoded–RNA Polymerase), ribosomes of prokaryotic structure and sequence, and lipid droplets. Within the chloroplast, the photosynthetic apparatus is sub-compartmentalized in thylakoid membranes.

The organization of these membranes resembles those of cyanobacteria, as they are composed of a single membrane compartment that forms interconnected flattened vesicles (Engel et al., 2015).

The photosynthetic machinery is embedded in the thylakoid membrane. In photosynthetic eukaryotes, it is composed of four major complexes called photosystem II, cytochrome b_6f, photosystem I, and ATP synthase (figure 1). These protein complexes function together to harvest light and use the energy to generate reducing power and ATP.

Photosystem II.

Photosystem II (PSII) is a light-driven water-plastoquinone oxidoreductase. This enzyme is composed of approximately 20 proteins (table 1) and 77 cofactors including P680 chlorophylls, the Mn cluster, pheophytins, and plastoquinones. This complex contains subunits that are nuclear encoded and some other chloroplast encoded (figure 1). Equimolar assembly of the subunits involves coordination of gene expression in the two genetic compartments .

The 3D structure of PSII from *C. reinhardtii* was obtained by averaging images obtained by transmission electron microscopy (Nield et al., 2000). This revealed a symmetrical dimerization of the core containing the proteins D1/D2, CP47, and CP43 in the center, and CP29, CP26, and LHCII trimers in the periphery. In higher plants, an additional CP24 is required to bind LHCII trimers (de Bianchi et al., 2008).

Electron transfer in photosystem II is well understood and was reviewed by (Diner and Rappaport, 2002). The D1–D2 heterodimeric reaction center of photosystem II contains two accessory chlorophylls and two central chlorophylls, called P680, which receive enough energy from the antenna to donate an electron and become oxidized. This highly reactive P680+ recovers an electron from the Y161 tyrosine of D1, which in turn is reduced by the oxygen evolution center (OEC). The latter is an oxo-bridged Mn_4Ca cluster arranged in a cuboidal shape and bound by several amino acids of D1 and CP43 (subunits of the PSII core). With its particular geometry, the OEC, oxidized 4 times by the Y161 tyrosine, is able to split two molecules of water into one O_2 molecule and four H^+, thus replacing the missing electrons. This reaction is at the origin of the oxygen that makes up 20% of our atmosphere.

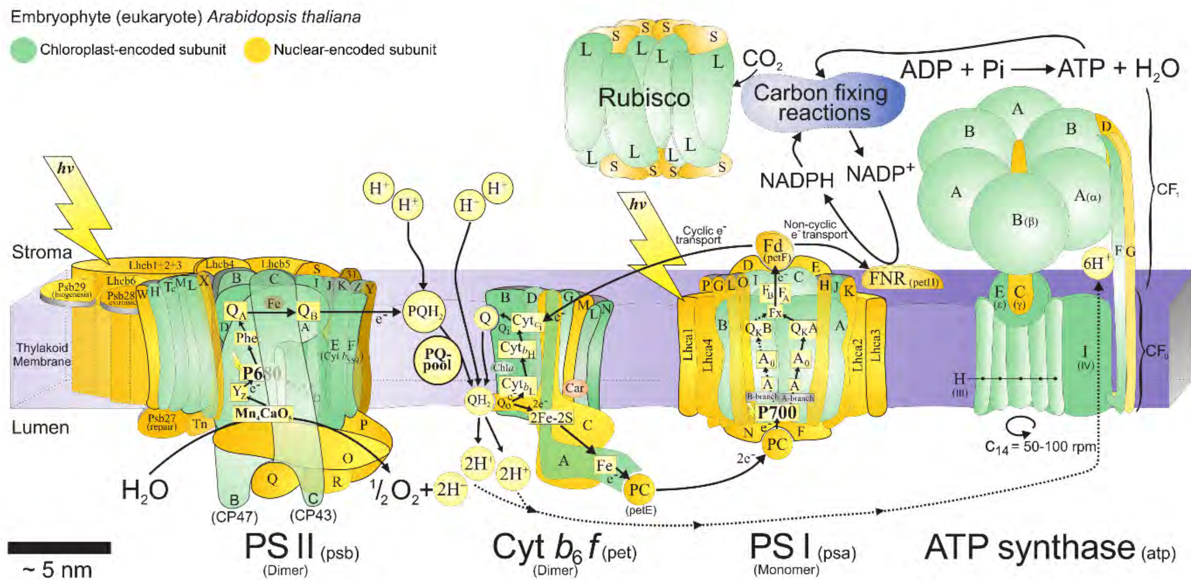


Figure 1: ***Schematic of the photosynthetic apparatuses from Arabidopsis thaliana***

Protein names are represented as a single letter code; their full names include the Psb prefix for the PSII subunits (for example A is PsbA), the Pet prefix for Cyt b6f, Psa for PSI, Atp for ATP synthase, and Rbc for rubisco. Linear and cyclic electron flow are represented by black arrows on the scheme (derived for Arabidopsis). All intermediates that participate in the Z scheme are represented. The proteins in yellow are nucleus-encoded while those in pale green are chloroplast-encoded. Modified from (Allen et al., 2011).

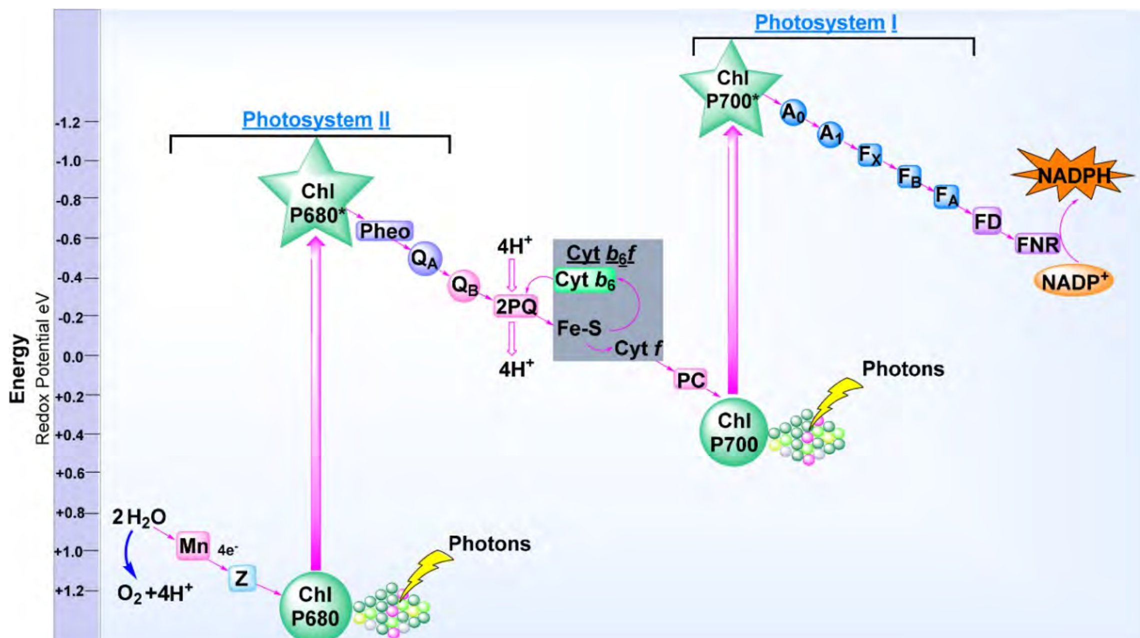


Figure 2: ***This Z-scheme illustration of oxygenic photosynthesis summarizes the redox reactions that take place in the photosynthetic electron transport chain.***

The redox potential of each intermediate corresponds to the scale on the left. Abbreviations: Mn = manganese complex; Z = tyrosine; Chl P680 = photosynthetic reaction center chlorophylls in PS II; Chl P680 = excited P680; Pheo = pheophytin; QA and QB = plastoquinones; PQ = oxidized plastoquinone; Fe–S = Rieske iron-sulfur protein; Cyt f = cytochrome f; Cyt b6 = cytochrome b6; Cyt b6f = cytochrome b6f complex; PC = plastocyanin; Chl P700 = photosynthetic reaction center chlorophylls in PS I; Chl P700* = excited P700; A0 = chlorophyll a; A1 = phylloquinone, FX, FB, FA = iron-sulfur centers; FD = ferredoxin; FNR = ferredoxin NADP oxidoreductase. Figure and legend modified from (Senge et al., 2014).*

On the other hand, the electron donated by the P680 chlorophylls reduces a pheophytin cofactor bound to D1, which is the starting point of a long cascade of oxydoreduction reactions as presented in the Z scheme (figure 2). The transfer continues to the Q_A site with a plastoquinone (PQ) bound to D2. The electrons are then sequentially transferred to the Q_B site in D1 where two of them are reduce PQH_2 with two protons taken from the stroma. PQ is an amphiphilic molecule that is soluble in the lipid membrane bilayer, its solubility in the thylakoids creates a redox link connecting PSII and b_6f complexes. The PQH_2 generated in the Q_B pocket is rapidly replaced by an oxidized PQ from the membrane pool. The reduced plastoquinone PQH_2 diffuses to the Q_o site of cytochrome b_6f.

Cytochrome-b_6f

The b_6f complex is a plastoquinone/plastocyanin oxidoreductase (Pierre et al., 1995). Its activity is affected by acidification of the thylakoid lumen in a process called photosynthetic control, which in turn influences the velocity of the whole electron transfer chain (Kramer et al., 1999; Rott et al., 2011).

The cytochrome-b_6f complex is composed of nine subunits in *C. reinhardtii* (table 2), all containing trans-membrane helices. This complex forms a dimer which is structurally similar to the well-known mitochondrial bc_1 complex, but presents supernumerary cofactors that are important for its specific role in the chloroplast (Xia et al., 1997; Stroebel et al., 2003). These are chlorophyll a, β-carotene, and a c-type heme. This complex is also composed of some subunits that are nucleus-encoded and others chloroplast-encoded (figure 1). This requires coordination of gene expression to perform equimolar assembly

The b_6f complex oxidizes PQH_2 to PQ at the Q_0 site, where it takes two electrons (one going to the b_L heme and the other to the iron sulfur center of the Rieske subunit) and releases two protons on the lumenal side, thus participating in the creation of a proton gradient across the thylakoid membrane. The electron routed to the b_L heme in the b_6 subunit is transferred to other hemes (b_H and c_i') close to the Q_i site where it forms a semiquinone from a PQ molecule, until a second electron allows the reduction of the semiquinone to PQH_2, taking up two protons from the stroma. This can diffuse to the Q_0 site thus liberating two more protons in the lumen. This second loop is important to increase the proton gradient across the membrane, the process is called the Q cycle (Cramer et al., 2011). The second electron taken from plastoquinone oxidation is accepted by the iron sulfur cluster in the Rieske subunit, leading to

Sub-unit	Role	Reference
D1 (PsbA) and D2 (PsbD)	Heterodimeric reaction center and electron transfer.	(Ferreira et al., 2004)
CP47 (PsbB), CP43 (PsbC)	Proximal antennae.	(Ferreira et al., 2004)
b_{559} α (PsbE) and β(PsbF)	Photoinhibition and assembly of PS II.	(Sugiura et al., 2015)
PsbH	Ensures a Qa binding site, helps repair and general stability of PSII.	(Summer et al., 1997)
PsbX	Close to the core and b_{559}, induced by light.	(Shi et al., 1999)
PsbZ	Stability of PSII-LCHII association.	(Swiatek et al., 2001)
PsbR	Assembly of the OEC.	(Suorsa et al., 2006)
PsbL	Involved in oxygen evolution in cyanobacteria.	(Anbudurai and Pakrasi, 1993)
PsbI	Mutant is autotrophic but hypersensitive to excessive light. low-level activity detected in the mutant. Single trans-membrane helix	(Künstner et al., 1995; Wang et al., 2015)
PsbK	Stability of the core photosystem II.	(Takahashi et al., 1994)
PsbJ, PsbW, PsbN	PsbW stabilizes dimeric PSII.	(García-Cerdán et al., 2011)
PsbO	Extrinsic protein, availability of calcium and chloride for water splitting.	(Popelkova et al., 2011)
PsbP	Extrinsic protein, (PsbP1 member of oxygen evolving complex, PsbP2 singlet oxygen signaling, and PsbQ binding).	(Brzezowski et al., 2012)
PsbQ	Extrinsic protein, stability, activity of Mn cluster.	(Ifuku, 2015)

Table 1 : ***Composition of photosystem II and known roles of the different subunits.***
This table groups results from different species.

the sequential reduction of the c-type heme in cytochrome f and of plastocyanin (Soriano et al., 2002).

Plastocyanin is a soluble protein found in the lumen of the thylakoids. It has a β-barrel structure that binds a single copper ion (Redinbo et al., 1993). Its reduction is characterized by the conversion of Cu^{2+} to Cu^{+} and followed by a diffusion to bind photosystem I, which acts as a light-driven plastocyanin-ferredoxin oxidoreductase. Higher plants exclusively use plastocyanin, while in *C. reinhardtii,* this can be replaced by an iron-heme-containing substitute, cytochrome c_6 (Merchant and Bogorad, 1986).

Even though their primary sequences are completely different, their redox potentials are very similar, and they have remarkably similar size, shape, charge distribution, and surface polarity patterns explaining why they can both be reduced by the b_6f complex and oxidized by photosystem I.

The evolution and conservation of two different electron transporters from b_6f to photosystem I can be rationalized by the fact that plastocyanin and cytochrome c_6 can replace each other when *C. reinhardtii* is subjected to iron or copper starvation.

Photosystem I.

Photosystem I (PSI) is a complex containing 14 proteins in *C. reinhardtii* (table 3) and numerous co-factors. Its crystal structure has been solved in *P.sativum* (Amunts et al., 2007; Mazor et al., 2015). This complex contains subunits that are nucleus-encoded and others chloroplast-encoded (*A.thaliana* complexes presented in figure 1). This involves coordinate expression of the two genomes to perform an equimolar assembly.

Ferredoxin and flavodoxin are PSI electron acceptors that are found in most photosynthetic bacteria and algal lineages. Flavodoxin is absent in land plants. They have a similar β-sheet structure but are distinguishable by the cofactor they use to carry redox charges, which are iron in ferredoxin or FMN in flavodoxin. Despite the different cofactors, the redundant function, has been retained throughout evolution and may offer adaptation to changing iron availability in aquatic environments. In contrast, flavodoxin has been lost during the evolution of plants that live in environments where iron is less limiting (Pierella Karlusich et al., 2015).

The light-harvesting apparatus of PSI forms a crown on one side of the PSI core (Kargul et al., 2005). The plants have a single crown made of 4 LHCA subunits while in *C. reinhardtii* they form 2 concentric crowns with at least 9 LHCA monomers. LHCA1 and 3 are conserved

Sub-unit	Role	Ref
PetA (cytochrome f)	On the lumenal side, anchored to the membrane with a single alpha helix. Single c-type heme involved in electron transfer to plastocyanin. Hosts Q_o and Q_i sites in association with PetD.	(Yamashita et al., 2007; Lemaire et al., 1986)
PetB (b_6)	Contains two b-type hemes and one c-type heme, binds PetD, embedded in the thylakoid membrane.	(Yamashita et al., 2007; Lemaire et al., 1986)
PetC (Rieske)	On the lumenal side, anchored to the membrane with a single alpha helix. Iron sulfur cluster. Redox link between PetA and PetB.	(Yamashita et al., 2007; Lemaire et al., 1986)
PetD (subunit IV)	Embedded in the thylakoid membrane bound to PetA. Hosts Q_o and Q_i sites in association with PetA.	(Yamashita et al., 2007; Lemaire et al., 1986)
PetG	Assembly of b_6f dimers, essential for accumulation. Single trans-membrane helix.	(Stroebel et al., 2003; Wang et al., 2015)
PetL	Stabilization of the dimer, conformation of Rieske subunit, and sensing of the redox plastoquinone state. Single trans-membrane helix.	(Breyton et al., 1997; Stroebel et al., 2003; Schwenkert et al., 2007)
PetM	Regulation of plastoquinone reductase activity in cyanobacteria, Single trans-membrane helix.	(Schneider et al., 2001; Stroebel et al., 2003)
PetN	Essential for accumulation. Single trans-membran2.3e helix.	(Schwenkert et al., 2007) (Stroebel et al., 2003)
PetO	On the lumenal side, anchored to the membrane with a single alpha helix phosphorylated upon state-transition.	(Hamel et al., 2000; Stroebel et al., 2003)

Table 2 : ***Composition of the b6f complex and known roles of the different subunits.***
This table groups results from different species.

among species, while the others seem to diverge. LHCA2 and 9 are thought to form the outer crown, which is more loosely associated and is involved in regulation *of* light absorption in *C. reinhardtii* (Drop et al., 2011). In addition, PSI is able to bind LHCII antenna under certain conditions, as detailed in the section on state transition. The electron pathway across PSI has been previously summarized (Berthold et al., 2012). When light energy is captured in the antenna, it travels to the core of the photosystem by the conjugation of the antenna pigments. At the core, central chlorophylls in P700 donate an electron to a proximal chlorophyll EC_2. The re-reduction of the P700 chlorophyll occurs via the oxidation of the Cu^+ in plastocyanin on the acceptor side. The EC_2 electron is transferred to a second chlorophyll, EC_3, followed by transfer to a phylloquinone co-factor. This pathway is present in duplicate, both in PsaA and PsaB. The P700 chlorophyll couple is in a pocket shared between the two subunits. Electron transfer through the A branch is on average 10 times slower than through the B branch (Redding et al., 1998; Guergova-Kuras et al., 2001) . From phylloquinone, electrons are transferred to a shared iron-sulfur cluster, F_X, and then to F_A and F_B, which are two other Fe-S-clusters located in PsaC. Finally, on the stromal side, a ferredoxin is reduced at PsaC via the F_B cluster and can then be used as a substrate by the FNR protein to produce NADPH.

The redox equivalents generated through the photosynthetic machinery are directed to several pathways. Different ferredoxins play a role in their partitioning (Blanco et al., 2013). The *C. reinhardtii* genome contains six ferredoxin genes (*FDX1–6*) and each protein has a specific interactome (Peden et al., 2013). Five of these proteins are predicted to be localized within chloroplasts (except FDX4). FDX1 is the predominant form present in the logarithmic phase in *C. reinhardtii* (90%). It is a substrate of the FNR that produces NADPH, whose reducing power is used throughout the cell. At the chloroplast inner membrane, a fraction of the NADPH and ATP can be exported to the cytoplasm through a dihydroxyacetone phosphate (DHAP)/phospho glyceric acid (PGA) shuttle. The other ferredoxins are induced by different stresses. Fdx3 and 6 are induced during iron starvation (Terauchi et al., 2009).

Light harvesting antennae

In photosynthetic organisms, the first players involved in harvesting light-energy are the antennae. In eukaryotes, these are chromoproteins of about 25 kDa that contain several chlorophylls and carotenes, are embedded in the membrane at the periphery of both photosystems, and are responsible for the transfer of light-energy to the core of the

Sub-unit	Role	Ref
PsaA and B	Components of the core, charge separation, and electron transfer.	(Milanovsky et al., 2014)
PsaC	Electron transfer through Fe-S clusters, ferredoxin/flavodoxin reductase activity. Its absence destabilizes the whole photosystem.	(Takahashi et al., 1991; Meimberg et al., 1999).
PsaD	Ferredoxin docking site, binds PsaC and ferredoxin.	(Cashman et al., 2014)
PsaE	Ferredoxin docking site and FNR binding, part of cyclic electron flow.	(Weber and Strotmann, 1993; Yu et al., 1993)
PsaF	PSI acceptor side; binds plastocyanin and LHCA1–4.	(Kargul et al., 2003)
PsaG	LHCA1–4 binding, may participate in the regulation of photosystem I.	(Kargul et al., 2003)
PsaH	Binds PsaB and PsaO.	(Yadavalli et al., 2011)
PsaI	Binding of PsaL and PsaM. In cyanobacteria, involved in trimerization, mutants make PSI monomers leading to faster state-transition (monomers more accessible for the antennae).	(Xu et al., 1995; Schluchter et al., 1996)
PsaJ	PsaF binding.	(Fischer et al., 1999)
PsaK	Light induced in barley, participates in state-transition induced LHCII binding.	(Kjaerulff et al., 1993; Jensen et al., 2007)
PsaL	PSI trimerization in cyanobacteria In wheat, linked to abiotic stress resistance. In *Arabidopsis thaliana*, involved in PSI-LHCI-LHCII complex formation.	(Chitnis et al., 1993; Seok et al., 2014)
PsaM	Trimerization in cyanobacteria, not found in *Viridiplantae*.	(Naithani et al., 2000)
PsaN	LHCI binding.	(Yadavalli et al., 2011)
PsaO	State-transition mechanism and stabilization of PSI-LHCI. Binds PsaB.	(Yadavalli et al., 2011)

Table 3 : ***Composition of photosystem I and known roles of the different subunits****.*
This table groups data from different species.

photosystems. Roles in photoprotection and in redistribution of excitation energy between the two photosystems via state-transition were also attributed to the antenna proteins mainly bound to PSII. Some of these light harvesting complexes II (LHCII) form trimers that bind the photosystems to generate supercomplexes of approximately 30 proteins (figure 3). The members of such supercomplexes are not perfectly defined and differ from one species to another. Some are specific to each photosystem, and others can be found under different conditions, sometimes replacing other subunits. LHCII is the most abundant membrane protein on Earth, and contains 50% of the total chlorophyll present in plants.

From a sequence point of view, LHC proteins share obvious homology (Wolfe et al., 1994). X-ray crystallography has revealed that they contain three trans-membrane alpha helices that bind most of the pigments. LHCII trimers isolated from *P.sativum* were crystallized and their structure was resolved at 2.5 Å (Standfuss et al., 2005). Each monomer binds eight chlorophyll a, six chlorophyll b, two luteins, and one 9′ *cis*-neoxanthine (Standfuss et al., 2005). Other cofactors were found in the monomer/monomer interface, including an additional chlorophyll b and violaxanthin, the latter being involved in the excess-light photoprotective mechanism via its de-epoxydation into zeaxanthin. In addition, phosphorylation of LHCII subunits is required for its connection to photosystem I to balance light harvesting according to the light quality and needs in reducing power and ATP (see section on state transition).

At PSII, the antennae (LHCII trimers) are coupled to the core heterodimer D1-D2 through CP47 and CP43, which are inner core antenna proteins, to form so called supercomplexes. The binding strength of LCHII trimers to the core define 2 classes, M-LHCII (for moderately bound) and S-LHCII (for strongly bound). The general organization of supercomplexes is thought to be $C_2S_2M_2$ (2cores, 2 S-LHCII, 2 M-LHCII). The minor peripheral antenna proteins, CP29, CP26 and CP24 encoded by the *Lhcb4*, *5*, *6* genes respectively, are required for the formation of PSII supercomplexes in plants (Ferreira et al., 2004; Sun et al., 2015). CP24 appears to be required for the binding of moderately attached M-LHCII trimers in *A.thaliana*, while it is replaced by special trimers in *C. reinhardtii* (Drop et al., 2014). PSBS and LHCSR1/3 proteins have been found in *A. thaliana* and *C. reinhardtii*, respectively, and are involved in the response to high levels of light for dissipation of excess energy (qE), and are transiently associated with PSII (Li et al., 2000) or with PSI (Bergner et al., 2015; Allorent et al., 2013). In *Chlamydomonas* both PSBS and LHCSR3 genes are present ; however, expression of PSBS at the protein level has not yet been observed in *Chlamydomonas* while both proteins are detected and active in *P. patens* (Alboresi et al., 2010; Pinnola et al., 2015).

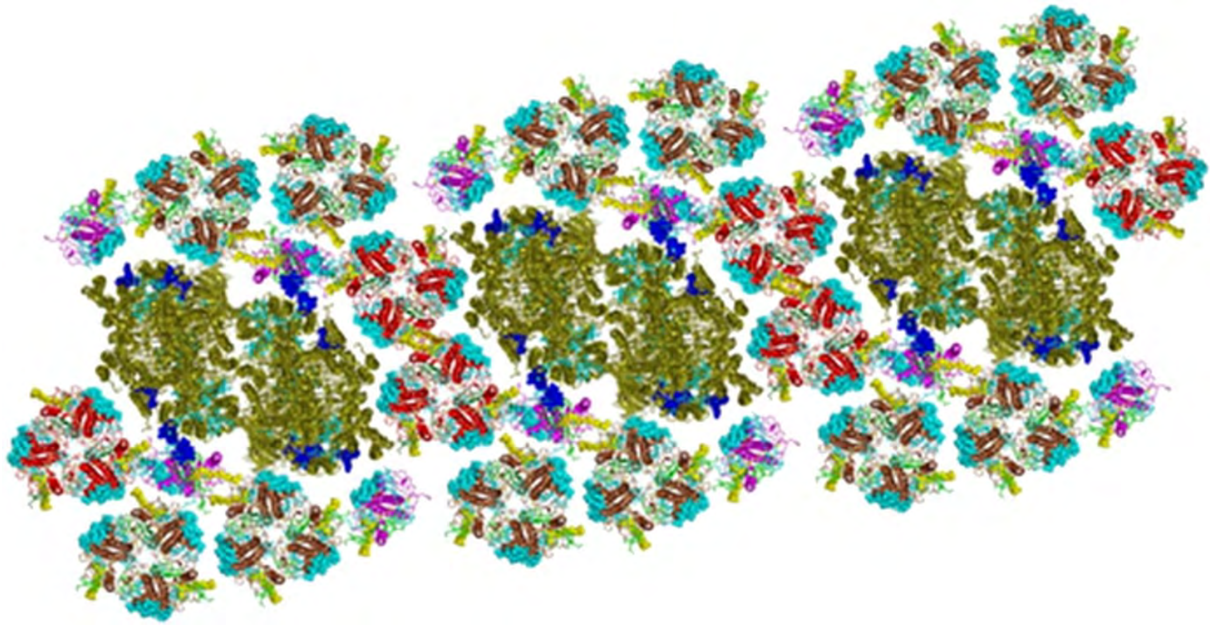


Figure 3 ***: Model of the tighter possible organization of the C2S2M2N2 supercomplexes of C. reinhardtii based on electron microscopy of isolated particles***. (Drop et al., 2014) *Proteins of the PSII core (lime green), LHCII-S and -M (brown), novel LHCII-N (red), CP29 and CP26 (magenta), Chls a (cyan), Chls b (green), neoxanthin (yellow spheres), lutein L1 (orange), lutein L2 (dark-yellow sticks).*

At PSI, the antennae (LHCI) are connected to the photosystem core via the PsaG/K/F subunits that ensure stable binding. Their direct role in energy transfer from LHCI to PSI has been reported recently (Le Quiniou et al., 2015)

ATP synthase

The proton gradient generated along the electron transfer chain is used by chloroplast ATP synthase to produce phosphorylation power in the form of ATP, which is used throughout the cell for thousands of different reactions. ATP synthase is commonly divided into F_1 and F_O subcomplexes. The first plays a role in the phosphorylation of ADP, which is powered by the movement of an axis linking it to a membrane embedded F_O subcomplex. In the latter, proton flow from the lumen to the stroma drives the rotation of the axis. ATP synthase is found in all kingdoms of life and has a conserved structure and sequence. Interestingly this structure is also conserved in ATP-dependent proton pumps (Beyenbach and Wieczorek, 2006). Chloroplast ATP synthase is composed of subunits encoded by the chloroplast genome (A, B, E, F, and I) and subunits encoded by the nuclear genome (C, D, G, and H) (figure 1)(table 4). As with the other major complexes, this requires coordinated gene expression. Like other thylakoid complexes, ATP synthase is essential for phototrophic growth and its assembly is strongly dependent on the availability of each subunit (Johnson and Melis, 2004).

Distribution of complexes among the thylakoid membrane domains

The PSII complex is located in a specific part of the thylakoid where membranes are stacked one over the other to form macrostructures called grana (Dekker and Boekema, 2005). PSII core complexes form dimers surrounded by monomeric antenna proteins (CP29, CP26, and CP24) and LCHII antenna trimers in arrangements called supercomplexes. The distance between the appressed membranes is significantly lower in the grana compared to the stromal lamella (Anderson, 2002; Dekker and Boekema, 2005). Such a confinement may be involved in limiting the access of stromal proteins or limiting the diffusion of PSII (with protein-protein interactions), PSI, and ATPase. Conversely, PSI and ATP synthase are mainly found in lamellae and grana margins. The organization of different complexes is flexible and under certain conditions, megacomplexes containing PSI-LHCI-LHCII, or PSI-b_6f have been observed (Rexroth et al., 2003; Heinemeyer et al., 2004; Takahashi et al., 2014; Bergner et al.,

Sub-unit	Role	Ref
AtpA	F_1 subcomplex, α subunit, binding and phosphorylation of ADP, forms a heterohexamer with AtpB.	(Weber et al., 1993)
AtpB	F_1 subcomplex, β subunit, binding and phosphorylation of ADP, forms a heterohexamer with AtpA.	(Weber et al., 1993)
AtpC	F_1 subcomplex, γ subunit, binds AptB and E. Is the rotary axis.	(van Lis et al., 2007)
AtpD	F_1 subcomplex, δ subunit, binds AtpA, B, C, and E. Role unclear but required for photosynthesis.	(van Lis et al., 2007)
AtpE	F_1 subcomplex, ε subunit, binds AtpC. Role unclear but required for photosynthesis and ATPase assembly.	(Johnson and Anastasios, 2004)
AtpF	F_O subcomplex, SuI, bound to psaG, constituent of the Stator.	(Allen et al., 2011)
AtpG	F_O subcomplex, SuII, bound to psaG, constituent of the Stator.	(Allen et al., 2011)
AtpH	F_O subcomplex, SuIII, structure of the membrane C ring in the Fo domain.	(Balakrishna et al., 2014)
AtpI	F_O subcomplex, SuIV, proton channel function, inserted in the membrane, binds AtpH.	(Allen et al., 2011)

*Table 4 : **Composition of ATP synthase and known roles of the different subunits**.*
This table groups results from different species

2015) (figure 4).

This compact organization can be modified in response to different signals, including an unbalanced ATP/NADPH ratio or changes in light quality (Nagy et al., 2014). Under the canopy, far-red enriched light or excessive light triggers re-arrangement of complexes in parallel with a change in the stacking of the thylakoid membrane, and a modification of the number of grana and their height. For example, far-red enriched light favors shorter grana with more layers (Pietrzykowska et al., 2014). Similarly, stacking of grana is modified during state-transition, which can be linked to far-red excitation (Chuartzman et al., 2008). It was demonstrated that mutants of STN8 (Fristedt et al., 2009) and PBCP (Samol et al., 2012), the kinase/phosphatase couple that controls phosphorylation of PSII core proteins, are affected in thylakoid membrane organization.

On the other hand, the CURT phosphoprotein family members are involved in the curvature of the thylakoids and are evolutionary conserved between cyanobacteria, green algae and higher plants. They form oligomers at the thylakoid margins to facilitate membrane curvature. The influence of CURT protein mutation is thought to override the influence of PSII phosphorylation on membrane architecture.(Armbruster et al., 2013)

Carbon fixation and other metabolic pathways (N reduction, biosynthesis)

In the chloroplast, CO_2 fixation is a major energy consumer, requiring two NADPH and three ATP per CO_2 molecule fixed. This anabolic process, the Calvin–Benson–Bassham cycle (CBB cycle), involves the most abundant protein on earth, ribulose-1,5-bis-phosphate-carboxylase-oxygenase (Rubisco). The CBB cycle generates the sugar precursor glyceraldehyde-3-P.
Nitrate assimilation and reduction also requires a lot of reducing power, since reduction of each NO_3 requires one NADPH and six FDX_{red} (FDX2 seems to be preferred to FDX1 [Terauchi et al., 2009]). Similarly sulfur assimilation is fed by ATP and reducing equivalents such as FDX_{red} , TRX_{red} and glutathione. FDX1 also interacts with, and in some cases reduces, FDX5 (induced during anaerobiosis and copper starvation,(Lambertz et al., 2010)), hydrogenase (acts as a valve to release excess reducing power in anaerobiosis), THI4 (vitamin B1 biosynthesis), METM (S-adenosyl-methionine synthase, produces the coenzyme for DNA or histone methylating enzymes), FLV3 (Flavodiiron protein involved in short-term photoprotection of PSI in cyanobacteria and in C.reinhardtii (Allahverdiyeva et al., 2014; Jokel et al., 2015)), SBE2/3 (starch catabolism), MDH4 (redox transport, (Scheibe, 1987)), LHCBM7 (light harvesting),

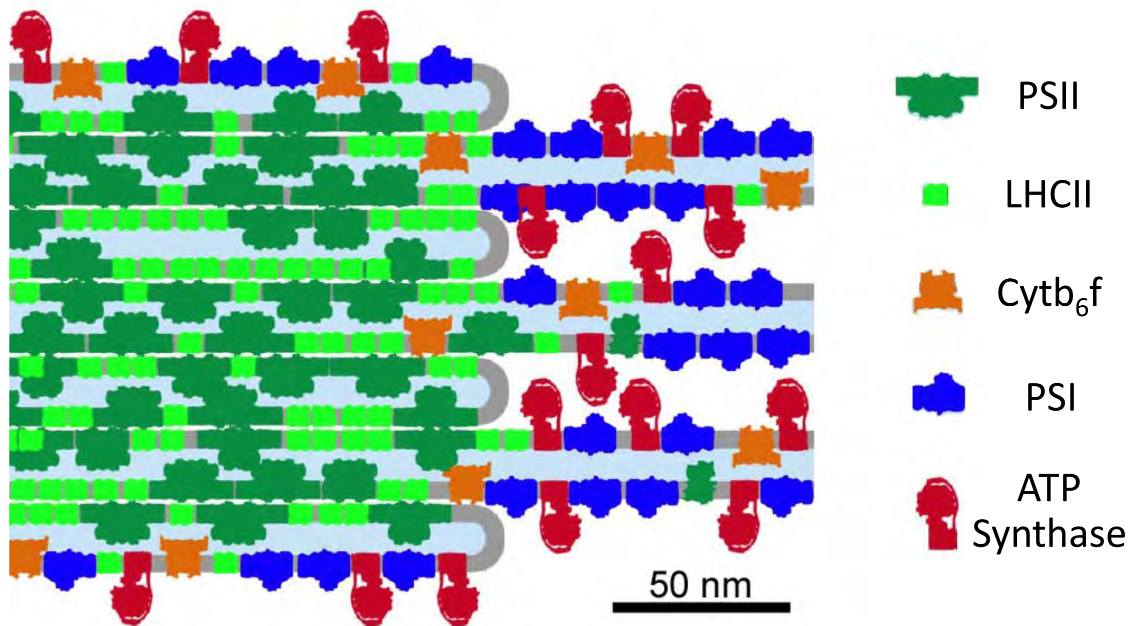


Figure 4 : ***Diagram of the photosynthetic complexes partitioned within the thylakoid membranes****, in the grana stacks (on the left) and the stromal lamellae (on the right). Modified from Dekker and Boekema, 2005.*

PSBW (stabilization of PSII dimers), LHCA5 (light harvesting), and DES6 (triglyceride metabolism, (Peden et al., 2013)).

Evolution of photosynthesis

The simplest form of photosynthesis is found in halobacteria, a subgroup of Archae that evolved a protein-pigment complex that uses light energy to create a proton gradient across the membrane that separates the cytoplasm from the S-layer space. The proton gradient powers an ATP synthase, which generates ATP to fulfill the energy needs of the cell. This complex, which is composed of bacteriorhodopsin homotrimers containing a single retinal molecule in each monomer, is a proton pump with an absorption maxima at 570 nm (Saeedi et al., 2012) which coincides with the maximum radiation level of the light spectrum received on earth (Wikipedia: Sunlight, 2015). In other Archae, carbon fixation is not linked to photosynthesis but to chemosynthesis. This is the process through which Archae obtain energy from the oxidation of sulfur, iron or H_2, and which utilizes simple carbon containing molecules such as CO_2 or methane to generate larger organic compounds (Cavanaugh, 1983; Edwards et al., 2011).

Carbon fixation and photosynthesis are combined in photosynthetic Eubacteria. Some studies suggest that both Archae Halobacteria and photosynthetic Eubacteria may have a common ancestor, a “Photocyte” that contained both photosynthetic and chemosynthetic abilities with a carbon fixation mechanism (Lake et al., 1985). Opposing findings support the possible horizontal transfer of photosynthetic genes through viruses, suggesting the separate evolution of photosynthesis and carbon fixation (Millard et al., 2004; Sullivan et al., 2006; Sharon et al., 2007; Hevroni et al., 2014).

The presence of two different photosystems (that might have originated from gene duplication followed by specialization) (figure5) with either proton-motive force (type II) or reducing functions (type I) also adds complexity to the system. These are precursors of photosystem II and bacteriorhodopsin or photosystem I, respectively. From an evolutionary point of view, it is likely that species with only one photosystem (losing one of the original duplicates) appeared much earlier than the species that were able to use both efficiently (Gupta et al., 1999).

Organisms carrying only photosystem I, like C*hlorobia* or *Heliobacteria*, (figure 6) do not seem to contain internal specialized compartments, but instead use their periplasm to

obtain electrons from cyt_C and to generate reduction potential in the cytoplasm through photosynthesis (figure 5). Cyt_c is then recycled using electrons from sulfur by sulfide quinone oxidoreductase (SQR) and different quinones. Carbon fixing abilities were found when the genome of *Chlorobium tepidum* was sequenced, and a reverse TCA cycle was also found to be functional in this organism (Hanson and Tabita, 2001).

On the other hand, organisms encoding only a PS II complex, like *Rhodobacter*, emerged. The thylakoids of these species have a spheroid shape and are widely distributed in the cytoplasm. Organisms with both photosystems evolved a way to functionally link them in order to optimize their cooperation. This is a function of the cytochrome b_6f complex which is also located within the thylakoid membrane (figure 7). These organisms present different kinds of thylakoids, such as flat vesicles stacked at the periphery, which, in cyanobacteria, form a spiral close to the plasma membrane. There is no evidence of thylakoid membrane continuity with the plasma membrane in cyanobacteria, but their different stacks seem to share a single continuous membrane (Liberton et al., 2011). In cyanobacteria, the carbon fixing pathway is linked to the reductive pentose pathway (Pelroy and Bassham, 1972).

Regulation of photosynthesis

Light intensity, quality

The successful evolution of photosynthesis is linked to its ability to adapt to fast and slow changes in the environment, particularly in the light regime. In the wild, under a canopy, light intensity and quality depends on shade from other plants. For plants on the forest floor, light intensity can vary 100-fold and its quality ranges from sunlight to leaf-filtered light, which is enriched in the far-red end of the visible spectrum (Kasperbauer, 1971). Changes in light intensity and quality are also observed between sunny and cloudy conditions, and between dawn and dusk.
To adapt to these changing conditions and to keep the ratio of reductants/ATP balanced with metabolic demands (Kramer et al., 2004), photosynthetic organisms have evolved strategies to compensate for the changes, either in the short-term or over the long-term.

The adaptation of photosynthetic organisms to changing conditions optimizes light absorption to fulfill the needs of metabolism. The efficiency of this adaptation is primordial, as too little light absorption and transformation into chemical energy would trigger over-oxidation

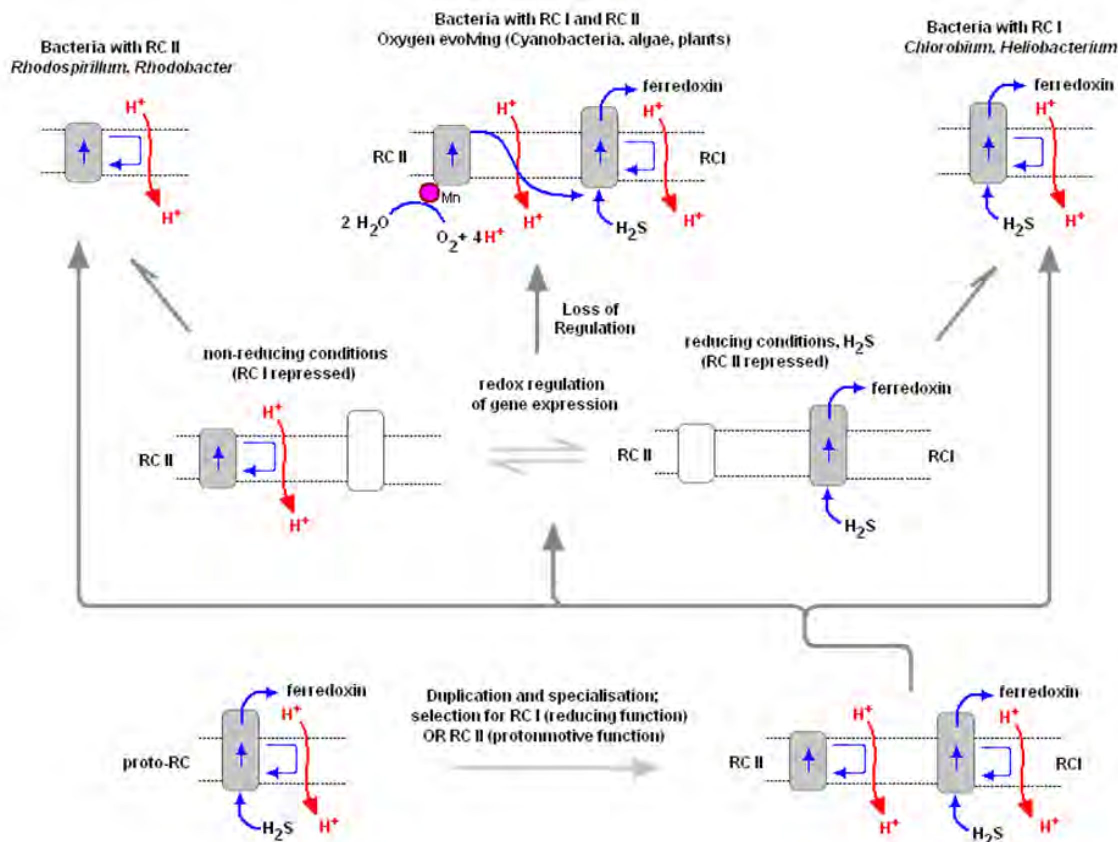


*Figure 5 : **Hypothetical evolutionary pathways leading to known phototrophic organisms**. A common ancestor for both PSII and PSI, which are homologous proteins, has been suggested. Some species may have lost one of the two complexes to adapt to particular environmental conditions, whereas others retained both (Johnson, 2006). Modified from (Allen and Martin, 2007).*

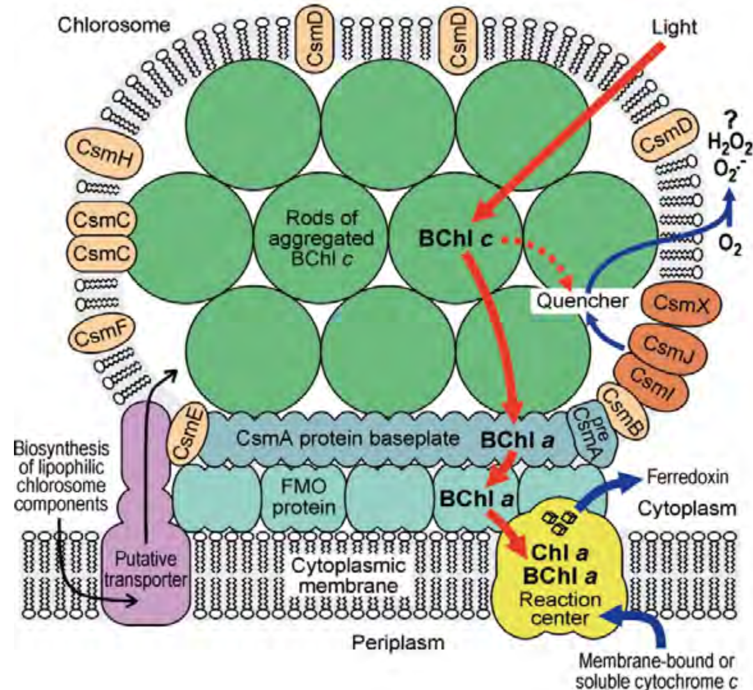


*Figure 6 : **Simplified model of the photosynthetic apparatus of the anaerobic bacterium Chlorobium tepidum**. The chlorosome harvests light energy, which is exported via a vesicle-type protein-lipid megacomplex, through different bacteriochlorophylls, to a photosystem I-like complex in the cytoplasmic membrane. Modified from Frigaard and Bryant, 2004.*

of cellular compartments, block many essential reactions, limit growth, and therefore, decrease the competitiveness and subsequent survival of the organism. Conversely, light absorbed in excess cannot be completely transformed into chemical energy. The excess energy is dissipated as heat but also can trigger the formation of reactive oxygen species (ROS), which cause damage to proteins and lipids (reviewed in Asada, 2006). The response of an organism to light depends on two main sets of variables. The first set of variables is external and depends on light availability (quantity and quality) as well as nutrient supply (CO_2, macro and microelements), toxic compounds, pathogens, water supply, and temperature. The second set of variables is internal and reflects the ability of the photosynthetic machinery to capture light and to transform it into chemical energy (Lalli and Parsons, 1997; Minagawa and Tokutsu, 2015).

Short term light acclimation.

Chloroplast movements, phototaxis

Chloroplast photo-relocation is an adaptive mechanism that can be observed in most plants, and some algae like *Zygnematales* (Suetsugu and Wada, 2009). In low light, the chloroplasts respond by aligning on the side of the cell that is illuminated to increase photosynthesis or, in high irradiance, away from light, to the sides of the cell, shading each other to decrease photo-damage (Higa and Wada, 2015). In *A.thaliana,* phototropins 1 and 2 are blue light receptors that contain a kinase domain and an oxygen and voltage sensing domain, and are mainly found in the chloroplastic outer membrane under normal light conditions (Kong et al., 2013). Phototropin 2 is responsible for the avoidance phenomenon. Both phototropins are found at the plasma membrane where they might be involved in regulating the accumulation response. (Jarillo et al., 2001; Kagawa et al., 2001). In *C. reinhardtii*, phototaxis is a similar adaptive mechanism that fits the light that is collected with the metabolic needs of the cell to maintain optimal photosynthesis while avoiding photodamage. This is directed via the eyespot, which is a unilateral macrostructure that involves the specialization of local membranes from several compartments. This ultrastructure contains pigments, including retinal, which is the prosthetic group of two light-gated ion channels called Channel Rhodopsin 1 (CHR1) and CHR2.

CHR1 can produce a photo-induced H^+ current (Nagel et al., 2002) and can transport

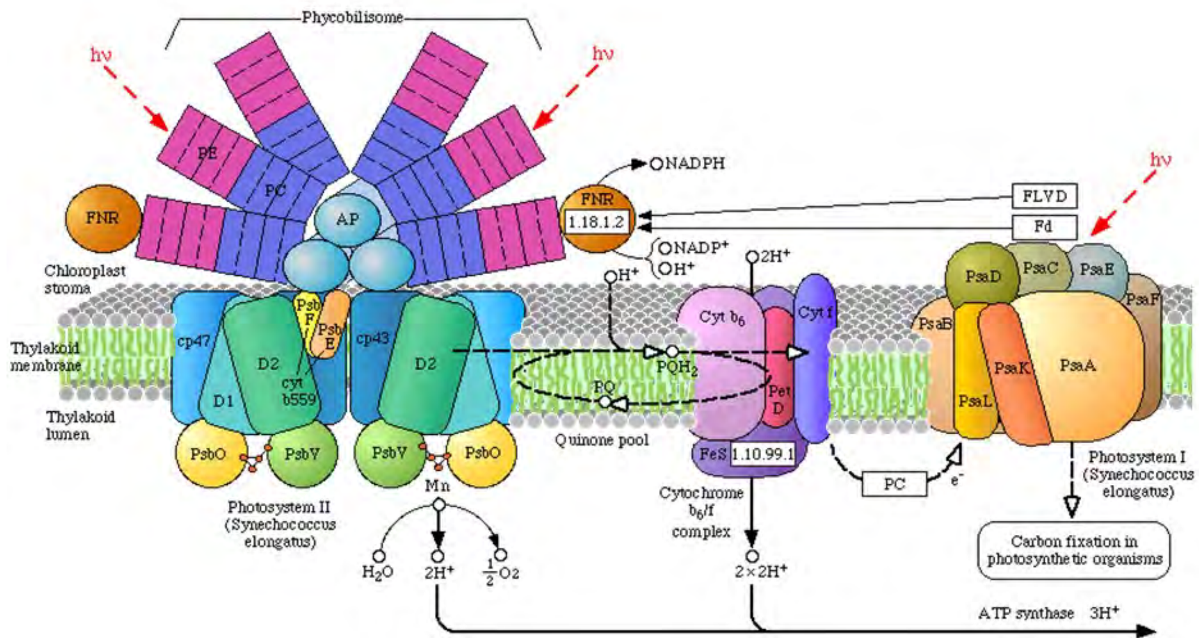


Figure 7 : ***Simplified model of the photosynthetic apparatus of the cyanobacterium Synechococcus elongatus****. The phycobilisome harvests light energy, which is then transfered by a protein megacomplex, through different pigments like phycoerythrin and phycocyanin, to a photosystem II-like complex in the thylakoid membrane. This contains PSII, b6f, and PSI as occurs in plant-type photosynthesis, but does not contain LHCII, which is replaced by the phycobilisome, or Lhca complexes, which are replaced by different PSI subunits. Modified from (Campbell et al., 1998)*

Ca^{2+}, K^+, Na^+, and Li^+ with different efficiencies under different pH conditions (Berthold et al., 2008). CHR2 is a light-gated cation channel that induces low intensity photocurrents (Sineshchekov et al., 2002), and is used in optogenetics (Müller et al., 2015). Phototropins are involved in the regulation of eyespot size. This modulation affects the accumulation of CHR1 only (Trippens et al., 2012). Positive and negative phototaxis are observed in *C. reinhardtii.* Even if the regulation of these processes is not fully understood, they seem to be affected by the calcium content of the media (Morel-Laurens, 1987). A low calcium concentration triggers positive phototaxis while a concentration over 10^{-5} M favors negative phototaxis. Pharmacological inhibition of calcium channels confirms these results. (Hegemann et al., 1990). There is also evidence showing the dependency of phototropism on photosynthetic activity (Takahashi and Watanabe, 1993).

Chlorophyll fluorescence and photochemistry.

To monitor photosynthesis, it is possible to observe the fluorescence emitted by pigments of the LHCII antennae (figure 8) in real time and at physiologically relevant temperatures (usually 20–25°C for *C. reinhardtii*). In these conditions fluorescence is mostly emitted by PSII and its antenna. The level of fluorescence reflects the level of reduction of the electron carriers downstream in the electron transfer chain. If they are oxidized, most of the energy captured by LHCII is transmitted to the chain via charge separation in PSII and fluorescence is low. Conversely, if the chain is reduced then more energy is dissipated as fluorescence or heat. figure 8 represents the kinetics of fluorescence induction upon a shift from dark to light in different mutants. Within the first second of illumination after a short period of dark incubation, fluorescence increases due to saturation of the electron transport chain caused by a reduced PQ pool (see section on “alternative electron pathways”). The fluorescence decreases thereafter and stabilizes when metabolism restarts and the electron transport chain is reoxidized. Actinic light and saturating pulses allow an estimation of photochemistry with the parameter qP (Fm (t)-Fs)/(Fm (t)-F0) (see figure 9).

qE or energy-dependent dissipation

In excess light conditions, non-photochemical energy dissipation pathways appear which are collectively called non-photochemical quenching (NPQ). The fastest is called qE, which occurs from the first few seconds of stimulation, and reflects in a first stage the heat

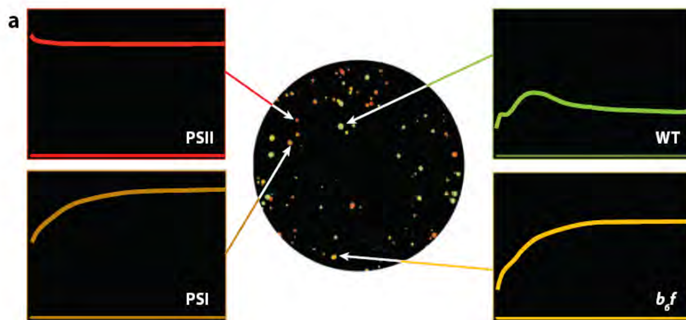


Figure 8 : ***Kinetics of chlorophyll fluorescence in colonies of wild-type and PSII, b6f, or PSI mutants of Chlamydomonas reinhardtii.*** *Fluorescence is monitored after a short dark-adaptation period and can be measured from transformed colonies on a plate by direct screening (courtesy of Xenie Johnson; Eberhard et al., 2008).*

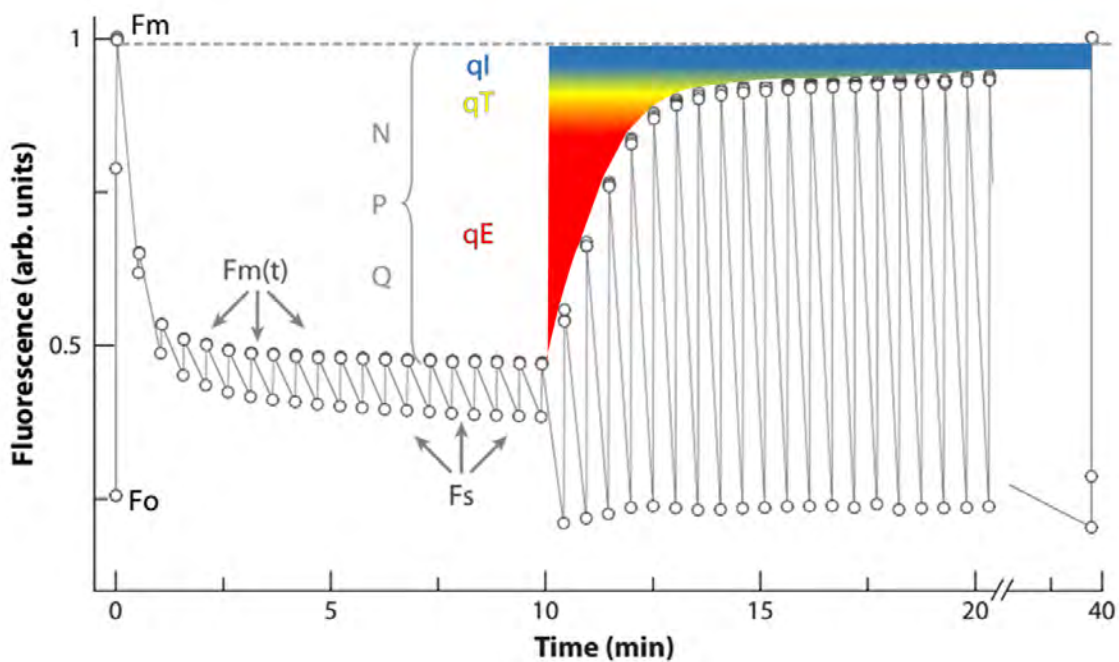


Figure 9 : ***Fluorescence kinetics at room temperature of a dark-adapted Arabidopsis thaliana leaf*** *subjected to continuous high light (red light, 500 µE·m^{-2}·s^{-1}) with saturating pulses (Fm(t)) followed by dark incubation with saturating pulses. NPQ relaxation can be observed and the recovery of each parameter, qE, qT, and qI can be annotated. Data were obtained using a JTS-10 spectrofluorimeter (Biologic, France). Figure and legend modified from Eberhard et al., 2008.*

dissipation induced by protonation at low luminal pH of PSBS in higher plants (Li et al., 2000) (or protonation of the high-light inducible LHCSR3 in *C. reinhardtii*, (Peers et al., 2009; Maruyama et al., 2014)). PSBS is thought to act by modifying the conformation of LHCII, making the process of energy transfer to the photosystem core less efficient. The *npq 4* mutant of *C. reinhardtii* is defective in LHCSR3 and shows a defect in NPQ at early timepoints of qE (figure 9). However, it has kinetics comparable to those of the WT for the late qE timepoints (Niyogi et al., 1997), suggesting that LHCSR3 has an additive role in association with another qE component. Energy dissipation is promoted in a second stage by the re-arrangement of LHCII antennae, within which the conversion of violaxanthin to zeaxanthin plays an important role. Zeaxanthin is present in both the LCHII antenna (LHCB1-6) and LHCI (LHCA1-4) in low quantities (for cells not adapted to excessive light) and favors energy dissipation as heat. The steady state of the second phase is obtained after 10 min in LHCB-3 and up to 120 min in LHCB4,5 and LHCA1,2,4 (Jahns et al., 2009). Violaxanthin de-epoxidase (VDE) is activated by a low pH in the lumen, and converts violaxanthin to zeaxanthin favoring zeaxanthin-dependent heat dissipation (Jahns et al., 2009). Zeaxanthin can be converted back to violaxanthin by the enzyme zeaxanthin epoxidase (ZE) (Sylak-Glassman et al., 2014). The relative activity of VDE and ZE determines the zeaxanthin content and is affected by acclimation to different light intensities. The *npq1* mutant in *C. reinhardtii*, which is defective in zeaxanthin production, shows a defect in NPQ kinetics at late qE timepoints (after 2 min, when the zeaxanthin-driven dissipation usually plays a significant role) (figure 9), whereas no defect is observed at early timepoints (Niyogi et al., 1997). In a knock-down mutant that is deficient in chloroplast ATPase, the consequent acidification of the lumen triggers qE even under low light conditions (Rott et al., 2011).

qT or state-transition.

Over a time scale of minutes, state transitions play an important role in the acclimation to light conditions. This process is also called qT and regulates the phosphorylation of antenna proteins to balance excitation of the photosystems via detachment or attachment of LHCII antennae to PSII or PSI. Phospho-LHCII is thought to be bound to PS I, but recent studies in *C. reinhardtii* have revealed that this may involve only a fraction of the antennae that disconnect from PSII (Ünlü et al., 2014, 2015; Nagy et al., 2014). The free antenna is believed to quench over-excitation energy, and this is likely to differ from species to species. The main

roles of state-transition are to balance excitation between the two photosystems, favor cyclic electron flow and limit the total energy and electrons injected into the photosynthetic electron transport chain, thus complementing qE(Allorent et al. 2013).

The two photosystem have different absorption spectra. Over-excitation of one photosystem compared to the other leads to an unbalanced redox status of the electron transfer chain (see section “alternative pathways”). For example, in orange-enriched light, PS II and its antennae (which are enriched in Chl_b compared to PS I), are more excited than PS I, thus triggering over reduction of the plastoquinone pool, which is limited by PS I activity. This unbalance is sensed at the Q_o site of the b_6f complex via the reduction state of the PQ pool, and activates a kinase that may be transiently bound to it. This is STT7 in *C. reinhardtii* (Depège et al., 2003) and STN7 in *A. thaliana* (Bellafiore et al., 2005). These kinases contain a trans-membrane helix that is responsible for their binding to the membrane and perhaps also to the b_6f complex. Phosphorylated LHCII detaches from PS II leading to a decrease in fluorescence. This phosphorylation is linked to the energy transfer from LHCII to PS I and increases its light capture and activity, bringing the over-reduced PQ pool to an adjusted level (Vener et al., 1997; Grieco et al., 2015). This phosphorylation is balanced by a protein phosphatase, PPH1/TAP38, which is mostly specific for LHCII (Shapiguzov et al., 2010; Pribil et al., 2010).

STT7/STN7 oxidoreduction, on two conserved cysteine residues, is thought to regulate its kinase activity (Lemeille et al., 2009). In high light, the disulfide bridge may be regulated by the ferredoxin-thioredoxin system while in low light regulation involves the binding of PQH2 to the b_6f complex (Rintamäki et al., 2000). CCDA and HCF164 may participate in this ferredoxin-thioredoxin driven regulation (Lemeille and Rochaix, 2010). Excitation of PS I favors cyclic electron flow (reducing equivalents generated by PS I can return to the b_6f complex increasing the proton gradient; see section on “alternative electron transfer”), thus producing more ATP rather than NADPH. As a consequence, adjusting the energy capture by each photosystem regulates the quantity and ratio of reducing equivalents/ATP, according to the needs of metabolism (Wollman, 2001; Grieco et al., 2012).

qI or photoinhibition

Photoinhibition is well documented for PS II. It is induced when light is in excess compared to that utilized in photochemistry, and first damages the Mn_4O_5Ca co-factor. This

cluster can absorb a photon and enter a reversible inactive state that blocks the OEC (Hakala et al., 2005). This complex is also inactivated by strong UV/blue light (Ohnishi et al., 2005). Second, the P680 center chlorophyll is blocked by the absorption of excess light (the efficiency of different wavelengths to induce qI reveals a photochemistry-like spectrum) and photochemistry is consequently prevented (Ohnishi et al., 2005). Both processes avoid the production of excess reducing equivalents that could block many metabolic reactions. This block of PS II favors the excited form of P680, which induces ROS production and affects D1 repair. D1 is the core protein of PSII supporting the reaction with the highest redox potential: at the level of P680, light energy forms P680*, which is converted to form $P680^+$, the reducer of the D1 pheophytins and the most powerful biological producer of ROS. When $P680^+$ accumulates (for example when the electron transfer pathway is saturated), a triplet chlorophyll can be generated that reacts with oxygen to produce singlet oxygen (Rutherford and Krieger-Liszkay, 2001). ROS (mainly singlet oxygen) are produced rapidly and irreversibly damage D1 (Kyle et al., 1984). D1 enters a protein turnover process and is degraded by FTSH and DEG1 proteases (Nixon et al., 2005). D1 being inaccessible in the grana, its degradation requires PS II migration and disassembly. The complex was found to move either to the margins of the grana or to the stromal lamellae membranes (Baena-González and Aro, 2002). The diffusion of D1 is highly dependent on the tightness of grana stacking (Fristedt et al., 2009). The damaged D1 is replaced by a newly synthetized D1, that assembles into PSII, which then re-integrates the grana.

High light also triggers phosphorylation of the membrane surface-exposed regions of the D1, D2, CP43, and PsbH proteins of PS II in plants (Rintamäki et al., 1997; Vener et al., 2001). Phosphorylation is thought to increase mobility of the damaged photosystem and to trigger their disassembly (Tikkanen et al., 2008; Goral et al., 2010; Fristedt and Vener, 2011; Herbstová et al., 2012; Puthiyaveetil and Kirchhoff, 2013). It favors PS II relocalization to the unstacked membranes. Phosphorylation is concomitant with the relaxation of grana membrane stacking (Nagy et al., 2014). The main kinase responsible for PS II phosphorylation is STN8 in *A. thaliana* (Bonardi et al., 2005; Vainonen et al., 2005), which is an ortholog of STL1 from *C. reinhardtii.* These kinases are paralogous to STT7 and STN7 (described in the section qT), respectively, sharing over 40% identity in the kinase domain (STN7 vs. STN8) (Vainonen et al., 2005; Grouneva et al., 2012). PBCP phosphatase was recently characterized and shown to be involved in the reverse reaction, dephosphorylation of PSII (Samol et al., 2012).

Long term acclimation

In Chlamydomonas after 10 divisions under high light, the quantity per cell of D1 and PsaA decreases while b_6f, Rubisco and ATPase stay stable. High light also induces some LHC rearrangements (Bonente et al., 2012). Light quality and intensity mainly affect transcription in the nucleus and translation, accompanied by posttranslational modifications in the chloroplast (Kloppstech, 1997; Ruban, 2009). Nuclear transcription is a major actor in adaptation as it influences all compartments of the cell such as mitochondria and chloroplasts. Its regulation requires the integration of signals from a broad range of environmental clues. Evidence for regulation was provided with the observation that the number of PSI centers increases to compensate for defective PS I excitation in the *stn7* mutant of *A. thaliana* (Grieco et al., 2012)(figure 10 and 11). Similarly, the number of photosystems per cell decreases under excessive light in *C. reinhardtii* (Bonente et al., 2012; Schöttler and Tóth, 2014).

The nucleus-encoded transcription factor AAG-box binding factor (AGF) (Shi et al., 1999) binds to a light-responsive *cis*-regulatory element upstream of the *psbD-psbC* operon in the barley chloroplast genome (Kim and Mullet, 1995; Hoffer and Christopher, 1997 ; Thum et al., 2001). The synthesis of AGF is blue-light dependent.

The expression of several nuclear genes encoding chloroplast proteins is regulated post-transcriptionally by the nucleus-encoded protein *Cen* in *C. reinhardtii* (Hahn et al., 1996). For long-term adaptation, the size of the light-harvesting antenna is a primary target and is controlled by proteases within the first hours of excess light treatment (Yang et al., 2000). The proteases FTSH and DEG1 are involved in this phenomenon. However, long-term adaptation also affects the levels of other photosynthetic proteins. Many chloroplast proteins are encoded in the nucleus (figure 1).

The regulation of chloroplast protein content in long-term acclimation is affected by protein import, in which the TIC/TOC machinery is a major player. The activity of these complexes is regulated, and is redox-controlled in *P.sativum* and *C. reinhardtii* (Yohn et al., 1998; Herrin, 1994). For example, ferredoxin import appears to be stimulated by the reducing agent DTT (Pilon et al., 1992). Conversely, protein import in isolated chloroplasts decreases with oxidizing $CuCl_2$ treatment (Seedorf and Soll, 1995). Similarly the long term acclimation of the electron transfer rate (ETR) to temperature has been demonstrated in winter wheat, and it affects the rate of electron transfer from plastocyanin to P700 and from water to P680. The highest ETR is observed at the growth temperature (Yamasaki et al., 2002).

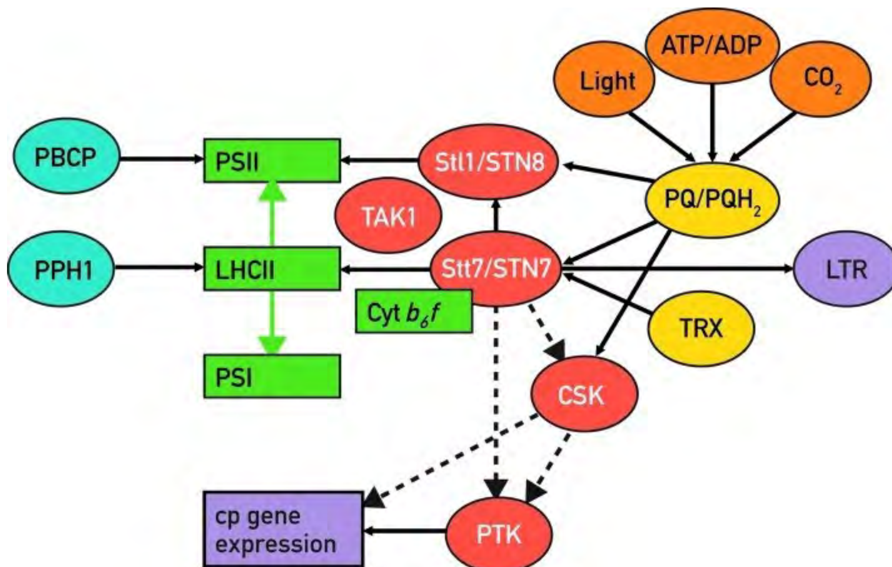


Figure 10 : ***Known players involved in STT7/STN7 and STL1/STN8 regulation****.*
Modified from Lemeille and Rochaix, 2010.

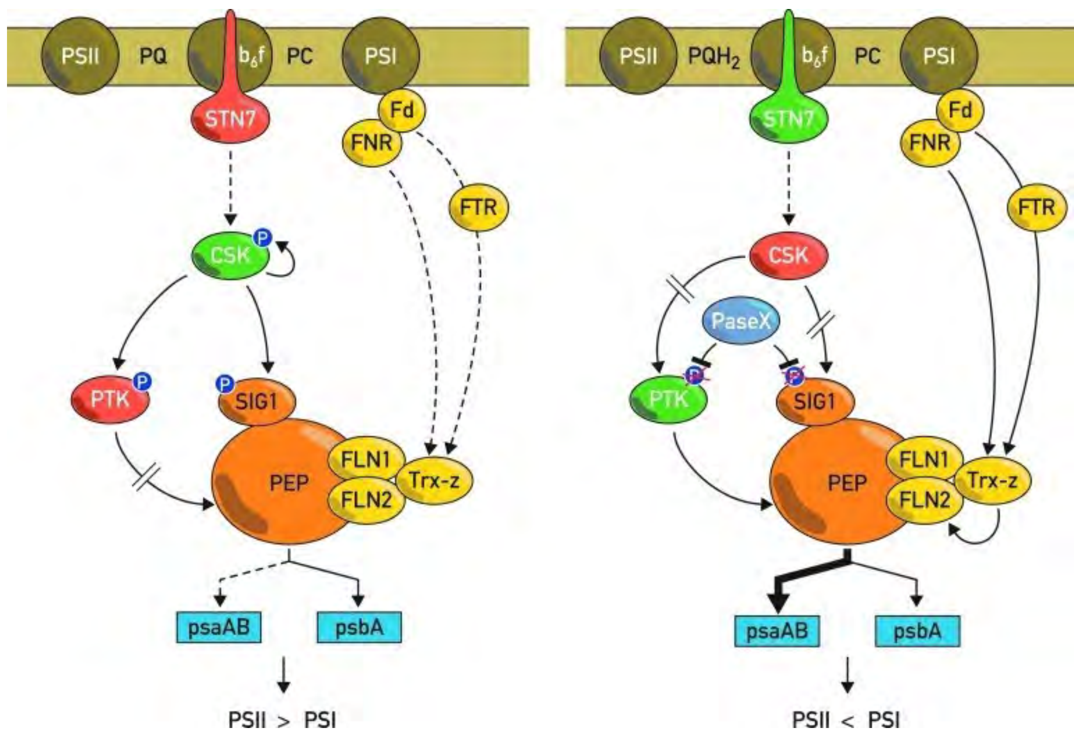


Figure 11 : ***Involvement of STN7 in the long-term acclimation of A. thaliana to different light or redox poise conditions****. Several potential partners in the signaling cascade involved in transcriptional control are depicted. Modified from Rochaix, 2013.*

There is evidence that Rubisco activity is continuously adjusted to the redox poise and is specifically related to the chloroplast glutathione pool (Sudhani and Moreno, 2015).

Alternative electron transfer pathways

Cyclic Electron Flow.

Photosynthetic ATP and NADPH production must constantly and precisely be adjusted to adapt to changing metabolic needs directed by fluctuating environmental conditions. Cyclic electron flow (CEF) around PSI is a major player in the adjustment that increases the ATP/NADPH ratio to fit the chloroplast energy output to the respective demands. ATP is produced by an ATPase using the proton gradient generated across the thylakoid membrane by two reactions, splitting of water by PS II and oxidation of PQH2 by the b_6f complex. The redox potential of the stromal carrier reduced by PS I, ferredoxin, is above that needed to reduce plastoquinone (see figure 2). With both molecules being mobile, it is easy to imagine that this reaction happens in nature with a protein catalyst. Indeed, in CEF around PS I, PQ is reduced to PQH_2, and protons are picked up in the stroma. The reduction reaction re-injects the electrons into the photosynthetic electron chain as reduced PQH_2 is re-oxidized by b_6f releasing two protons in the lumen.

This cyclic flow, which is powered by the light harvested at PS I, increases the proton gradient thus favoring ATP production, with no net production of NADPH,(Kramer et al., 2004). This regulated process occurs with a big amplitude in green algae, thus favoring adaptation to a broad range of conditions. (Lucker and Kramer, 2013). For example, the carbon concentrating mechanism in *Chlamydomonas* is required to reduce the level of photorespiration when the CO_2 concentration is low. This requires much more ATP than is needed when the CO_2 concentration is high. Under CO_2-deficient conditions, increased cyclic electron flow was observed (Lucker and Kramer, 2013).

State-transition is induced by the PQ redox pool and was proposed to lead to CEF as it favors light capture by PS I in spinach (Vener et al., 1997) and in *Chlamydomonas* (Zito et al., 1999; Iwai et al., 2010; Kukuczka et al., 2014). This state-transition re-mobilizes the antenna proteins and creates a super-complex containing PS I, b_6f, FNR, and PGRL1. This complex has been purified and its activity has been observed in vitro (Iwai et al., 2010). State-transition is indeed needed to balance ATP production when mitochondria are impaired and

vice-versa, as a double *stt7–9 dum22* mutant had a strongly impaired fitness in photoautotrophy (Cardol et al., 2009). PGRL1 was identified by its role in a first pathway of CEF, it is sensitive to antimycin, and is thought to be a PGR5-dependent ferredoxin-plastoquinone reductase (Hertle et al., 2013). The *pgr5* mutant was shown to have the same phenotype as a *pgrl1* mutant suggesting their role in the same pathway in *A. thaliana.* Furthermore, a split ubiquitin assay revealed that they interact directly as well as with PsaD and cytochrome b_6. In addition, PGRL1 binds FNR and ferredoxin (DalCorso et al., 2008).

The second main route involved in CEF is antimycin-independent and involves an NADPH/plastoquinone oxidoreductase. This circuit is called the NDH route (Shikanai, 2014). A recent finding suggests that a third CEF pathway exists, which is antimycin-dependent but PGR5-independent (Nellaepalli et al., 2015). In addition, another recent report suggested a link between NPQ and the regulation of CEF, whereby the overexpression of PsbS induces overoxydation of the PQ pool and blocks CEF independently of the lumenal pH (Roach and Krieger-Liszkay, 2012).

The Mehler reaction

The Mehler reaction was described by Mehler in 1951, and offers a “safety valve” that reduces the risk of damage to PS I under excessive light conditions. PS I can produce $O_2^{\cdot -}$ from O_2 on the acceptor side to dissipate excess excitation. This ROS is then converted to H_2O_2 via superoxide dismutase (SOD) (Asada et al., 1974). The reaction is active at the beginning of light treatment after a long dark incubation or when there is a lack of oxidized $NADP^+$. There is evidence that CO_2 might intervene in the regulation of the Mehler reaction, maybe through NADPH consumption in the CBB cycle (Roach et al., 2015).

Chlororespiration

In a pioneering paper, Bennoun (1982) described a respiratory process within the thylakoids. The proposed model involved NAD(P)H dehydrogenase (NDH), the thylakoid plastoquinone pool, and an unknown oxidase that reduced O_2 to water, suspected to generate at the same time a proton gradient. The process was called chlororespiration due to its analogy with the mitochondrial respiratory chain. The role of plastid terminal oxidase (PTOX) was later discovered with the *A. thaliana* variegated mutant *immutans*, in which a chloroplast-localized oxidase homologous to mitochondrial alternative oxidase (AOX) was affected (Carol et al.,

1999; Wu et al., 1999). Chlororespiration is thought to maintain the ATP/NADPH ratio (complementary to CEF), and to maintain a balanced PQ/PQH_2 ratio. The latter is involved in the early development of thylakoids for oxidizing phytoene, a precursor of ++carotenoids (Carol and Kuntz, 2001). PTOX may also have a role in photoprotection by dissipating excessive harvested energy (Sun and Wen, 2011). PTOX is similar to AOX, and a PTOX mutant can be complemented with AOX directed to the chloroplast (McDonald et al., 2011).

As a non-photochemical reduction of the plastoquinone pool had been observed (Mus et al., 2005), an NAD(P)H oxidoreductase was suspected of being expressed and functional within chloroplasts of higher plants and green algae. Characterization of the *C. reinhardtii* type II NDH (CrNDA2) revealed that this enzyme is located in the thylakoids. In vitro, recombinant NDA2 preferentially oxidizes NADH (50 times more activity at physiological pH compared to NADPH) and reduces the plastoquinone pool(Desplats et al., 2009). This enzyme is involved in reduction of the plastoquinone pool from cytoplasmic reductants that originate from catabolic reactions such as starch degradation. It is possible that its affinity towards NADPH in vivo might be different, thus explaining its role in cyclic electron flow around PS I.

Hydrogenase

Hydrogenases belong to a large family of enzymes that catalyze the following reversible reaction: $2H^+ + 2e^- \Leftrightarrow H_2$

They are found in extremely diverse populations of living organisms (Vignais et al., 2001) from the methanogens, which use H_2 as a substrate, to *C. reinhardtii,* which uses it to release excess reducing power(Ghysels et al., 2013). They are highly sensitive to O_2 and have only been described in organisms that have an anaerobic metabolism, even though genomic approaches have identified hydrogenase-like proteins in fungi, plants, and animals. Two hydrogenase-like proteins were discovered in *Homo sapiens*, one being involved in the modulation of a hypoxia response factor (Huang et al., 2007). They all have in common the use of an iron atom, which is differentially coordinated and involves either nickel, selenium, a second Fe, or sulfur from a protein cysteine cluster. Another aspect of this large diversity is that hydrogenases can be found in monomeric or multimeric forms. The NiFe hydrogenase class is the most common and most studied, and is found in *Desulfovibrio gigas*. In *C. reinhardtii,* hydrogenase activity was discovered by Gaffron (1939). The two hydrogenases HYDA1 and HYDA2, both of the [FeFe] type, are involved in the generation of H_2 from the

photosynthetic machinery, with HYDA2 being four times less active than HYDA1 (Meuser et al., 2012). HYDEF and HYDG are important for the correct assembly of HYDA1 and HYDA2 (Posewitz et al., 2005).

These hydrogenases are soluble proteins that compete with FNR to utilize reduced ferredoxin as an energy input. FDX1 is thought to be the main reduced ferredoxin produced by the electron transfer chain and atomic-resolution modeling in silico (Chang et al., 2007) confirms that a docking site for FDX1 on hydrogenase is possible. Mutations in these potential docking sites modulate the electron transfer and permit the improvement of affinities for light driven H_2 production (Winkler et al., 2009). Biotechnological applications that engineer hydrogenase for photobiohydrogen (biofuel) production are limited because of two main regulatory mechanisms that are common to all known hydrogenases. The first is that these enzymes remove the photosynthetic reducing power in anaerobiosis, thus limiting the cellular damage caused by excessive light absorption. In aerobiosis, this enzyme would lead to the leakage of reducing power, thus providing a rationale for its tight inactivation by oxygen. Expression of hydrogenase is rapidly activated under anaerobiosis (Happe et al., 1994) and its activity is inhibited by oxygen (Swanson et al., 2015). This means that the photobiological production of H_2 is rare, because during the day, PS II emits O_2 in response to light and there is no photosynthetic electron transfer at night, although a small amount of hydrogenase activity is observed due to oxidative carbon metabolism (Healey, 1970). To achieve conditions under which hydrogenase can take up reducing power from the photosynthetic chain, a perfect balance between respiration (i.e., oxygen uptake) and photosynthesis (i.e., oxygen production) is required (Scoma et al., 2014). During sulfur starvation, protein synthesis is greatly impaired thus affecting proteins with a short half-life such as D1. Likewise, under low oxygen and sulfur-limited environments, the water splitting activity of PS II is reduced, triggering hypoxia that favors hydrogenase requirement, expression, and activity. This enzyme provides a target for the improvement of H_2 production yields leading to the light driven production of biofuel(Tsygankov et al., 2006). Trials were performed with hydrogenases from different species and it appeared that some are more resistant to the oxygen inhibitory effect(Ciaccafava et al., 2013; Kwan et al., 2015). They were expressed under aerobiosis, and used to engineer the active site with higher affinity for hydrogen and lower affinity for oxygen. Other optimization trials were done to increase the proportion of detoured ferredoxin, to modify the carbon metabolism or to decrease D1 (lowering oxygen production) by repression of gene expression or sulfur starvation. So far, photo-production of hydrogen is not high enough to be

commercially viable.

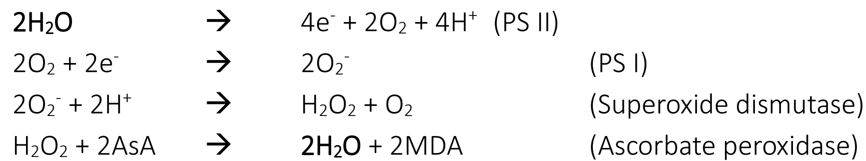
ROS metabolism

Photosynthetic organisms have to withstand the production of ROS formed at PS I and PS II. Even though they have a signaling role, ROS are harmful and quickly damage surrounding molecules such as membrane lipids, proteins, and nucleic acids. Toxicity is determined by the reactivity of each ROS. Singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$) are all produced in *C. reinhardtii* chloroplasts at different levels (reviewed in Asada, 2006).

Singlet oxygen is produced mainly in PS II and LHCII by chlorophylls (Rinalducci et al., 2004). Under excessive light, chlorophyll molecules from both core and antenna proteins can create a triplet state chlorophyll that is at the origin of singlet oxygen production. Acclimated cells can partially quench this over-excitation, materialized as chlorophyll triplet state, using different carotenoids (Glaeser et al., 2011). However, under very high light conditions, the non-quenched chlorophylls inevitably produce singlet oxygen (Fischer et al., 2007). This particular ROS has a short half-life as it can react with many molecules in its surroundings, primarily damaging D1 and antennae. As described above, ROS produced at PSI through the Mehler reaction participate in a so called "water-to-water" cycle (Asada 2006) (figure 12).Superoxide dismutase (SOD) is a metallo-enzyme found in almost all aerobic species and is associated with Mn and Fe in one family, Cu and Zn in a second, and Ni in a third. Some of these enzymes retain their activity when they bind the other metal species, thus avoiding significant oxidative stress that would follow starvation of any one metal (Meier et al., 1982). In *C. reinhardtii*, three isoforms of Mn-SOD and one isoform of Fe-SOD are found (Sakurai et al., 1993), the latter being responsible for 40% of the soluble SOD activity observed in the conditions tested.

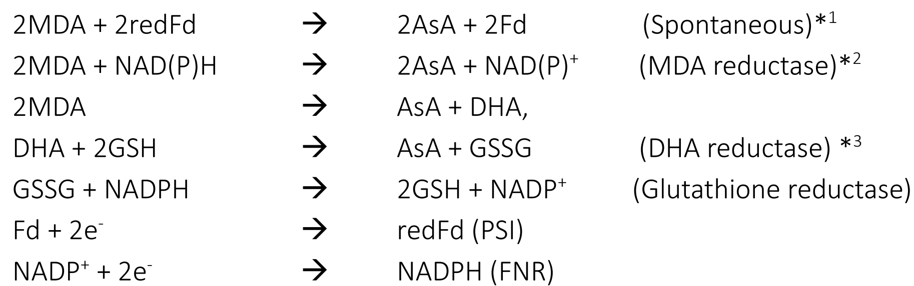
Furthermore, side degradation pathways are also known, including the breakdown of H_2O_2 by UV light, which generates $OH^{\cdot}$. However, whether this reaction plays a role in UV protection or signaling remains unknown. H_2O_2 is also known to induce carotenoid accumulation, which protects against singlet oxygen production (Chang et al., 2013). This phenomenon has been attributed to chloroplast to nucleus retrograde signaling in plants (Maruta et al., 2012).

Water to water cycle



$2H_2O$	→	$4e^- + 2O_2 + 4H^+$ (PS II)	
$2O_2 + 2e^-$	→	$2O_2^-$	(PS I)
$2O_2^- + 2H^+$	→	$H_2O_2 + O_2$	(Superoxide dismutase)
H_2O_2 + 2AsA	→	**$2H_2O$** + 2MDA	(Ascorbate peroxidase)

Regeneration of AsA



2MDA + 2redFd	→	2AsA + 2Fd	(Spontaneous)*1
2MDA + NAD(P)H	→	2AsA + $NAD(P)^+$	(MDA reductase)*2
2MDA	→	AsA + DHA,	
DHA + 2GSH	→	AsA + GSSG	(DHA reductase) *3
GSSG + NADPH	→	2GSH + $NADP^+$	(Glutathione reductase)
Fd + $2e^-$	→	redFd (PSI)	
$NADP^+ + 2e^-$	→	NADPH (FNR)	

Figure 12 : ***Reactions involved in the water to water cycle (Asada, 2006)*** *AsA: Ascorbic acid, MDA: monodehydro ascorbate, Fd: ferredoxin, DHA: Dehydro ascorbate, GSH: reduced glutatione, GSSG: oxydized glutatione. *1 (Miyake and Asada, 1994) *2 (Sano et al., 2005) *3(Shimaoka et al., 2003).*

CAS and Ca^{2+}

A calcium sensor protein (CAS) was found in the thylakoid membrane of *C. reinhardtii* (Terashima et al., 2010) and *A. thaliana* (Peltier et al., 2004) by different proteomic approaches. Knock-down of *C. reinhardtii* CAS revealed light sensitivity due to the absence of qE induction and the failure to activate the expression of high-light responsive genes encoding LHCSR3 (Petroutsos et al., 2011). This may imply that calcium signaling is involved in a route of signaling from the chloroplast to the nucleus.

Accumulation of LHCSR3.1 and 3.2 mRNA and protein under conditions of excessive light is abolished in CAS knock-down strains, in response to the calmodulin inhibitor W7, and to the photosynthesis inhibitor DCMU (Petroutsos et al., 2011; Maruyama et al., 2014), implying that calcium signaling and a signal from the photosynthetic electron transport chain are linked in *LHCSR3* regulation. The defect of CAS in the knock-down strain can be rescued by increasing the calcium concentration in the medium (Petroutsos et al., 2011). A recent report noted that the three LHCSR genes can be transcriptionally induced by high light intensity and that LHCSR1 induction is not sensitive to DCMU, and is only slightly sensitive to W7. (Maruyama et al., 2014). Deletion of LHCSR3.1 and 3.2 triggers an NPQ-deficient phenotype that cannot be rescued by wild-type levels of LHCSR1 (Peers et al., 2009).

Calcium signaling has been shown to affect the response to excessive light as the eye-spot, which is the master regulator of positive and negative phototactism, contains cation channels transporting light is too strong, thus limiting oxidative stress. Furthermore, the calcium concentration in the media also affects photophobic and phototactic responses. Likewise, measurements of ion content revealed that dark-induced increases in stromal Ca^{2+} levels precede the generation of cytosolic Ca^{2+} pulses in tobacco (*Nicotiana tabacum*) leaf cells (Sai and Johnson, 2002), validating the possibility of calcium communication between chloroplasts and the cytosol.

Calcium signaling has also been implicated in the heat shock response (HSR) (Schmollinger et al., 2013) as treatment of *Physcomitrella* with BAPTA, a calcium-specific chelator, abolishes the HSR. The same treatment only delays the HSR in *C. reinhardtii.* It was hypothesized that a temperature change might affect membrane fluidity causing calcium pores to open, with the subsequent calcium influx activating a calcium-dependent kinase (Saidi et al., 2010).

Nutrient limitation

Sulfur

Sulfur deprivation induces general responses that are common to a number of stress conditions and include cessation of cell division, accumulation of storage starch, and a decrease in metabolic processes including photosynthesis. Three specific responses occur: the first is an increased ability to transport and metabolize the missing nutrient.

In *A. thaliana,* sulfate transporters are induced upon starvation (Takahashi et al., 1997). In *C. reinhardtii*, the affinity of sulfate transporters is greatly increased upon starvation (Yildiz et al., 1994). *C. reinhardtii* also excretes an ARS (extracellular arylsulfatases) that hydrolyzes soluble SO_4^{2-} esters in the medium, releasing free SO_4^{2-} for uptake and assimilation (Yildiz et al., 1994). Second, sulfide reductase and numerous enzymes involved in sulfur metabolism are upregulated during sulfur starvation (Zhang et al., 2004). Finally, a recycling and economy process is established to decrease the need for the missing element. For example, proteins rich in sulfur are downregulated (González-Ballester et al., 2010; Aksoy et al., 2013), and non-essential proteins and sulfolipids are degraded (Ferreira and Teixeira, 1992).

In *A. thaliana* sulfur starvation has a high impact on the growth rate, while the chloroplast ultrastructure appears unchanged and the mitochondria are dilated, showing a lower matrix density (Ostaszewska et al., 2014). In *C. reinhardtii*, sulfur starvation is the subject of many studies as it affects biohydrogen production. The fast rate of D1 turnover makes this protein highly sensitive to the lack of sulfur-containing amino-acids. The subsequent decrease in D1 diminishes oxygen production at PS II and induces favorable conditions for light driven hydrogen production. Similarly, sulfur starvation appears to favor degradation of the $b_6$fcomplex (Malnoë et al., 2014). The process behind this degradation involves both FTSH and CLPP proteases (Malnoë et al., 2014) (Majeran et al., 2000).

Chloroplast translation is also affected under sulfur starvation. In addition to the effect of the lack of sulfur-containing amino-acids, the level of the bacterial-type sigma transcription factor RPOD (SIG 1) decreases due to the effect of the lack of sulfur on SAC3, which is a nucleus-encoded serine/threonine kinase known to affect the expression of several nuclear genes (Irihimovitch and Stern, 2006).

Iron

In nature, oceans contribute around 1/2 of the total CO_2 uptake via photosynthesis,. For photosynthetic organisms living near the ocean surface (2/3 of ocean photosynthesis), the limiting resource for growth is not light but the availability of nutrients, particularly iron (Boyd et al., 2000). Adaptation to this lack of nutrient implies that decreased photosynthetic efficiency occurs in order to balance the low energy needed for iron-limited growth.

In the early 1990's, John Martin (Martin, 1990) hypothesized that increasing levels of photosynthesis could fix huge quantities of CO_2 in the oceans. The sequestration of such a large amount decreases atmospheric CO_2 and subsequently the greenhouse effect. Since then, several experiments have been carried out to confirm the limited availability of iron for ocean phytoplankton. These experiments are depicted in figure 13 and 14. More recently, in 2012, Russ George and collaborators dumped 100 tons of iron sulfate in the ocean (Lukacs, 2012), (west of Haida Gwaii). This resulted in an algal bloom that was followed by an increase in the fish population.

The way in which iron starvation is perceived remains unknown, but evidence from different species would suggest that it has an effect on transcriptional regulation. This was revealed by transcriptomic (Castruita et al., 2011) and proteomic data (Naumann et al., 2007; Hsieh et al., 2013). In plants the signal involves several transcription factors acting concomitantly on gene expression (Kobayashi and Nishizawa, 2012).

Iron is often available as Fe^{3+} and is used as Fe^{2+}. Its uptake is coupled with copper-dependent metallo-reductases at the cell surface (De Silva et al., 1995; Kosman, 2002). Evidence shows that in *C. reinhardtii* a coupled FOX 1 - FTR 1 complex at the plasma membrane is responsive to reduced-iron levels, and import is induced during iron starvation (Moseley et al., 2002). This high-affinity transport is dependent on copper availability (Herbik et al., 2002) since FOX 1 is a copper-containing protein and the complex is physically associated with a copper chaperone, ATX1, and a copper-transporting ATPase (La Fontaine et al., 2002).

The largest difference between strains in terms of iron nutrition is the presence or absence of a cell wall. Some researchers have used strains lacking a cell wall in their studies to avoid overestimation of the rate of iron uptake caused by iron bound to the cell wall (Lynnes et al., 1998). However, it was later found that two key components of the iron assimilation pathway are soluble proteins that are secreted into the periplasmic space between the plasma membrane and cell wall. Additionally, siderophores were found to be produced by

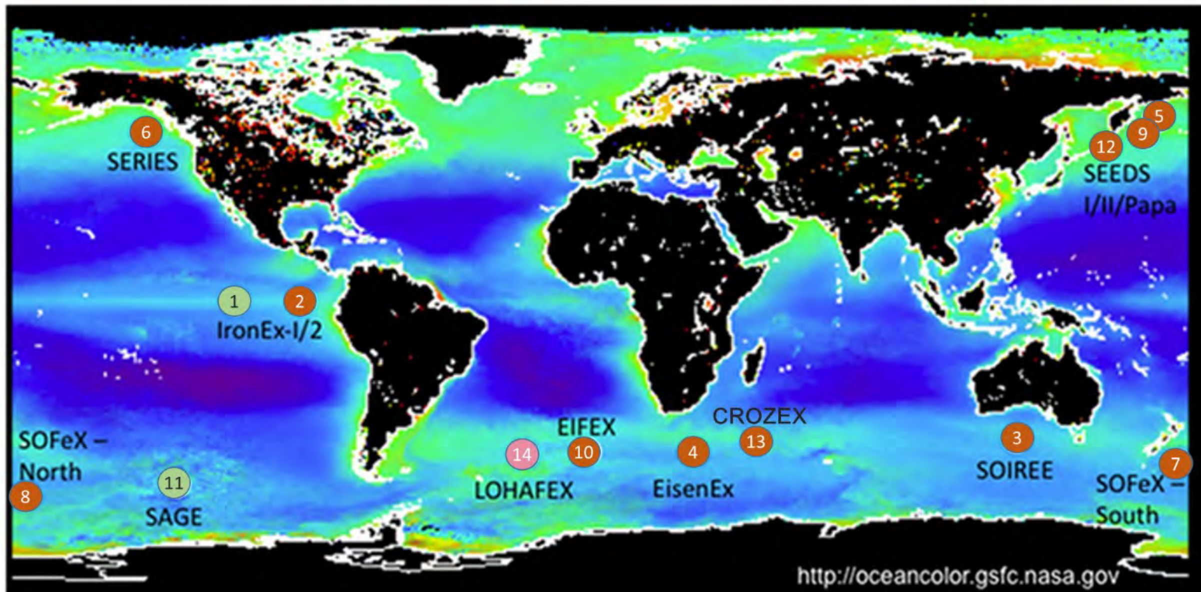


Figure 13 : ***Iron release experiments. Chlorophyll fluorescence map of oceans****. The chlorophyll fluorescence is coloured in green. Colored circle indicates dominant plankton in resultant blooms: orange – diatoms; green – picophytoplankton; pink – zooplankton. 1 – IronEx-I, 1993; 2 – IronEx-II, 1995; 3 – SOIREE, 1999; 4 – EisenEx, 2000; 5 – SEEDS-I, 2001; 6 – SERIES, 2002; 7 – SOFeX North, 2002; 8 – SOFeX South, 2002; 9 – SEEDS-II, 2004; 10 – EIFEX, 2004; 11 – SAGE, 2004; 12 – PAPA-SEEDS, 2006; 13 – CROZEX, 2005; 14 – LOHAFEX, 2009. Adapted from Trick et al. (2010).*

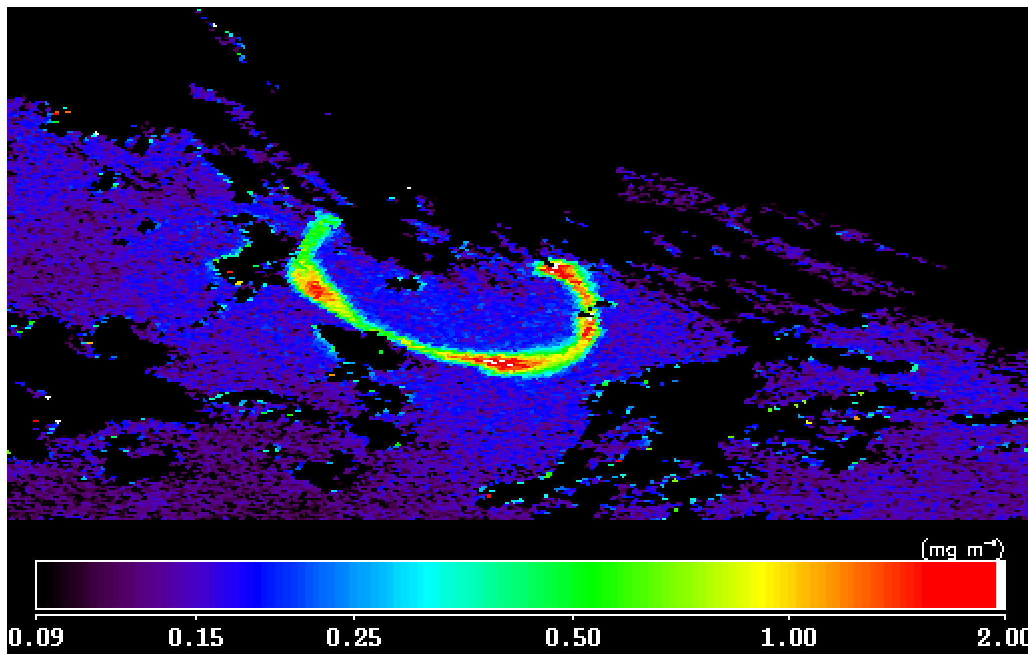


Figure 14 : ***SOIREE algal bloom****. Captured by the Sea-viewing Wide Field-of-view Sensor (SeaWiFS). The bright comma in the above image indicates phytoplankton growth stimulated by iron added during the course of the experiment. (Image courtesy Jim Acker, Goddard Distributed Active Archive Center, the SeaWiFS Project,NASA/Goddard Space Flight Center, and ORBIMAGE).*

Synechococcus to assist with iron scavenging but have not been further characterized (Boiteau and Repeta, 2015). Expression of genes encoding the algal-specific proteins, FEA1 and FEA2, is significantly induced during iron deficiency. In strains lacking cell walls, these proteins are lost as they diffuse in the media (Allen et al., 2007). A significant consequence of losing FEA proteins into the medium is increased sensitivity of these strains to iron depletion (Glaesener et al., 2013). As the function of these periplasmic proteins has not been characterized, it is hypothesized that they may bind iron and increase its concentration at the proximity of the plasma-membrane assimilatory transporters. Their relative binding affinity for Fe(II) or Fe(III) is not known. In *C. reinhardtii,* iron starvation also triggers remodeling of PS I with destabilization of PsaA, PSAF, plastocyanin, LHCA3, and CytF as determined by western-blot (Moseley et al., 2002).. In terms of the antennae, LHCA1 and LHCA9 show the most dramatic decrease after 24h, while the decrease of other antennae is slower (Yadavalli et al., 2012). After 5 days in iron deficient medium, Lhca3 is processed at the N-terminus, LHCA5 is depleted, LHCA 1, 7, 8 are reduced and LHCA4 and 9 are induced relative to PSI (Naumann et al., 2005) Recent experiments using western-blotting showed that after 72 h in iron-free acetate-containing media, the amount of PsaC and PSAD decreased 2-fold and that PSAE disappeared.

In *C. reinhardtii,* iron starvation triggers overaccumulation of lipid bodies made of saturated fatty acids (Urzica et al., 2013). In addition, it also induces the loss of function of iron-containing enzymes in chloroplasts and mitochondria, including cytochromes, iron-sulfur proteins, and Fe superoxide dismutase (FeSOD). ROS in the *C. reinhardtii* chloroplast are then detoxified by a recently discovered MnSOD that accumulates at high levels upon starvation, while FeSOD is preferentially retained over other iron-containing proteins. (Page et al., 2012).In contrast, ferredoxins are also depleted, and their lack is compensated for by the induction of flavodoxin in *Phaeodactylum tricornutum* (Yoshinaga et al., 2014). No large induction in flavodoxin expression was observed by RNAseq upon iron starvation in mixotrophy in *Chlamydomonas* (Urzica and Merchant, 2009).

Nitrogen

C. reinhardtii preferentially uses ammonium as a nitrogen source, but can also use nitrate and nitrite, which are converted into ammonium (Navarro et al., 2000). It can also use external amino acids. Nitrogen starvation induces accumulation of starch and lipid bodies

(Wang et al., 2009) which are of great interest for bio-diesel production. A mutation in *STA1*, which encodes an enzyme required to accumulate starch, doubles lipid accumulation under nitrogen starvation. It does not exclude a concomitant increase in phytoglycogen (Dauvillée et al., 1999). Nitrogen starvation also induces autophagy which seems to be regulated by calcium, calnexin, and calreticulin, which are ER proteins that bind Ca^{2+} and are involved in routing towards apoptosis (death)/autophagy (survival) upon aging and nitrogen starvation in *Schizosaccharomyces* (Núñez et al., 2015).

Nitrogen starvation also induces gametogenesis (Goodenough et al., 2007) and decreases the accumulation of the b_6f complex (Bulte and Wollman, 1992). It appears that degradation of the b_6f complex under nitrogen starvation involves the general FTSH and CLPP proteases (Majeran et al., 2000)(Wei et al., 2014). This degradation appears to be dependent on nitric oxide signaling (Wei et al., 2014).

Phosphorus

Low phosphorus availability in soil and its deficiency represent a major constraint to global crop production (Cordell et al., 2009). Like other types of deprivation, the response to phosphate starvation involves remobilization from the growth medium. *C. reinhardtii* shows high-velocity phosphate uptake following 24 h of starvation due to accumulation of high-affinity phosphate transporters. In addition, it secretes alkaline phosphatase (Shimogawara et al., 1999).

In *A. thaliana* and tomato, it was also shown that nucleases and phosphatases are secreted during phosphorous starvation (Nürnberger et al., 1990; Robinson et al., 2012). *A. thaliana* also secretes organic acids to increase the solubility of mineral phosphates (Raghothama, 1999) and modify its root architecture (Svistoonoff et al., 2007). Reorganization of internal storage represents a second step towards acclimation to low phosphate availability. The chloroplast membranes are rich in phospholipids, but under phosphate starvation, these phospholipids are hydrolyzed and substituted by galactolipids (Essigmann et al., 1998). In senescent leaves, nucleases and phosphatases are expressed and are involved in the remobilization of phosphoassimilates to more demanding plant organs such as young leaves and flowers (Arlen, 2013).

Transcriptional regulation following phosphate deficiency in *A. thaliana* (Bustos et al., 2010) and *C. reinhardtii* (Wykoff et al., 1999) is under the control of PSRI, a putative

transcription factor which is located in the nucleus and contains a MYB1 DNA-binding domain and a glutamine-rich domain. Phosphorus starvation also induces the accumulation of lipid bodies in *A. thaliana*. This response is directed by the PHR1 transcription factor (Pant et al., 2015). In *C. reinhardtii*, the accumulation of lipid bodies is reliant on an acyltransferase driven by a phosphorous-inducible promoter (Iwai et al., 2014).

In *C. reinhardtii* chloroplasts, the genome is present in approximately 80 copies. As much as 75% of this DNA is degraded following 48 h in phosphate-free medium while mRNAs are overstabilized (Yehudai-Resheff et al., 2007); these changes are linked to a decrease in chloroplast PNPase (polynucleotide phosphorylase), which is a target of the PSRI regulator.

Genes for photosynthesis

The chloroplast proteome

The chloroplast proteome is a mosaic of proteins, many of which are arranged in complexes that enable the organelle to transform light into chemical energy and power the whole cell. The correct integrity of the plastid depends on the assembly of complexes that contain subunits from both the nuclear and chloroplast compartments. Proteomic studies have shown that the chloroplast contains at least 996 proteins that have an experimentally confirmed localization (Terashima et al., 2011). Estimations of the total number in *A. thaliana* chloroplasts reveals a range of 2100–3600 proteins (Abdallah et al., 2000; the *A. thaliana* Genome Initiative, 2000).

The best known posttranslational modification in the chloroplast is phosphorylation. In the A. thaliana chloroplast, mass spectrometry analyses led to the identification of 905 putative kinases substrates (Schönberg and Baginsky, 2015) while only 45 kinases and 21 phosphatases are predicted (Schliebner et al., 2008).

Another posttranslational regulation is the creation of disulfide bridges that play important roles, for example in regulating the activity of enzymes as a function of the redox poise. The thylakoid kinase STT7 is an example of such a protein (see section on State Transitions), it contains two cysteine residues exposed to the stromal side of the thylakoid membrane. The redox state of these cysteins is thought to be directly involved in modulating the activity of the kinase (Lemeille et al., 2009). Similar mechanisms has been reported for other chloroplast enzymes (Ruelland and Miginiac-Maslow, 1999). These cases involve the

ferredoxin - thioredoxin redox pathway. Some cysteine residues are thought to be subjected to a possible regulation by glutathionylation (Zaffagnini et al., 2012). The *C. reinhardtii* phospho-ribulo kinase activity was demonstrated to be modulated to a large extent by glutathionylation in vitro (Thieulin-Pardo et al., 2015). Similarly cysteins can be subjected to posttranslational modifications that involve nitric oxide. This compound participates in different regulatory mechanisms in the chloroplast, playing an important role in the induction of thylakoid protein degradation under nitrogen starvation (Wei et al., 2014) Nitric oxide is thought to regulate enzyme activity through cystein-S-nitrosylation, and indeed chloroplastic triosephosphate isomerase that was found among nitrosylated proteins in proteomic studies in *A. thaliana*, *O. sativa*, and *C. aurantium* (Lindermayr et al., 2005; Tanou et al., 2009; Lin et al., 2012; Tanou et al., 2012).

Nuclear genes

Among the nuclear genes required for chloroplast maintenance and biogenesis, many are components of the complexes themselves while others are required for the expression of the chloroplast encoded genes, specifically targeting one or a few of them (table 6 part 1 and 2). From the endosymbiotic event(s), when the "endosymbiont" was autonomous, to the complex machinery required to make a functional, non-autonomous chloroplast, genetic transfer from the endosymbiont to the nucleus played an important role (Martin et al., 1998).

Expression of nucleus-encoded chloroplast proteins is subjected to differential regulation that might be coordinated with the response of the chloroplast to different stimuli. The molecular ratio between different subunits within chloroplast complexes is tightly controlled at the level of their expression and involves feedback from the chloroplast to the nucleus called retrograde signaling.

Negative feedback from unassembled subunits on translation allows an equimolar association of subunits in a mechanism that is known as CES regulation (Control by Epistasy of Synthesis, see the dedicated section below). Protein degradation also plays a role in the regulation. In *A. thaliana* a *psad1*-2 mutant was not photosynthetic and failed to accumulate other nuclear-encoded subunits of PS I (Ihnatowicz et al., 2004), while in contrast *psag*, *psah*, and *psak* mutants were still able to grow with a weak impairment and were not affected in the accumulation of other subunits (Varotto et al., 2002).

Other examples of retrograde signaling regulating the expression of nucleus-encoded

chloroplast proteins were found. The retrograde signals includes ROS (Maruta et al., 2012), Mg-protoporphyrin IX and other heme derivatives (Strand et al., 2003)(Zhang et al., 2015) linked to the *GUN* genes (Brzezowski et al., 2014), and the redox state of the plastoquinone pool via an unknown mechanism (Kimura et al., 2003).

Chloroplast genes

The first chloroplast genome to be sequenced was from tobacco (Shinozaki et al., 1986), which revealed a 155-kbp sequence containing two large inverted repeats. Sixteen-years later, an assembly of the *C. reinhardtii* chloroplast genome was published for the first time (Maul et al., 2002) and was reported to have a full size of around 203 kbp with similar features. It contains large inverted repeats and a relatively high AT content, which is common to many chloroplast genomes. Comparison of sequenced chloroplast genomes reveals that most species share a surprisingly conserved complement of genes (Raubeson et al., 2007). The chloroplast genome of *C. reinhardtii* only contains 99 identified ORFs plus 8 ORFs (table 5) with unknown functions. Although the chloroplast retains its own transcription and translation apparatus, gene regulation is predominately conferred by nucleus-encoded proteins targeted to the chloroplast (Barkan, 2011).

The chloroplast found in *Viridiplantae* has retained only a few genes, but interestingly each of the main complexes contains a few hydrophobic subunits that are chloroplast-encoded. Are there any selective pressures against the complete loss of chloroplast genes?

A. thaliana contains a copy of 75% of the mitochondrial genome near the centromere of chromosome 2 (Lin et al., 1999); however the mitochondrial genome was not subsequently shortened and retains the expression of some genes.

Three main hypotheses were proposed to explain the retention of few genes within these organelles. The first is the hydrophobicity theory proposing that, as the retained proteins in the organelles are highly enriched for hydrophobic proteins that are integral to the membranes, their hydrophobicity prevents correct targeting if expressed in the nucleus, some of them being toxic if integrated in other compartments. The second hypothesis is that, from a regulatory point of view, an organelle such as a single chloroplast that is subject to more oxidative stress than others in the same cell would have different regulatory needs that it can meet individually while a nucleus-encoded protein would go indiscriminately to every chloroplast within the cell. The co-localization of a whole set of genes in a single organelle

Photosystem I	Photosystem II	Cytochrome b_6f	ATP synthase	Transcription	Translation	Others
PsaA	PsbA	PetA	AtpA	RpoA	Rpl2	CcsA
PsaB	PsbB	PetB	AtpB	RpoB	Rpl5	CemA
PsaC	PsbC	PetD	AtpE	RpoC1	Rpl14	ChlB
PsaJ	PsbD	PetG	AtpF	RpoC1	Rpl16	ChlL
	PsbE	PetL	AtpH	RpoC2	Rpl20	ChlN
	PsbF		AtpI		Rpl23	RbcL
	PsbH				Rpl36	Ycf3
	PsbI				Rps2	Ycf4
	PsbJ				Rps3	Ycf12
	PsbK				Rps4	ClpP
	PsbL				Rps7	
	PsbM				Rps8	
	PsbN				Rps9	
	PsbT				Rps11	
	PsbZ				Rps12	
					Rps14	
					Rps18	
					Rps19	
					TufA	
tRNA		rRNA	Splicing	Uncharacterized		
trnA(UGC)	trnM1(CAU)	23S	tscA	ORF50		
trnA(UGC)	trnM2(CAU)	16S		ORF58		
trnC(GCA)	trnM3(CAU)-fMet	7S		ORF59		
trnD(GUC)	trnN(GUU)	5S		ORF112		
trnE1(UUC)	trnP(UGG)	3S		ORF140		
trnE2(UUC)	trnQ(UUG)			ORF271		
trnF(GAA)	trnR1(ACG)			ORF1995		
trnG1(GCC)	trnR2(UCU)			ORF2971		
trnG2(UCC)	trnS1(UGA)					
trnH(GUG)	trnS2(GCU)					
trnI(GAU)	trnT(UGU)					
trnI(GAU)	trnV(UAC)					
trnK(UUU)	trnW(CCA)					
trnL1(UAG)	trnY(GUA)					
trnL2(UAA)						

Table 5 : ***List of all identified chloroplast genes in C. reinhardtii*** (Harris, 2009, 230).

could offer the possibility of them being regulated together. It was postulated that a broad redox-regulation affects the organellar genomes, termed CoRR (colocalisation of redox regulation), in which ancestral two-component regulatory systems could play a role (Allen, 2015). These theories have been reviewed by Daley and Whelan (2005).

In *C. reinhardtii,* the chloroplast genome is present in 80 copies, and DNA replication is regulated by redox signaling that is independent of chloroplast division (Kabeya and Miyagishima, 2013). The existence of highly packed DNA within the chloroplast has been shown. In *C. reinhardtii,* a histone like protein (HLP) originating from the bacterial ancestor is required for nucleoid organization. Its absence significantly decreases the genome copy number and rate of transcription but does not affect mRNA accumulation (Karcher et al., 2009; Eberhard et al., 2002). Conversely, the gene copy number in *Z. maize* seems to have a proportional impact on mRNA accumulation (Udy et al., 2012). The copy number of chloroplast genomes is 100–200 on average, but this may change depending on the developmental stage and may exceed several thousands in developing plant tissues (Day and Madesis, 2007). The high copy number of chloroplast genomic DNA is thought to guarantee correct gene expression even during genome replication and chloroplast scission.

Chloroplast gene expression

Nuclear genes assist in chloroplast gene expression. The nucleus encodes proteins that participate in the assembly of the chloroplast photosynthetic machinery at different steps. They are involved in the transcription and translation apparatuses such as in the protein composition of the chloroplast ribosomes.

A high proportion of nuclear genes not contributing to the structure of the photosynthetic machinery, are required for transcription, mRNA splicing, for stabilization, maturation, edition, or translation of specific chloroplast mRNAs (table 6). Other proteins ensure proper assembly and insertion of proteins into the thylakoid membrane.

In addition to the proteins listed in table 6, in *A. thaliana* CSP41 regulates chloroplast transcription and translation (Bollenbach et al., 2009). RBF1 is involved in 16s ribosome processing in both *C. reinhardtii* and *A. thaliana* (Fristedt et al., 2014a).

MCD3, MCD4, MCD5,	Pleiotropic effects, stability of the 3′ end of mRNA.	(Levy et al., 1999) (Rymarquis et al., 2006)
ALB3	Insertion into the membrane.	(Göhre et al., 2006)
PSII		
NAC2	(TPR), stability of *psbD* mRNA.	(Kuchka et al., 1989)
RB38, RB47 RB55, RB60	(Long repeated motifs), binds the *psbA* 5′UTR in vitro. Motifs of RB38 and 47 do not resemble TPR, PPR, or OPR, but they contain an RNA binding domain.	(Danon and Mayfield, 1991),
MBB1	(TPR), stability of *psbB*, *T*, *H.*	(Vaistij et al., 2000)
MBC1,	Stability of *psbC* mRNA.	(N. Gumpel, J. Girard-Bascou, F. A. Wollman, S Purton. Unpublished data)
NAB1	Repress stability of some LHCBM mRNAs.	(Berger et al., 2014)
MBI1	(OPR), *psbI* mRNA stability.	(Wang et al., 2015)
RB complex	Translational activation of several mRNAs.	(Trebitsh and Danon, 2001)
ACC115, RBP40, RB38 MBD1	*psbD* translation, binds the 5′UTR.	(Schwarz et al., 2007)
RB47	PolyA in 5′UTR *psbA*, needed for translation.	(Yohn et al., 1998)
RBP63	PolyA in 5′UTR *psbA.*	(Ossenbühl et al., 2002)
TBA1, TBA2,	Pre elongation translation of *psbA.*	(Yohn et al., 1996)(Somanchi et al., 2005)
TBC1, TBC2, TBC3,	(39 amino acid repeats), translation of *psbC* .	(Zerges et al., 2003)
NAC1	Post initiation in *psbD* mRNA.	(Cohen et al., 2001)
b_6f		
MCA1	(PPR),stability of *petA* mRNA, needed for efficient translation.	(Gumpel et al., 1995; Raynaud et al., 2007)
MCB1, MCG1	Stability of *petB* and *petG* mRNA, respectively, MCG1 is an OPR.	Wollman F.A. in (Rochaix et al., 1998)(Wang et 2015)
MCD1	Stability of *petD* mRNA.	(Murakami et al., 2005)
TCA1	Translation of *petA*	(Raynaud et al., 2007)
CCB1.2.3.4	Assembly of the Heme.	(Lezhneva et al., 2008)(Kuras et al., 1997)
CCS 1.2.3.4.5.6.A	Assembly of the Heme.	(Xie et al., 1998)

*Table 6 part 1 : **Table summarizing nuclear genes with known involvement in biogenesis and assembly of the photosynthetic machinery in C. reinhardtii** .*

Transcription

The vestigial genome of plastids has retained many typical prokaryotic features from its bacterial ancestor. A bacterial-type RNA polymerase (plastid-encoded RNA polymerase, PEP), whose core subunits are encoded by the plastid genome, transcribes most plastid genes. Together with nucleus-encoded sigma factors of the σ70 type (RPOD), the plastid-encoded RNA polymerase enzyme recognizes promoters that contain conserved −10 (TATA) and −35 boxes, and thus resemble bacterial promoters (Liere and Börner, 2007).

Transcription in *C. reinhardtii* is reported to be regulated by the circadian clock (Hwang et al., 1996). It appears that the circadian regulation of chloroplast transcription is nucleus-dependent (Matsuo et al., 2006). The gene for chloroplast-encoded elongation factor *(tuf A)*, and *atpB*, *psbA*, *psaA* and *rrn* were demonstrated to have circadian transcription (Hwang et al., 1996; Kawazoe et al., 2012). A nuclear mutant with an impaired *roc81* gene failed to regulate the chloroplast circadian rhythm (monitored via a reporter with the *tufA* promoter driving the luciferase lucCP). This is an example of anterograde signaling. The precise regulation of this signaling mechanism is still unknown (Matsuo et al., 2008).

Chloroplast transcription of *psaA* and *psbA* in *A. thaliana* was reported to be affected by the phosphorylation status of SIG1 and by the photosynthesis inhibitors DCMU and DBMIB. These drugs are reported to affect the redox pool of the plastoquinone pool, whereby the first causes its oxidation it and the second its reduction. These drugs had opposing effects on transcription (Shimizu et al., 2010).

In *C. reinhardtii,* only PEP and RPOD were found to ensure chloroplast transcription. The latter does not show any circadian behavior at the level of its accumulation (Kawazoe et al., 2012), while its mRNA varies from 2 to3-fold during light-dark cycles (Carter et al., 2004). However, no studies have been conducted to investigate the rhythmicity of its activity. Similarly, PEP levels did not change during light dark cycles, leaving the rhythmicity of chloroplast transcription unsolved. Recent work demonstrated that the circadian regulation triggers first the transcription of genes that are involved in transcription followed by those involved in translational (Idoine et al., 2014).

Around 2.6% of nuclear genes are regulated with a circadian rhythmicity (Kucho et al., 2005), and *Lhca1* is an example (Hwang and Herrin, 1994). A transcriptomic analysis with a cDNA macro-array confirmed that several nuclear genes are regulated in this manner, highlighting the observation that 16 chloroplast ribosomal proteins are co-expressed and their

PSI		
MAB1	Stability of *psaB.*	(unpublished data)
RAA1	Splicing both introns of *psaA* (OPR).	(Hahn et al., 1998) (Merendino et al., 2006)
RAA2	Splicing of second intron.	(Perron et al., 1999, 2004)
RAA3	Splicing of the first intron	(Rivier et al., 2001)
RAT1, RAT2,	Splicing of the first intron	(Balczun et al., 2005)
RAA4	Splicing of first intron.	(Glanz et al., 2012)
RAA6		(O. Reifschneider, U. Kuck unpublished data)
RAA7	Splicing of the second intron	(Lefebvre-Legendre et al., 2015)
RAA8	Splicing of the second intron (OPR)	(Marx et al., 2015)
TAA1	Translation of psaA. (OPR)	(Lefebvre-Legendre et al., 2015)
TAB1/3, TAB2	Translation of *psaB.*	(Barneche et al., 2006; Stampacchia et al., 199
TLA1	Correct assembly of LHCs.	(Tetali et al., 2007)(Mitra and Melis, 2010)
NAB,	LHC translation.	(Mussgnug et al., 2005)
ATPase		
THM24	*atpB* transcription impaired mutant.	(Drapier et al., 1992)
MDH1	*atpH* transcription impaired mutant.	(Majeran et al., 2001)
MDA1	*atpA* mRNA stability.	(Drapier et al., 2002)
TDA1	(OPR), *atpA* translation.	(Drapier et al., 1992)
Rubisco		
MRL1	(PPR) *rbcl mRNA stability*	(Johnson et al., 2010)
TAS1, TAS2	Processing 3'UTR *rbcL.*	(Goldschmidt Clermont et al., 2008)

Table 6 part 2 : ***Table summarizing nuclear genes with known involvement in biogenesis and assembly of the photosynthetic machinery in C. reinhardtii****.*

RNA accumulation follows a circadian rhythm (Kucho et al., 2005)

In *A. thaliana* several nucleus-encoded sigma factors affect the transcription of chloroplast-encoded genes. A SIG2-PEP holoenzyme specifically transcribes some of the *t*RNA genes (Kanamaru et al., 2001) and *psaJ* (Nagashima et al., 2004). SIG3-PEP specifically transcribes *psbN*, but can also influences the *psbB* operon via antisense RNA from the *psbN* locus (Zghidi et al., 2007). SIG4 is of notable importance for *ndhF* gene transcription (Favory et al., 2005). SIG5 has been shown to play an important role in recognition of the blue-light dependent promoter of the *psbD* gene (Tsunoyama et al., 2002; Nagashima et al., 2004). SIG 6 has a more general role during early plastid differentiation and plant development (Ishizaki et al., 2005). In addition, it was found that these nucleus-encoded sigma factors play a role in chloroplast to nucleus retrograde signaling (Woodson et al., 2013).

The transcription of chloroplast-encoded genes is also affected by light. For example, in mustard, *psaA/B* and *psbA* transcription, mRNA accumulation, levels of P700, and the chlorophyll a/b ratio are affected by light favoring PS II or PS I, mediated through the PQ redox state (Pfannschmidt et al., 1999). The CSK protein is thought to be involved in this process, and may, sense the redox state of the PQ pool (Puthiyaveetil et al., 2012)The model proposed suggests a CSK-dependent phosphorylation of SIG1 and PTK (Puthiyaveetil et al., 2012). As a consequence, this affects PEP transcription activity on chloroplastic PS I genes (Allen et al., 2011; Puthiyaveetil et al., 2013). CSK is thought to be one of the rare example of gene regulation by a bacterial-type two-component system, derived from a sensor kinase were the histidine phosphorylation site has been mutated to a phosphomimetic glutamic acid (Puthiyaveetil et al., 2008)

However, the amount of accumulated mRNAs was observed not to be a limiting factor for protein accumulation (see section on RNA granules). It is thus legitimate to ponder what role plays the regulation of mRNA accumulation in response to light quantity (Salvador et al., 1993), nutrient stresses, and photosynthetic redox poise (Salvador and Klein, 1999).

Perhaps distinct processes regulate RNA and protein contents in the chloroplast. RNA half-lives differ, such that *psbA* mRNA is turned-over in 8 hours whilst *tufA* takes less than 30 minutes (Zicker et al., 2007). Degradation processes that implicate an attack from the 5′UTR and 3′UTR were demonstrated by the addition of poly (G) loops, which blocked degradation by exonucleases (Drager et al., 1999). Processing of the 3′ end is another determinant of mRNA stability, and in the case of *atpB* in Chlamydomonas, is thought to be initiated by endonucleolytic cleavage (Hicks et al., 2002).

Splicing

The chloroplast has retained traces of its prokaryotic origins in the organization of its genes. In bacteria and Archae, many functionally related genes are organized into operons that are transcribed and translated as a unit. Operons are rarely seen in eukaryotes except in the trypanosome and nematode (Pi et al., 2009). In the chloroplast, most genes are transcribed in polycistronic units that are trimmed to form monocistronic messengers and their maturation involves a cohort of RNA binding proteins, maturases, endo- and exonucleases, and stabilization factors, … Mutants involved in mRNA stabilization suggest a 5′ to 3′ and 3′ to 5′ degradation of polycistronic mRNA, unless they are protected at both their 3′ and 5′ ends, thus forming the mature transcripts (Barkan, 2011; Germain et al., 2013).

As in most genetic systems, the chloroplast also contains introns and fractionated genes. Intriguingly, the chloroplast presents around 20 different introns but they are rare in extant cyanobacteria . Introns exist in different classes, spliceosomal, tRNA, group I and II. Some can self-splice, and others require proteins to assist with folding or trans-esterification. Only group I and group II introns were found in the chloroplast.

Group I introns are self-splicing catalytic introns, which are widely distributed in the genomes of prokaryotic and eukaryotic organisms, but are not found in Archae (Haugen et al., 2005). Five group I introns are reported in the *C. reinhardtii* chloroplast genome, four in *psbA,* and one in the large rRNA (*crLSU*) (Holloway et al., 1999). These catalytic introns, called ribozymes, are sometimes dependent on maturases to assist with splicing (Belfort, 2003). This observation provides insight into the evolution of group II introns as the autocatalytic activity can be lost when a protein-dependent splicing is set-up. Furthermore, group I introns from *psbA* were shown to undergo homing through a large ORF that is encoded inside the intron itself (Odom et al., 2001), even if its deletion does not impair splicing (Lee and Herrin, 2003). Intron splicing in *psbA* is dispensable, and the replacement of the split gene with an intron-less cDNA is phenotypically silent (Johanningmeier and Heiss, 1993). The inefficient in vitro splicing of the *psbA* introns would indicate the need for protein assistance. Furthermore, the amount of unspliced precursors of *psbA* increase 6–10-fold under light in response to the redox poise, suggesting that a regulation event might be protein-directed (Deshpande et al., 1997).

Group 2 introns are restricted to chloroplasts and mitochondria of lower eukaryotes, plants, and in some rare cases, prokaryotes. These prokaryotes belong to cyanobacterial and proteobacterial lineages and are believed to be potential ancestors of chloroplasts and

mitochondria. Most group 2 introns are dependent on proteins for splicing, some non chloroplastic examples were shown to self-splice in vitro. Their processing is thought to involve a large ribonucleoprotein complex (Glanz and Kück, 2009).

Chloroplast mRNA splicing sometimes involves a single precursor transcript molecule (cis-splicing) or several precursor transcript molecules (trans-splicing). Trans-splicing occurs in the chloroplast of several species (Glanz and Kück, 2009). In *C. reinhardtii*, the two examples of trans-splicing in the chloroplast are in the *psaA* gene, as shown in figure 15.

The assembly of the mRNA for the PsaA subunit of PS I needs four different transcripts—three exon-containing precursors and *tscA*, which is part of the first intron. The transcripts are assembled together through the splicing of two group II introns (Goldschmidt-Clermont et al., 1991). These splicing events require at least 14 independent nuclear loci (Goldschmidt-Clermont et al., 1990) suggesting a coordinated role of multiple proteins . Later, the existence of such a complex was confirmed using yeast 2- hybrid assays in which a 500 kDa membrane-associated complex that binds *tscA* RNA and contains the characterized splicing factors RAA1, RAA3, RAA4, RAT2, and RAB1 was found (Jacobs et al., 2013). The same team also confirmed that an even bigger complex regroups both intron1 and intron2 splicing factors (personal communication). Similarly RAA8 is associated with RAA1, RAA2 and RAA7 and is involved in splicing the second split intron (Legendre et al, in press). Evidence suggests that the total size of the complex might be around 1700 KDa (Rivier et al., 2001). Similarly to splicing of the group I introns of *psbA*, *psaA* trans-splicing can be by-passed by inserting an intron-less copy of the gene in the chloroplast genome (Lefebvre-Legendre et al., 2014).

Editing

In *A. thaliana* chloroplasts and mitochondria, numerous editing events have been reported. Modification of cytidines to uridines in mRNA are the only reported examples of chloroplast editing, affecting start codons, conserved amino acids, and stop codons. These post-transcriptional modifications depend on a complex of around 400 KDa called the editosome. This oligomer contains several proteins with specific domains such as PPR, RIP/MORF, ORRM, and zinc finger (Sun et al., 2015; Germain et al., 2013). Many instances of RNA editing in *A. thaliana* are dependent on PPR proteins (Fujii and Small, 2011),.In the *A. thaliana* chloroplast, a zinc finger protein was found to be required for RNA editing in

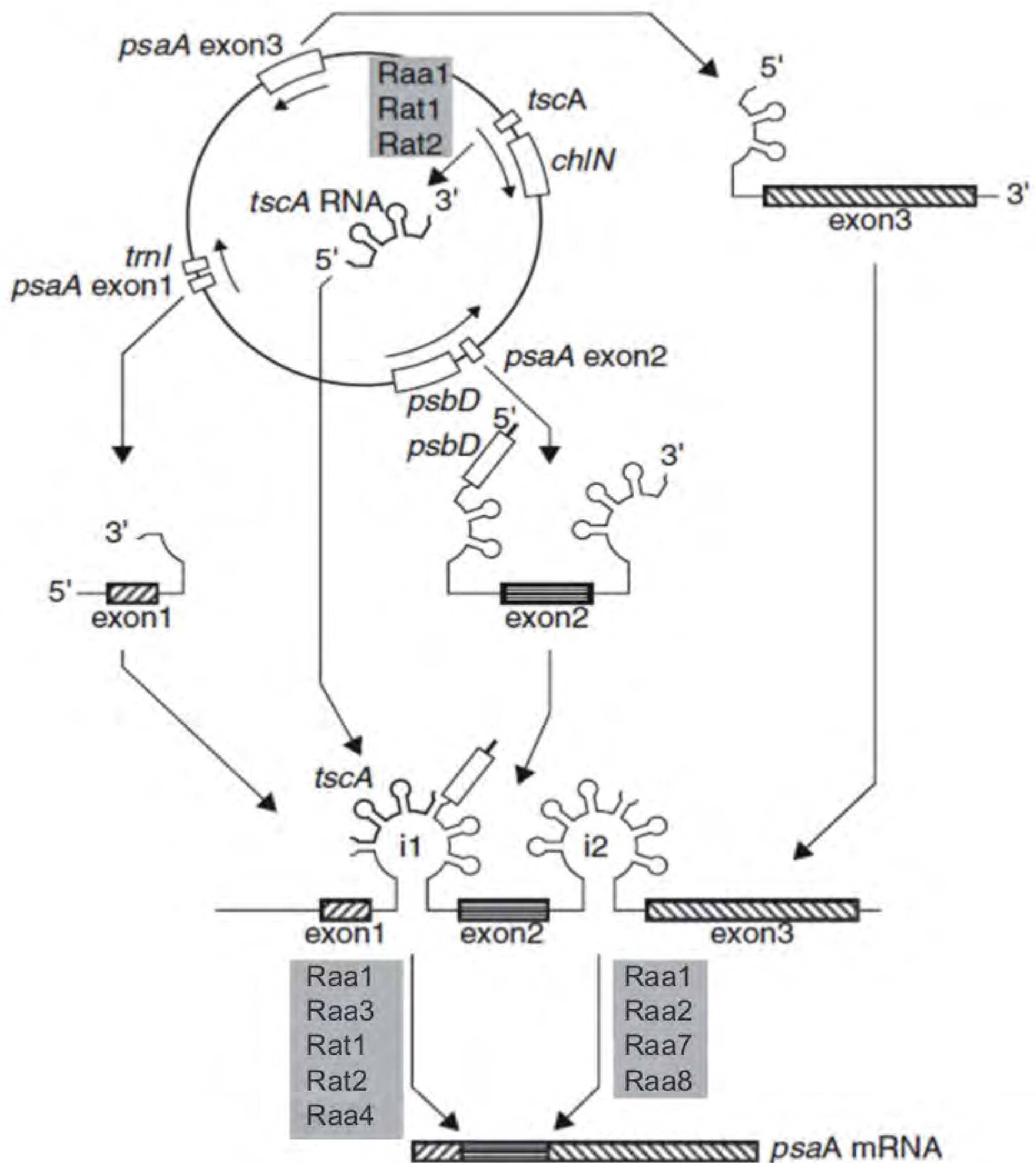


Figure 15 : ***Scheme of psaA trans–slicing in the Chlamydomonas chloroplast and the different known nuclear genes involved in the process****. The different transcripts appear with their co-transcribed genes. Modified from Goldschmidt-Clermont in Stern, 2009.*

association with a more specific RNA binding protein from the PPR family (see below) (Sun et al., 2015). However there are no reports showing a clear role for these proteins in regulation of gene expression. While RNA editing occurs in many plants and mosses, no editing has been reported in *C. reinhardtii* or *V. carteri* chloroplast, which questions the relevance of such a process.

Processing of mRNA

Both mRNA termini ensure its stability in the chloroplast. Unlike the processing of nucleus-encoded transcripts that involve a poly-A tail at the 3′UTR, processing of chloroplast mRNA involves the RNA secondary structure, RNA-binding proteins and nuclease(s). In the chloroplast, the transcriptional stop signal is not perfectly defined and does not seem to be critical for mRNA stability. In contrary, a hairpin structure in the 3′UTR was found in most chloroplast mRNAs, which might play an important role in their correct trimming and stability.

Processing of the 3′ end is thought to be initiated by endonucleolytic cleavage, it is for example the case of *atpB* (Hicks et al., 2002). This was studied in detail in the *rbcL* mRNA, which has a double hairpin structure in its 3′UTR. Although the presence of such structures is essential, their deletion destabilizes the mRNA (Goldschmidt-Clermont et al., 2008). Even if the hairpin structure could act as a transcription stop signal, *atpB* mRNA shows a low transcription termination efficiency at the hairpin, and 50% of the primary transcripts are longer and retrimmed by an endonuclease, and subsequently maturated by a 3′–5′ exonuclease to obtain the final termini in vitro (Stern and Kindle, 1993). The hairpin structure sometimes requires external proteins to be correctly shaped and functional. This is the case in the *clpP* 3′UTR in moss, which requires a nuclear encoded PPR protein called PpPPR38 (Hattori and Sugita, 2009). This system seems to work differently for *tufA*, which contains hairpins within its 3′UTR that are followed by a single stranded region (Zicker et al., 2007) containing a short dispersed repeat (SDR) sequence that might be involved in 3′UTR maturation. Correct 3′UTR maturation is required for translation (Rott et al., 1998); however, this may not play any role in translation regulation (Barnes et al., 2005). Conversely, the endoribonuclease P54 involved in the 3’ end processing of messengers appears to be regulated by phosphorylation and highly sensitive to the redox state of glutathione in mustard (Liere and Link, 1997). There are numerous protein coding genes in the chloroplast that encode mature mRNAs via a polycistronic precursor cleaved by endonucleases and matured. The intergenic regions can be

diverse but usually contain a cleavage site and translational regulatory elements. The *atpA* cluster in *C. reinhardtii* has been well characterized (Drapier et al., 1998) revealing a complex pattern of RNA processing of the four genes: *atpA*, *psbI*, *cemA*, and *atpH*. Three of these genes possess their own 5′UTR even though their translation can be achieved on polycistronic precursors. In contrast, *cemA* does not have a 5′UTR region and can only be translated from polycistronic mRNA.

In addition, hairpin structures have been found in the 5′UTR of *rbcL*. Experiments strongly suggest that this element interacts with a trans-acting factor that protects transcripts from rapid degradation in chloroplasts. (Suay et al., 2005). The 5′UTRs are often protected by RNA-binding proteins, many of them belonging to the PPR or TPR families. Those nuclear encoded factors are specific for a few mRNAs. For example, the TPR/HAT protein MBB1 was proven to bind to *psbB* and *psbH* mRNAs in vitro, and the two binding sites were found to immediately follow the 5′UTR end of the respective mature mRNAs. This fits with the model predicting that they determine the 5′ end of mRNA (Loizeau et al., 2014). Small non coding RNAs, were also found to match the 5′-ends of chloroplast tRNAs and 3′-ends of chloroplast ribosomal RNAs in *Brassica rapa* (Wang et al., 2011)and in *Zea Z. maize* (Schmitz-Linneweber et al., 2006). Some of these were characterized and found to match target sites on specific RNA binding proteins(Pfalz et al., 2009). This is indeed the case for the two MBB1 binding sites, which are thought to be protected from RNAse digestion by the highly specific interaction with MBB1 in vivo. Similarly, closely related protein classes like PPRs, produce RNA "footprints" (Ruwe and Schmitz-Linneweber, 2012). For example, in Maize, PPR10 is involved in the binding of UTRs between *atpI-atpH*, and *psaJ-rpl33.* Small non coding RNAs found in databases match the recognition sites. PPR10 is thought to stabilize both downstream mature RNAs and upstream mRNAs substituting the need for a hairpin structure (Pfalz et al., 2009). Repeat-containing proteins are found in association with other proteins, D.L. Herrin (Stern, 2009, p959) summarized "The protective proteins are often in large complexes that include PPR/TPR proteins. It also appears that these RNA-binding complexes may contain common as well as unique proteins. A more complete catalog of the complexes, their subunits, and dynamic interactions with the mRNA targets will be necessary to fully understand this important aspect of RNA stabilization. This is particularly obvious comparing results on MBD1 and MBB1, involved in the maturation of *psbD* and *psbB* mRNA respectively, both part of complexes but of different sizes. This suggests that the complexes are specific for each mRNA and vary in most of their components.

Translation

Translation in the chloroplast involves ribosomes that are composed of proteins encoded by both the nuclear and chloroplast genomes. The plastidic 70S ribosomes share many properties with bacterial ribosomes. The small subunit of the plastid ribosome (30S subunit) contains the 16S ribosomal RNA (rRNA) and twenty one protein subunits, most of which have, clear orthologs in *Escherichia coli*. Twelve of these subunits are encoded by the chloroplast genome, and the remaining nine are encoded by the nuclear genome (Yamaguchi et al., 2000). The large subunit of the plastid ribosome (50S subunit) consists of three RNA subunits (23S rRNA, 5S rRNA, and 4.5S rRNA) and 31 ribosomal proteins , most of which have orthologs in *E. coli*. Nine of these proteins are encoded by plastid genes, whereas the remaining twenty two are encoded by nuclear genes (Yamaguchi and Subramanian, 2000). A few of these ribosome proteins are specific for plastids and do not show any homology with *E. coli* proteins. Mutants or knock-downs have revealed that they are dispensable for survival (Tiller et al., 2012), even if their absence reduces the fitness.

Translation of chloroplast-encoded proteins might be coordinated with the insertion of certain proteins into the thylakoid membrane, because polysomes could be extracted from a thylakoid membrane preparation (Bolli et al., 1981). Interestingly the transcription in the chloroplast shows a day-night rhythmicity. A study on the relative role of the circadian clock showed that translation is globally induced by light rather than by circadian signals (Lee and Herrin, 2002).

In the chloroplast, varying the 3′UTR has little impact on mRNA and protein accumulation, as long as a 3′UTR is present. On the other hand, stability and translation are mainly determined by the 5′UTR and its interaction with the coding sequence (Barnes et al., 2005). Chimeric constructs of *psbA* or *psbD* 5′UTR with *gfp* suggest that translational efficiency and light-regulated translation are separate.

TAA1 provides an example of how translation may be regulated in the chloroplast. TAA1 is a nucleus-encoded protein involved in the translation and stability of *psaA*. This protein is down-regulated during iron starvation (Lefebvre-Legendre et al., 2015) via decreased translation in the cytoplasm and selective degradation in the chloroplast. This down-regulation is concomitant with a loss of *psaA* mRNA and decreased PsaA protein accumulation.

Translation initiation, which involves ribosomes binding to the 5′UTR, is likely to be a

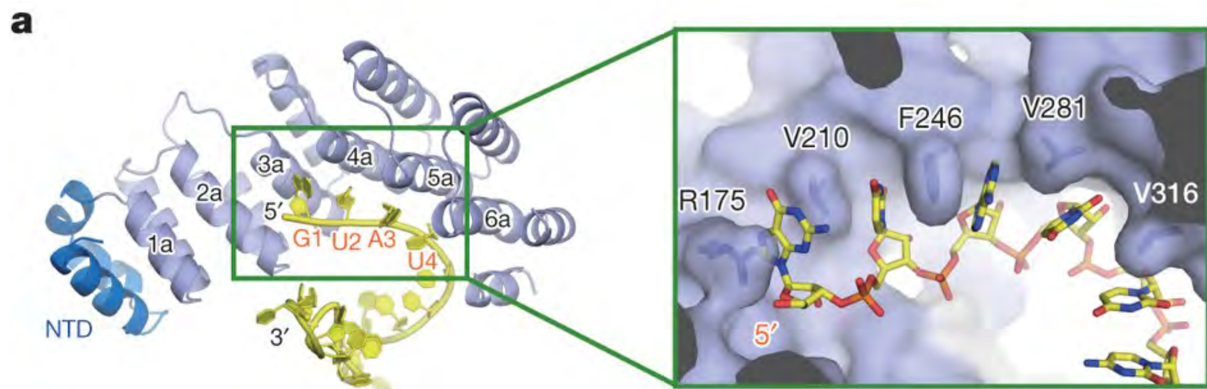


*Figure 16 : **Crystal structure of recombinant PPR10 of Zea mays co crystallized with its target RNA, an atpI/atpH intergenic fragment**. The figure shows the binding surface and the residues of PPR10 involved in RNA recognition (Yin et al., 2013).*

modulated step as was suggested by findings on the control by epistasy of synthesis (CES, see later section) (Choquet et al., 2001). Redox regulation is known to affect translation. For example, translation of the chloroplast *psbA* mRNA is redox-regulated through its 5′UTR via an unknown mechanism (Trebitsh et al., 2000). This was confirmed to affect translation of D1 following reduction by the addition of DTT during a pulse-chase labeling experiment (Zhang et al., 2000). Similarly, a protein disulfide isomerase was found to regulate the activation of chloroplast translation. This protein is redox regulated (Kim and Mayfield, 1997).

Evidence also suggests that D1 biogenesis is regulated according to the energy source via the protein RBP 63/cpDLA 2. RBP 63 was first identified by its binding to thylakoid membranes and to a small poly-A stretch in the 5′UTR of *psbA*. It was then thought to be involved in the insertion of D1 into the membrane (Ossenbühl et al., 2002). A recent discovery demonstrated that this enzyme is a subunit of the pyruvate decarboxylase complex (Bohne et al., 2013), suggesting crosstalk between carbon metabolism and photosystem biogenesis. It appears that this feature is not an isolated case as dihydrolipoamide acetyltransferases from different organisms (*C. reinhardtii, Saccharomyces cerevisiae, Synechocystis* sp. *6803,* and *Homo sapiens*) seem to bind the *Chlamydomonas psbA* mRNA in vitro with different affinities (higher for the two photosynthetic organisms), while PRAT A, which is a TPR (TetratricoPeptide repeat domain) involved in the Cter processing of PsbA, does not show any binding (Bohne et al., 2013).

Codon usage

Codon use in *C. reinhardtii* chloroplasts is highly biased (Nakamura et al., 2000). The AT richness reflects the preferential use of an A or T at the third codon position. Likewise, the expression of the exogenous gene *gfp* in the chloroplast, driven by *rbcL* 5′ and 3′UTRs, is enhanced 80-fold when encoded with a chloroplast codon bias (Franklin et al., 2002) compared to a native codon bias.

CES

Control by epistasy of synthesis (CES) is one of the regulating mechanisms observed in the assembly of all photosynthetic macrocomplexes in *C. reinhardtii* (Drapier et al., 2007). Through this interesting mechanism, the coordination required for the equimolar assembly of

subunits within a complex is perfectly orchestrated. While assembly-driven regulation of nucleus-encoded subunits is unclear, the regulation of chloroplast-encoded subunits revealed an assembly feedback mechanism called control by epistasy of synthesis (CES) discovered in Chlamydomonas. This modulation affects protein synthesis on a translational level when the unassembled subunit accumulates. Initially revealed for subunits of the b_6f complex (Choquet et al., 2001), it turned out that the main chloroplast complexes PS I (Wostrikoff et al., 2004), PS II(Bennoun et al., 1986; Erickson et al., 1986; Minai et al., 2006), ATPase (Drapier et al., 2007) and Rubisco (Khrebtukova and Spreitzer, 1996) are also concerned. This process involves the chloroplast-encoded core subunits of the complexes that usually assemble in a 1:1 ratio with other subunits. When a core protein is unassembled because another subunit is lacking, its levels increase. This accumulation is rapidly compensated at a translational level via negative feedback on the initiation step; however, the precise mechanism remains unknown. Conversely, when a subunit is missing, the initiation of translation at the 5′UTR is enhanced (Michelet et al., 2011), demonstrating that normal levels of translation require equilibrium between assembly needs and production.

Degradation of mRNA

An important insight into the degradation of chloroplast RNA concerns the role of polyadenylation at the 3′end. Unlike that observed for cytoplasmic messenger RNA, polyadenylation of chloroplast mRNA, tRNA, and rRNA was demonstrated to favor their degradation in vivo with different efficiencies depending on the transcript (Komine et al., 2002). In addition, these authors demonstrated that this polyadenylation-dependent enhanced degradation is effective only at aberrant 3′ termini.

Only a few RNAses have been described in the *C. reinhardtii* chloroplast. Polynucleotide phosphorylase (PNPase) was first described to affect the stability of polyadenylated RNA and in the knock-down strain its deficiency impairs the resistance to phosphate starvation (Yehudai-Resheff et al., 2007). The authors postulated that PNPase activity is required to remobilize phosphorus. It has also been suggested that this enzyme might play a role in RNA adenylation. The same study presented evidence showing that another exonuclease, RNB2, could play a role in the 3′–5′ degradation as it over-accumulates in PNPase knock-down strains. Another RNAse was characterized, RNASEJ, which seems to be involved in 5′ maturation in the *A. thaliana* chloroplast (Sharwood et al., 2011).

RNA degradation is also a target of regulation. In *Chlamydomonas*, the accumulation of *psaB*, *atpB*, and *rbcL* mRNAs is affected by treatment with DCMU, which modifies the redox state of the photosynthetic electron transport chain (Salvador and Klein, 1999). In addition, the redox regulated kinase, STT7 possesses a potential target sequence in NAC2, which is involved in *psbD* mRNA processing and stability (Rochaix et al., 2012), and in MBB1, which is involved in *psbB* mRNA stabilization (Lemeille et al., 2010).

Interestingly, the quantity of mRNA was observed not to be a limiting factor for protein accumulation (see section on RNA granules). It is then legitimate to ponder why mRNA accumulation responds to light quantity (Salvador et al., 1993), nutrient stresses, and photosynthetic redox poise (Salvador and Klein, 1999).

Perhaps distinct processes regulate protein content in the chloroplast. RNA half-lives differ, as *psbA* is turned over in 8 hours whilst *tufA* takes less than 30 minutes (Zicker et al., 2007). Degradation processes that implicate an attack from the 5′UTR and 3′UTR were demonstrated by the addition of poly (G) loops, which blocked degradation by exonucleases (Drager et al., 1999).

RNA-binding proteins in chloroplasts

It was previously demonstrated that RNA-binding proteins can be involved in post-transcriptional regulation in bacteria (Van Assche et al., 2015). In eukaryotes, an RNA molecule transcribed in the nucleus rarely remains free. As soon as it is transcribed, ribonucleoproteins (RNPs) form on the nascent transcript and participate in its processing, nuclear export, transport and localization, half-life, and translation rate (Dreyfuss et al., 2002). The RNA binding domains and their functions are extremely diverse. Among the known domains are, DBD (DNA Binding Domain), zinc fingers, PIWI (P-element Induced Wimpy testis), PAZ (Piwi Argonaut and Zwille) and SAM (Sterile Alpha Motif), which mainly bind to backbones or to the ends of single-stranded RNA. Others are specific for a certain sequence (for example, RRM(RNA Recognition motif), KH (K Homology), S1, TRAP (thrombospondin-related anonymous protein), and Pumillio). Identification of such domains allows an inference to be made between the primary structure and the role of a given protein (Lunde et al., 2007).

RNA-binding proteins that belongs to the superfamily of helical repeats proteins play important roles in the chloroplast. The first example from this superfamily was Pumilio which binds a specific RNA in metazoans (Zamore et al., 1999). It has a structure composed of

tandem helical repeats that form a superhelical structure , each repeat being involved in the binding of a single nucleotide (Wang et al., 2002). Similarly, the human mitochondrial regulator mTERF, binds to a specific DNA sequence via tandem helical repeats (Jiménez-Menéndez et al., 2010). The mTERF family also contains members that are located within the chloroplast. In *Z. Z. maize*, Zm-mTERF4 was found to be required for the splicing of certain group 2 introns (Hammani and Barkan, 2014). In *C. reinhardtii*, MOC1 is a mitochondrial mTERF factor whose inactivation alters respiration and also light acclimation. This was found to bind specifically to the rRNA module S3 (Wobbe and Nixon, 2013).

PPR proteins are another family of RNA binding factors, they contain tandem helical repeats of 35 amino-acids and are found in all eukaryotes. The PPR family has expanded in photosynthetic organisms. While in Chlamydomonas only few PPR family members are found, in land plants, the nuclear genome contains up to 600 different PPR genes (450 in *A. thaliana)*, many of which are essential for plant development (Tourasse et al., 2013). As noted, PPR10 is involved in the binding of intergenic regions in the polycistronic mRNA precursors, *atpI-atpH* or *psaJ-rpl33* in a sequence specific manner. PPR10 also exists as a dimer, but the physiological significance is still unknown (Li et al., 2014). The “code” underlying the sequence specificity was elucidated through computation (Yagi et al., 2013) and by in vitro protein binding assays (Barkan et al., 2012) allowing the use of this discovery for engineering of RNA-binding proteins.

In the *C. reinhardtii* genome*,* only 14 PPRs have been found. These are mainly involved in post transcriptional processes in organelles (Delannoy et al., 2007). PPR7 is the smallest and most highly expressed PPR found in *C. reinhardtii.* It is part of a high molecular weight ribonucleoprotein complex in the stroma. RIP-chip analysis revealed that it is associated with several chloroplast RNAs — *rrnS*, *psbH*, *rpoC2*, *rbcL*, *atpA*, *cemA*-*atpH*, *tscA*, and *atpI*-*psaJ* (Jalal et al., 2015). RNA-interference lines show that a reduction of PPR7 to 25% causes excessive light sensitivity, but that protein accumulation of D2 (PS II), Cytb6 (b_6f), AtpB (ATPase), and RbcL (Rubisco) seems unimpaired. Conversely, decreased PsaA accumulation can be attributed to the 30% decrease in *tscA* RNA followed by a decrease in the levels of other *psaA* mRNA precursors to the same degree.

Among tandem helical-repeat, OPR (octotricopeptides repeats) proteins also play an important role in *Chlamydomonas.* These tandem repeats can be found in at least 43 nuclear encoded genes (Rahire et al., 2012). The OPR proteins, with repeats of 38 amino-acids residues, also include TBC2, which was the first OPR protein found to be involved in mRNA

metabolism in the chloroplast. It affects *psbC* mRNA accumulation and translation (Auchincloss et al., 2002; Zerges and Rochaix, 1994) and is a 114 kDa protein that is part of a 400 kDa complex.

TAB1 is another OPR protein. Its deficiency reduces the accumulation of *psaB* mRNA, and the trace amounts of remaining translation are not sufficient to support phototrophic growth. Similar to other RNA binding proteins, TAB1 is associated with a large protein complex. The target of TAB1 was mapped to the 5′UTR of *psaB*, to which it can bind directly (Rahire et al., 2012). Similarly RAA1 and RAA8 are other examples of OPR proteins involved in a complex that trans-splices introns of the *psaA* precursor transcripts (Marx et al., 2015).

Finally, some members of a class of 34 amino-acid repeat proteins, TPRs, which were first known to be involved in protein-protein interactions, were shown to be involved in mRNA metabolism, forming a subclass dubbed “Half a TPR (HAT)”. Examples of HAT-domain proteins include UTP6, which is involved in nuclear pre-rRNA processing, PRP6, which is involved in nuclear pre-mRNA splicing, and CSTF-77, which is involved in pre-mRNA cleavage and polyadenylation. As for PPRs and OPRs, TPR motifs are typically found in a tandem repeat structure, in which they stack to form a broad surface that can bind protein ligands. Crystal structures of CSTF-77 confirmed that HAT repeat motifs can adopt a TPR-like structure (Bai et al., 2007). Some TPR or HAT proteins are involved in the stabilization of chloroplast mRNA encoding photosynthetic proteins in *A. thaliana* (Hammani et al., 2012).

In Chlamydomonas, NAC2 is a TPR protein that is involved in the stability, processing and translation of *psbD* mRNA, and directly binds to the 5′UTR, as determined by electro mobility shift assay (EMSA) (Boudreau et al., 2000). NAC2 was also found to be associated with a large complex. The TPR protein MBB1 in *C. reinhardtii* is homologous to HCF107 in *A. thaliana* and is part of a 300 kDa complex (Vaistij et al., 2000). It is required for the stability of *psbB* and *psbH* RNAs (Monod et al., 1992; Loizeau et al., 2014). MBB1 immunoprecipitates are able to associate with the *psbB* 5′UTR in EMSA experiments (Loizeau et al., 2014). HCF107, the ortholog of MBB1 in *A. thaliana*, was expressed in *E.coli* and shown to bind the *psbH* and *atpF/A* 5’UTRs in vitro (Hammani et al., 2012).

RNA granules

GRSF1, an RNA binding protein, was found to be located to discrete foci in the mitochondrial matrix of HeLa cells. Further analysis showed that these foci also contain RNA

and RNAse P and are important structures for transcript processing. They have been called mitochondrial RNA granules (Jourdain et al., 2013). Similarly, several studies have shown that large complexes could exist in the chloroplast and that they are involved in transcript processing. CSP41a and b are involved in *A. thaliana* chloroplast rRNA maturation (Beligni and Mayfield, 2008). Later findings revealed a role for CSP41b as an essential component of a specific subset of RNA binding protein complexes that stabilize non-translated mRNA in the dark and disassembly in the light (Qi et al., 2012). CSP41 a and b are homologous proteins of RAP38 and RAP41 in *C. reinhardtii*. PPR7 is a stromal chloroplast protein that is found in large ribonucleoprotein complexes involved in mRNA maturation/stability. The existence of such complexes in several species suggests that they might have an important conserved role. *A. thaliana* CP31A and CP29A associate with multiple transcripts in the ribonucleoprotein complex and play an important role in the response to cold stress (Kupsch et al., 2012). In *C. reinhardtii* chloroplasts, the quantity of *atpA* mRNA does not seem to be a limiting factor for protein accumulation (Drapier et al., 1998). Most of the mRNA is trapped and stored in big ribonucleic complexes containing TDA1 when it is not translated (Eberhard et al., 2011).

In the chloroplast of Chlamydomonas, these ribonucleoprotein complexes were observed by fluorescence in situ hybridization (FISH) and immunofluorescence experiments under different stress conditions and were therefore named cpSGs (chloroplast stress granules). The mRNAs of *psbA, psbC, rbcL*, and *psaA* were seen in cpSGs after high-light treatment (Uniacke and Zerges, 2008).

Import of nucleus-encoded proteins

As noted above, the chloroplast genome contains around a hundred of genes while its proteome is estimated to approximately 3000 proteins, implying that nucleus-encoded proteins are imported from the cytoplasm for the function of the chloroplast. The synthesis of nucleus-encoded proteins in the cytoplasm means they face a barrier between compartments and need to cross two biological membranes, which is not possible without assistance.

Nucleus-encoded proteins contain a transit peptide extension at their N-terminus, which targets them towards their destination. This short sequence is highly variable between proteins, but shares characteristics of hydrophily, and the presence of positively charged amino acids. In addition, they are enriched in hydroxylated amino-acids such as serine (Bruce, 2001). While they do not share a conserved sequence, one might hypothesize that their secondary structure

is conserved, but that is not the case as the transit peptide is largely unstructured in aqueous environments (Wienk et al., 1999; Krimm et al., 1999). The transit peptide was thought to have evolved to maximize a secondary structure with a low degree of order (von Heijne and Nishikawa, 1991). An alpha-helix structure was found in lipidic/detergent environments (Endo et al., 1992).

For different transit, sequence-dependent targeting, a guidance complex is required. It seems that transit peptides are first recognized by the TOC translocon at the outer chloroplast membrane and this recognition requires the hydrophobic environment of the membrane bi-layer (Bruce, 1998), correlating with the alfa helix that the transit peptide might form in lipidic environments. TOC34, TOC86/159/132/120, TOC64, and TOC75 are involved in the early steps of protein import (Perry and Keegstra, 1994; Ma et al., 1996; Sohrt and Soll, 2000). This first recognition stage involves GTPase activity (Kessler et al., 1994), which most likely concerns TOC34 or TOC159. The TOC complex forms a press-stud with TIC (Translocon on the inner chloroplast membrane) joining the two membranes of the envelope thus favoring translocation. TIC20 and 22 were proposed to functionally link the TIC and TOC complexes (Kouranov et al., 1998). TIC21, TIC32, TIC40, TIC55, TIC56, TIC62, TIC100, TIC110, TIC214 and YCF1 are thought to be part of the chloroplast TIC complex (Nakai, 2015), but its exact composition is still unknown.

Similar to mitochondria, in the inner plastid envelope membrane, the import of transit sequence-less proteins was shown to be dependent on the guidance factor PRAT (Rossig et al., 2014).

Insertion and import into thylakoid membranes.

Different pathways were found to be involved in the integration of proteins into the thylakoid membranes, dependent on a signal at the N-terminus of each protein. The first pathway is the simplest, as some proteins can spontaneously integrate into the membrane co-translationally or post-translationally. The second is a pathway related to the SEC system of the bacterial export machinery. This pathway requires ΔpH and ATP, for which requirements are protein specific, and more likely concerns proteins localized to the lumen (Mant et al., 1995). In the third identified pathway, folded proteins use the twin-arginine translocation (TAT) system, in which the trans-thylakoid pH gradient is used as the sole energy source in vitro, but which is not mandatory in vivo (Theg et al., 2005). Light-harvesting proteins mostly use the fourth

pathway, which is a GTP-dependent signal recognition particle (SRP) system in which ATP hydrolysis by SECA drives the transport of the substrate protein through the membrane in an unfolded conformation. In *A. thaliana*, in addition to the SRP this pathway requires FTSY in the stroma and ALB3 in the thylakoid membrane (Bals et al., 2010). In *C. reinhardtii*, two members of the ALB3 family, ALB3.1 and ALB3.2 are involved in the translocation of LHC and PS I/PS II core proteins, respectively (Göhre et al., 2006). ALB3.1 and 3.2 were found to interact with VIPP1, a protein involved in the biogenesis of thylakoid complexes that forms a rod like structure located at the thylakoid center, a place in which thylakoids converge and is thought to be the structure involved in their biogenesis (Rütgers and Schroda, 2013).

The insertion of subunits into the membrane is led by a number of factors such as PPD1 (Roose et al., 2014), Ycf3, and Ycf4 (Naver et al., 2001; Rochaix et al., 2004). Evidence suggests that a 250 kDa complex harboring PSA2 (chaperone-like protein) and PSA G (PSI subunit) mediates interactions in the thylakoid lumen that are required for PS I assembly via PSA2 disulfide-isomerase activity and a chaperone domain (Fristedt et al., 2014b). PS I mutants do not affect PSA2, but YCF3 mutants have reduced levels of PSA2, suggesting some level of communication between these proteins.

Proteases

Degradation of proteins also plays an important role in regulating the protein content of the chloroplast. Unassembled subunits are degraded to ensure stoichiometry within complexes (Adam, 2007). Chloroplast protein balance is regulated by semi-specific proteases that recycle damaged and unassembled proteins. One such protease is FTSH, which was shown in Chlamydomonas to play an important role in PS II and b_6f turnover under stress conditions (Malnoë et al., 2014). CLP protease (CLP) is a predominantly nucleus-encoded complex with a single chloroplast encoded subunit, ClpP1, and is essential for cell viability. The induced knock down of this subunit triggers an autophagy-like response through the disrupted accumulation of a subset of chloroplast proteins (Ramundo et al., 2014). The deficiency of this protease also induces other proteases such as FTSH and DEG suggesting an “unfolded protein signal” and/or autophagy-regulated expression.

DEG belongs to the HTRA protease family, which includes serine-type endopeptidases that are involved in quality control (Clausen et al., 2011). These proteins are present in different isoforms in A. thaliana chloroplasts. DEG1, 5, and 8 are located on the lumenal side of the

thylakoids while DEG2 and 7 are located in the chloroplast stroma. As for FTSH proteases, evidence suggests that they play a role in D1 turnover induced by photo-damage (Kato and Sakamoto, 2013).

DEG5 in A. thaliana forms trimers that bind calcium, an ion involved in light signaling in the stroma (see section on calcium signaling). This suggests that excessive light might regulate their activity (Sun et al., 2013). In contrast to other HTRAs, DEG5 does not contain the PDZ domain that is required for their regulation. Instead, the crystal structure of its trimers revealed a triad conformation similar to that found in the hexameric structure of DEG8 which harbors a single PDZ domain.

C. REINHARDTII AS A MODEL ORGANISM

C. reinhardtii is a eukaryotic photosynthetic green alga that can complete its vegetative growth cycle under both autotrophic and heterotrophic conditions. It is able to grow on agar plates and in liquid media and has an average doubling time of 8 hours, although this can be shorter under optimal conditions. It measures around 10 µm, has two flagella, and a cell wall containing hydroxyprolin-rich glycoproteins.

WT laboratory strains are derived from 137 C, which was isolated near Amherst, Massachusetts, in 1945 by Gilbert M. Smith. Its unique chloroplast contains around 80 copies of the 200 kb genome. The nucleus contains a 120 Mb haploid genome, with 17 chromosomes. Its genome was first sequenced and assembled in 2007 (Merchant et al., 2007). The fifth and last version of the genome assembly was released in December 2011. *C. reinhardtii* is used as a model organism for studies on flagellum function, chloroplast dynamics, and photosynthesis (as it can grow heterotrophically in the dark, the photosynthetic machinery is not essential). *C. reinhardtii* is at the origin of the discovery of extra chromosomal DNA in chloroplasts (Sager and Ishida, 1963).

Under specific conditions such as nitrogen starvation, *C. reinhardtii* is able to reproduce through a sexual cycle. Mating is generally induced by nitrogen starvation and specific light conditions (Beck and Acker, 1992) and leads to the formation of a diploid zygospore. After meiosis, the progeny forms tetrads that can be dissected and analyzed. The mating type of strains is genetically determined at the *mat* locus. While the nuclear genome heredity follows Mendelian rules, organellar heredity is uniparental, with the *mt+* genotype transmitting the chloroplast to the progeny (Nishimura et al., 2002) and the *mt-* genotype giving the

mitochondria (Beckers et al., 1991). The specific degradation of non-inherited organelle DNA is thought to be led by methylases that protect the chloroplast DNA from the *mt+* strain and the mitochondrial DNA from the *mt-* strain prior to mating.

Genetic manipulation of *C. reinhardtii* is possible in both the chloroplast and nucleus. Chloroplast transformation is achieved using glass beads for cell wall-less mutants (Economou et al., 2014) or a particle gun for both cell-wall phenotypes (Boynton et al., 1988). Transformation involves the homologous recombination system, allowing precise modifications to be made. In the chloroplast, the spectinomycin resistance marker "*aadA*" is often used (Goldschmidt-Clermont, 1991)., Phototrophic rescue is also an advantageous selection marker, such as complementation with wild-type atpB of a strain deleted for this gene in the chloroplast genome of FUD50 (Boynton et al., 1988). Marker-free chloroplast transformation is also possible using a psbA deletion strain (Bertalan et al., 2015) or an rbcL mutant (Chen and Melis, 2013). A recent study revealed that it is feasible to apply the negative selection marker, cytosine deaminase (Young and Purton, 2014).. It is also possible to use reporter genes in the chloroplast, with the most commonly used being firefly luciferase (Matsuo et al., 2006), Renilla luciferase (Minko et al., 1999), and the bacterial luciferase *luxCt* (Mayfield and Schultz, 2004). Amazingly, it is possible to modify several loci at the same time via the in vitro assembly of the whole chloroplast genome (O'Neill et al., 2012). When undertaking chloroplast transformation, the fact that the genome is present in approximately 80 copies must be considered. To successfully transform all copies of the genome, the transformants have to remain under selection during a few subcultures, and homoplasmy can be assessed by PCR. A system was recently developed to control the expression of both nuclear and chloroplast transgenes via the thiamine riboswitches (Ramundo and Rochaix, 2015).

Random point mutagenesis is possible and mostly uses EMS (Lee et al., 2014) or UV (Girard et al., 1980). Random insertions of DNA into the nucleus of *C. reinhardtii* are an important technique for both mutagenesis and exogenous gene expression. The drawback of this method of transformation is that it induces "collateral damage" (mutations not directly linked to the DNA insertion but appearing during the transformation process) that require backcrosses to resolve. It is possible to insert DNA into cell wall-less strains via the shot-gun method, the glass-bead technique (Kindle, 1990), silicon carbide whiskers (Dunahay, 1993), or electroporation (Ladygin, 2003). Use of the electroporation technique to transform cell wall containing strains is the most efficient, but differs from earlier techniques as cells require either pre-treatment with autolysin (digests the cell wall, secreted during mating), cold incubation

(Shimogawara et al., 1998), or the specific pulse pattern generated by the Nepa21 electroporator (Yamano et al., 2013).

Mutant isolation in *C. reinhardtii* is more direct compared to other species, as vegetative cells are haploid, allowing the phenotype of mutations to be immediately revealed. Directed mutagenesis in the nucleus has successfully been employed, but it still involves the random insertion of the cassette that encodes for components needed for the targeted mutagenesis .This technique remains difficult in *C. reinhardtii*. Artificial miRNAs were successfully used to knock down LHCBM proteins (Molnar et al., 2009; Oey et al., 2013). TALENs were also considered for use in *C. reinhardtii*, but no success has yet been reported. Zinc-finger nucleases were used successfully for targeted mutagenesis but the efficiency of this strategy was too low to be routinely used (Sizova et al., 2013). Finally, the promising CRISPR-Cas9 system was reported to induce targeted cleavage but the toxicity of Cas9 was a major hurdle and a very low efficiency was obtained even when transiently expressed (Jiang et al., 2014).

C. reinhardtii is also able to stably express numerous nuclear and chloroplast reporters and markers, most of which have recently been reviewed (Jinkerson and Jonikas, 2015).The nuclear transformation markers successfully used to date include the paromomycin resistance gene *aphVIII* (Sizova et al., 2001), zeocin resistance *ble* (Lumbreras et al., 1998) (Stevens et al., 1996), arginine autotrophy *ARG7* (Debuchy et al., 1989), tetracycline resistance *tetx* (Garcia-Echauri and Cardineau, 2015), and hygromycin resistance *hyg, aphVII* (Berthold et al., 2002). In addition, many fluorescent or luminescent proteins are available, they have been optimized for nuclear codon usage. These include venusYFP, mCHERRY, GFP, mCerrulean, (Rasala et al., 2013), and *Gaussia* luciferase (Shao and Bock, 2008).

Transgene expression that is not maintained under selection is silenced in *C. reinhardtii* (Cerutti et al., 1997b), and silencing is epigenetically inherited via histone methylation (Casas-Mollano et al., 2008). A strategy to counteract this silencing is the use of the HSP70A promoter, which suppresses silencing of the adjacent promoter (Schroda et al., 2000). UV mutagenesis and genetic screens allowed the isolation of two strains that displayed less silencing of the transgenes, UVM4 and UVM 11 (Neupert et al., 2009). Another strategy used to avoid silencing is to fuse the gene of interest translationally using a self-cleavable FMDV 2A fragment to a marker gene that can be kept under selection (Plucinak et al., 2015).

RESULTS Chapter I: MAC1

Introduction

The chloroplast is an organelle that is able to produce and accumulate massive amounts of proteins. For example, it hosts Rubisco, which is the most abundant protein on Earth. From a biotechnological point of view, the chloroplast can be seen as an interesting production platform presenting both the advantage of being present in organisms with a fast growth rate and also of being powered by light. Later use of this production platform implies a full understanding of its physiology and regulation, and of the relationship between the nucleus and the chloroplast. The model organism *C. reinhardtii* allows the genetic manipulation of both the chloroplast and nuclear compartments. The accumulation of many chloroplast proteins is already known to be regulated under different conditions of light, CO_2, sulfur, copper, nitrogen, phosphorus, and iron supply. Protein accumulation results from a balance between their biogenesis and degradation, and little is known about the detailed mechanism behind their regulation and what really triggers these processes.

While in bacteria, transcription is most often the target of biosynthesis regulation, in the chloroplast there are only a few examples of transcriptional regulation, with the circadian effect being the best known in *C. reinhardtii,* or blue light responses and plastid development in plants. In contrast, there is more evidence that the chloroplast undergoes regulation based on RNA stability and/or translation. The CES process is an example of regulation that affects translation initiation (see section on CES). TAA1, which is required for *psaA* mRNA stability and translation, is regulated in response to iron availability, MCA1, TCA1 and other b_6f biogenesis factors respond to nitrogen deficiency (Raynaud et al., 2007). Amongst the numerous nucleus-encoded genes that are involved in chloroplast gene expression, only a few are characterized as being regulated, raising the question of whether the others are constitutively expressed and active, or involved in fine-tuning the biogenesis of the chloroplast machinery. In many cases of regulation triggered by environmental stimuli, the nucleus is subjected to transcriptional changes, but does regulation of the chloroplast also pass through a nuclear step? Is this regulation directly triggered in the chloroplast and if so, how? Is it an issue of degradation, post translational modification(s), phosphorylation, or association of different protein partners? Another interesting aspect of chloroplast RNA metabolism is that in different species, maturation of homologous mRNAs uses different strategies. For example, in

C. reinhardtii, the *psaA* gene is split across four different loci and the maturation of *psaA* mRNA requires trans-splicing and involves numerous proteins.

However, these are absent in land plants as the *psaA* gene is present in a single transcript without intron. Conversely, *rps12* has no intron in Chlamydomonas but is spliced in trans from separate precursors in *Z. maize* (Schmitz-Linneweber et al., 2006).

The primary goal of my research project was to identify, in *C. reinhardtii,* new factors that are involved in photosystem I biogenesis and subsequently determine whether they are implicated in regulatory mechanisms. The strategy used for this identification was to use random insertional mutagenesis and screen for PSI deficient strains. This mutagenesis approach has already been used to identify numerous factors involved in chloroplast gene expression (table 6 page 37) (Goldschmidt-Clermont et al., 1990). Random insertional mutagenesis induced by electroporation of a WT strain derived from 137C with a paromomycin resistance cassette called *AphVIII* was used here. The mutant screen was based on fluorescence induction kinetics at room temperature (which can be observed on colonies growing on a plate) to identify mutants that were defective in electron flow, as distinguished by the shape of the fluorescence induction curve. PS I mutants have a typical fluorescence phenotype, similar to b_6f mutants, that allows their identification. Xenie Johnson and coworkers at the “Institut de Biologie Physico-Chimique” in Paris used a similar screen, and through their collaboration, I obtained several PS I mutants in addition to those I generated myself.

Amongst the PSI mutants that were initially characterized, one was chosen for further study as described below. The other mutants were mainly defective in *psaA* splicing: there were 14 class C mutants (affected in trans-splicing of exons 1 and 2), 2 class B mutants (defective in trans-splicing of both introns) amongst which a new allele of *raa1*, and 4 class A mutants (impaired in trans-splicing exons 2 and 3). There were also 2 mutants defective for *psaB* mRNA accumulation (amongst which an allele of *tab1*), a mutant affected in both PsbA (D1) and PsaA accumulation, and another pleiotropic mutant. The *mac1* mutant isolated by Xenie Johnson specially affected the accumulation of *psaC* mRNA. Because of its novel phenotype, it was selected for further study. The characterization of the *mac1* mutant is described in a manuscript that constitutes the next section. This is followed by further experiments that were not included in the manuscript.

For this manuscript, I contributed most of the experimental work, with the exception of genetic crosses which were performed by Dr. Linnka Legendre-Lefebvre, and of the analysis of small sRNAs which was conducted by Yujiao Qu in the lab of Prof. Christian Schmitz-

Linneweber. I collaborated with Dr. Paolo Longoni for the optimization of the PhosTag gel electrophoresis.

Manuscript

A nucleus-encoded chloroplast phosphoprotein governs expression of the photosystem I subunit PsaC in *Chlamydomonas reinhardtii*

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Short title: Anterograde control of *psaC* expression

One sentence summary: The stability of *psaC* RNA in the chloroplast is under the control of a nucleus-encoded protein which is differentially phosphorylated in response to environmental cues.

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SUMMARY

The nucleo-cytoplasmic compartment exerts anterograde control on chloroplast gene expression through numerous proteins that intervene at post-transcriptional steps. The *maturation of psaC* mutant (*mac1*) of *Chlamydomonas reinhardtii* is defective in photosystem I and fails to accumulate *psaC* mRNA. The *MAC1* locus encodes a member of the Half-A-Tetratricopeptide (HAT) family of super-helical repeat proteins, some of which are involved in RNA transactions. The Mac1 protein localizes to the chloroplast in the soluble fraction. *MAC1* acts through the 5' untranslated region of *psaC* transcripts and is required for their stability. Small RNAs that map to the 5'end of *psaC* RNA in the wild type but not in the *mac1* mutant are inferred to represent footprints of *MAC1*-dependent protein binding, and Mac1 expressed in bacteria binds RNA in vitro. A coordinate response to iron deficiency, which leads to dismantling of the photosynthetic electron transfer chain and in particular of photosystem I, also causes a decrease of Mac1. Overexpression of Mac1 leads to a parallel increase in *psaC* mRNA but not in PsaC protein, suggesting that Mac1 may be limiting for *psaC* mRNA accumulation but that other processes regulate protein accumulation. Furthermore Mac 1 is differentially phosphorylated in response to iron availability and to conditions that alter the redox balance of the electron transfer chain.

INTRODUCTION

The photosynthetic electron transfer chain in the thylakoid membrane comprises several large pigment-protein complexes that function together with remarkable efficiency to convert light energy into chemical energy which is in turn used to fuel metabolism. The assembly of the complexes of the photosynthetic electron transfer chain requires the concerted expression of genes in two separate compartments, the nucleus and the chloroplast. These genes encode not only the subunits of the photosynthetic complexes, but also a large cohort of proteins that are required for gene expression and complex assembly.

Photosystem I is composed of 12 to 19 polypeptide subunits, depending on the organism, that bind approximately two hundred pigments and cofactors. In *Chlamydomonas reinhardtii* four components of PSI are encoded in the chloroplast: the larger PsaA and PsaB subunits, as well as the smaller PsaC and PsaJ subunits (Redding, 2009). Ten other polypeptides are encoded in the nucleus and imported in the chloroplast where they assemble with the chloroplast-encoded subunits. Two other chloroplast-encoded proteins, Ycf3 and Ycf4, facilitate the

assembly of the PSI complex (Boudreau et al., 1997; Naver et al., 2001; Ozawa et al., 2009). The expression of the chloroplast *psaA* and *psaB* genes is controlled at the post-transcriptional level by a set of nucleus-encoded factors that are transcript-specific. The *psaA* mRNA is assembled in two steps of *trans*-splicing from three separate precursors (Choquet et al., 1988; Kück et al., 1987). Trans-splicing of *psaA* depends on a chloroplast-encoded RNA, *tscA*, and on at least fourteen nucleus-encoded proteins (Goldschmidt-Clermont et al., 1991; Goldschmidt-Clermont et al., 1990). The stability and translation of *psaA* mRNA further depends on Taa1, a nucleus-encoded member of the OPR family (octatrico peptide repeat) of RNA-binding helical-repeat proteins (Lefebvre-Legendre et al., 2015). The stability and translation of *psaB* mRNA depend on Tab1, another OPR protein, and on Tab2, a protein which is widely conserved in oxygenic phototrophs but has no previously described RNA-binding motifs (Dauvillee et al., 2003; Rahire et al., 2012; Stampacchia et al., 1997).

The expression of chloroplast genes encoding subunits of the other photosynthetic complexes similarly depends on numerous transcript-specific nucleus-encoded proteins. Likewise in flowering plants a large group of nucleus-encoded proteins govern chloroplast gene expression at the levels of transcript processing, splicing and stability, C-to-U editing and mRNA translation (Barkan, 2011; Stern et al., 2010). The somewhat surprising complexity of chloroplast gene expression and the large number of nuclear genes that are involved raises the question of whether this provides regulatory control by the nucleus on the plastid. Alternatively it has been argued that part of the complexity may have arisen in a process of constructive neutral evolution, where pre-existing nucleus-encoded proteins can suppress new mutations that appear in the chloroplast (Barkan and Small, 2014; Gray et al., 2010; Lukes et al., 2011; Maier et al., 2008). A typical example where this might be the case is provided by the nucleus-encoded editing factors that make specific C-to-U changes in the sequence of chloroplast mRNAs at a post-transcriptional step (Schmitz-Linneweber et al., 2005a). Another example may come from the numerous factors that are required for splicing *in trans* of the *psaA* mRNA in Chlamydomonas (Lefebvre-Legendre et al., 2014). In these cases, the nucleus-encoded proteins could be needed constitutively and would not be involved in chloroplast gene regulation in the strict sense.

This does not exclude the possibility that some of the nucleus-encoded proteins do participate in the regulation of chloroplast gene expression in response to environmental or developmental cues. For example in Chlamydomonas, nitrogen deprivation leads to rapid decrease in the amount of cytochrome *b_6f* complexes. This coordinate response involves the proteolytic

degradation of its subunits and also of Mca1 and Tca1, nucleus-encoded proteins that control the stability and translation of *petA* mRNA, which encodes the Cyt*f* subunit (Boulouis et al., 2011; Raynaud et al., 2007; Wei et al., 2014). The concerted response to nitrogen deprivation also involves other nucleus-encoded proteins that take part in the assembly of the complex and its hemes (Wei et al., 2014). An early response of Chlamydomonas to iron deprivation is the down-regulation of PSI and the remodeling of its light-harvesting antenna (Moseley et al., 2002; Naumann et al., 2005). There is a concomitant down-regulation of Taa1, which is required for the stability and translation of *psaA* (Lefebvre-Legendre et al., 2015).

Here we describe the identification of Mac1, a nucleus-encoded protein that localizes to the chloroplast where it is required for the expression of *psaC*. Mac1 belongs to the TPR / HAT (tetratrico peptide repeat / half a tetratrico peptide) family of helical repeat proteins, whose members are involved in RNA transactions (Hammani et al., 2014). Mac1 acts through the 5'UTR on the stability of *psaC*, and Mac1 protein expressed in bacteria binds RNA in vitro. The amount of Mac1 is regulated in response to iron deprivation. We present evidence that Mac1 is phosphorylated and that this post-translational modification is modulated by iron availability and other environmental conditions.

RESULTS

Identification of *MAC1*

A collection of random insertional mutants of *Chlamydomonas reinhardtii* was previously generated by transformation with an expression cassette containing the *aphVIII* gene which confers paromomycin resistance (Houille-Vernes et al., 2011; Johnson et al., 2010). The mutants were screened for defects in photosynthesis based on chlorophyll fluorescence induction kinetics (Figure 1A). One of these mutants, which had an apparent defect in Photosystem I (PSI) and could not grow photo-autotrophically, was chosen for further study. Immunoblotting with antibody against the PsaA subunit of PSI, which accumulated in the mutant to less than 30% of the wild-type level, confirmed that it has a defect in PSI (Figure 1B). The loss of one subunit of a photosynthetic complex can lead to the degradation of the other subunits. This applies in particular to chloroplast mutants lacking PsaC (Takahashi et al., 1991). To investigate which subunit is primarily affected in the PSI-deficient mutant, wild-type and mutant RNA were analyzed by agarose gel electrophoresis and blot hybridization with probes for the chloroplast genes encoding three major subunits of PSI, *psaA*, *psaB* and *psaC* (Figure 1C). This revealed a defect in the accumulation of *psaC* transcripts in the mutant. The *psaC*

Figure 1

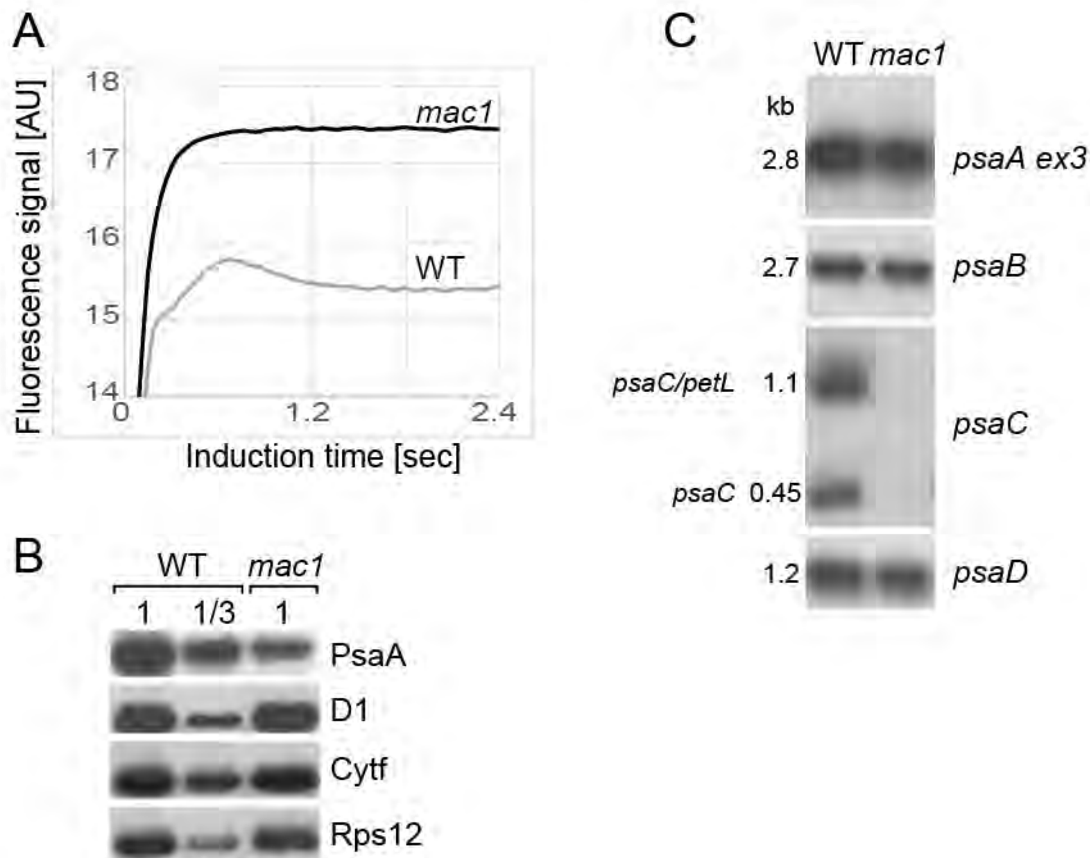


Figure 1. Characterization of the *mac1* mutant

A. Chlorophyll fluorescence induction kinetics. The induction of chlorophyll fluorescence in the wild type and the *mac1* mutant was monitored upon a transition from dark to light using a video-imaging system. The cells were grown in the dark on a plate containing acetate media.

B. Protein analysis. Total protein extracts of the wild type (WT; 25 µg and 1/3 the amount) and of the *mac1* mutant (25 µg) were analyzed by SDS-PAGE against the proteins indicated on the right.

C. RNA analysis. RNA extracts of the wild type (WT) and of the *mac1* mutant were analyzed by denaturing gel electrophoresis, blotting to nylon membranes and hybridization with the radiolabelled probes indicated on the right. The sizes of the transcripts are shown on the left.

gene is upstream of *petL*, with which it is co-transcribed in the Chlamydomonas chloroplast genome. The slower-migrating band is the di-cistronic *psaC-petL* RNA (1.1 kb), while the faster-migrating band is the monocistronic *psaC* RNA (0.45 kb) (Takahashi et al., 1991). Both of these transcripts were missing in the mutant, while the monocistronic *petL* RNA was present at elevated levels relative to wild type (Figure 1C). In contrast *psaA* was *trans*-spliced normally and *psaB* mRNA accumulated to wild-type levels. Because of its specific defect in the accumulation of *psaC* RNA, the mutant was called *mac1* (mRNA of *psaC*). For further analysis, the *mac1* mutant was backcrossed three times to the wild type. The wild-type and *mac1* progeny segregated 2:2, indicating a nuclear mutation, and paromomycin sensitivity or resistance segregated with the wild-type or PSI-deficient phenotype respectively in 92 progeny from 24 tetrads (some of them incomplete), suggesting that the *aphVIII* insertion was linked to the *mac1* mutation.

To identify the site of the *aphVIII* insertion in the *mac1* mutant, reverse PCR and sequencing were used to obtain a flanking sequence tag, which corresponded to gene Cre02.g124700 (g9646.t1) in Version 10 of the Phytozome database (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). The insertion maps to exon 2 of the predicted gene (Supplemental Figure 1) and *mac1* is thus most likely a null mutant. Genomic transformation with both BAC (bacterial artificial chromosome) clone 23A16 and a 5.6 kb subclone (*pMAC1_gen3*) containing the Cre02.g124700 gene efficiently rescued the photosynthetic deficiency of *mac1* (Supplemental Figure 2). In representative transformants, the accumulation of the PsaC protein and the *psaC* transcripts were restored (Figure 2 A,B). Thus mapping of the insertion site and complementation with the wild-type gene identify Cre02.g124700 as the *MAC1* gene. To raise a rabbit antiserum against Mac1, a C-terminal domain of the protein was expressed in *Escherichia coli*. Although the serum recognized Mac1, it was not monospecific and also recognized several non-specific bands including one that nearly co-migrates with Mac1 (Figure 2B) or can sometimes barely be resolved (Figure 2C, marked with an asterisk). Therefore to facilitate the detection of Mac1, the *MAC1* gene was tagged with a triple hemagglutinin (HA) epitope (*MAC1-HA*). This construct rescued the *mac1* mutant with high efficiency (Figure 2 A,B and Supplemental Figure S2), showing that Mac1-HA is functional. As expected the HA-tagged protein was also detected by the Mac1 antiserum and migrated more slowly than the un-tagged form (Figure 2B, *mac1; MAC1-HA*).

MAC1 encodes a predicted polypeptide of 982 amino acids. A striking feature of the predicted Mac1 polypeptide is the presence of two adjacent domains each with six or seven

Figure 2

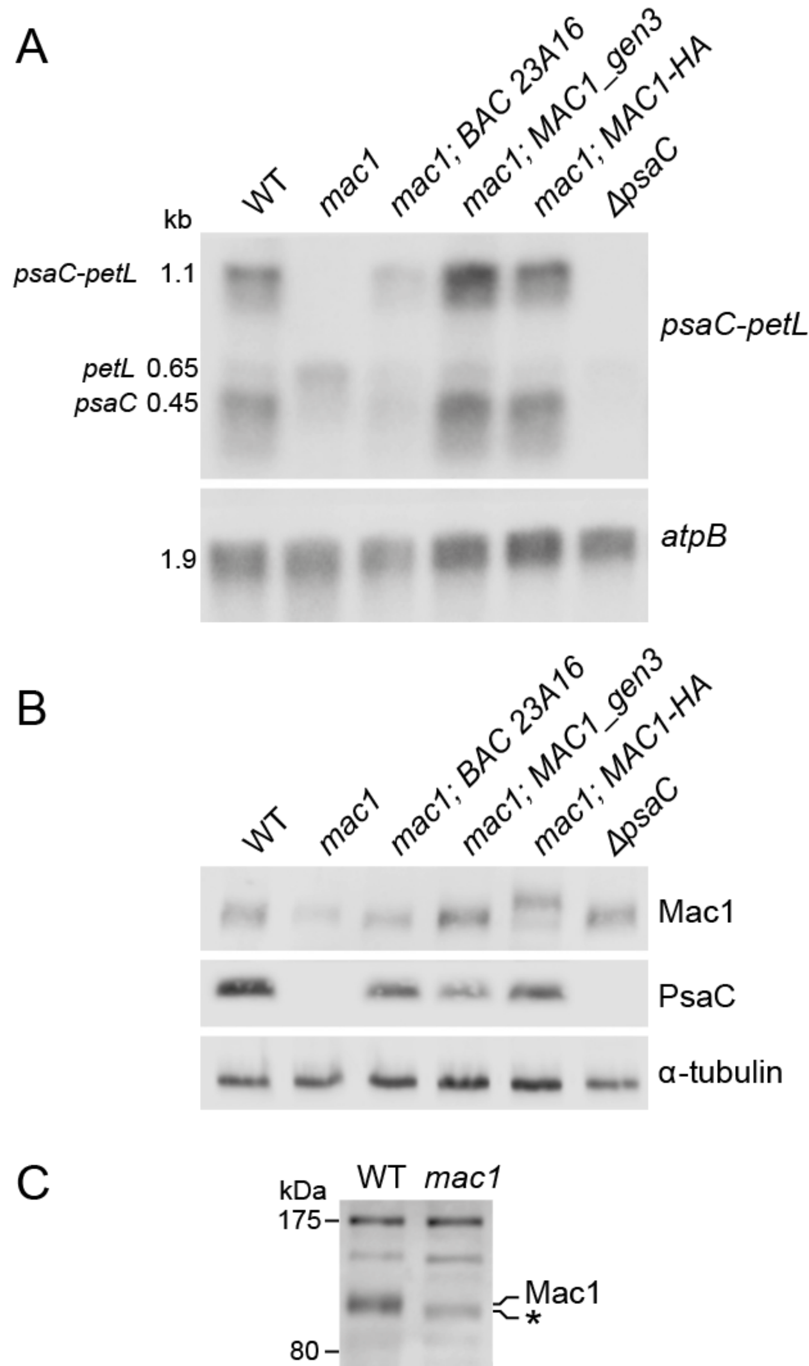


Figure 2. Complementation of the *mac1* mutant

A. RNA blot hybridization analysis of the *mac1* mutant and complemented strains. RNA was extracted from the wild type (WT), the *mac1* mutant, the *mac1* mutant rescued by transformation with the genomic BAC clone (*BAC23A16*), with the genomic subfragment containing *MAC1* (p*MAC1_gen3*) or with a derivative of the latter carrying a triple HA epitope (*MAC1-HA*), and a chloroplast mutant with an insertion in the *psaC* gene (*ΔpsaC*). The samples were analyzed by denaturing gel electrophoresis and RNA blot hybridization with radiolabelled fragments containing *psaC* and *petL* (upper panel) or *atpB* as a control (lower panel). The identities of the transcripts and their sizes are shown on the left.

B. Protein analysis. Immunoblot analysis of total proteins extracts from the same strains as in panel A. The antisera used for immunoblotting are shown on the right.

C. Specificity of the antiserum against Mac1. Total protein extracts of the wild type (WT) and of the *mac1* mutant were analyzed by SDS-PAGE and immunoblotting with a rabbit polyclonal antiserum raised against recombinant Mac1. A minor non-specific band which is present in the mutant (marked with an asterisk) runs just below Mac1.

tetratricopeptide (TPR) repeats, most of which have features of the HAT subfamily (Half-A-TPR) (Supplemental Figure 1C). Members of this subfamily are implicated in RNA metabolism and some have been shown to associate with RNA *in vivo* or to bind RNA *in vitro* (Hammani et al., 2014; Hammani et al., 2012; Loizeau et al., 2014). Indeed the closest paralog of Mac1 in Chlamydomonas is Mbb1, a TPR/HAT repeat protein involved in the stabilization or maturation of the *psbB/T* and *psbH* mRNAs (Loizeau et al., 2014; Vaistij et al., 2000a) (Supplemental Figure 3). The orthologue of Mbb1 in higher plants, HCF107, is required for the stability of *psbH* transcripts (Felder et al., 2001; Hammani et al., 2012; Sane et al., 2005).

Mac1 is a chloroplast protein

Immunofluorescence and confocal microscopy were used to determine the subcellular localization of Mac1. In the *mac1*;*MAC1-HA* strain, immunolabelling with a monoclonal anti-HA antibody gave a signal in the chloroplast, which was absent in the wild-type control (Figure 3; Supplemental Figure 4). The chloroplast localization of Mac1-HA was confirmed by co-labelling with a polyclonal antibody against the chloroplast stromal protein DnaK. Furthermore the localization of Mac1-HA was distinct from Rpl37 (Figure 3A) or Rpl4 (Supplemental Figure 4), subunits of cytoplasmic ribosomes.

Cell fractionation experiments were used to confirm the localization of Mac1. The rabbit polyclonal antibody against the Mac1 protein was used to probe immunoblots of Chlamydomonas subcellular fractions (Figure 3B). Mac1 was found in the chloroplast fraction, together with the chloroplast markers PsaC and DnaK, but not in the supernatant of the lysate which contained the Rpl37 subunit of the cytosolic ribosomes. Further fractionation of the chloroplasts yielded a stromal fraction with soluble proteins such as DnaK and a membrane pellet which contained the PsaC subunit of PSI, an integral protein of the thylakoid membrane. The Mac1 protein co-fractionated with DnaK in the soluble fraction and showed no indication of membrane association.

To investigate whether Mac1 is part of a ribonucleoprotein complex, an extract of total soluble proteins was prepared from a strain expressing HA-tagged Mac1 (*mac1*;*MAC1-HA)*. Half of the extract was treated with RNase while the rest was mock-treated, and then the two samples were fractionated by sucrose gradient sedimentation (Figure 3C). Most of Mac1 remained close to the top of the gradient, in a position consistent with the expected sedimentation of the monomer. A minor fraction of Mac1 was distributed in larger complexes. Their sedimentation was not significantly affected by the RNase treatment.

Figure 3

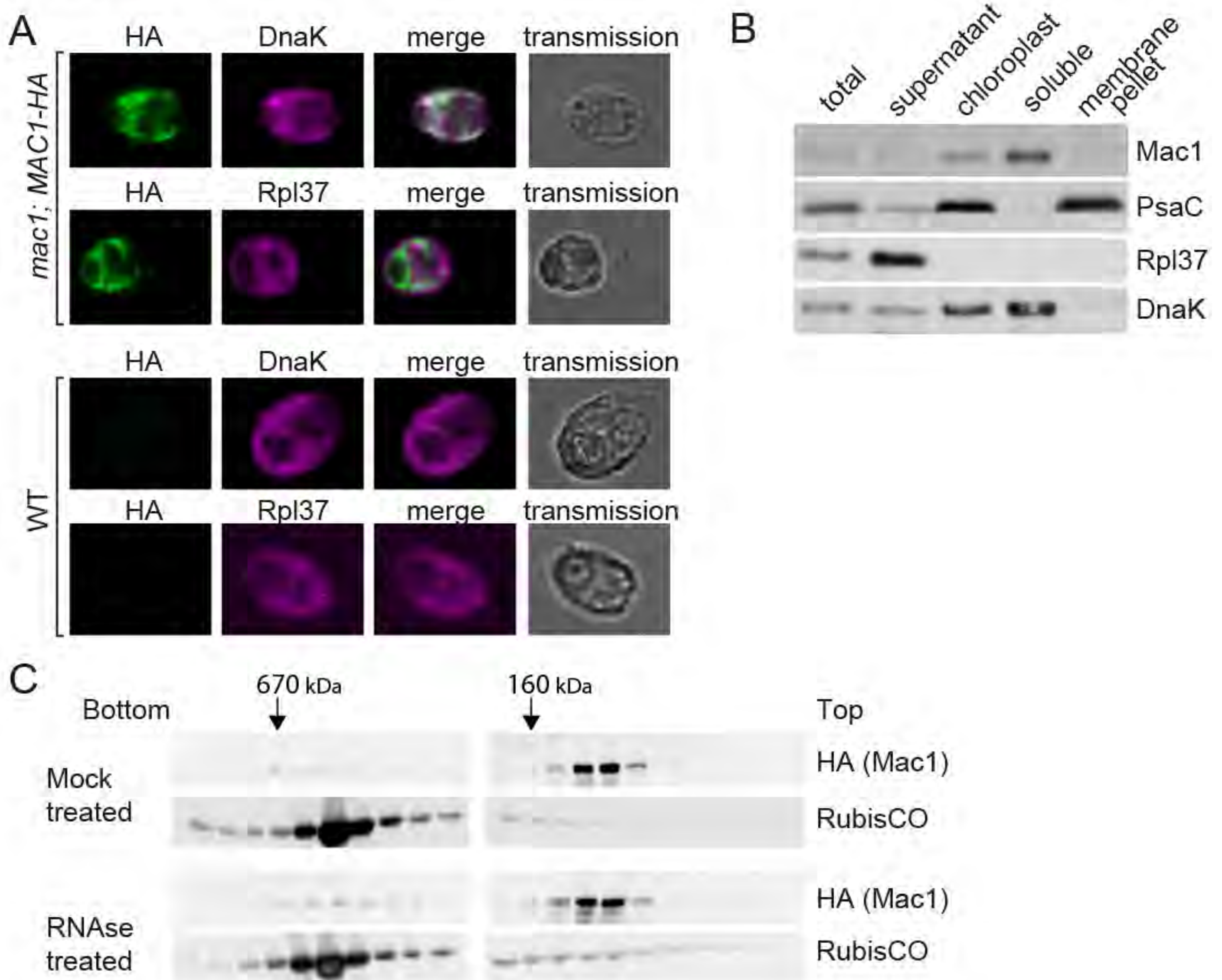


Figure 3. Mac1 localizes to the chloroplast

A. Chlamydomonas *mac1* mutant cells complemented with an HA-epitope tagged *MAC1* gene (*mac1;MAC1-HA*), or wild-type cells as a control (WT), were fixed in methanol to remove chlorophyll. They were then decorated with both a monoclonal mouse antibody against the HA epitope (HA) and a rabbit polyclonal antibody against either the chloroplast protein DnaK or the cytosolic subunit of the ribosome Rpl37. The anti-mouse and anti-rabbit secondary antibodies were labelled with Alexafluor 647 or Alexafluor 546 respectively. Immunofluorescence was observed by confocal microscopy and is shown separately (green and magenta respectively) or as a merged image (green + magenta in white; third panels). An image of the same cells observed by transmission microscopy is also presented (fourth panels).

B. Cells from the cell-wall deficient mutant *cw15* were lysed by nebulization. The lysate (total) was subjected to low-speed centrifugation, the supernatant was collected (supernatant) while the chloroplasts in the pellet were further purified by Percoll gradient centrifugation (chloroplast). The chloroplast were further lysed by sonication and fractionated by high-speed centrifugation into a supernatant fraction (soluble) and a pellet (membrane pellet). Equal amounts of protein from each fraction were subjected to SDS PAGE and immunoblotting with the polyclonal antisera indicated on the right.

C. Sucrose gradient sedimentation analysis of Mac1. An extract of total soluble protein from the *mac1; MAC1-HA* strain was split in two aliquots, one was mock-treated and the other was treated with RNAseA. The protein complexes were then separated by sedimentation in sucrose density gradients. Twenty fractions were collected from the bottom, and analyzed by SDS PAGE and immunoblotting with either anti-HA monoclonal antibody or anti-RubisCO antiserum as indicated. The sedimentation of markers used for calibration in a parallel gradient is indicated at the top (thyroglobulin, 670 kDa; aldolase, 160 kDa). RubisCO holoenzyme has a molecular mass of ~ 550 kDa.

Mac1 is required for *psaC* RNA stability

The absence of detectable *psaC* transcripts in *mac1* could be due to a defect either in transcription or in RNA stability. To distinguish between these two possibilities, transcription of *psaC* was evaluated in run-on transcription assays. Wild-type and *mac1* cells were permeabilized and incubated with radio-labelled α-^{32}P-UTP for 5 or 15 minutes to allow extension of nascent transcripts (Klinkert et al., 2005). Under such conditions, there is no transcription re-initiation and the amount of radiolabel incorporated in nascent transcripts reflects the density of transcribing polymerases on the respective gene (Guertin and Bellemare, 1979; Monod et al., 1992). The radiolabelled RNA was extracted and hybridized to DNA probes spotted on a nylon membrane (Figure 4). There was no significant difference in the radioactive signal for *psaC* between the *mac1* mutant and the wild type. These results indicate that transcription of *psaC* proceeds at normal rates in *mac1* and hence that it is the stability of *psaC* RNA that is compromised in the mutant.

Some helical-repeat proteins are known to stabilize specific chloroplast mRNAs by tightly binding to defined sequences in the 5'UTR or the 3'UTR, and protecting the target transcript against exonucleases (Pfalz et al., 2009; Prikryl et al., 2011). This mechanism can lead to the accumulation of RNA footprints, small RNA fragments that are protected by the respective RNA-binding proteins (Loizeau et al., 2014; Pfalz et al., 2009; Ruwe and Schmitz-Linneweber, 2012; Zhelyazkova et al., 2012). Footprints that map to the 5'end of the *psaC* transcripts could be identified in Chlamydomonas small-RNA databases (Figure 5 and Supplemental Figure 5). This was confirmed by RNA-blot hybridization which detected a small RNA of approximately 50 nt that was present in the wild type, but absent in the *mac1* mutant (Figure 5B, marked with an arrow). This suggests that Mac1 may bind the 5'end of the *psaC* transcripts, or may indirectly promote the binding of a protein to this transcript, two alternatives which are not mutually exclusive.

Mac1 acts through the 5'UTR of *psaC* RNAs

Mbb1, the closest paralog of Mac1 in Chlamydomonas, protects the *psbB* transcript by associating with its 5'UTR (Loizeau et al., 2014; Vaistij et al., 2000b). This precedent and the presence of Mac1-dependent footprints matching the 5' end of *psaC* transcripts suggest that the target of Mac1 may be the 5'UTR of *psaC*. To test this hypothesis, a chimeric reporter gene was constructed (*psaC::lucCP*) with the promoter and 5'UTR of *psaC* fused to the coding sequence of firefly luciferase (Matsuo et al., 2006) followed by the 3'UTR of *rbcL*. For biolistic

Figure 4

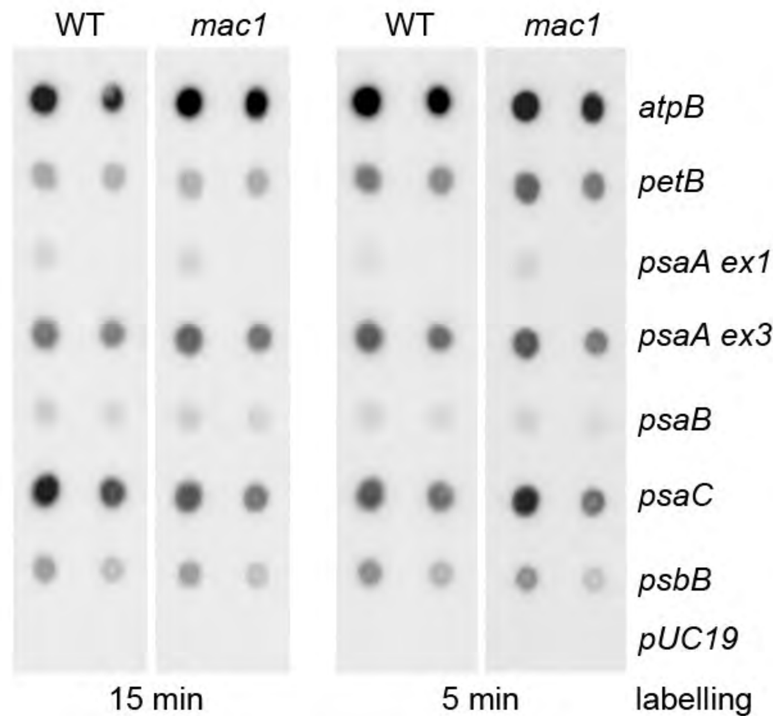


Figure 4. Analysis of *psaC* transcription in the *mac1* mutant

For the run-on transcription assay, duplicate nylon membranes were decorated with spots of PCR fragments derived from the chloroplast genes indicated on the right (0.6 and 0.3 µg DNA of each probe, except a single spot of 0.25µg for *psaA ex1*), or the bacterial plasmid vector *pUC19* as a negative control. Wild-type (WT) or mutant *mac1* cells were permeabilized by freezing and thawing, and then radiolabeled with ^{32}P-UTP for 15 min (left panel) or 5 min (right panel). The labelled RNAs were extracted and hybridized to the membranes (the same pair of membranes was used first with the 5 min samples, and after stripping used again with the 15 min samples). In the run-on transcription assay elongating RNA polymerases insert radiolabel in the nascent transcript, but transcription initiation does not occur. The hybridization signal is thus a measure of the density of transcribing polymerases on the respective gene.

Figure 5

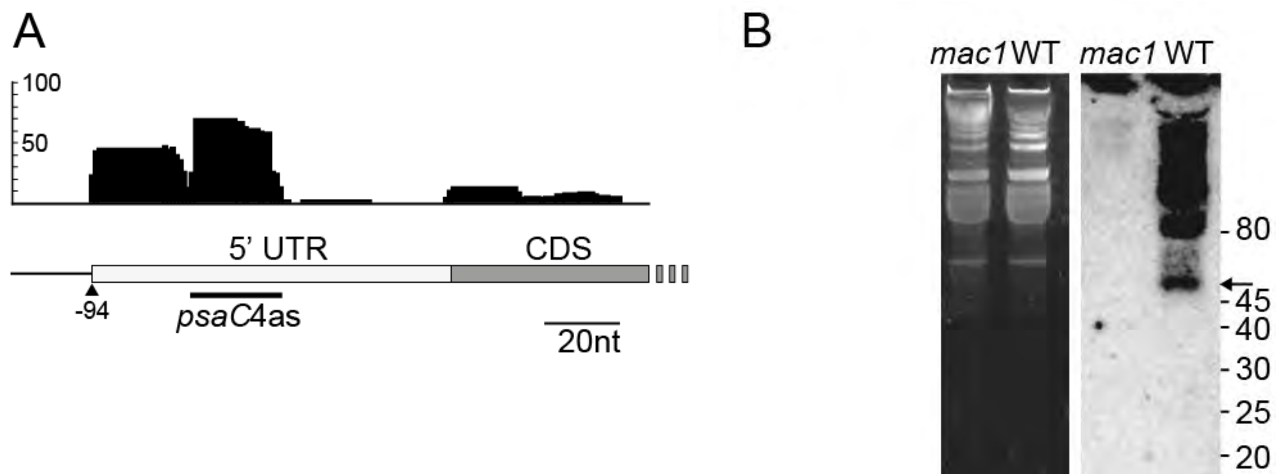


Figure 5. Small RNA footprints in the 5' UTR of *psaC*

A. Small RNA sequence-coverage graph of the *psaC* 5'-region. Per-base read coverage within this region was extracted from small-RNA sequencing databases (Ibrahim et al., 2010; Loizeau et al., 2014). Bars represent the *psaC* 5'UTR and the coding sequence (the actual sequence is provided in Supplemental Figure 5). The arrowhead marks the 5'-end of the *psaC* 5'-UTR. A line labelled *psaC*4as denotes the antisense probe used in panel B.

B. Low-molecular-weight enriched RNA extracted from the *mac1* mutant and the wild type (WT) was subjected to gel electrophoresis (left panel, ethidium bromide fluorescence image), transferred to nylon membranes and hybridized with the radiolabeled anti-sense probe. The arrow marks a small RNA of approximately 50 bases that is present in the wild type but not in the *mac1* mutant.

chloroplast transformation, the chimeric luciferase reporter was introduced in the atpB-INT vector (Michelet et al., 2011), which carries a modified *atpB* gene as selectable marker, allowing selection of photoautotrophic transformants in a *ΔatpB* mutant host (Figure 6). The strain with the *psaC::lucCP* reporter was then crossed to the *mac1* mutant, so that sibling progeny with either the wild-type or the *mac1* nuclear genome were recovered for analysis. Luciferase activity in the *mac1* mutant (*mac1/psaC::lucCP*) was at background level, and thus at least 30-fold lower than in the wild type (Figure 6B). Luciferase activity was restored to wild-type levels by transforming the *mac1/psaC::lucCP* progeny with the wild-type *MAC1* gene (*mac1;MAC1/psaC::lucCP*). The *mac1* mutation did not significantly affect the expression of a control reporter with the *psaB* promoter and 5'UTR (*mac1/psaB::lucCP*). Luciferase expression with the *psaB* construct was much stronger than with the *psaC* construct, but such differences between chimeric genes expressed in the chloroplast are commonly observed in Chlamydomonas (Michelet et al., 2011). The results indicate that a genetic target of Mac1 is found in the promoter or 5'UTR of *psaC*. Since the *mac1* mutation affects *psaC* transcript stability, it is most likely that the target of Mac1 is in fact in the 5'UTR rather than the promoter.

Mac1 binds RNA in vitro.

To investigate the RNA-binding properties of Mac1, a recombinant protein corresponding to the full sequence (except for the predicted transit peptide) was expressed in *Escherichia coli*, with a tag of six histidines at the C-terminus. After nickel-affinity chromatography, the preparation still contained contaminants, some of which were found to also bind RNA in preliminary experiments. Therefore the proteins were further separated by gel filtration chromatography (Figure 7). In the eluted fractions, recombinant Mac1 protein was identified by SDS-PAGE by its expected size (96 kDa; Figure 7A) and its recognition by Mac1 anti-serum in immunoblots (Figure 7B). Mac1 peaked in the fraction expected for a monomer of its size (lane 8). To assay RNA binding, a radiolabeled probe was prepared corresponding to 51 nucleotides at the 5'end of *psaC* mRNA, matching the footprint observed in vivo. For the electro-mobility shift assay (EMSA), aliquots of each protein fraction eluted from the gel filtration column were mixed with the radiolabeled probe, and the RNA-protein complexes that formed were separated from the unbound probe by non-denaturing gel electrophoresis. The major RNA-protein complex that formed matched exactly the elution profile of Mac1 protein, peaking in lane 8 (B_2, Figure 7C). Minor complexes (B_1 and B_3, Figure 7C) with other proteins were clearly separated from Mac1, peaking in fractions 5 and 12 respectively. Mac1 bound the

Figure 6

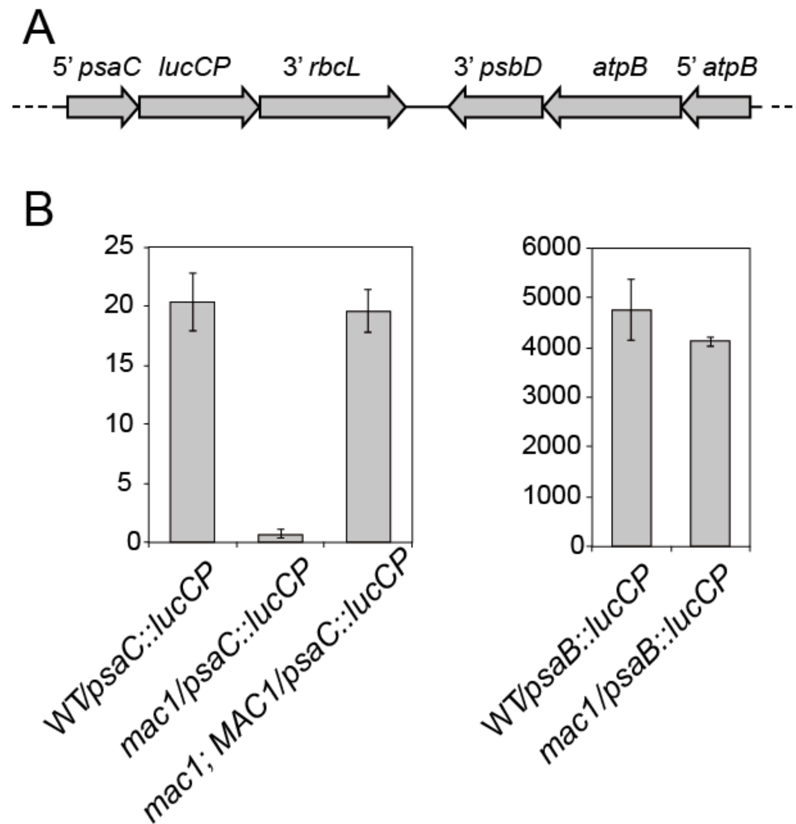


Figure 6. The 5'UTR of *psaC* contains a target of *MAC1*

A. Schematic map of the chimeric *psaC::lucCP::rbcL* reporter inserted in the chloroplast genome next to the *atpB* gene which served as a selection marker for transformation of a *ΔatpB* host strain. In this construct the *psaC* promoter and 5'UTR are fused to a codon-optimized coding sequence of the firefly luciferase, followed by the 3'UTR of *rbcL*. A similar construct where the *psaB* promoter and 5'UTR replace those of *psaC* was used as a control. In the atpB-INT transformation vector that was used, the 3'UTR of *atpB* has been replaced with the 3'UTR of *psbD* (Michelet et al., 2011).

B. Luciferase assays of the Chlamydomonas strains indicated at the bottom. The nuclear genotype is indicated first, followed by a slash (/) and the chloroplast genotype. Nuclear genotypes: WT, wild type; *mac1*, *mac1* mutant line; *mac1;MAC1*, *mac1* mutant line complemented with the *MAC1* gene. Chloroplast genotypes: *psaC::lucCP*, chimeric *psaC::lucCP::rbcL* reporter; *psaB::lucCP*, chimeric *psaB::lucCP::rbcL* reporter. Three independent lines of each genotype were assayed in three technical replicates (luminescence is indicated with arbitrary units), error bars represent the standard error of the three lines.

RNA probe with high affinity. The estimated Kd was 40-60 nM for two independent preparations, comparable to the Kd observed in vitro for HCF107 (70 nM) (Hammani et al., 2012)

Regulation of Mac1 in response to iron limitation

In Chlamydomonas, early responses to iron limitation under mixotrophic conditions involve the dismantling of the photosynthetic electron transfer chain, and in particular of PSI and the cytochrome b_6f complex (Hohner et al., 2013; Moseley et al., 2002). PsaC is the subunit of PSI that directly binds two of its three 4Fe4S iron-sulfur centers, F_A and F_B. To investigate whether the response to iron limitation also involves the regulation of Mac1 protein accumulation, *mac1/Mac1-HA* cultures were grown through ten division cycles in acetate-containing medium at three different iron concentrations: 20 µM Fe (iron replete), 1 µM Fe (limited) and 0.2 µM Fe (deficient) (Glaesener et al., 2013). As expected, in the iron-deficient culture (0.2 µM Fe) PSI decreased approximately four-fold as estimated from the reduced accumulation of PsaA and PsaC compared to a dilution series of proteins from the iron-replete culture (Figure 8A, Supplemental Figure 6). There was a concomitant approximately 50 % decrease in the abundance of Mac1-HA (Supplemental Figure 6 D). Similar results were obtained with a wild-type strain using the Mac1 antibody (Figure 8B). However the accumulation of ATP synthase was not reduced under iron limitation (Figure 8A, CF1 antiserum), confirming previous observations that the response to iron starvation is not a general dismantling of the thylakoid membrane (Hohner et al., 2013; Moseley et al., 2002). Under the same conditions, the abundance of the *psaC* transcripts decreased approximately 30 % as estimated from a comparison to a dilution series of RNA from iron-replete conditions (Figure 8 C, Supplemental Figure 6 B). The amounts of *atpB* mRNA remained unchanged, indicating that the response is not a general degradation of chloroplast mRNA. The lack of PsaC protein in the *ΔpsaC* mutant (Takahashi et al., 1991) did not affect the accumulation of Mac1 (Figure 2 B), suggesting that the decrease of Mac1 in iron-deficient conditions is not a consequence of the reduced levels of PsaC.

Under iron deficiency, we observed a concomitant decrease in Mac1-HA and *psaC* mRNA accumulation, with the former more pronounced than the latter (Supplemental Figure 6 D,F). The question thus arises, whether the amount of Mac1 could be limiting for *psaC* expression. To investigate this point, different transformants of *mac1* with the *MAC1::HA* construct, expressing different levels of Mac1-HA, were compared under iron-replete conditions (Figure

Figure 7

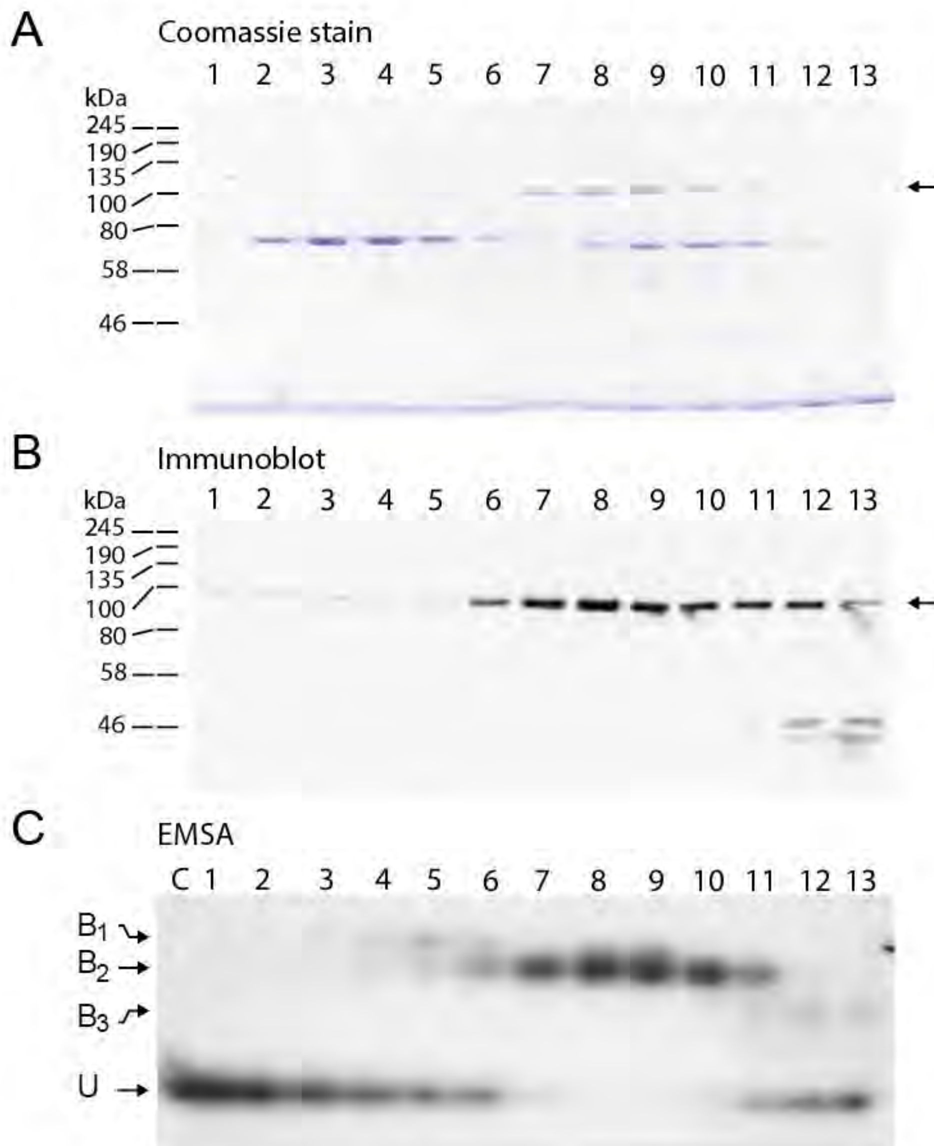


Figure 7. Mac1 binds RNA in vitro

A. Following Ni-NTA affinity chromatography, Mac1 was further purified by gel filtration chromatography. Selected fractions were analyzed by SDS PAGE and staining with Coomassie R250. The band corresponding to Mac1 is indicated with an arrow.

B. The same fractions as in panel A were analyzed by SDS PAGE and immunoblotting with Mac1 antibody.

C. The same fractions as in panels A and B were used for Electro Mobility Shift Assays with a radiolabelled RNA probe corresponding to the first 51 nucleotides from the 5'UTR of *psaC* mRNA. The positions of three RNA-protein complexes (B_1, B_2, B_3) and the unbound RNA (U) are indicated on the left. The peak fractions that form the major complex (B_2) coincide with the peak of elution of Mac1 centered on lane 8. The free probe was run alongside in lane C.

9). In strains that expressed different levels of Mac1-HA, the accumulation of the *psaC* transcripts paralleled the amounts of Mac1-HA (Figure 9 A). However the amount of PsaC protein in the different transformants remained constant and similar to the wild type (Figure 9 B). These observations suggested that Mac1-HA may be limiting for *psaC* mRNA accumulation, but that increased levels of *psaC* mRNA do not lead to increased accumulation of PsaC. This could be due to translational or post-translational regulation of PsaC amounts.

Phosphorylation of Mac1

Phosphorylation is a post-translational modification that is involved in the regulation of numerous proteins. To analyze whether Mac1 could be subject to protein phosphorylation, protein extracts of *mac1*;*MAC1-HA* were analyzed using for electrophoresis in polyacrylamide gels containing a Phos-tag™ gradient (Phos-tag PAGE, Supplemental Figure 7). When chelated with a divalent cation such as Zn^{2+}, Phos-tag™ binds phosphate groups and retards the migration of phosphorylated polypeptides (Kinoshita and Kinoshita-Kikuta, 2011; Kinoshita et al., 2006; Longoni et al., 2015). Immunoblotting with monoclonal anti-HA antibody revealed two bands for Mac1, suggesting that the upper one (P_1) could represent phosphorylated Mac1 (Figure 10). Treatment of the sample with λ protein-phosphatase led to the disappearance of the slowly migrating band, confirming that it corresponds to a phosphorylated form of Mac1. As a control, a strain expressing HA-tagged Sedoheptulose Bis Phosphatase (Sbp-HA) (Loizeau et al., 2014) was analyzed in parallel and there was no evidence that this protein was phosphorylated.

The availability of iron had a strong impact on the phosphorylation of Mac1 (Figure 10 B). In cells grown under iron-replete mixotrophic conditions, Mac1 was strongly phosphorylated. After growth under conditions of iron limitation, the ratio of phosphorylated ($P_{1)}$ to non-phosphorylated Mac1(U) decreased, and was even lower under iron depletion. As already observed in the previous section (Figure 8), the total amount of Mac1-HA concomitantly decreased under iron depletion compared to iron sufficiency. To control that the lower apparent ratio of phosphorylation, which paralleled the reduction in total amount of Mac1, was not due to an artefact of Phos-tag PAGE and immunoblotting, a dilution series of the sample from iron-replete condition was similarly analyzed (Figure 10 C). The ratio of the phosphorylated band to the unphosphorylated form did not change significantly at lower total protein concentrations, thus consolidating the validity of the observation that phosphorylation of Mac1-HA is lower under iron limitation.

Figure 8

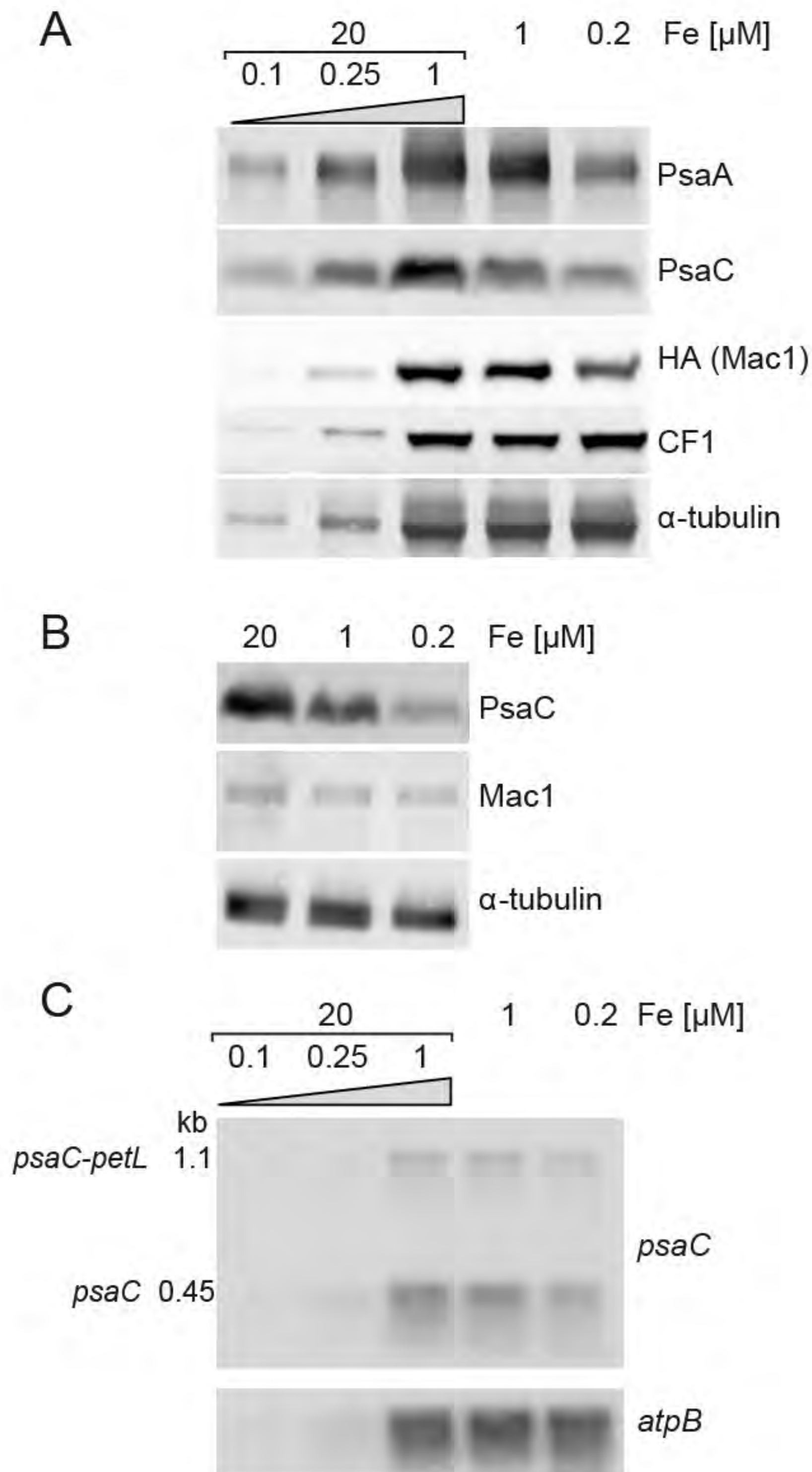


Figure 8. Mac1 and PsaC levels coordinately respond to iron availability

A. Cultures of the *mac1* mutant complemented with an HA-epitope tagged *MAC1* gene (*mac1;MAC1-HA*) were grown mixotrophically (acetate-containing medium in the light) through ten doublings in the presence of different concentrations of Fe: 20 µM, 1 µM or 0.2 µM. Total cell extracts were analyzed by SDS PAGE and immunoblotting with the antisera indicated on the right. A dilution series of the iron-replete sample (20 µM) is presented in the first three lanes.

B. Wild-type cells were grown and analyzed as in panel A, Mac1 was detected with the polyclonal antiserum (see Figure 2C).

C. RNA was extracted from the same wild-type cultures as in panel B and subjected to denaturing gel electrophoresis and blot hybridization with radiolabeled probes for *psaC* (upper panel) or *atpB* as a control (lower panel).

Phosphorylation of Light Harvesting Complex II (LHCII) subunits in the thylakoid membrane by the kinase Stt7 is regulated by the redox state of the electron transfer chain (Lemeille and Rochaix, 2010). Under anaerobic conditions in the dark, which lead to a reduction of the PQ pool (state 2), Mac1-HA was largely phosphorylated (Figure 10 D, lane 2). The two slowly migrating bands (labelled P_1 and P_2) could represent different degrees of phosphorylation of Mac1. Conversely under aerobic conditions in low light, which favor PQ oxidation (state 1), Mac1 was partly un-phosphorylated (Figure 10 D, lane 1, band labelled U). To determine whether this phosphorylation depends on the Stt7 kinase, the *MAC1::HA* construct was transformed into the *stt7-7* mutant. In this strain, the phosphorylation patterns of Mac1-HA in state1 and state 2 conditions were the same as in the wild type *MAC1::HA* strain, indicating that Stt7 is not involved in the phosphorylation of Mac1 (Figure 10 D, lanes 3 and 4).
Proteomic surveys of Chlamydomonas protein phosphorylation have indicated that Mac1 can be phosphorylated at serines 137 and 139 (Wang et al., 2014). To determine whether these are the residues that account for the phosphorylation of Mac1 detected by Phos-tag™ electrophoresis, a mutant version of *MAC1-HA* where both serines 137 and 139 are replaced by alanines was constructed, *MAC1-AA-HA*. The *mac1* mutant strain transformed with *MAC1-AA-HA* grew normally on minimal medium, indicating that the modified protein is functional. The migration of phosphorylated Mac1-AA-HA in Phos-tag™ gels was unaffected compared to wild-type Mac1-HA in state1 or state 2 conditions (Figure 10 D, lanes 5 and 6), indicating that the major sites of phosphorylation that can be detected in this way are not serines 137 or 139.

DISCUSSION

Mac1 controls the stability of *psaC* mRNA

Mac1 was identified through the analysis of a non-photosynthetic mutant deficient for PSI. The primary defect in *mac1* is its failure to accumulate *psaC* mRNA (Figure 1 C, Figure 2 A), and the PsaC subunit (Figure 2 B). This has ensuing consequences on the accumulation of PsaA (Figure 1 B) and presumably other PSI subunits, which are known to be destabilized in the absence of PsaC (Takahashi et al., 1991). Mac1 localizes to the chloroplast, where it is found in the soluble, stromal fraction (Figure 3).
In the chloroplast genome of *Chlamydomonas reinhardtii*, the *psaC* gene is transcribed as a polycistronic unit which also contains the downstream *petL* gene (Takahashi et al., 1994). In

Figure 9

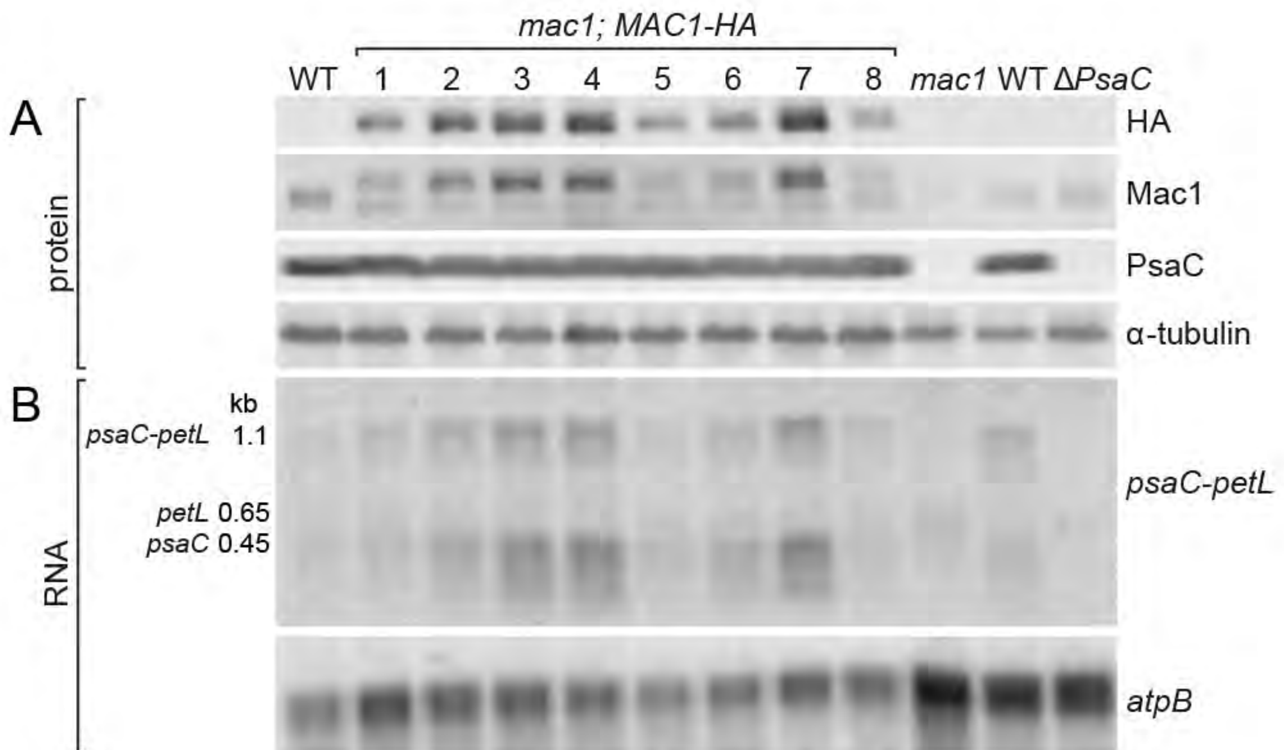


Figure 9. *psaC* RNA accumulation correlates with Mac1 levels

A. The wild type (WT) and a series of independent *mac1* mutant lines complemented with *MAC1-HA* (*mac1; MAC1-HA* # 1 to 8) were grown in iron-replete mixotrophic conditions under normal light (60 µmol m^{-2} sec^{-1}). For comparison *mac1*, the wild type and the *ΔpsaC* mutant (with an insertion disrupting the *psaC* gene) are also shown, grown in low light (6 µmol m^{-2} sec^{-1}) because PSI-deficient mutants are light-sensitive. Total proteins were extracted and analyzed by SDS PAGE and immunoblotting with the antisera indicated on the right.

B. Total RNA was extracted from the same cultures as in panel A, and analyzed by denaturing gel electrophoresis and blot hybridization with radiolabelled probes containing *psaC* and *petL* (upper panel), or *atpB* as a control (lower panel).

the *mac1* mutant, the accumulation of the monocistronic *psaC* mRNA and the dicistronic *psaC-petL* transcript are specifically affected, while the amount of monocistronic *petL* mRNA is slightly elevated. This suggests that the *mac1* mutation affects the stability rather than the transcription of *psaC*. Indeed, in a run-on transcription assay the activity of *psaC* appears comparable in the mutant and in the wild type (Figure 4). Furthermore a fragment containing the promoter and 5'UTR of *psaC* is sufficient to confer dependence on *MAC1* to a chimeric *psaC-lucCP* luciferase reporter (Figure 6). Although an accessory effect on transcription cannot be ruled out, taken together these results indicate that Mac1 is involved, directly or indirectly, in stabilizing transcripts containing *psaC* through its 5'UTR. This interpretation is supported by the identification in existing databases of small RNAs (sRNA) corresponding to the 5' end of the *psaC* transcripts. Using RNA blot hybridization, the existence of a sRNA from *psaC* (ca. 50 nt) could be confirmed *in vivo* and was shown to depend on the presence of Mac1 (Figure 5). Such sRNA footprints were first identified in plant chloroplasts at the position of RNA-binding proteins such as PPR10, which protect the bound RNA against exonucleolytic degradation from both sides (Pfalz et al., 2009; Prikryl et al., 2011). Likewise in Chlamydomonas, small RNAs at the 5'end of *psbB* and *psbH* are formed in the presence of Mbb1, a nucleus-encoded protein which is required for the stable accumulation of the two mRNAs (Loizeau et al., 2014) and is the closest paralog of Mac1 (Supplemental Figure 3). Interestingly HCF107, the orthologue of Mbb1 in Arabidopsis and maize, can bind *psbH* RNA *in vitro* and forms a sRNA footprint *in vivo* (Hammani et al., 2012). Mac1 is only found in Chlorophyta, whereas in maize the PPR protein CRP1 binds the 5'UTR of *psaC*, where it generates a sRNA footprint, and is required for its efficient translation (Fisk et al., 1999; Ruwe and Schmitz-Linneweber, 2012; Schmitz-Linneweber et al., 2005b).

Mac1 contains two domains with tandem repeats of 34 amino-acid residues that belong to the HAT / TPR family. Members of the HAT repeat proteins, which in turn belong to the helical-repeat superfamily, are involved in RNA interactions (Preker and Keller, 1998). Indeed, Mac1 protein expressed in bacteria binds RNA with high affinity in vitro (Figure 7). As mentioned above, HCF107 also belongs to this family and binds RNA *in vitro* with similar affinity (Hammani et al., 2012). In the pentatricopeptide repeat proteins (PPR), which form the most prevalent helical-repeat protein family in plant organelles (Barkan and Small, 2014), the 35 amino-acid repeats are composed of two anti-parallel α-helices which stack onto one another to form a superhelical backbone (Ke et al., 2013; Yin et al., 2013). Each modular repeat of a PPR protein interacts with one nucleotide of the bound RNA through specific residues that determine the

Figure 10

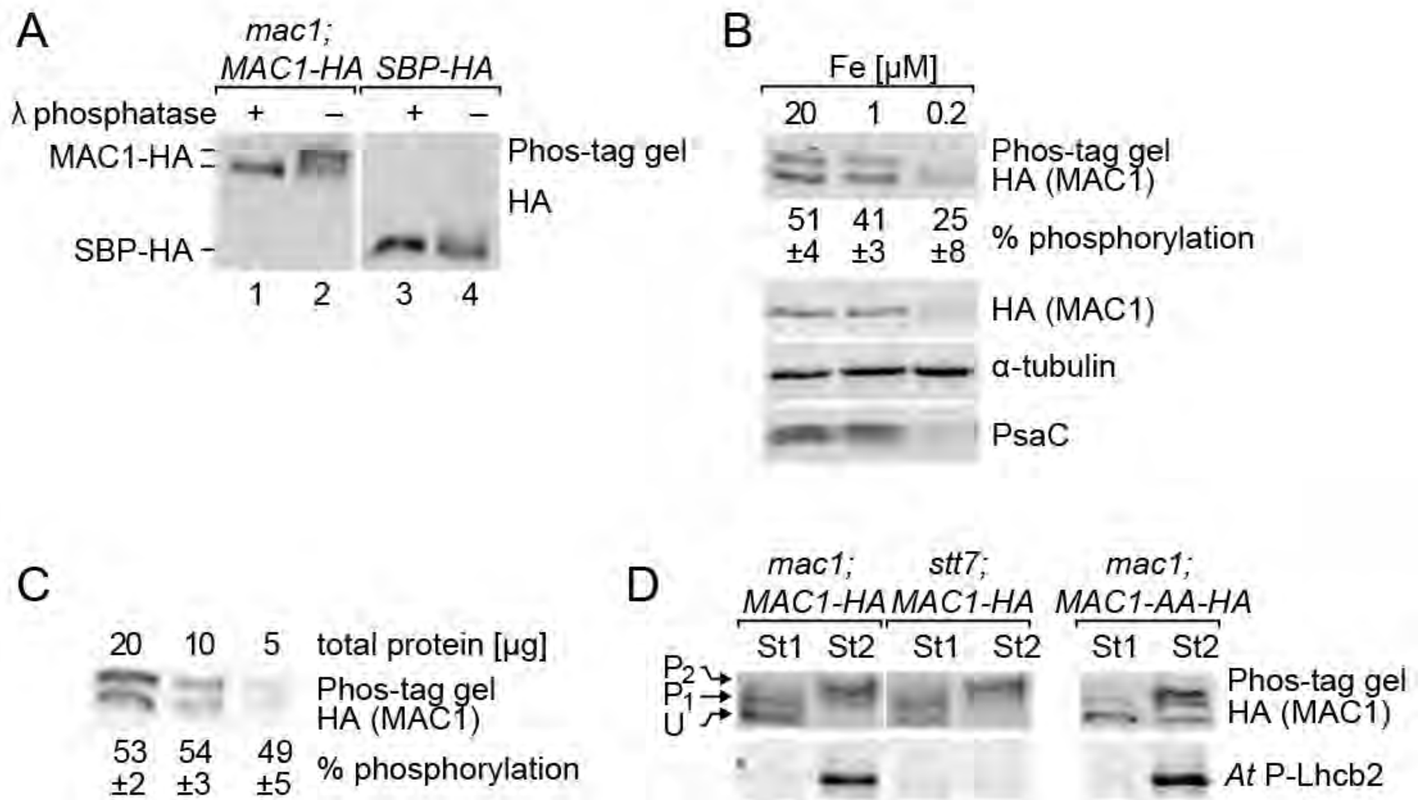


Figure 10. Mac1 is phosphorylated in response to environmental conditions

A. Protein extracts of *mac1;MAC1-HA* (the *mac1* mutant complemented with an HA-epitope tagged *MAC1* gene) grown mixotrophically were analyzed by electrophoresis in polyacrylamide gels containing a gradient of Phos-tag™. Prior to loading on the gels, the samples were incubated in the presence (+) or absence (-) of λ protein phosphatase as indicated at the top (lanes 1 and 2). For comparison, protein extracts of *SBP-HA* were analyzed after incubation with (+) or without (-) λ protein phosphatase (lanes 3 and 4).

B. Top panel: cultures of *mac1;MAC1-HA* were grown mixotrophically in the presence of different initial concentrations of Fe as described for Figure 8A. Protein extracts were analyzed by Phos-tag™ gel electrophoresis and immunoblotting with HA antibodies. The extent of phosphorylation (estimated as the intensity of the upper band divided by the sum of the intensities of both bands) is shown below each lane as a percentage (± SD, n=3).

Lower panels: the same samples were analyzed by SDS-PAGE in normal gels and immunoblotting with the antisera indicated on the right.

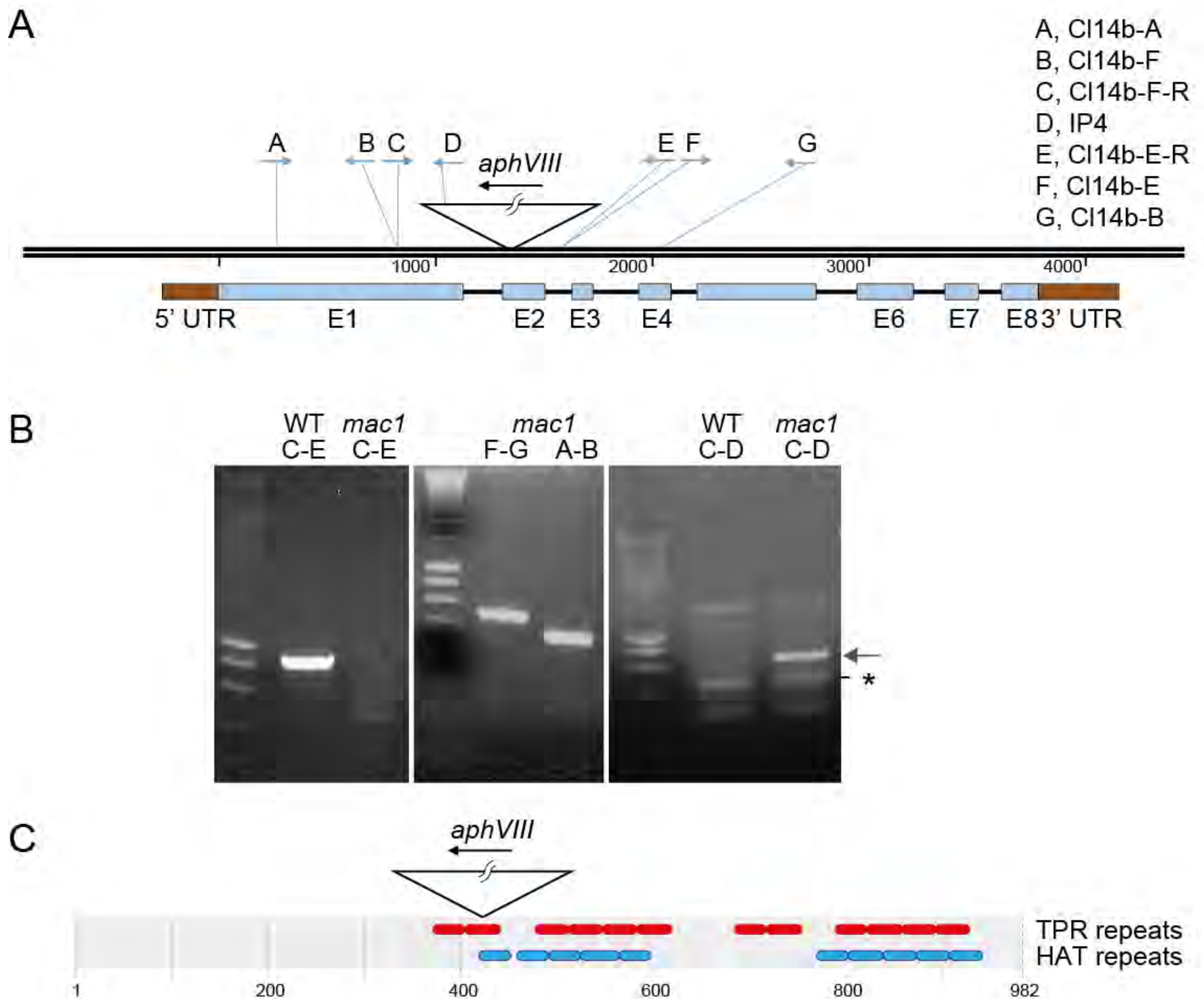
C. Three different amounts of total protein from cultures with 20 μM Fe were analyzed by Phos-tag™ gel electrophoresis and immunoblotting with HA antibodies, and phosphorylation was calculated as in panel B. The estimated phosphorylation is approximately constant over the 4-fold range of total protein, indicating that in panel B the decreased phosphorylation of Mac1 observed with the 0.2 μM culture is not an artefact due to the lower amount of Mac1.

D. Cultures of *mac1;MAC1-HA*, of *stt7-7;MAC1-HA* (the *stt7-7* kinase mutant transformed with *MAC1-HA*) or of *mac1;MAC1-AA-HA* (with serines 137 and 139 changed to alanines) were treated under conditions that favor oxidation of the plastoquinone pool (state 1, 10 μM DCMU in the light; lanes 1, 3 and 5) or its reduction (state 2, anaerobiosis in the dark; lanes 2, 4 and 6). Protein extracts were analyzed by Phos-tag™ gel electrophoresis and immunoblotting with HA antibodies (upper panel), or by SDS-PAGE in normal gels and immunoblotting with phospho-specific antiserum against phosphorylated Lhcb2 of Arabidopsis (*At* P-Lhcb2, lower panel). That the conditions were effective in promoting state 1 or state 2 is shown by the widely different levels of phosphorylation of LHCII. The exposure shown for *stt7-7;MAC1-HA* is longer than for *mac1;MAC1-HA* because of different levels of Mac1-HA expression in the two strains.

recognition of the target base, allowing the definition of a “PPR code”. The HAT repeats also form a superhelical scaffold, and it can be predicted that TPR / HAT domains of Mac1 will bind RNA in a similar way (Bai et al., 2007; Hammani et al., 2014). In the Chlamydomonas sRNA databases that we interrogated (Figure 5), a series of sRNAs corresponding to the 5’end of *psaC* delineate a relatively long footprint (ca. 50 nt) which is roughly the size of the sRNA detected *in vivo*, and two subpopulations of shorter small RNAs map to the same region. There are two TRP/HAT domains in Mac1 (Supplemental Figure 1C), each of which consists of six or seven TRP repeats and is thus expected by analogy with PPR proteins to bind 6-7 bases in the RNA (Barkan and Small, 2014). The relatively large size of the protected RNA fragment could be due to binding of two adjacent sites by the two TPR/HAT domains of Mac1 with a possible intervening RNA loop, such as was proposed for the binding of CRP1 to the *petB-petD* site in maize (Barkan et al., 2012). Alternatively, it is also possible that the large footprint is generated by one or more other partner proteins in a Mac1-dependent manner. It is conceivable that the minor fraction of Mac1 that sediments with large complexes in sucrose gradients could reflect its transient or labile association with other partners.

In Chlamydomonas there are several examples of nucleus-encoded proteins that bind the 5’end of their respective target transcripts and offer protection against 5’ to 3’ exonucleolytic degradation. Apart from Mbb1, the closest paralog of Mac1 mentioned above, these also include the TPR/HAT protein Nac2 which is required to stabilize *psbD* RNA (Kuchka et al., 1989; Nickelsen et al., 1999). Other examples are provided by members of the OPR (Octotrico Peptide Repeat) family of helical-repeat proteins such as Taa1 and Tab1, which are required for the stability and translation of *psaA* and *psaB* respectively, and Mbi1, which is necessary for the stable accumulation of *psbI* mRNA (Lefebvre-Legendre et al., 2015; Wang et al., 2015). Also of interest is another OPR protein, Mcg1, which is required for stabilization of *petG* mRNA and generates a corresponding sRNA footprint at its 5’end (Wang et al., 2015). There are also examples amongst the relatively few PPR proteins of Chlamydomonas: Mca1 binds the 5’UTR of *petA*, hinders its 5’ to 3’ exonucleolytic degradation and, in association with Tca1, promotes its translation (Loiselay et al., 2008), while Mrl1 ensures the stability of *rbcL* mRNA (Johnson et al., 2010). The properties of Mac1 are consistent with the model that emerges from these comparisons: Mac1 could bind the 5’UTR of *psaC* and protect the downstream transcripts from exonucleolytic degradation. The combined action of 5’ and 3’ exonucleases would eventually generate a sRNA footprint. It remains an open question whether Mac1 is also involved in translation.

Supplemental Figure 1



Supplemental Figure 1. Identification of the *MAC1* gene

A. Map of the *MAC1* gene and the *mac1* insertion. The exons of the *MAC1* gene are represented as bars, with the 5' and 3' untranslated regions (UTR) in brown and the coding sequence in blue. The site of insertion in exon 2 of the *aphVIII* cassette that was used for insertional mutagenesis is shown above. Primers used for genotyping are shown as small blue arrows (A-G). The primers are listed on the right and their sequences are provided in Supplemental Table 1.

B. PCR genotyping of the *mac1* mutant. The PCR primers (A-G) used for genotyping are shown above the map in panel A, and their names are listed on the right (for their sequences see Supplemental Table 1). The left panel shows the absence of wild-type exon 2 in the *mac1* mutant, indicating that no intact copy of *MAC1* is present in the mutant. The middle panel shows that PCR fragments on either side of the insertion can be amplified from the *mac1* mutant, indicating that the insertion of *aphVIII* was not accompanied by a major deletion. The right panel shows a PCR with one primer in the *MAC1* gene and the other in the *aphVIII* insertion. The band specific to *mac1* (arrow) was sequenced and confirmed the insertion in exon 2. The asterisk marks a non-specific band.

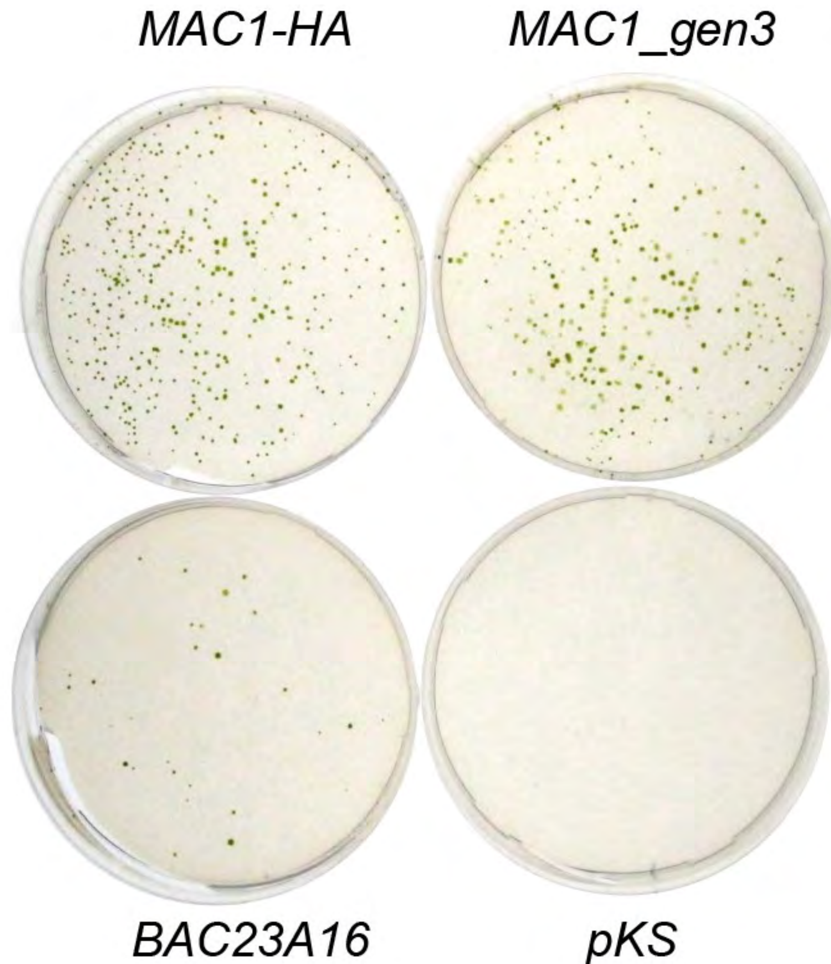
C. Domain structure of the predicted Mac1 protein. The protein domains were predicted using the InterPro database (http://www.ebi.ac.uk/Tools/pfa/iprscan5/). TetratricoPeptide Repeats are shown in red (TPR), Half-A-Tetratricopeptide (HAT) repeats are shown in blue.

Mac1 is down-regulated in response to iron deficiency

The low availability of iron can be a severe limitation for the growth of photosynthetic organisms, be it in aqueous environments or on land. Both the mitochondrial respiratory chain and the photosynthetic electron chain comprise proteins that contain iron, heme and irons-sulfur clusters as cofactors. In Chlamydomonas grown in photoheterotrophic conditions, where both respiration and photosynthesis are normally active, iron deficiency leads to the preferential allocation of the metal to mitochondrial respiration at the expense of photosynthesis (Moseley et al., 2002; Terauchi et al., 2010; Urzica et al., 2012). PSI, which contains three 4Fe4S centers, is an early target of this response: it is disconnected from its light-harvesting antenna and rapidly dismantled, like other complexes of the photosynthetic electron chain, while ATP synthase remains more stable (Moseley et al., 2002; Naumann et al., 2005). The iron that is released is bound by ferritin, which is upregulated at the level of translation (Busch et al., 2008). The response to iron deficiency also involves changes in the abundance of numerous other proteins, and in particular of Taa1 which is degraded (Hohner et al., 2013; Lefebvre-Legendre et al., 2015). As previously mentioned, the latter is a nucleus-encoded protein of the OPR family that is specifically required for the stability and translation of *psaA* mRNA in the chloroplast. There is thus a clear parallel with Mac1 which controls *psaC* mRNA stability and is down-regulated under iron deficiency. Hence the response to iron limitation involves not only the degradation of the PSI complex, but also the down-regulation of nucleus-encoded factors that control the expression of chloroplast-encoded PSI subunits. As was observed with Taa1, the decrease in Mac1 abundance is most likely regulated at the post-transcriptional level, since data from high-throughput RNA sequencing indicate that under iron limitation there is a moderate increase in *MAC1* RNA levels ((Urzica et al., 2013) Phytozome v11 at https://phytozome.jgi.doe.gov/). It is interesting to compare this response to a similar response that occurs under nitrogen limitation in Chlamydomonas, with the down-regulation of the cytochrome b_6f complex. This response involves the coordinate degradation of the b_6f complex itself, of proteins that control the expression of its chloroplast genes, and of proteins involved in its biogenesis (Raynaud et al., 2007; Wei et al., 2014).

In response to iron deficiency, there is a correlation between the reduced accumulation of PSI subunits and of factors that govern their expression. This correlation raises the question whether the decrease of the nucleus-encoded factors has a causal role in the decrease of the chloroplast-encoded proteins. Alternatively, it cannot be excluded that the nucleus-encoded factors and the PSI subunits could be responding independently to the same nutritional cues.

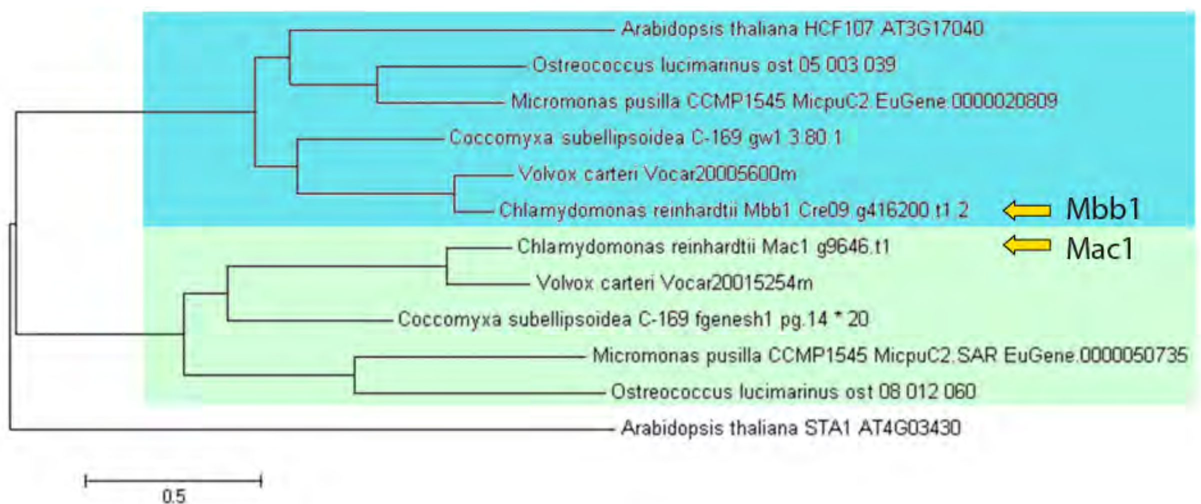
Supplemental Figure 2



Supplemental Figure 2. Complementation of the *mac1* mutant

The *mac1* mutant was transformed with the BAC clone *BAC23A16*, its subclone *MAC1_gen3*, a derivative of the latter with an HA epitope tag, *MAC1-HA*, or the empty plasmid vector pBluescritp KS+ (*pKS*). The transformants were selected for photoautotrophic growth on minimal medium (HSM) under 60 µmol m^{-2} sec^{-1} white light.

Supplemental Figure 3



Supplemental Figure 3. Phylogenetic analysis of Mac1

A maximum likelihood tree was derived using the MEGA6 sofware with 500 boostraps (Tamura et al., 2013). Mac1 and Mbb1, its closest paralog in *Chlamydomonas reinhardtii*, form two separate phylogenetic clades. Mac1 has no clear orthologue in plants, while Mbb1 is orthologous to HCF107 of *Arabidopsis thaliana*.

In other words, the question is whether Mac1 truly exerts anterograde regulation on *psaC* expression in response to iron availability. As a step towards answering this question, we sought to determine whether the amounts of Mac1 can be limiting for the accumulation of *psaC* mRNA and PsaC protein. In an allelic series of transformants over-expressing different levels of Mac1-HA, we observed that the accumulation of *psaC* mRNA parallels that of Mac1-HA (Figure 9). Thus the amounts of Mac1 do seem to be limiting for the accumulation of *psaC* mRNA. However the amounts of PsaC protein were similar in all the strains of the allelic series. This may be due to the negative feedback control that is exerted by unassembled PsaC on translation of its own mRNA (Control by Epistasy of Synthesis, CES) (Wostrikoff et al., 2004). In the Mac1-HA over-expressors, any excess of PsaC that cannot be assembled with PsaA and PsaB is expected to inhibit its own translation and eventually to be degraded (Choquet and Vallon, 2000). Thus the synthesis of PsaC would not be affected by increases in the amount of *psaC* mRNA above what actually is required for translation (Hosler et al., 1989).

Mac1 is differentially phosphorylated in response to environmental changes

Protein phosphorylation is a prevalent post-translational modification that plays an important role in the regulation of numerous processes in biology. Hundreds of phosphoproteins that are known or predicted to localize to the chloroplast have been identified in large-scale proteomic studies, both in Chlamydomonas and in higher plants (Lohrig et al., 2009; Reiland et al., 2009; Wang et al., 2014). In plastids, protein phosphorylation plays regulatory roles in photosynthesis, gene expression and metabolism (Baginsky and Gruissem, 2004). Thylakoid proteins such as Light Harvesting Complex II (LHCII) and Photosystem II (PSII) are some of the most abundant chloroplast phosphoproteins. The phosphorylation of LHCII, which is largely dependent on the protein kinase Stt7 in Chlamydomonas and STN7 in plants, plays a role in a regulatory response to changing light quality and to metabolic demands of the cell known as state transition (Depege et al., 2003). Reversible phosphorylation regulates the dynamic allocation of LHCII to PSI or PSII. The phosphorylation of the PSII core subunits by the protein kinase STN8, a paralog of STN7, plays a role in the organization of the thylakoid membranes and in the repair cycle of photo-damaged PSII in Arabidopsis (Bonardi et al., 2005; Fristedt et al., 2009; Tikkanen et al., 2008).

Focusing on the role of protein phosphorylation for gene expression in the chloroplast of higher plants, there is evidence for regulation both at the level of transcription and at post-transcriptional steps. The chloroplast sensor kinase (CSK), which is related to bacterial two-

Supplemental Figure 4

HA DnaK merge transmission

HA Rpl4 merge transmission

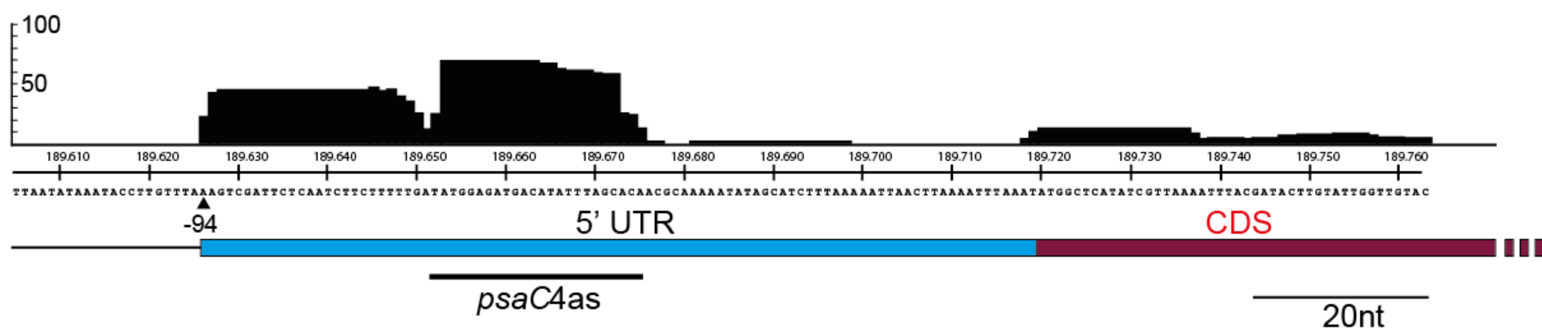
mac1; MAC1-HA

WT

Supplemental Figure 4. Localization of Mac1-HA by immunofluorescence confocal microscopy

Chlamydomonas *mac1* mutant cells complemented with an HA-epitope tagged *MAC1* gene (*mac1;MAC1-HA*), or wild-type cells as a control (WT), were fixed and decorated (as described in the legend of Figure 6) with both a monoclonal mouse antibody against the HA epitope (HA) and a rabbit polyclonal antiserum against either the chloroplast protein DnaK or the Rpl4 protein of the large subunit of the cytosolic ribosome. The anti-mouse and anti-rabbit secondary antibodies were labelled with Alexafluor 647 or Alexafluor 546 respectively. Immunofluorescence was observed by confocal microscopy and is shown separately (green and magenta respectively) or as a merged image (third panels). An image of the same cells observed by transmission microscopy is also presented (fourth panels).

Supplemental Figure 5



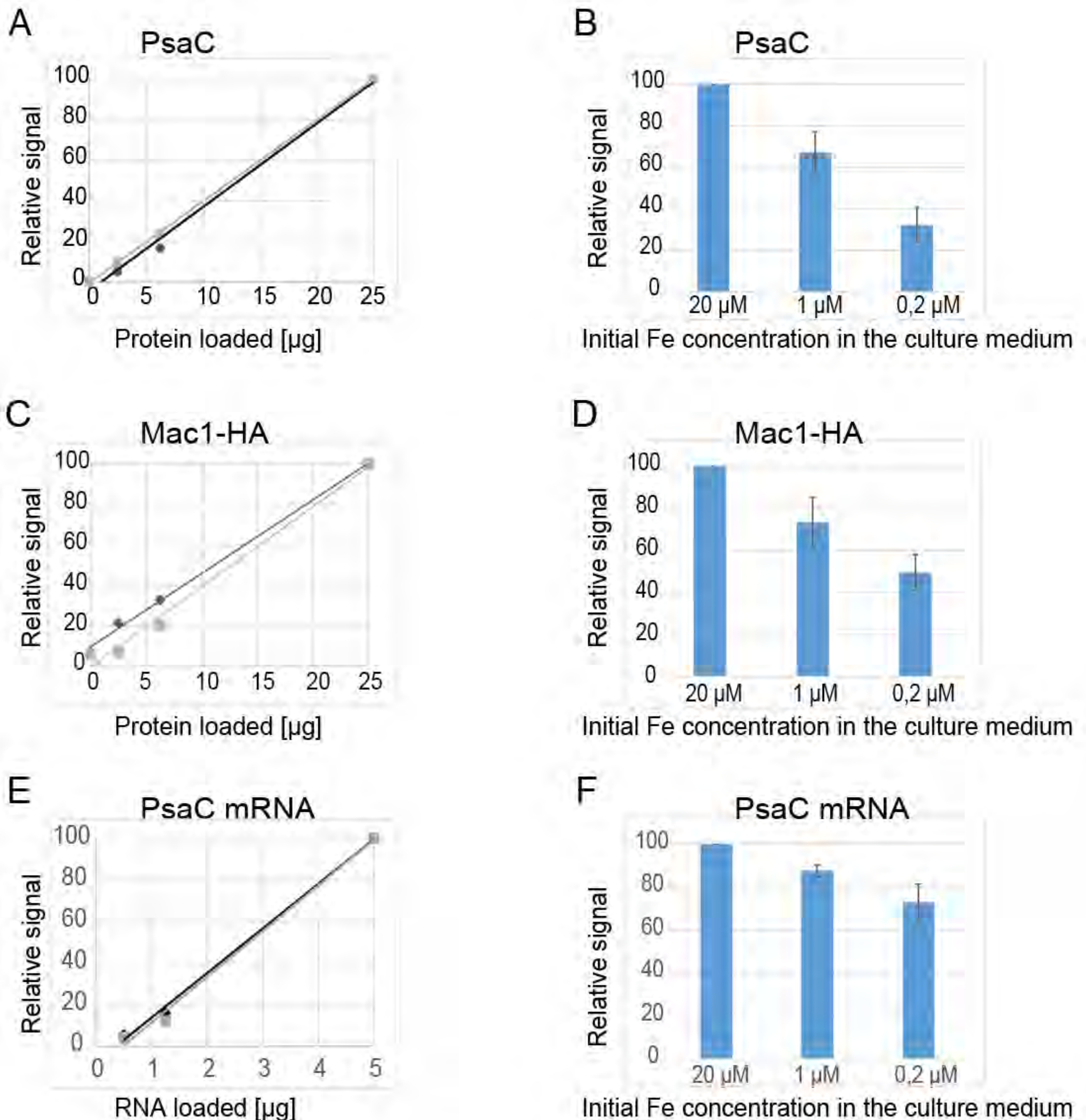
Supplemental Figure 5. Small RNA footprints in the 5' UTR of *psaC*

The sequence of the *psaC* 5'UTR and the beginning of the coding regions are shown. The per-base read coverage is shown as in Figure 5. Numbers refer to the position within the Chlamydomonas chloroplast genome sequence in the GenBank record FJ423446.1

component sensor kinases, is reported to play a role in the regulation of plastid transcription as a function of photosynthetic activity (Puthiyaveetil et al., 2008). The chloroplast casein kinase 2 (cpCK2), also known as Plastid Transcription Kinase (PTK) because of its association with RNA polymerase complexes, is involved in the regulated phosphorylation of sigma factors which play a role in promoter recognition (Schweer et al., 2010). At the post-transcriptional level, chloroplast ribonucleoproteins are phosphorylated by cpCK2, and in the case of 28RNP, phosphorylation can influence RNA binding *in vitro* (Kanekatsu et al., 1995; Lisitsky and Schuster, 1995). Phosphorylation by cpCK2 / PTK also regulates the activity of the chloroplast endoribonuclease p54 *in vitro* (Liere and Link, 1997).The targets of cpPK2 are not limited to proteins involved in gene expression, and for example also include the β-subunit of ATP-synthase in the thylakoid membrane (Kanekatsu et al., 1998). The fact that the kinase STN7 is itself a phosphoprotein, and the presence in the plastid of numerous other protein kinases are suggestive of a complex regulatory phosphorylation network in the chloroplast of higher plants (Baginsky and Gruissem, 2004; Bayer et al., 2012; Reiland et al., 2011).

Little is known on the phosphorylation of proteins involved in chloroplast gene expression in Chlamydomonas. Using electrophoresis in Phos-tag gradient gels and immunoblotting, we could reveal that Mac1 is phosphorylated (Figure 10). Two phosphorylated forms of Mac1-HA were resolved with this gel system (P_1 and P_2), which could represent mono- and di-phosphorylated Mac1-HA, or alternatively two different forms with different degrees of multiple phosphorylation. A first indication that phosphorylation of Mac1 changes with the environmental conditions came with the observation that under conditions of iron deficiency, there was a decrease in the proportion of the phosphorylated form, concomitant with a reduction in the total amount of Mac1-HA. A second indication came with Chlamydomonas cells that were incubated under anaerobiosis in the dark, a condition that induces state 2, with the reduction of plastoquinone and the consequent activation of Stt7 (Wollman and Delepelaire, 1984). In this condition, a strong phosphorylation of Mac1-HA was observed, with the appearance of the slower-migrating form (P_2). Conversely in a condition that induces the oxidation of the plastoquinone pool and state 1, incubation in dim light in the presence of DCMU, phosphorylation of Mac1 was less extensive. However in the *stt7-7* mutant background, the strong phosphorylation with the presence of two phosphorylated forms of Mac1-HA was still observed in state 2 conditions. Hence the kinase, or possibly the kinases, responsible for Mac1 phosphorylation remain elusive. A possible candidate could be Stl1, the paralog of Stt7 in Chlamydomonas, for which no mutant has yet been described. The physiological

Supplemental Figure 6



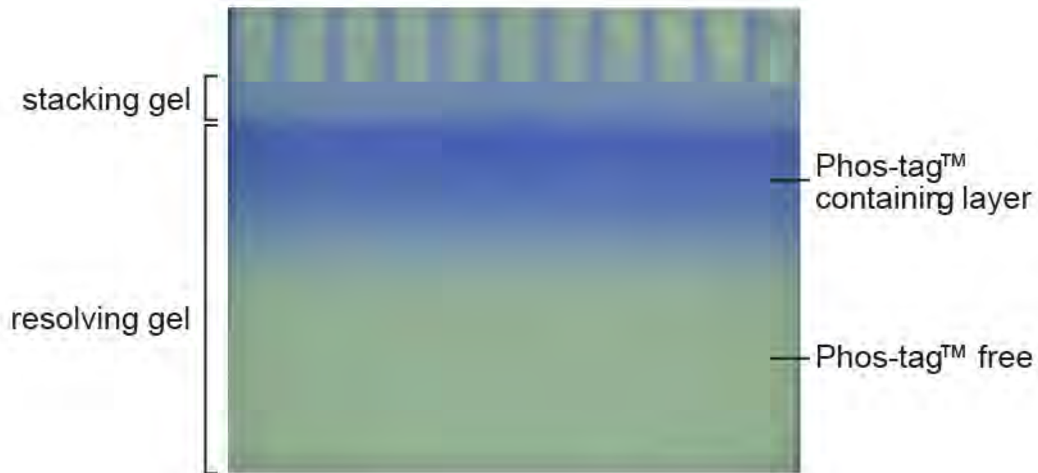
Supplemental Figure 6. Quantification of PsaC, Mac1 and *psaC* mRNA under iron limitation.

A – D. The relative amounts of the Mac1 and PsaC protein after acclimation to different iron content was estimated in immunoblots such as the example shown in Figure 8 (n=3). The ECL signal digitized with the LAS 4000 (GE Healthcare). The integrated density of the bands was measured with FIJI (Image-J 1.48C, Wayne Rasband, National institute of Health, USA). Dilution series of the samples were used to obtain the calibration curves shown in panels A and C for Mac1 and PsaC respectively. The estimated amounts of PsaC and Mac1 after growth with different Fe concentrations, relative to the amount in the iron-replete cultures (20 µM Fe), appear in B and D respectively.
E – F. The relative amount of *psaC* mRNA was estimated from RNA blot hybridization such as the example shown in Figure 8 (n=3). The RNA extracts were prepared from the same cultures as the immunoblots in panels B and D. The signal was detected with a PhosphorImager (BioRad) and quantified as described for the immunoblots. Dilution series of the samples were used to obtain the calibration curve shown in panel E. The signal for *psaC* mRNA was normalized to the signal for *atpB* mRNA, which was used as loading control, and the amount of *psaC* mRNA in 20 µM Fe was set to 100 (panel F).

consequences of Mac1 phosphorylation will need to be investigated in future experiments, in particular to determine whether phosphorylation might affect its RNA-binding activity or its proteolytic turnover.

Among the proteins involved in post-transcriptional steps of chloroplast gene expression, two general groups can be distinguished. One includes the proteins that bind a large set of chloroplast RNAs with little gene specificity, such as the cpRNP proteins which intervene at multiples steps of gene expression (Kupsch et al., 2012). The other consists of the proteins that bind to a restricted subset of plastid transcripts or to a single one and have more specific roles. As discussed above, it is known that some of the proteins of the former group are phosphorylated. However to our knowledge, Mac1 is the first member of the latter group where regulated phosphorylation is demonstrated. This observation opens many new questions on the post-transcriptional regulation of chloroplast gene expression.

Supplemental Figure 7



Supplemental Figure 7. Phos-tag™ gel electrophoresis

The gel was prepared in 0.35 M Bis-tris buffer system pH6.8, and contained a Phos-tag™ gradient gel. The bottom part of the resolving gel (1 mm thickness) contained 6% (w/v) acrylamide, 0.016% (w/v) APS, 0.008% (v/v) TEMED, and 20% (v/v) glycerol without Phos-tag. Before polymerization, the upper part of the resolving gel was also poured (6% (w/v) acrylamide, 50 μM Phos-tag, 100 μM $Zn(NO_3)_2$, 0.016% APS, 0.016% (v/v) TEMED and 10 μg/mL Coomassie brilliant blue G250 (BioRad)). A gradient was formed by loading 300 μL aliquots and gentle partial mixing with the previous layer using a nylon net, visualized by the Coomassie pigment. After polymerization, the stacking gel containing 3.5% (w/v) acrylamide, 0.15% (w/v) of poly-N-acrylamide (Sigma-Aldrich), 0.016% (w/v) APS, 0.016% (v/v) TEMED and 1 μg/mL Coomassie was poured. The running buffer contained 50 mM MOPS, 50 mM Tris, 142 mM Glycine, 5 mM $NaHSO_3$, 0.1% (w/v) SDS. The gel was run at 120V until the marker band at 46 kDa (New England Biolabs, P7708) ran out. The transfer to nitrocellulose was done in 25 mM Bicine, 25 mM bis-Tris free base, 1 mM EDTA, 10% (v/v) MeOH, 5 mM $NaHSO_3$ in a 1L wet-transfer unit (Hoefer Mighty Small) at 4°C for 16 hours at 150 mA.

MATERIALS AND METHODS

Strains and growth conditions

Cells were grown in Tris Acetate Phosphate (TAP) or High Salt Minimal (HSM) media (Kropat et al., 2011) in the dark, under low light (6 µmol m^{-2} s^{-1}) or normal light (60 µmol m^{-2} s^{-1}) from fluorescent tubes. The original mutant isolate was backcrossed three times to a wild type strain (137c), and a spore from the last cross was used in this work as *mac1* (mating type minus).

For growth under iron limitation, the glassware was treated with sterile 10mM EDTA for 15 min and rinsed 3 times with sterile MilliQ water before use. Precultures were grown for at least 10 doublings under normal light in TAP medium, collected by centrifugation and washed 3 times in TAP without Fe. Cells were then diluted to 2 x 10^5 cells / mL, supplemented 0.2, 1.0, or 20 µM $FeCl_3$ and grown for 10 doublings with intermediate dilutions to maintain the concentration below 2 x 10^6.

The state transitions were obtained with cells grown in TAP and normal light. The cells were shifted to HSM medium with three steps of centrifugation and washing, and incubated with shaking in the dark for 2 hours. They were then collected by centrifugation and resuspended in HSM medium to a concentration of 2 x 10^7 cells / mL and split in two aliquots. State 1 was induced during 1 h 30 min by gentle agitation in low light with 10µM DCMU. State 2 was obtained by incubating 1 h 30 min in the dark in a sealed 2 mL syringe without air bubbles on a rotating wheel.

Identification of *MAC1*.

The mutant was generated by random insertional mutagenesis of an *aphVIII* cassette into the wildtype line *jex4* and screened for aberrant chlorophyll fluorescence kinetics as described previously (Houille-Vernes et al., 2011). The mutation was identified by inverse PCR as follows. Total DNA from the mutant was extracted with the DNeasy plant mini kit (Qiagen) and 100 ng were digested with BstU1 for 5 hours. The enzyme was inactivated by heat treatment and the DNA was purified through phenol-chloroform extraction followed by ethanol precipitation. The DNA from the pellet was ligated with T4 ligase overnight at 16°C in a volume of 70 µL and 5µL aliquots were used as templates for nested PCR. The first PCR was with primers IP2 and IP3 (Supplemental Table 1) in a final volume of 25 µL (45 sec at 95°C, 45 sec at 55°C, 2min at 72°C, 35 cycles). A 1 µL aliquot was then used for a second round with primers IP1 and IP4. Sequencing of the fragment and a BLAST search of the Chlamydomonas genome showed

Supplemental Figure 8

Supplemental Figure 8. Construction of DNA clones and vectors

A. Construction of the pMAC1_gen3 plasmid and of its derivative where MAC1 is tagged with a triple HA-epitope. For details see Materials and Methods.

B. Construction of the pET28Mac1cter vector used for expression of the C-terminal domain of Mac1 in E. coli as a source of antigen for raising an anti-Mac1 serum.

that the insertion mapped to exon 2 of Cre02.g124700 (g9646.t1) in Version 10 of the Phytozome database (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). This locus is included in the BAC clone 23A16 (Lefebvre and Silflow, 1999).

DNA constructs.

The plasmids MAC1_gen3 and MAC1-HA were obtained as illustrated in Supplemental Figure 6A. A 4 kb BspH1 fragment containing the 3' flank and most of the CDS was cloned in the TOPO vector pCR2.1 from a digest of BAC 23A16. This plasmid (Mac1-genomic-TOPO) was then extended with a PCR fragment (prepared with primers IF-EcorV-Sbf1-for, and IF-Rev, Supplemental Table 1) containing the beginning of the gene and the 5' flanking promoter region using Gibson assembly (Gibson et al., 2009). The final construct (MAC1-gen3) contains approximately 1 kb upstream of the predicted coding sequence and 1 kb downstream. To insert the triple HA (haemagglutinin) epitope tag at the C-terminus of the coding sequence, a synthetic Bsm1-BsiW1 fragment (obtained from Biomatik) was cloned into the corresponding sites of Mac1-genomic-TOPO. The beginning of the gene and the 5' flanking promoter region were then added as above. A transformant expressing the HA-tagged Mac1 at a level similar to the wild type (#6 in Figure 9) was selected and designated in this work as *mac1;MAC1-HA.* The *MAC1-AA-HA* mutant plasmid was derived from *MAC1-HA* by replacing the BstEII-NruI fragment with two overlapping PCR fragments (obtained with oligonucleotides mut5'macfor with S137A_S139A_R and S137A_S139A_F with mut-mac-rev respectively, and *MAC1-HA* as template; Supplemental Table 1) using Gibson assembly.

The *psaC::lucCP::rbcL* reporter construct was obtained by replacing the *acrV* gene in the atpB-INT-psaA::acrV vector (Michelet et al., 2011) with the *lucCP* gene (Matsuo et al., 2006) using NcoI and SphI. The *psaC* 5' flank (promoter and 5'UTR) was amplified by PCR with primers (psaCprom3'BspH1 and psaCprom5, Supplemental Table 1), subcloned in the topo vector pCR 2.1 and inserted as a XbaI-BspHI fragment in a XbaI-NcoI digest of the vector.

The transformation vector containing *MAC1-HA* used for transformation of *stt7-7* was obtained by inserting the *aph 7"* hygromycin resistance cassette (Berthold et al., 2002) as a PCR fragment (obtained with primers MacHA_hyg_F and MacHA_hyg_R, Supplemental Table 1) at the XbaI site of MAC1_gen3 using Gibson assembly. The final construct was transformed using a helium gene gun into the *stt7-7* mutant (Depege et al., 2003) with selection on 20 µg / mL hygromycin (Calbiochem). A transformant expressing Mac1-HA was identified by

Supplemental Table 1. Primers used in this work

Primers used for cloning and DNA constructs		
mac1 half for	AGGCCATGGCGGGGAACGGGTACTTG	
rev xho	TTACTCGAGGACCGTGGCACTGGGCCCAGCCGA	
IF-EcoRV-SbfI-for	GCCGCCAGTGTGATGGATCGTCAATCAGCAAGG	
IF-Rev1	CTCGCGCGGCCGACCC	
IP1	CGCCTCCATTTACACGGA	
IP2	GGCAAGGCTCAGATCAAC	
IP3	TGACGAACGGCGGTGGATGGAAG	
IP4	TACTGCTCTCAAGTGCTGAAG	
MacHA_hyg_F	TGGCGGCCGCTCGAGCATGCATCTTTCTTGCGCTATGACACTTCCA	
MacHA_hyg_R	ACTATAGGGCGAATTGGGCCCTGGTACCCGCTTCAAATACGCCCAGCC	
Cl14b-A	GCAACTTCAGAGCAGTTAGGTG	
Cl14b-F	GCTCTTCCATGCCCTGGTAA	
Cl14b-F-R	TTACCAGGGCATGGAAGAGC	
Cl14b-E-R	TCTGCTTGTCGTGGTTCAGCTCAAAC	
Cl14b-E	GTTTGAGCTGAACCACGACAAGCAGA	
Cl14b-B	ATGCGCATACCGCTCTTGCATA	
mut5'macfor	CGCGGCGCAAATGGGCCTCAGTG	
S137A_S139A_R	CTGTCCCGTCTGCGGCCGTGGCGGCAGGCTCGAGCTCGAGCA	
S137A_S139A_F	TGCTCGAGCTCGAGCCTGCCGCCACGGCCGCAGACGGGACAG	
mut-mac-rev	CACATGCAGCACGTAGGCATTG	
cdna_pet_ inf_for	AAGGAGATATACCATGGTCGCTGGGGCCC	
cdna_pet_inf_rev	GTGGTGGTGGTGCTCGAGGACCGTGGCACT	
Primers used for the preparation of hybridization probes		
psaA ex3	TTAACCTACAGAAATGATACGTG	AAATTTTAGAAGCTCACCGT
psaB	AACTGTTTCCAAAATTTAGC	AATGAGTACATGTGTTGTG
psaC	CCCATTAGCCGTGGTTTTACTCAT	ATTCCCTAATGGACCAAAAGCAGTCATTCAA
psbB	TGAATGATGCGTGACCTAA	CTTGGTATCGTGTACATACAG
petB	GGGGTGTAGATTGTGTTGAAGCTG	CGTGGAATAACTCCTTCTGGTTCTG
atpB	AATATCTTGTAATTCTTTGTAACGTT	CATATTAATTCCACTTACTATGAGTGA
psbD	GATGACTATGCACAAAGCAG	ACATTGCGTGTATCTCCAAAA
*psaC*4as	TGTGCTAAATATGTCATCTCCATA	

immunoblotting.

Transformation

Nuclear transformation by electroporation was modified from (Shimogawara et al., 1998). A volume of 300 µL of cells suspended at 1 x 10^8 cell/mL in either HSM + 40 mM sucrose or TAP + 40 mM sucrose (for selection for photoautotrophy or for antibiotic resistance respectively) were incubated with 2µg of DNA at 16°C for 20 min, and then 250 µL of the mix was transferred to a 4 mm-gap electroporation cuvette and pulsed at 750 V (C = 50 µF). After 2 min incubation at room temperature, the cuvettes were transferred to 16°C for 20 min. The cell suspension was plated with 1mL of HSM containing 25% starch (for photoautotrophy) or transferred to 50mL of TAP (without sucrose) overnight in low light and collected by centrifugation prior to plating with TAP starch (for antibiotic resistance).

Chloroplast transformation was described previously (Vaistij et al., 2000b).

Mac1 antiserum

The Mac1 antiserum was raised in a rabbit with the C-terminal region of Mac1 prepared as follows. The *MAC1* cDNA was amplified by PCR from a cDNA library (with the primers mac1-half-for and rev-xho, Supplemental Table 1), cloned in TOPO vector pCR2.1 and transferred to pET28a (Novagen) (Supplemental Figure 8B). This vector (pET28Mac1cter) was used to transform *E.coli* BL21 cells and the protein (tagged with 6 histidines from the vector) was purified using Ni-NTA affinity chromatography and imidazole elution, followed by gel filtration on Sephadex S200. The rabbit serum was used at 1/5000 dilution for immunoblots.

Immunoblotting

Cell pellets were resuspended in lysis buffer containing 50mM Tris pH 6.8, 5% SDS, 10mM EDTA and 1 x Protease inhibitor cocktail (Sigma). 25µg of each sample were then supplemented with 0.2 volumes of sample buffer (10% SDS (w/v), 250 mM Tris pH 6.8, 50% glycerol (v/v), 500 mM DTT, bromophenol blue) and heated at 55°C for 15 min. Proteins were then separated by SDS-PAGE on 15 or 7 % acrylamide gels and transferred to nitrocellulose membranes. The total protein on the membrane was visualized by amido black staining, and the membrane was blocked in Tris Buffered Saline Tween (TBST: 20 mM Tris pH 7.5, 150 mM NaCl, 1% Tween 20 (v/v)) supplemented with 5% (w/v) non-fat milk for 1h. The membrane was then incubated with primary antibody in TBST, 1% milk. The primary antisera (and their

sources) were: monoclonal anti-HA (Covance, MMS-101R), anti phospho-Lhcb2 (Agrisera AS13-2705), anti Rpl37 and Rpl4 (gifts of W. Zerges), anti PsaA, PsaC, Cyt_f , D1, α-tubulin and Rps12 (gifts of J.-D. Rochaix). The membranes were washed 3 times for 10 min and then incubated for 2 h with HRP-conjugated secondary antibody (Promega). After 3 washes, the detection was performed by Enhanced Chemi-Luminescence (ECL) and imaging with the GE LAS4000 system (General Electric).

RNA hybridization.

Cells were grown in the indicated conditions until they reached 2 x 10^6 cells / mL. They were then centrifuged 2000 g for 5 min, aliquoted as pellets of 2 x 10^7 cells, frozen in liquid nitrogen and kept at -80°C until their use. RNA from the frozen pellet was extracted using the RNeasy Kit (Qiagen) and analyzed by 1.2% agarose MOPS formaldehyde gel electrophoresis and capillary transfer to nylon membranes Hybond N^+ (Amersham) (Sambrook et al., 1989). The hybridization was done in Church and Gilbert hybridization buffer with ^{32}P-labeled probes as described (Rio et al., 2011) The probes were obtained by PCR with the oligonucleotides listed in Supplemental Table 1 and total genomic DNA as template, except for the *psaA exon1* probe which was a 280 bp HindIII fragment.

Probes were stripped in 0.1% SDS, 1mM phosphate buffer pH7, 1 mM EDTA at 98°C for 5 min, and the membranes were checked for residual signal by phosphorimaging. The RNA blots presented in figures 1, 2, 8 and 9 and the run-on transcription assay figure 4 were all obtained using one membrane for each that was repeatedly stripped, checked for absence of residual signal and reprobed .

Immunofluorescence

The protocol for immunofluorescence was described previously (Lefebvre-Legendre et al., 2015). The HA signal was revealed with goat anti-mouse antibodies coupled to Alexafluor 647, (excitation: 647 nm, emission: 660-750 nm). The other antibodies were revealed with goat anti-rabbit antibodies coupled to Alexafluor 546 (excitation: 546nm, emission : 560-600nm).

Cell fractionation and sucrose gradient sedimentation analysis

Cell fractionation was described previously (Lefebvre-Legendre et al., 2015), using the protocol of Percoll-gradient chloroplast isolation from (Rivier et al., 2001).

For sucrose gradient sedimentation analyisis, cells from a 250ml culture of *mac1; MAC1-HA*

(2.10^6 cells mL^{-1}) were collected and resuspended in 1.1 mL of HKM buffer (20 mM HEPES pH 7.2, 50 mM KCl, 10 mM $MgCl_2$) supplemented with protease inhibitor cocktail (Roche , EDTA-free tabs). The sample was frozen as 100µL drops in liquid nitrogen, and ground in a 50 mL compartment of a MM400 bead-beater (Retsch) for 2 min at 30 cycles s^{-1}.The powder was then collected, melted on ice and centrifuged for 20min at 15000 g. Aliquots of the supernatant (800 µL) were mixed with 100 µL of RNAse buffer (100mM Tris pH7.5, 10mM sodium acetate) with or without (mock treatment) 10 µg mL^{-1} of RNAseA and incubated for 10 min at 22°C. The samples were loaded on 10 mL sucrose gradients (HKM buffer, 5-45% sucrose) as described previously (Lefebvre-Legendre et al., 2015). Twenty fractions (500 µL) were collected from the bottom. Aliquots (150 µL) were precipitated (Wessel and Flugge, 1984) by sequential addition with thorough mixing of 600 µl methanol, 150 µl chloroform and 450 µl water, followed by centrifugation for 5 minutes at 14 000 g. The clear upper aqueous layer was discarded and the bottom phase with the white protein interface was washed with 1mL of 50% (v/v) methanol, vortexed and centrifuged again. The bottom phase with the protein interface was supplemented with 650µL methanol, inverted 3 times and centrifuged for 5 minutes. The pellets were dried and resuspended in 100µL of buffer (100 mM Tris-HCl pH 7, 3% SDS (w/v), 10% glycerol (v/v), 80mM DTT) , treated 10 min at 55°C, and finally analyzed by SDS-PAGE (7% acrylamide) and immunoblotting with anti-HA monoclonal antibody (Covance, www.covance.com/) or anti-RubisCO (gift of Jean-David Rochaix, U. of Geneva).

Run on transcription assay

The protocol was modified from (Klinkert et al., 2005). A total of 10^8 cells in early exponential phase were harvested by centrifugation and washed with resuspension buffer (10 mM Hepes pH7.5, 150 mM KCl, 250 mM sucrose, 1mM EDTA, 0.1mM PMSF). The pellet was adjusted to 100 µL with the same buffer and frozen in liquid nitrogen. 40µL of Run-on buffer 4X (100 mM Hepes pH7.5, 1 M Sucrose, 120 mM $MgCl_2$, 30 mM DTT, 100 mM NaF) were mixed with 5µL each of 10 mM rATP, rGTP and rCTP, 5µL RNasin (Promega), 20 µL ^{32}P-rUTP (200 µCi, 5 µM final) and 80 µL of cell pellet (defrozen in a waterbath at 20 °C).The reaction mixture was incubated at 26°C for 5 or 15 min. Total RNA was immediately extracted with TriReagent (Sigma), precipitated with isopropanol and resuspended in TE (10 mM Tris, 1mM EDTA pH 8) prior to separation on a Sephadex G50 column. The RNA fractions were then used for hybridization. The membrane was prepared by spotting DNA probes prepared by PCR (see

above) onto a Hybond N^+ nylon membrane (Amersham). The membrane was then dried and crosslinked using the automatic mode of a UV crosslinker (Stratalinker). Hybridization of the labelled RNA probes to the membrane was performed as described above for RNA blots.

Small RNA

Published Chlamydomonas small RNA sequencing data (Ibrahim et al. 2010) were mapped to the chloroplast genome (NC_005353) as described (Loizeau et al 2014). Read coverage within the *psaC* 5'-UTR and the 5' end of the *psaC* CDS were visualized using the Integrated Genomics Viewer (Figure 5A). For experimental verification of identified sRNAs, total RNA from dark-grown cells was extracted with TriReagent (Sigma). Small-RNA enrichment was carried out as described (Loizeau et al., 2014). 10 µg of enriched small RNAs were separated by denaturing PAGE, blotted and hybridized to an end-labelled DNA oligonucleotide named *psaC*4as (Supplemental Table 1) situated antisense to the putative sRNA within the 5'-UTR of *psaC*.

Luciferase assay

The different strains were grown in TAP medium under normal light to 2 x 10^6 cells / mL. For transformants of *psaB::lucCP*, 50 µL aliquots of each culture were placed in the wells of a white-walled microtiter plate sitting on dry ice. Cultures of *psaC::lucCP* were concentrated 10-fold by centrifugation before freezing. Frozen plates were kept at -20°C prior to the measurement. For the assay, 100 µL of luciferase reagent (50 mM potassium phosphate buffer pH7, 150 mM NaCl, 1 x Protease inhibitor cocktail (Sigma), 2.5 mM ATP, 2.5 mM $MgCl_2$, 4 mM luciferin (Promega)) were added to the frozen sample and the luminescence emission kinetics monitored at 28°C for 30 min using a Synergy 2 plate reader (Biotek). The maximum luminescence was used for the quantification.

Mac1 purification and RNA binding assays

The coding sequence of MAC1 lacking the transit peptide was amplified by PCR with the oligonucleotides cdna_pet_ inf_for and cdna_pet_inf_rev (Supplementary Table 1). The PCR fragment (2695 bp) was cloned between the *Nco*I and *Xho*I sites of pET28a using Gibson assembly. The construct was freshly transformed into *E. coli* BL21(DE3) cells for each purification. The bacteria were cultivated in 2 L of LB medium containing 50 µg mL^{-1} kanamycin until the culture reached an absorbance of 0.25 at 600 nm. Cultures were transferred to ice for 15min, IPTG was added to 100 µM and ethanol to 1% (v/v). After 15 more minutes the culture

was transferred to an incubator at 18°C and agitated for 20 h. Cells were harvested by centrifugation and the pellet was used immediately. The cells were resuspended on ice in 25mL lysis buffer (50 mM Hepes pH 7.7, 750 mM NaCl, 5 mM MgCl2, 4 mM DTT) containing proteinase inhibitors (Roche, EDTA-free tabs), and lyzed by three passages in the EmulsiFlex C-3 at 20 000 psi. The resulting extract was centrifuged 30 min at 12 000 g and the supernatant was loaded on a 1mL Ni-NTA, washed with lysis buffer supplemented with 30mM imidazole, and eluted with 5mL of 25mM Hepes pH7.7, 750 mM NaCl, 5 mM MgCl2, 4 mM DTT, 300mM imidazole. The extract was then directly injected onto the size exclusion column (HiLoad Sephadex 200 16/60, AKTA system, GE Healthcare) and eluted in column buffer (50 mM Tris pH 7.7, 250 mM NaCl, 4 mM DTT, 10% (v/v) glycerol) at 0.6 mL.min^{-1}.

A vector for the preparation of the RNA probe was obtained by cloning the following sequence in the TOPO pCR2.1 (Invitrogen) vector:

TAATACGACTCACTATAGGGAGA**AAGTCGATTCTCAATCTTCTTTTTGATATGGAGATGA CATATTTAGCACA***A**TCGAT*.

The T7 promoter is underlined, the 5' part of the *psaC* UTR is highlighted in bold, and a *Cla*I site is shown in italics. The vector was digested with *Apa*I (in the vector) and *Cla*I (shown above in italics) leading to a fragment of 139bp, which was purified by agarose gel electrophoresis and transcribed in vitro with T7 RNA polymerase(Promega) for 2 hours at 30°C in a 20 µL reaction mixture containing: 4µL of Transcription buffer (Promega), 1µL of 1mM rUTP, 1µL each of 10 mM stocks of the three other nucleotide triphosphates, 3µL ^{32}P-rUTP at 10 mCi mL^{-1} and 3000 Ci mmol^{-1}, 1µL RNAse-in (Promega), 1µL DTT 20mM, 1µL T7 polymerase and 200ng of probe. This produced a 62 nucleotide RNA containing the first 51 nucleotides of the *psaC* 5'UTR. After treatment with RQ1 DNAse (Promega) for 30 min, the labelled RNA was separated from the free nucleotides on a size exclusion column (Sephadex G25 fine).

The binding conditions for EMSA were previously described in (Williams-Carrier et al., 2008). The buffer used was the column buffer supplemented with 0.04mg/mL of BSA (Applichem), 0.5mg/mL of Heparin (Sigma-Aldrich) and 4000 cpm of radiolabeled RNA probe (40 pM) in a final volume of 20µL. The Kd was determined as the Mac1 concentration at which half of the probe is bound by the protein, which is a valid approximation when the protein is in large excess over the RNA in the assay (30 – 100 nM versus 40 pM).

Phos-tag™ gel electrophoresis

10 mL of Chlamydomonas culture were added to 40 mL of cold acetone and precipitated

overnight at -20°C. After centrifugation at 3000 g for 20 min, the pellet was resuspended in 200 µL of sample buffer (50 mM Hepes pH 7.8, 200 mM NaCl, 1x complete protease inhibitor (Roche, ETDA-free), 0.5% Triton X100). The suspension was transferred to a 1.5 mL microtube, 500µl of acid-washed glass beads (0.4-0.6mm) were added, and the samples were homogenized for 2 times 15 seconds in a Silamat© shaker. The mixture was then centrifuged at 20 000g for 20min, and the supernatant was transferred to a new tube. For de-phosphorylation, an aliquot containing 25 µg protein was completed to 50 µL in lambda protein phosphatase reaction mix following the instructions of the manufacturer (New England Biolabs) and treated for 1 hour at 30°C.

For Phos-tag™ (Wako, N-(5-(2-aminoethylcarbamoyl)pyridin-2-ylmetyl)-N,N′,N′-tris(pyridin-2-yl-methyl)-1,3-diaminopropan-2-ol) gel electrophoresis (Kinoshita and Kinoshita-Kikuta, 2011), a modified protocol was used that improves the migration, separation and electrophoretic transfer of Mac1 to the nitrocellulose membrane (Longoni et al., 2015). The gel was prepared in 0.35 M Bis-tris buffer system pH6.8, and contained a Phos-tag gradient prepared as described in Supplemental Figure 7.

SUPPLEMENTAL DATA

Supplemental Figure 1. Identification of the *MAC1* gene
Supplemental Figure 2. Complementation of the *mac1* mutant
Supplemental Figure 3. Phylogenetic analysis of *Mac1*
Supplemental Figure 4. Localization of Mac1-HA by immunofluorescence confocal microscopy
Supplemental Figure 5. Small RNA footprints in the 5’ UTR of *psaC*
Supplemental Figure 6. Quantification of PsaC, Mac1 and *psaC* mRNA under iron limitation.
Supplemental Figure 7. Phos-tag™ gel electrophoresis
Supplemental Figure 8. Construction of DNA clones and vectors
Supplemental Table 1. Oligonucleotides used in this work

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AUTHOR CONTRIBUTIONS

DD, YQ, PL, LL-L, XJ, CSL and MGC designed the research; DD, YQ, PL, LL-L, XJ performed research; DD and MGC wrote the paper.

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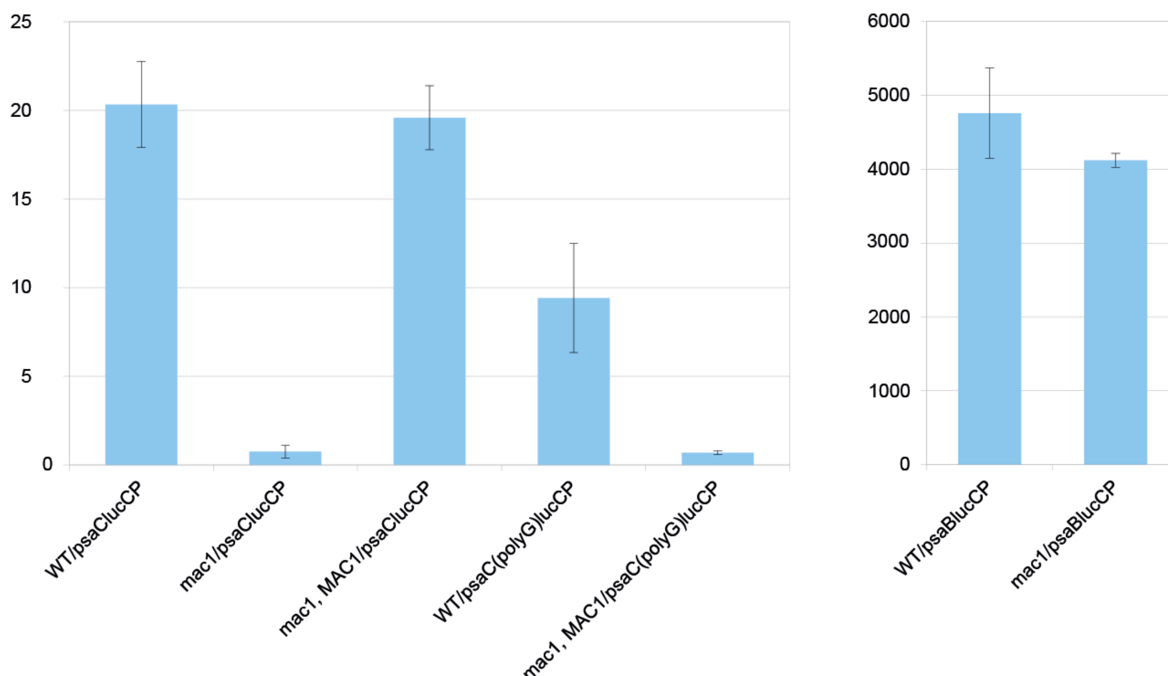


Figure 17 : ***A polyG insert in the 5'UTR fails to restore expression of psaC in mac1****.*
The wild-type mt+ strain with a psaC :: luciferase or psaC(polyG)::luciferase chimeric gene in its chloroplast was crossed with a mac1,mt- strain. Three of the resulting mac1/psaC ::lucCP progeny were further transformed with the MAC1 gene using BAC 23A16 (mac1, MAC1/psaC ::lucCP).
The nuclear genotype is indicated first, followed by a slash (/) and the chloroplast genotype. Nuclear genotypes: WT, wild type; mac1, mac1 mutant line; mac,;MAC1, mac1 mutant line complemented with the MAC1 gene. Chloroplast genotypes, chimeric reporters: psaC::lucCP for psaC::lucCP::rbcL; psaB::lucCP, for psaB::lucCP::rbcL . Three independent lines of each genotype were assayed with three technical replicates, error bars represent the SD of the three lines. Progeny from a cross between a wild-type strain expressing the chimeric psaB::lucCP construct with the mac1 mutant is presented as control on the right. (figure 6B of the manuscript).

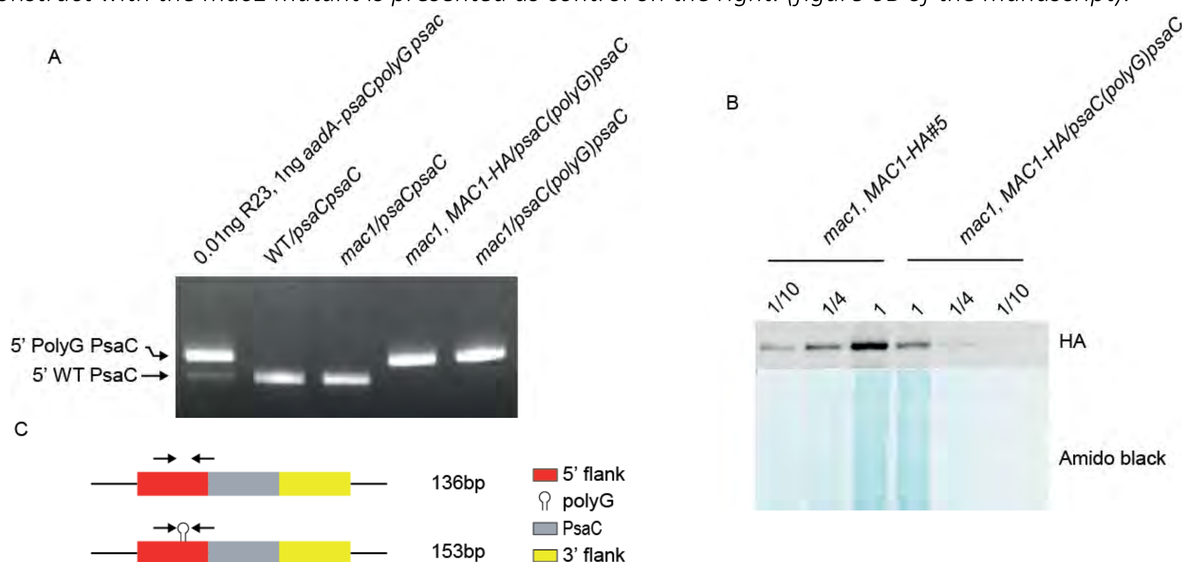


Figure 18 : ***Characterization of the strains containing a polyG tract in the 5'UTR of the endogenous psaC gene***
A : Genotyping of strains with a polyG tract in the 5'UTR of psaC. PCR-based genotyping over the poly G using the primers drawn in C. The strain with the rescued nuclear background and a polyG tract in the 5'UTR of psaC in the chloroplast was obtained by transformation of the mac1 /psaC(polyG)psaC line with genomic MAC1-HA fused to a gene encoding Hygromycin resistance. B : MAC1 expression levels were evaluated by immoblotting. The strain HA#5 appears in figure9 of the manuscript. C : Map of the psaC locus, detailed maps of the vectors used are presented in Material and Methods.

ADDITIONAL DATA

In this part, I describe additional experiments on MAC1 and *psaC* that were not included in the manuscript presented in the previous section.

Is a 5'-3' exonuclease involved in psaC mRNA *degradation?*

To determine whether MAC1 may function in protecting the *psaC* transcripts against 5'-3' exonucleolytic degradation, a polyG tract was introduced in the 5'UTR of the luciferase reporter (at position -60 relative to the AUG codon; *psaC(polyG)::lucCP*)(figure 26). The polyG tract forms a stable RNA structure, which was previously shown to impede exonuclease progression (Drager et al., 1998; Drager et al., 1999; Loiselay et al., 2008; Nickelsen et al., 1999; Vaistij et al., 2000b). In the wild-type background (*WT/psaC(polyG)::lucCP*), the polyG tract allowed expression of the reporter to approximately half the level of the WT/*psaC::lucCP* (figure 17) However the polyG tract did not rescue expression of the luciferase in the mutant background (*mac1/psaC(polyG)::lucCP*). The expression was restored when the mutant was rescued by the wild-type *MAC1* gene (*mac1, MAC1 /psaC(polyG)::lucCP)*. The data suggest either that the polyG tract is inefficient for stabilizing *psaC* mRNA in the absence of MAC1, or that MAC1 is further required for translation.

To investigate whether the stability of the chimeric reporter mRNA was restored by the polyG track in a *mac1* mutant context, RNA blot hybridization experiments were attempted. Unfortunately they did not lead to any conclusive result because even in the wild-type background, only low amounts of highly heterogeneous *lucCP* transcripts were observed.

An attempt was then made to introduce the polyG-modified *psaC 5'UTR* in the native environment of *psaC*. To do so a construct was assembled with a spectinomycin resistance cassette (*aadA*) upstream of *psaC*. This construct inserts at the *psaC* locus to replace the endogenous gene. (see figure 26). Both the WT strain and the *mac1* mutant were transformed either with the polyG-modified *psaC 5'UTR* or the wild-type version of *psaC* and homoplasmy was assessed. The WT *psaC 5'UTR* was inserted and became homoplasmic without any problems (as genotyped by PCR with one primer in *aadA* and the other primer in the insertion flank). However the homoplasmic polyG version could only be obtained in the *mac1* mutant background but not in the wild type despite numerous trials. To circumvent this problem, the homoplasmic *mac1/ psaC(polyG)psaC* mutant strain was transformed by electroporation with

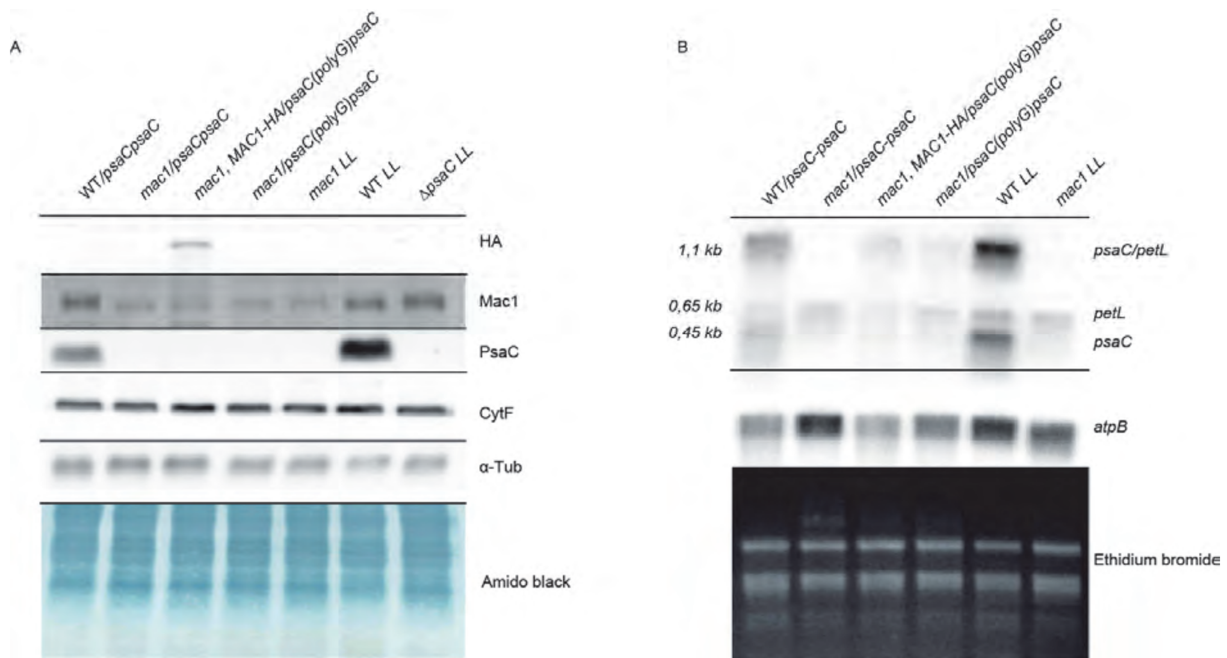
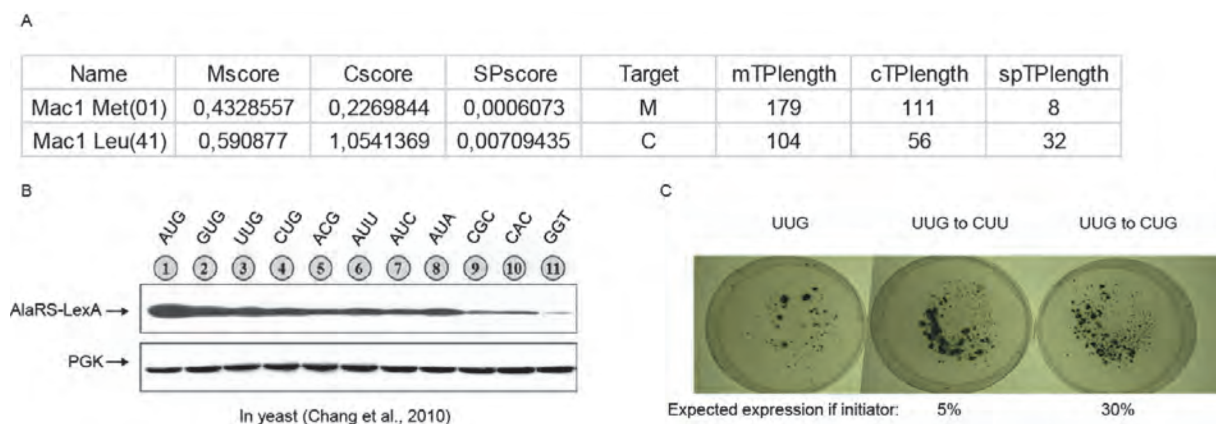


*Figure 19 : **Phenotyping of the strains containing a poly G in the psaC 5'UTR grown in the dark**. Immunoblot analysis on the left and RNA blot analyses on the right. Mutant and WT lines grown in low light are presented for comparison.*



A

Name	Mscore	Cscore	SPscore	Target	mTPlength	cTPlength	spTPlength
Mac1 Met(01)	0,4328557	0,2269844	0,0006073	M	179	111	8
Mac1 Leu(41)	0,590877	1,0541369	0,00709435	C	104	56	32

*Figure 20 : **Investigations on the start site***

A, Comparison chloroplast localization predictions obtained with "PredAlgo" for the AUG and the putative UUG start sites. B, reported translation initiation efficiency in yeast of different start codons used to replace the native start of the LexA gene. PGK is a loading control. C, Complementation for phototrophy of the mac1 mutant with genomic constructs of MAC1 carrying a mutation of the putative UUG start site. Transformation plates are shown.

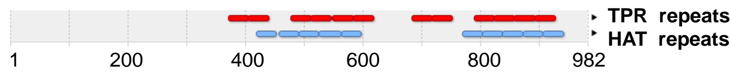
a plasmid containing *MAC1-HA* and a selectable marker conferring resistance to Hygromycin. This led to a homoplasmic strain with weak but detectable expression of MAC1 HA, about four times lower compared to the rescued strain HA#5 (figure 18). Furthermore HA#5 was one of the low-expressing strains presented in the manuscript (figure 9 of the manuscript).

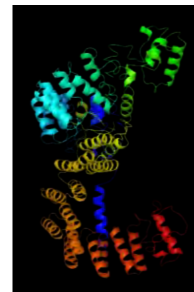
Immunoblots with the polyG strains grown in the dark revealed that PsaC protein, absent in the *mac1* mutant, was not restored by the polyG track, even in the strain were a low level of MAC1-HA was restored (figure 19 A). RNA blot hybridization showed that the polyG track does not restore the mature *psaC* mRNA but partially restores the dicistronic *psaC/petL* RNA (figure19 B), suggesting that 5' degradation plays a partial role in the destabilization of the precursor. The *psaC* mRNA in the control transformant with only the *aadA* spectinomycin resistance cassette behaves as in the wild type. Thus the expression of a low level of MAC1-HA in a *psaC (polyG)* strain does not restore *psaC* mRNA suggesting either that the quantity of MAC1 is bellow a minimal level required to stabilize the monocistronic *psaC* RNA, or that the polyG track prevents MAC1 activity without improving RNA stability.

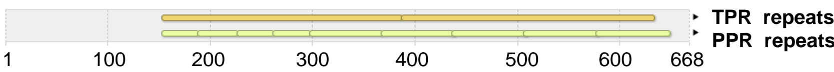
In further experiments, transformation of the *mac1* mutant with a polyG-tagged *psaC* gene and selection on minimal medium did not yield any phototrophic colonies (data not shown).It could be speculated that the polyG-tagged *psaC 5'UTR* is toxic in a wild-type background, even if the cells are kept in the dark as no homoplasmy could ever be obtained in a wild-type background. The role of MAC1 on translation and/or on mRNA stabilization cannot be resolved from these results.

Mutants of a putative alternative translation start codon

The predicted sequence of MAC1 has two methionine residues at the predicted start codon. Its target is a chloroplast mRNA, *psaC,* and it was shown by cell fractionation and immunofluorescence microscopy to be located within the chloroplast. It is thus expected to have a N-terminal transit peptide. PredAlgo software can be used to predict the target compartment. This software was optimized using databases of Chlamydomonas protein sequences with a confirmed subcellular localization (Tardif et al., 2012). The PredAlgo algorithm predicts, with only a low score, an abnormally long transit peptide for MAC1. In contrast, a potential non-AUG initiation codon, UUG, was found in frame 40 codons downstream from the AUG. In *E. coli*, 130 UUG start sites were predicted to function in translation initiation (Blattner et al., 1997). In yeast, GLS1 and ALA1, two amino-acid tRNA







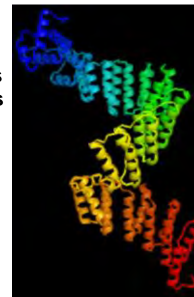


Figure 21: ***Interpro domain prediction and Phyre2 3D-structure prediction of the two proteins stabilizing psaC mRNA in C. reinhardtii*** *MAC1 (TPR/HAT, top) and Zea mays Crp1 (PPR, bottom).*

```
chlamydomonas_reinhardtii 100.0%   AAATGCTTGGGTCACCGGTTCAAGTCCGGTTCCTGGCAATTCCCTAATGG
volvox_carteri             46.2%   CTAAACTA----AGTCGCTTTCGTAGGTGCGCGTCCTTAGGCCATAAGTC
coccomyxa-sp-c169          39.2%   ----GCTT----CATCGCTTTCTGATA----CATGGAAAAGGCTTGCATC
consensus/100%                     ....uCTs....susCGsTTsssssss....CsTssssAsssCsTussss

chlamydomonas_reinhardtii 100.0%   ACCAAAAGCAGTCATTCAAAATATAGGTA-TGAGGAGAGAAATATATGGCC
volvox_carteri             46.2%   ACCT-ACGTTGTGATCTAACGCGAACACCTTAGGTGT-GAACGTAT--CT
coccomyxa-sp-c169          39.2%   GCATTAGGAAGCGATGCA--GAGAAAA-----GGTGGAGAACAATT--CC
consensus/100%                     uCss.AsGssGssATssA..sssAuss.....GGsGs.uAAsussT..Cs

chlamydomonas_reinhardtii 100.0%   GCAAGG---CGCACTGCCACT----GCTTAATATAAATA---CCTTGTT-
volvox_carteri             46.2%   TACAGGGCTTGTGCTGTAAATGTTGGCTTTGCCAAAGTA--GCCTAACTA
coccomyxa-sp-c169          39.2%   CAACG---TTGTGGTATAA-TATTAACCAACTCAGTGCATTGATTTGCAA
consensus/100%                     ssssG....sGsusTussA.T....uCsssssssususA...ssTsuss.

chlamydomonas_reinhardtii 100.0%   --TAAAGTCGATTCTCAATCTTCT--TTTTGATATGGA-GATGACATATT
volvox_carteri             46.2%   CGTTAGGGACGAAGTTAAGCTACT---TTAGCTAAAAAAGGTCACGTGCT
coccomyxa-sp-c169          39.2%   TAGAATGCCCGTGCTCAACCCAATGCATCATCGATGCATGGGAAATTCTC
consensus/100%                     ..ssAsGsssusssTsAAsCsssT...TsssssAsusA.GussAssTsss

chlamydomonas_reinhardtii 100.0%   TA-GCACAACGCAAAAATATAGC----ATCTT---TAAAAATTAACTT--
volvox_carteri             46.2%   AA-GTACTACGTTGTAGTTTAACTGCGTTCTT--ATGAAATTTCATA---
coccomyxa-sp-c169          39.2%   AATGAACAACCATTGAGGAACACCTCAATCTTGAATCAAAAC-CATAGAG
consensus/100%                     sA.GsACsACsssssAussssuC....sTCTT...TsAAAss.sAss...

chlamydomonas_reinhardtii 100.0%   AAAA--TTTAAAT--
volvox_carteri             46.2%   AAAA---TTAAAAAT
coccomyxa-sp-c169          39.2%   AAAACCCTTAAC---
consensus/100%                     AAAA...TTAAs...
```

Figure 22 : ***Alignment of the psaC 5′ sequence in species that encode an orthologue of MAC1 but not of CRP1****. The black boxes represent sequence identity, the grey boxes, sequence differences. The black line below the consensus represents the first bases at the 5′ end of C. reinhardtii psaC mRNA which is the beginning of a small-RNA footprint (~ 50 bases). The grey line represents the hairpin predicted in C. reinhardtii. Both V. carteri and Coccomyxa also have a hairpin structure around this position, slightly longer in V. carteri and shorter in Coccomyxa.*

synthases and CARP2A (ribosomal acidic protein) are translated from a non-AUG codon (Chang and Wang, 2004; Tang et al., 2004; Abramczyk et al., 2003). It has also been shown that the activity of translation initiation from non-AUG start codons is context-dependent (Chang et al., 2010).

If the translation initiation site of *MAC1* were the UUG codon, PredAlgo would predict chloroplast localization with a much better score (figure 20 A). The putative translation initiation activity of this UUG site was evaluated by replacing the Leu41 UUG codon by other leucine codons that would fail to initiate translation, according to studies done in yeast (Chang et al., 2010). These showed that replacing the AUG initiation codon with other codons changed the initiation efficiency, which was always lower than that observed with the original start site. Changing the third base resulted in a more severe phenotype (figure 20 B). I decided to replace the potential leucine UUG initiation site in *MAC1* with a CUG or CUU, to generate other leucine codons that could have different translation initiation efficiencies and thus different abilities to rescue *mac1* by transformation.

Complementation of the *mac1* deficient strain was performed by the shot-gun method with genomic fragments containing the HA-tagged*MAC1* gene in which the start codon has been engineered. Both genes with modified UUG codons (were able to efficiently rescue the mutant. Because the wild-type control was a BAC which is eight times larger, for the same amount of DNA an 8-fold difference in the number of colonies was expected, which is consistent with the difference observed in the experiment. This is comparable to the relative transformation frequencies with *MAC1-HA* and the BAC clone reported in the manuscript (figure 20 C) . Further characterization at the level of *MAC1* expression in these strains were not performed. It can be tentatively concluded that the modification of this leucine codon does not affect MAC1 expression and therefore that the UUG codon is not used for translation and that the AUG codon is the likely start site.

Discussion

MAC1 is a TPR/HAT repeat protein encoded by the nucleus and targeted to the chloroplast were it binds and stabilizes the 5' end of the *psaC* messenger RNA. This function is required for photosynthetic activity, as a *mac1* mutant does not produce the PsaC subunit, causing the degradation of unassembled PSI. Despite its essential function in Chlorophytes,

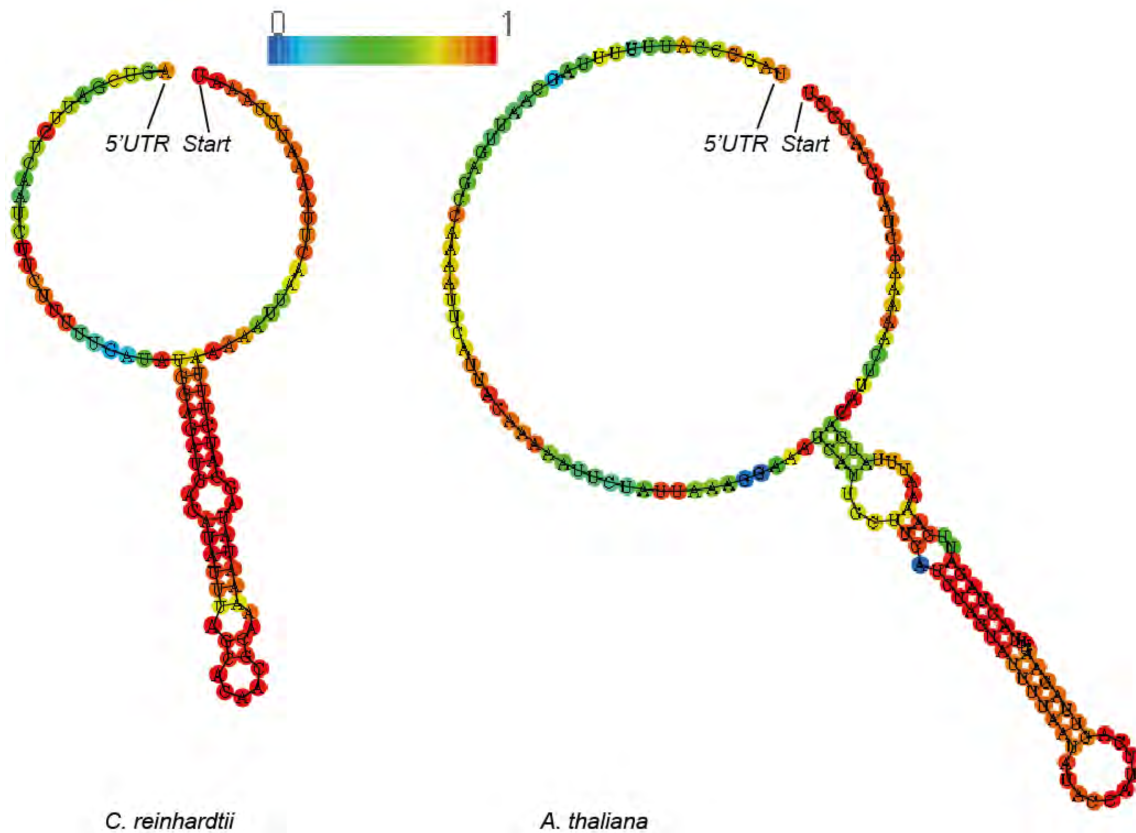


Figure 23: ***Predicted secondary structure obtained with RNA-fold using the experimentally determined 5'UTR of psaC immediately before the AUG start codon in Chlamydomonas reinhardtii and in Arabidopsis thaliana.***

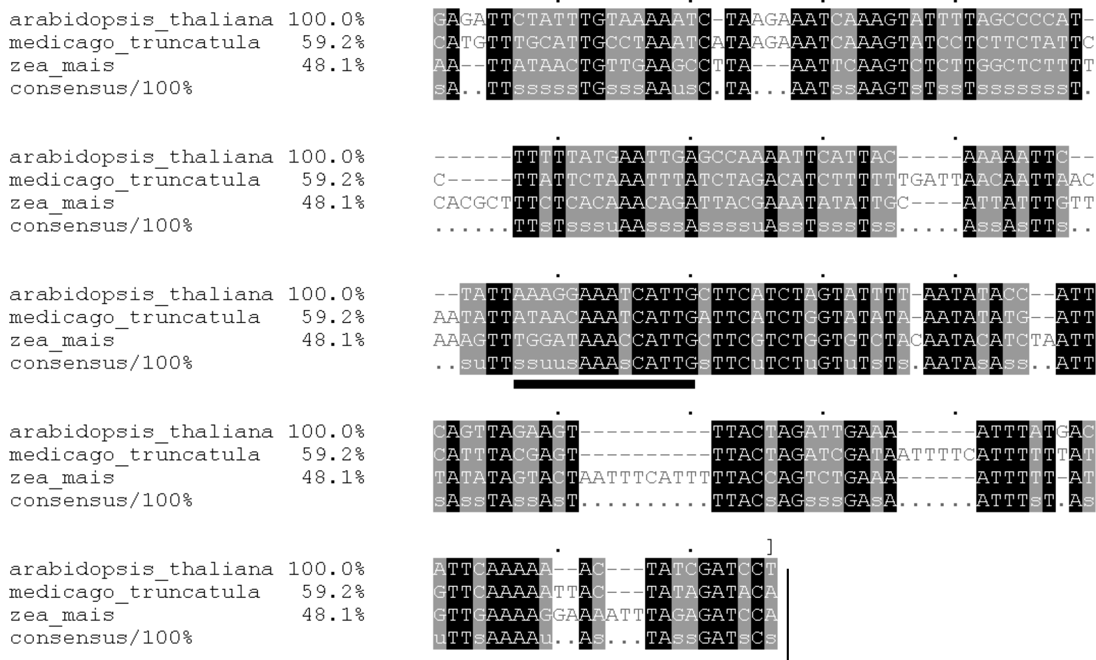


Figure 24 : ***Alignment of the psaC 5' sequence in species that do not have a MAC1 orthologue but have a CRP1 orthologue****. The black line represents the CPR1 binding sequence closest to the ATG. The black boxes represent sequence identities, the grey boxes sequence differences.*

MAC1 is absent from land plants. In *Zea mays*, the nucleus-encoded PPR protein CRP1 is required for stability of *psaC* mRNA (Schmitz-Linneweber et al., 2005). CRP1 binds *psaC* mRNA via two small sequences separated by 51 nucleotides, based on immunoprecipitation and hybridization to chloroplast microarrays (RIP-chip (Schmitz-Linneweber et al., 2005). This protein has a structure similar to MAC1 (figure 21) but their sequences are very different, sharing only 50% average identities on several small segments representing only 7% of the MAC1 sequence, as determined with the BLAST algorithm.

To search for a possible conserved binding site of MAC1 in *psaC* genes from different species, I aligned sequences of the *psaC 5'UTR* from 3 sequenced chlorophyte chloroplast genomes They do not share a conserved sequence at their 5'end, where the small RNA footprint was found (figure 5 of the manuscript and figure 22). Interestingly the AUG start-codon environment shows a higher conservation.

The sequence divergence being consequent, I looked for structural conservation using the RNAfold server (Gruber et al., 2008) and found a hairpin in some sequenced chloroplast genomes mapping to the same region, with varying length from species to species (figure 24). Interestingly the hairpin sequence corresponds perfectly to the small RNA footprint reported in the manuscript. This raises the question about the link between this footprint and the precise MAC1 binding site. Is this small RNA a MAC1 footprint or/and a left-over from RNA degradation, more resistant because of its secondary structure? Is the structure important for MAC1 binding?

Similarly, the *psaC 5'UTR* from higher plants can also form a hairpin loop psaC. In A. thaliana, the UTR seems more structured than in Chlorophytes (figure 23). Spermatophytes species have a much more conserved *psaC 5'UTR* than what can be observed in the Chlorophytes (figure 24). This close sequence conservation may be explained by their late evolutionary divergence. The hairpin predicted in all *psaC* mRNAs is suggestive of a PPR10-like mechanism (Prikryl et al., 2011) in which it is proposed that the RNA-binding protein blocks the formation of a hairpin, promoting the release of the ribosome binding site.

To address the role of MAC1 in stabilization and/or translation, a polyG tract was inserted in the 94 base-long 5'UTR of *psaC*. It was observed that the polyG tail placed 60 bases before the start codon does not fully restore the stability of the dicistronic *psaC-petL* mRNA while it completely fails to stabilize the monocistronic *psaC* RNA, suggesting that the mechanism behind their stability is different and seems to be more complex than expected. Starting from the hypothesis that the 5'UTR of the precursor and the mature mRNA are identical, the dicistronic precursor mRNA contains a polyG blocking degradation from the 5'

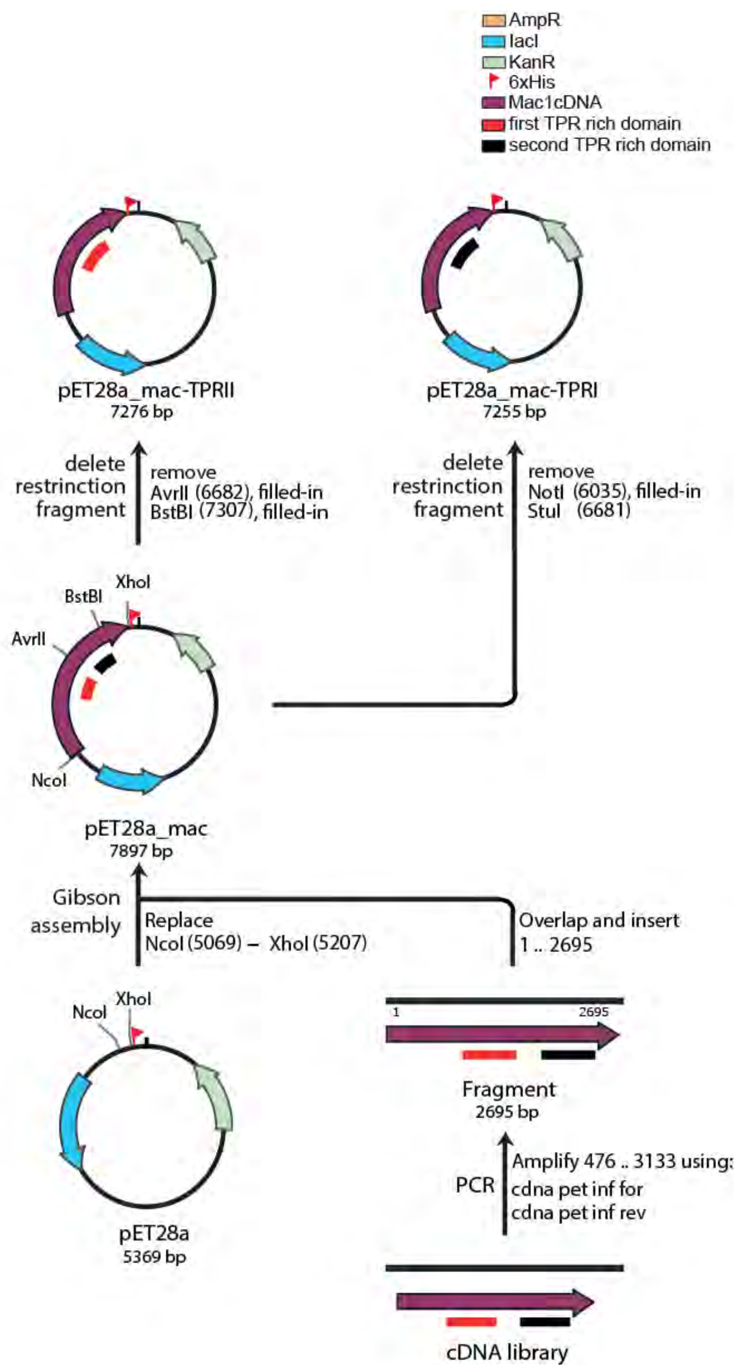


*Figure 25 : **Construction of the plasmid used to express MAC1 in E. coli***

UTR and all the stability factors of *petL* that can block its degradation through the 3'UTR. The new psaC 3'UTR produced by the maturation of the precursor mRNA is maybe not protected against degradation in the *mac1* mutant therefore explaining the difference between the precursor and the mature *psaC* mRNA and suggesting a role of MAC1 in 3', direct or indirect. At the opposite, the hypothesis could be made that the precursor and mature mRNA have a 5'UTR that differ by a few dozen of bases. This would explain why the precursor mRNA, containing the polyG is more stabilized than the mature mRNA that would not retain the polyG. The difference of size between the two hypothetical 5' variants would not be resolved on a RNA-blot. Likewise the mature RNA would be further processed endonucleolytically 44 bases upstream of the start codon, releasing a small RNA of approximately 50nt detected as the sRNA footprint. The shorter 5'UTR would be stabilized by MAC1 and, as a consequence, be unstable in the *mac1* strain

It could be also proposed that either the 3′UTR or the coding sequence has a regulatory role affecting endonucleases or 3′-5′ exonucleases activities. As an example it was shown that MBB1 has two targets (Loizeau et al., 2014) and that PPR10 binding can protect RNA from both 5' and 3' exonucleases (Pfalz et al., 2009). It could be hypothesized that MAC1 is involved at another stabilization site, in addition to the 5'end of psaC. This hypothesis can be tested by identification of other footprints, modification of the psaC 3'UTR, or engineering differently the 5'UTR.

MAC1 accumulation decreases in response to iron limitation. It could be postulated that its transcription is the direct target of iron limitation as was described for *FOX1*, *FTR1*, *FER1*, *ATX1*, and *FEA1 which all have an iron-responsive element* in their promoter (Fei et al., 2010). The iron responsive element in the FOX1 promoter does not have a stringent consensus, "CRCRCK". It can be found at -115, -262, and -338 relative to the MAC1 translation start site. The sequence "AGCGATTGCCAGAGCGC", the iron response element of FTR1, can be aligned with "AGTGCTGGGCAGTGTGC" at -395 from the ATG. Whether MAC1 transcription is regulated by iron availability and whether these elements play a role in the regulation still needs to be further investigated.

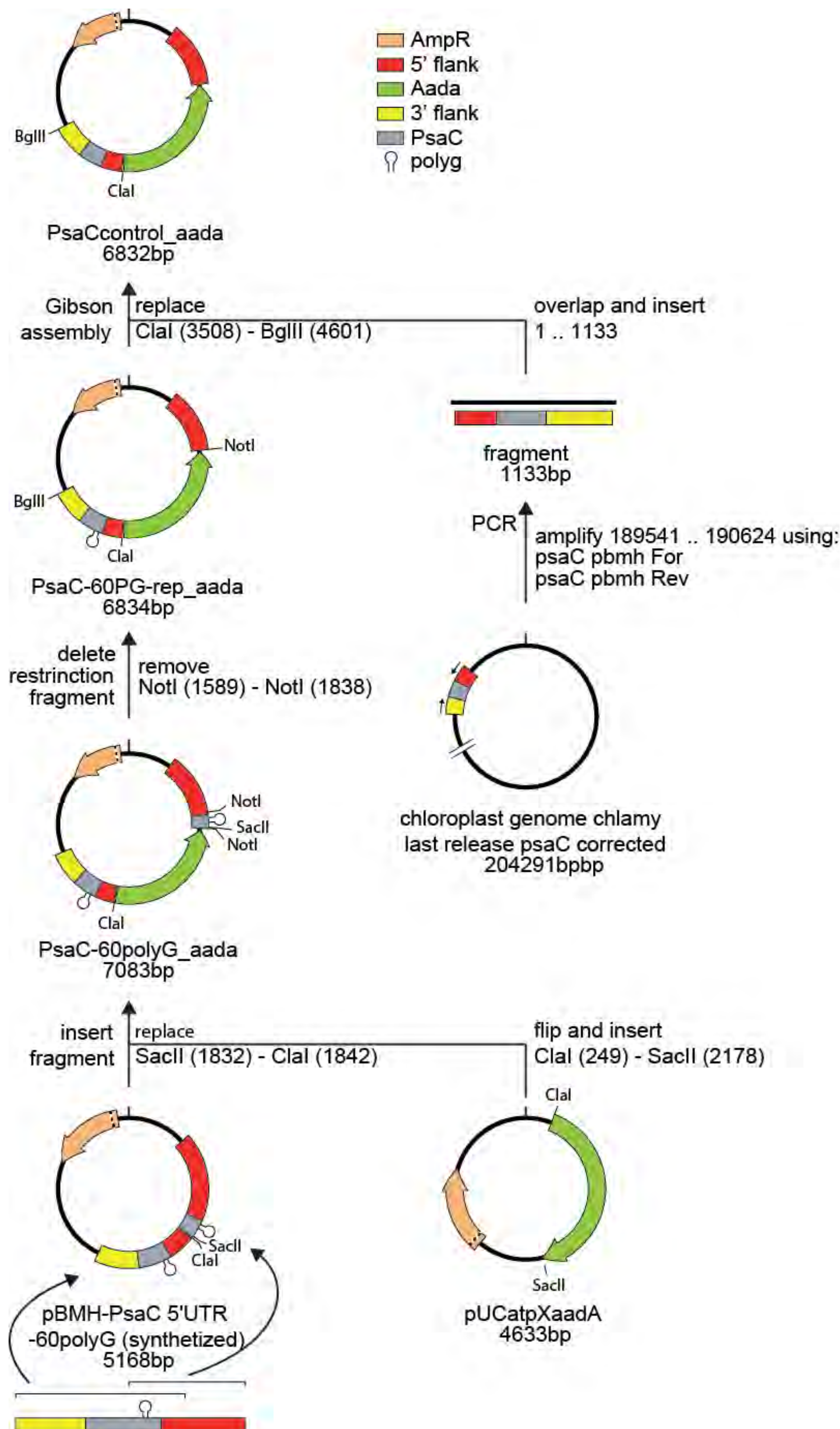


Figure 26 : Construction of the plasmids used to replace the native *psaC* 5'UTR with a polyG-containing *psaC* 5'UTR

Material and methods

MAC1 constructs with modified leucine codon.

They were produced by replacing the NruI Sbf1 in the pMAC1-HA fragment by PCR fragments obtained with oligonucleotides containing the mutation. The fragments were fused and integrated in the NruI-SbfI digested vector (9550 bp) using Gibson assembly. Positive clones were verified by sequencing.

Oligonucleotides used for PCR amplification:

Mutfor	cgcggcgcaaatgggcctcagtg
mutCUUrev	gtgtgagggtcaggaagagctcgcgcgg
mutCUGrev	gtgtgagggtcaggcagagctcgcgcgg

Complementation by transformation

The nuclear transformation was done using the gene gun following the protocol described for chloroplast transformation (Vaistij et al., 2000b) except that the construct was designed for a nuclear expression. Transformants were selected either for autotrophy on minimal medium HSM in moderate light (60µE), or for antibiotic resistance in heterotrophic conditions on TAP medium in the dark .

Insertion of a polyG tract in the 5′UTR of *psaC*.

The construction of the vectors was done using a synthetic fragment (Biomatik) containing the polyG (18bases) inserted at -60 relative to the ATG start codon in *psaC 5'UTR*. An *aadA* cassette was then inserted in reverse orientation 386 bp before the psaC ATG start codon. For the wild-type construct, the polyG was replaced by a wild-type fragment as described in figure 26.

Homoplasmy for the insertion of the construct with *aadA* inserted in reverse orientation, upstream of psaC with the native or polyG-containing 5'UTR, was checked by PCR using the oligonucleotides polyG_dualscreen_for PsaC Prom Rev ..

PCR amplification with these primers led to a 136 bp fragment in the wild type and a 153 bp

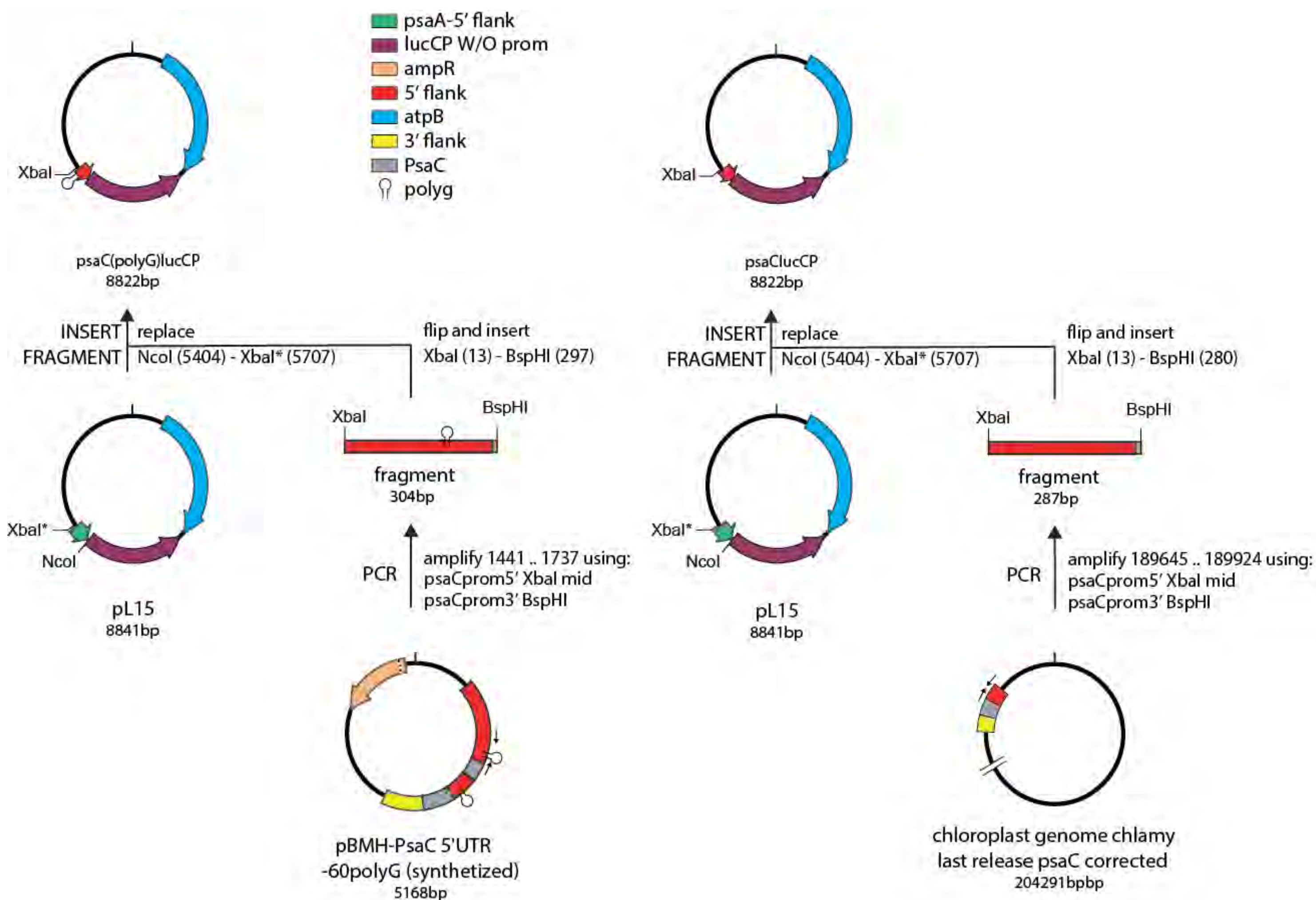


Figure 27 : ***Construction of the psaC::lucCP vectors***
from a synthetic psaC(polyG)::psaC aadA vector

fragment in the polyG-containing fragment.

Primers used for the experiments presented in the Additional Material

Name	Sequence
polyG_dualscreen_For	CTGCCACTGCTTAATATAAATAC
PsaC_Prom_Rev	CGATATGAGCCATATTTAAATTTTAAG
PsaCprom5'XbaI mid	GATAGTTCAGTTTCTAGAGCAAAGC
PsaCprom3'BspHI	GGTCATGATTTAAATTTTAAGTTAATTTTTAAAGAT
psaC pbmh For	CTGCCTCTAATAAAGTCATCGATTTACATTAGAAAAGTTATATA
psaC pbmh Rev	AGCTTCACGTGATACTTAAGATCTTCTAACTGTCATTGGCATCC

RESULTS Chapter II: Acclimation to excess light

Introduction

In nature, light conditions vary significantly in both time and space. Photosynthetic organisms have to constantly regulate their metabolism, and in particular, the way they collect light energy. The strategy used by photoautotrophs to adapt to light conditions is dependent on many other environmental factors including temperature, CO_2 and O_2 concentrations, availability of water, or supply of macro and micro-nutrients.

The photosynthetic apparatus is regulated at two levels, the first level occurs within the first minutes (short term), and the second within hours or days (long term). Short-term acclimation involves biochemical processes that occur in the thylakoids and affect the use of light excitation by the photosystem antennae. This type of regulation involves the NPQ mechanisms (Goss and Lepetit, 2015), which dissipate excess light as heat (qE), re-arrange the antenna distribution between photosystems (qT), or causes photodamage to a core sub-unit of PS II (qI) (detailed in the Introduction).

The activity of the b_6f complex is also regulated over the short-term, as it is known to be affected by thylakoid lumen acidification via a process called photosynthetic control, thus affecting the speed of the whole electron transfer chain (Rott et al., 2011; Kramer et al., 1999). Finally, light absorption can be modulated by chloroplast movements within the cell (for non-motile photoautotrophs) or by phototaxis (for motile organisms). A more detailed description of these types of regulation can be found in the “Short term acclimation” section of the Introduction.

Over the long term, from hours to weeks, photosynthetic organisms are subject to varying light intensity, from night (0.005 µmol photons.$m^{-2}.s^{-1}$ at full moon) to 2000 µmol photons.$m^{-2}.s^{-1}$ (light on a sunny day at noon) during the day. Under such day-night cycles, regulation of the photosynthetic machinery is needed to keep metabolic homeostasis. In pine for example, it was reported that the photosynthetic machinery is further adjusted with the seasons (Verhoeven et al., 2009). In bacteria, most regulation occurs on a transcriptional level. In eukaryotic cells, acclimation requires coordination of both nuclear and chloroplast gene

expression.

How light regulates nuclear gene expression is not entirely understood. Light signals are perceived in the cytoplasm through photoreceptors such as phototropins (Briggs and Christie, 2002), and by retrograde signaling from the chloroplast. Numerous molecules are thought to transmit information from the chloroplast to the nucleus, including ROS (Maruta et al., 2012), Mg-protoporphyrin IX and other heme derivatives (Strand et al., 2003)(Zhang et al., 2015) linked to the *GUN* genes (Brzezowski et al., 2014), and the redox state of the plastoquinone pool via an unknown mechanism (Kimura et al., 2003).

In the chloroplast, regulation of gene expression was demonstrated with a few examples of transcriptional regulation. Chloroplast transcription is affected by light and by the PQ redox state, which both affect *psaA/B* and *psbA* transcription (Pfannschmidt et al., 1999). These cues also modulate accumulation of *psaA/B* and *psbA* mRNA, the amount of P700, the quantity of Q_A, and the chlorophyll a/b ratio in mustard (Pfannschmidt et al., 1999). Translation and mRNA stability are examples of regulation that control *psbA* (Trebitsh et al., 2000; Zhang et al., 2000).

The objective of this project was to understand the molecular mechanisms involved in the regulation of PS I accumulation by comparing conditions in which the change is maximized. PSI is ideal for this study as it is much less sensitive to photodamage and photoinhibition than PS II, leading to phenotypes that are easier to understand. For these experiments, the chosen model organism was again *Chlamydomonas reinhardtii,* which is a photosynthetic haploid microalga that presents the advantage of also being able to grow independently of photosynthesis, using acetate as a carbon source.

While this work was in progress, similar data were published by (Bonente et al., 2012) and the project was discontinued. Thus only preliminary experiments will be presented hereafter.

Results

Conditions that modulate PsaA accumulation

Light quantity represents the environmental parameter that fluctuates most often and with a high amplitude, which might affect photosystem accumulation. Tests revealed that gas supply is also important, as the doubling time of *Chlamydomonas reinhardtii* can fluctuate from

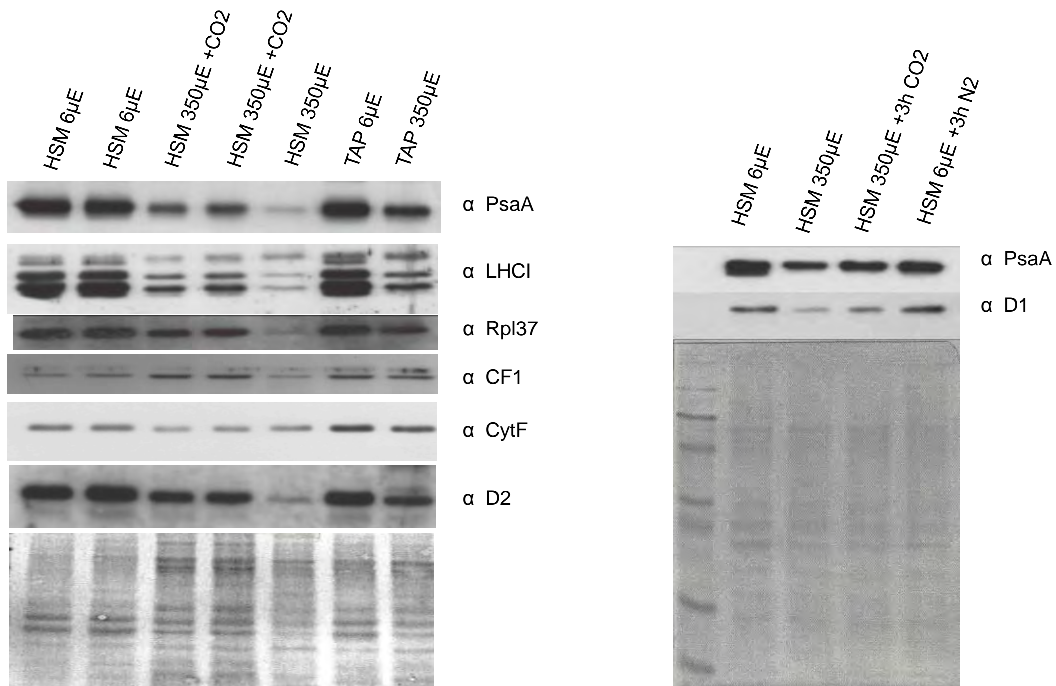


Figure 28: ***Immuno-blot analysis of protein accumulation in different growth conditions****.*

Total proteins were extracted from cells following 24-h acclimation under different light and gas conditions, and analyzed by SDS PAGE and immunoblotting with the antibodies shown on the right of the panels. Amido-black staining of the immuno-blot membranes is shown at the bottom as a loading control. Samples were loaded on a protein basis (25 ug / lane).

8 h observed in mixotrophy at 60 µmol photons m^{-2} s^{-1}, to 6 h when the culture is bubbled with air. Several tests were performed to determine conditions in which there is differential accumulation of PS I during long-term acclimation without excessive ROS production. The effects of light intensity, medium composition, temperature and the addition of CO_2 (bubbling of air enriched with 5% CO_2) were investigated. However, temperature influences the rate of intracellular metabolism and the kinetics of photosynthesis and as a consequence, hinders the identification of regulation specific to photosynthesis. Experiments were performed at 25°C by growing cells in minimal media with bubbling air under 6 µmol photons m^{-2} s^{-1} or under 350 µmol photons m^{-2} s^{-1}. Differential accumulation of PS I was evaluated by immunoblotting (figure 28). Supplemental tests were performed using different light conditions and in different gas conditions to determine whether the responses are driven by light, CO_2 availability, or energetic metabolism.

The results (figure 28) show that light intensity affects the accumulation of both photosystems and their antennae, while cytochrome f and ATPase are not visibly affected by light. These results were confirmed by Bonente et al. (2012) who showed a decrease of both PsaA and D1, while ATPase and b_6f were stable based on the cell count. Surprisingly, ATPase seems to be affected by both CO_2 and acetate, suggesting that it is also regulated, and that the presence of acetate has an effect on chloroplast regulation. It also seems that the bubbling of N_2 to remove CO_2 and O_2 affects the regulation.

Because excess light is harmful for photosynthesis; photo-oxidative stress was evaluated by first measuring the Fv/Fm ratio. The maximum quantum yield of PSII (Fv/Fm), can be estimated by measuring the increase in fluorescence emission from dark-adapted minimal fluorescence (F_0) to maximal fluorescence (Fm), which is associated with the closure of reaction centers under saturating light conditions (Fv = Fm - F_0). D1 is a protein of the PS II core that is very sensitive to photoinhibition, when thylakoid membranes are under stress conditions this protein is damaged and there is a decrease in the Fv/Fm ratio. Cells in the conditions described above had Fv/Fm around 0.6 in high light and 0.72 in low light and can be considered as weakly stressed.

During high-light stress, photosynthetic electron transport produces toxic ROS. In order to determine if cells were under oxidative stress in these experiments, nitrobluetetrazolium (NBT) was used. This molecule reacts with superoxide ions to form an insoluble formazan precipitate that indicates ROS stress (figure 29).

Cells acclimated to low light produced superoxides when they were placed under high

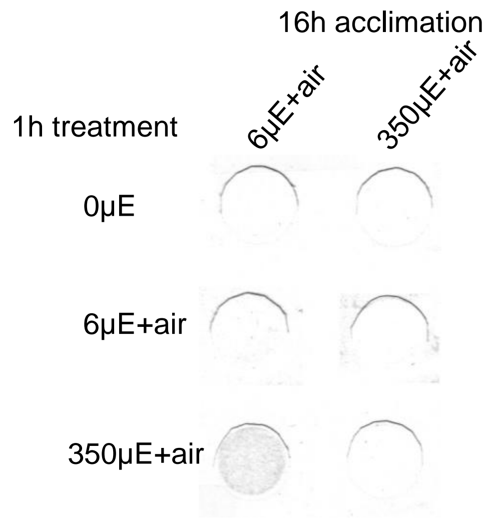


Figure 29: ***Superoxide production measured with NBT.***

Cells pre-acclimated to different light condition were treated with high light or low light in the presence of 50µM NBT. The precipitate formed was separated from the culture by filtration and washed with HCl and methanol

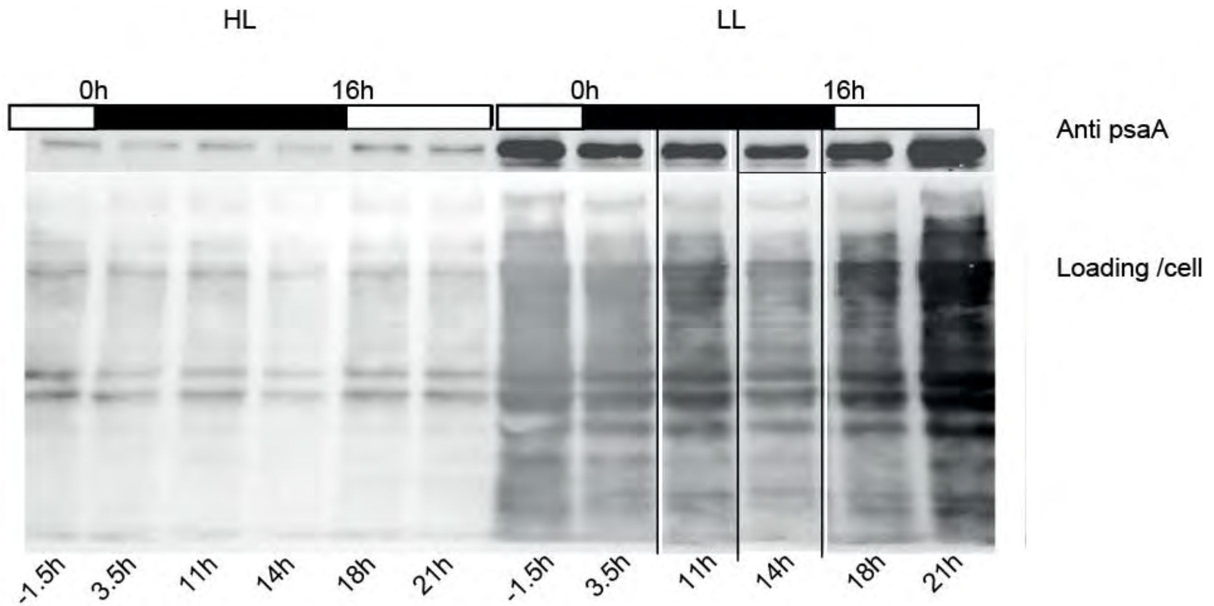


Figure 30 : ***Western blots using protein extracted from 2 cultures separately acclimated for 3 days under 8h/16 h light /dark cycles*** *either to high light (left, 350 µmol photons m^{-2} s^{-1}) or to low light (right, 6 µmol photons m^{-2} s^{-1}) and harvested during the fourth day. Samples from equal numbers of cells were loaded.*

light, while the high-light acclimated cells did not produce any detectable superoxide under the same condition. These data suggest that when *C. reinhardtii* is acclimated to the high-light conditions used in this work it does not endure oxidative stress, in contrast to non-acclimated cells.

Diurnal effects on acclimation

Circadian regulation was found to affect chloroplast transcription (Hwang et al., 1996). A bacterial type sigma factor (RPOD) which is nucleus-encoded was thought to be an important player in this regulation as its mRNA fluctuates during light-dark cycles (Carter et al., 2004). The RPOD protein accumulation was controversially reported to be stable in light dark cycles (Kawazoe et al., 2012).

To investigate the interplay with light intensity an experiment was performed under light-dark cycles in *C. reinhardtii* adapted to low and high light. (figure 30).

In light-dark cycles cells divide at night, and the decrease in total protein per cell can be used as a control to confirm synchronous division. It appears that PsaA exhibits a light-dark pattern under low light, which peaks in the middle of the light period and is at its lowest at the end of the night. This variation follows the cellular protein content. High-light day-night cycles also led to discrete light-dark behavior in the level of PsaA accumulation, which peaked during the middle of the light period, but again followed the cellular protein content. In conclusion, it appears that light-dark cycles do not cause variation in the level of PsaA accumulation on a protein basis, even during the night period when PsaA might expected to increase since it was observed to increase in constant low light (figure 30). This suggests that the day-night regulation is separate from the daylight intensity regulation.

Regulation of PsaA accumulation is not at the level of RNA maturation

Precursor RNAs are maturated and/or spliced to give the final translatable mRNA; therefore, these steps are also a potential target of regulation. For example, for *psbA* the amount of released introns, assumed to reflect the rate of splicing, increase 6–10-fold under light in response to the redox poise, suggesting possible regulatory control at the level of splicing. This suggests that, as psbA introns are from the group I, protein dependent, a regulatory event might be protein-directed (Deshpande et al., 1997). Maturation also involves

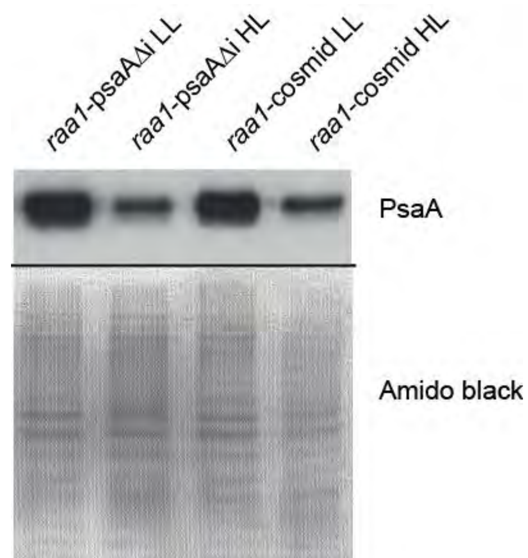


Figure 31: ***Immuno blots using protein extracts from cells acclimated for 24 h to different light conditions*** *(LL: 6µmol.m^{-2}s^{-1}, HL: 350µmol.m^{-2}s^{-1}) and bubbled with air in minimal medium (HSM). The strains used were the psaA splicing mutant raa1 rescued with an intron-less psaA construct (psaA-Δi) or with a cosmid containing the RAA1 gene.*

the activity of endo- and exonucleases. There are a few examples of regulation at the maturation step. One of them is the report that P54, a 3′-endoribonuclease, appears to be phospho-regulated and highly sensitive to the redox state in mustard (Liere and Link, 1997). Similarly, MCA1, a limiting factor in the accumulation acting at the 5’end of *petA* mRNA, is down-regulated under nitrogen starvation (Raynaud et al., 2007)

The trans-splicing of *psaA* is a complex mechanism involving many proteins. It was not clear whether the process could provide a target for regulation. The trans-splicing of *psaA* is defective in the *raa1* mutant of *Chlamydomonas reinhardtii*. This mutant was successfully rescued by expression of intron-less *psaA* cDNA *(psaA-Δi)* in the chloroplast, or as a control by nuclear insertion of a cosmid containing the *RAA1* gene (Lefebvre-Legendre et al., 2014). There was no evidence for a regulatory role of trans-splicing in response to iron availability, which is known to affect *psaA* accumulation.

Similarly, the high light versus low light acclimation of both intron-less and cosmid rescued strains did not show any difference (figure 31). Thus the regulation of PsaA accumulation under different light intensities is not at the trans-splicing step.

Regulation of mRNA accumulation / translation

In the chloroplast, mRNA degradation is an important player in the modulation of biogenesis (Salvador and Klein, 1999). To determine whether mRNA accumulation differed under the two light regimes, samples from adapted cultures were analyzed by RNA blot hybridization .

It appeared that high light did not have a striking impact on the accumulation of selected mRNAs of photosystem I and II core subunits (figure 32), or on the correct splicing of *psaA* (figure 31). Because the HL lane is a little overloaded, a small decrease in HL may be inferred.

Similarly the fusion of the *psaA, psaB*, and *psaC 5’UTR*s to the luciferase reporter gene revealed that the 5’UTRs of *psaA*, and *psaB* are not targets of regulation mechanism according to the light intensity in phototrophic conditions. Conversely the *psaC 5’UTR* seems to react to the light intensity (figure 33). This suggests a regulation of translation as *psaC* mRNA accumulation does not appear to be influenced by light intensity (figure 32). This regulation could affect in cascade the translation and assembly of the whole photosystem through the CES.

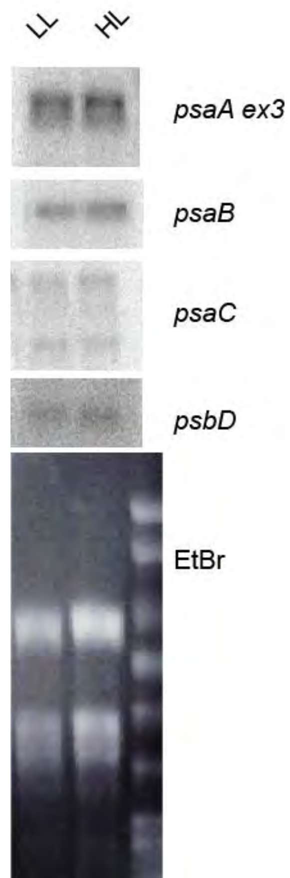


Figure 32: ***RNA-blots of cultures acclimated for 24 hours in different light conditions and bubbled with air.***

The probes used are indicated on the right, and the picture of the gel stained with ethidium-bromide (EtBr) is shown at the bottom.

Regulation through translation or degradation.

In most reported cases, the 5′UTR is the target of regulation; therefore to investigate translational regulation we decided to use constructs containing the chloroplast codon-optimized firefly luciferase reporter (*lucCP,* (Matsuo et al., 2006)) fused to the promoters of *psaA*, *psaB,* and *psaC*. The constructs contained *atpB* as a selectable marker for photoautotrophic growth and were introduced in the FUD50 strain, which carries a deletion of *atpB*. The transformants were acclimated for 24 h under low or high light with air bubbling and collected for a luminescence assay.

There were large differences in the absolute values of luciferase activity in the different constructs. This was surprising as all PSI sub-units are synthetized in equimolar ratios. The promoters/5′UTR may have lacked some regulatory sequences. Alternatively, the relative translation or mRNA stability of the respective endogenous genes may be diverse. In any event, only the activity of *psaC* 5′UTR appeared to be slightly affected by high light.(P-value=0.0387)(figure 33).

As there was no significant decrease in luciferase activity under high light, these data also suggest that regulation is not at the level of translation. Therefore the proteolytic turnover of the protein should be considered under the different light conditions. To investigate this process, acclimated cultures were incubated with either protease inhibitors to block the activity of proteases, or chloramphenicol to block chloroplast translation.

The protease inhibitor cocktail and chloramphenicol were previously used to investigate the stability of recombinant proteins (Michelet et al., 2011). It is not clear whether the protease inhibitor treatment was efficient since the proteases involved in D1 turnover were apparently not inhibited. Similarly the regulation of PsaA core protein accumulation was not affected. With the chloramphenicol treatment in high light, the D1 subunit of PSII, which is normally actively renewed because of photoinhibition, strikingly decreased as expected. In contrast PsaA appears to have a long half-life in high light. (figure 34).

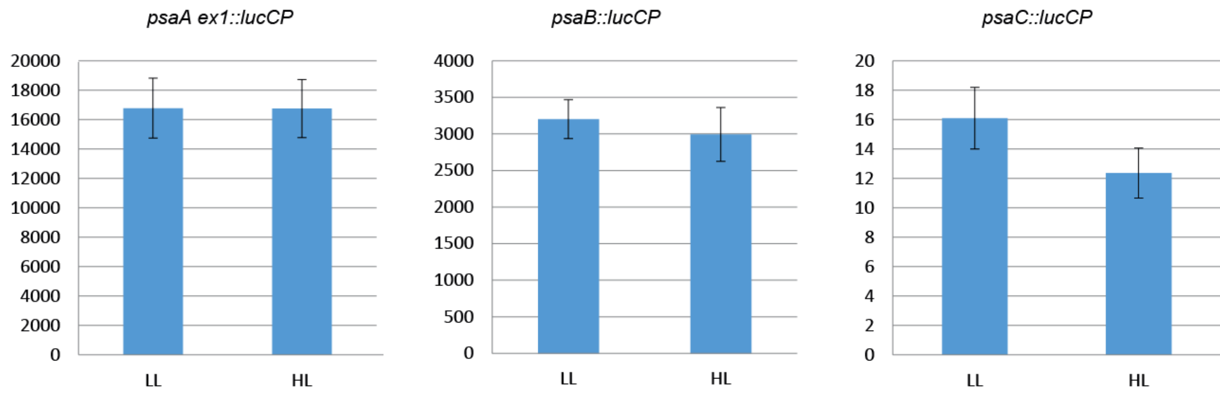


Figure 33: ***Luciferase activity assays on strains expressing luciferase under the control of the psaA exon1, psaB, or psaC 5'UTR in the chloroplast****. Each strain was submitted to either low ligh (LL, 6µmol.m^{-2}s^{-1}) or high light (HL, 350µmol.m^{-2}s^{-1}) with bubbling for 24 h*. The determined p-value for the *psaC::lucCP* experiment is 0.0387.

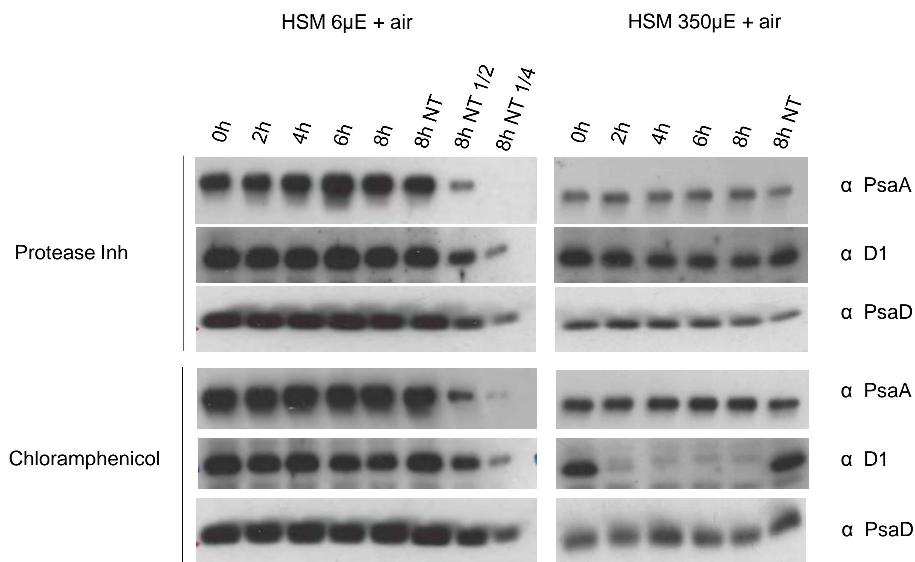


Figure 34: ***Western blots on protein extracts from cultures acclimated for 24 h in low light or high light with bubbling****. The cultures were then treated for 8 h with either the chloroplast translation inhibitor chloramphenicol or a broad-range protease-inhibitor cocktail and compared to the non treated cells (NT) kept under the same conditions.*

Discussion

Acclimation to light intensity

The data suggest that light intensity affects the quantity of photosystems present in long-term acclimated states. These states were obtained under low and high light with bubbling the cultures with air to standardize gas availability. The long term acclimation to high light involves a decrease in PS I and PSII levels compared to total proteins. The amount of PSI antenna protein LHCI follows the decrease in PS I, indicating a concomitant adaptation of the whole complex to the new quantities of the PS I core. Such acclimation influences the light harvesting capacities of the photosynthetic chain, decreasing when light intensity goes over what is needed for growth. The growth rate is determined by three parameters: the first is the intrinsic speed of metabolism that depends on the temperature and on an organism-dependent constant, the second parameter would be the availability of energy sources, in this case acetate or light, and finally the third is the availability of external nutrients such as CO_2. In low light, the availability of energy limits growth and cells tend to harvest as much light as possible and therefore invest in building larger quantities of photosynthetic machinery. In high light, the availability of nutrients is limiting, and harvesting useless energy is detrimental; the cell therefore degrades its supernumerary photosynthetic apparatus.

These data are consistent with a study that reported a similar acclimation after 10 division cycles under high or low light (Bonente et al., 2012). Those authors demonstrated that the number of photosystem core proteins PsaA and D1 per cell decreases in high light compared to low light. This decrease is also seen in the antenna of both photosystems as the harvesting capacity per reaction center is stable and the abundance of reaction centers is lower. The short-term acclimation to high intensities was also reported to drastically affect PsaC and the oxygen-evolving complex in mixotrophy (Nama et al., 2015).

Effect of electron sinks on regulation

In the present experiments, the addition of CO_2 to minimal medium decreased the effect of high light, suggesting a role for electron acceptance in this regulation. One could imagine that the accumulation or the redox state of one or more intermediate in the chain of electron transfer from the photosystems to the CBB cycle is sensed, subsequently triggering the

regulation. High CO_2 levels that enhance activity of the CBB cycle, would increase the ATP and NADPH demand thus decreasing the overaccumulating proton gradient at the thylakoid membrane and the reduction of the acceptors. This proton gradient is also known to have regulatory effects as it enhances the qE component of NPQ by activating VDE (Jahns et al., 2009) and protonating different antenna protein residues such as in LHCSR3 (Tokutsu and Minagawa, 2013), The proton gradient also affects the rate of plastoquinol oxidation by the b_6f complex (photosynthetic control). In tobacco the build-up of a proton gradient does not affect the accumulation of the photosynthetic machinery (Rott et al., 2011). In *C. reinhardtii,* the mutant *mda1-ncc1* is defective in the stabilization of the *atpA/psbI/cemA/atpH* messenger, thus showing a defect in ATPase. This deficiency induces an increase in the proton gradient. The mutant does not show any difference in *psbC* and *petA* translation (Drapier et al., 2002). Accumulation of the *psbD* mRNA is also normal (Boulouis et al., 2015).

The presence of acetate in the medium also lowers the effect of light, suggesting a role for carbon metabolism, possibly through mitochondrial respiration or chloroplastic photorespiration. In mixotrophy, acetate in the media is catabolized and respired at the level of the mitochondria, releasing substantial amounts of CO_2 (Singh et al., 2014). There is a reported link between mitochondrial CO_2 production and its incorporation in the chloroplast (Singh et al., 2014). The results shown here show that addition of CO_2 or acetate into the media under high light have similar effects on the photosynthetic apparatus. It is possible that CO_2 which is produced by acetate respiration, is used in the CBB cycle as with CO_2 bubbling, therefore promoting a comparable response.

Furthermore, the bubbling of nitrogen, chasing O_2 and CO_2, changes the signal that triggers PSI and II long-term acclimation. Anoxia was shown to limit the electron flow downstream of PSI (Alric, 2014) by increasing the reducing pressure on the chain,. As a consequence, reducing power accumulates in the photosynthetic chain even in low light and triggers a high-light type response, therefore decreasing the accumulation of photosystems.

The acclimation to high light requires the electron transport chain to readapt to excitation energy. Under these conditions, when the cells are not acclimated, electron transport machinery initially suffers from too many electrons being injected in the chain by PS II, which disrupt the balance within the redox couples that are essential in the chain. An electron-saturated chain is more likely to react with molecular oxygen and produce ROS in non-acclimated cells (figure 29). To decrease the number of electrons injected in the chain and avoid an unbalance of the redox equivalents, photosynthetic organisms have developed

protective mechanisms, including NPQ that dissipates the extra energy absorbed in the short term and photosystem accumulation adjustment that decreases light absorption in the long term. The latter plays a major role in decreasing the light energy input into the chain. It was observed that the relative light absorbed per cell decreases in excessive light, consistent with the decreasing levels of the photosystem core.

Role of the redox poise

Among the electrons acceptors, ferredoxin which is the first intermediate downstream the photosynthetic machinery, is seen as a major player in energy distribution (Peden et al., 2013). Ferredoxin may have a role in regulation as its overreduction triggers cyclic electron flow (Johnson et al., 2014) and regulates *psbA* translation (Trebitsh and Danon, 2001). Redox poise sensing via ferredoxin is thought to go through thioredoxin and STT7 as suggested (Lemeille and Rochaix, 2010). Unfortunately, no technique is available to investigate the redox state of the different ferredoxins, but western-blotting analysis could help to show if there is a correlation between their accumulation and the regulation of several chloroplast proteins. In addition, an acclimation test using different ferredoxin, thioredoxin, and flavodoxin mutants may be attempted, but only if the mutant is photosynthetic. This also points to the problem of the redundancy of the ferredoxins. In vitro, the Glu-91 residue in FDX1 (PETF) was found to be critical for reduction of thioredoxin by FTR (Jacquot et al., 1997). The rescue of an *fdx1* mutant by a modified ferredoxin would show a different long term acclimation phenotype if thioredoxin was involved in the pathway, and if flavodoxin does not compensate the effect. Overexpression of this ferredoxin leads to markedly better resistance to heat stress, suggesting that it has a role in pre-acclimation and/or ROS scavenging (Lin et al., 2013). Studying light acclimation in this strain would help our understanding of the regulation.

At the level of b_6f complex, the redox state of the plastoquinone pool was postulated to affect gene expression via STT7, CSK and PTK in *C. reinhardtii* (Rochaix, 2013). The *stt7* mutant, which is unable to sense the plastoquinone redox poise and maybe also the ferredoxin redox state as seen in plants (Rintamäki et al., 2000), shows a modified accumulation of PSAD, PsbA and LHCSR3 compared to the wild type, affecting the acclimation needed to grow under different light irradiances (Bergner et al., 2015). This illustrates the regulatory role of the redox poise on protein biogenesis or degradation. In *A. thaliana,* the *stn8* mutant which is affected in phosphorylation of PSII and therefore in regulating the photosynthetic apparatus, shows

decreased accumulation of *psaA, PSAD, LHCB2.1,* and *rbcL* mRNA (Bonardi et al., 2005). In addition, the *pgrl1* mutant, impaired in the cyclic pathway around PSI which normally reoxidizes ferredoxin and reduces the plastoquinone pool, shows a decreased accumulation of PSAD. In Chlamydomonas, *npq4* is a mutant impaired in LHCSR3 , which is involved in energy dissipation in high light. Its absence leads to over reduction of the photosynthetic chain in high light, therefore inducing ROS production. In *npq4*, the accumulation of PSAD is not modified while *pgrl1*/*npq4* shows a bigger loss of PSAD than the *pgrl1* single mutants (Bergner et al., 2015). Taken together, these data suggest that several signals might be detected separately, which subsequently trigger different effects on the accumulation response of the photosystems.

Identification of the regulation targets

The target of regulation induced by high light is unknown. Because the circadian rhythm is one of the best-characterized light regulatory responses at the level of transcription, I decided to examine the interaction between the high-light response and the diurnal rhythm. The amount of PsaA per cell fluctuated in line with the total protein quantity, suggesting that light/dark cycles do not affect the quantity of PsaA with respect to total protein. However on a per cell basis, total protein amounts as well as PsaA increase gradually and then drop because of cell division.

The lack of PsaA overaccumulation during the dark phase in high-light acclimated cells suggests that down-regulation of PsaA accumulation is inhibited during the night in synchronized cells. This contrasts with the acclimation of continuously illuminated cells, in which the change in conditions already triggers visible effects within the first 3 hours (figure 28). The first hypothesis that can be made explaining the lack of PsaA overaccumulation would be that protein synthesis is slowed down at night due to the lack of available energy. Another hypothesis would be that an unknown component of the signaling pathway can perceive whether cells are in day or night conditions and can block regulation at night. Jacobshagen and Johnson (1994) reported that some proteins have a continuous rhythmicity entrained by cell division even if the cells have not been exposed to light/dark cycles. , Other proteins stay perfectly constant in continuous dim light and oscillate in light/dark cycles, demonstrating that not all proteins are affected in the same way by light/dark rhythmicity and suggesting the existence of acclimation to rhythmic conditions.

Chloroplasts of many species contain introns in their genome. While splicing varies

between species on the base of the length of their introns, and in the genes involved, the presence of splicing in the chloroplast is conserved. An evolutionary advantage of splicing was proposed to occur at the regulatory level as *psbA* mRNA, which has four group I introns, has a light-dependent splicing rate (Deshpande et al., 1997) while the replacement of the *psbA* gene with an intron less copy is phenotypically silent in laboratory conditions (Johanningmeier and Heiss, 1993). In contrast, trans-splicing of the *psaA* mRNA, which is matured from four different transcripts, was not found to have a regulatory role during iron deprivation (Lefebvre-Legendre et al., 2014). The results presented here demonstrate that a strain with an intron-less version of *psaA* still exhibits high-light regulation of PsaA accumulation. This is a further situation in which the presence of splicing does not present a visible advantage over the non-spliced situation.

Moreover, the fact that *psaA* splicing is not affected by light, directed the search of the regulation toward investigations on mRNA accumulation in response to light treatment. RNA-blot experiments on cells acclimated to the different light intensities indicated that the *psaA, psaB,* and *psaC* mRNAs are not significantly affected by light treatment. Therefore, it could be assumed that messenger quantity does not reflect protein quantity, which depends on translation and protein degradation (Drapier et al., 1998)(Eberhard et al., 2002). Drapier et al. (1998) demonstrated that accumulation of *atpA* mRNA is not limiting for protein accumulation. In order to determine a possible role of mRNA accumulation and of translation, on the expression of the chloroplast encoded PSI subunits A, B, and C, the promoters and 5′UTRs of *psaA*, *psaB,* and *psaC* were fused to the luciferase ORF. There were no significant differences of the luciferase activity for both *psaA* and *psaB* 5'UTR while a small but significant difference can be observed for *psaC 5'UTR*. This observation would suggest a role of *psaC* in the regulation of the stability of the photosystem I in response to different irradiances. Of course, the reliability of the luciferase reporter depends on the construct, which may need further improvement, such as a longer 5′ fragment. The 3′UTR is sometimes required for correct translation (Rott et al., 1998). Association of the luciferase with the endogenous 3′ of the respective genes might show whether acclimation to these different conditions involves the 3'UTR. In addition, several publications have reported that the coding sequences can also have roles in translational regulation, as well as the context of the start codon, the presence of introns or the codon usage (Chaney and Clark, 2015; Richter and Coller, 2015). Fusion of the luciferase as a C- terminal tail to the gene of interest could yield an even more reliable reporter construct but may also induce the co-degradation of both the endogenous and the luciferase

proteins, therefore affecting the reliability of the reporter . Use of a self-cleavable FMDV-2A peptide as a spacer might solve the problem (this peptide was successfully used in E.coli (Dechamma et al., 2008)). A protein pulse-labelling experiment with $^{35}SO_4$ or ^{14}C-acetate could also be used to monitor translation of PsaA/B. but in high light, D1 (and maybe others) is highly translated, thus creating a high level of background and making other proteins difficult to visualize.

Translation was monitored by blocking degradation with protease inhibitors. This showed that PsaA appears to be stable when treated with a protease inhibitor, suggesting that either It is not stable and the degrading protease is not sensitive to the treatment, or that the rate of degradation is slow and PsaA stable, or that the treatment did not work, although it was reported to increase VapA accumulation (Michelet et al., 2011). Following the same treatment, D1 levels were slightly decreased. In contrast, chloramphenicol treatment, which prevents chloroplast translation, drastically lowered D1 levels, demonstrating that it has a high turnover rate in high light (turnover rate defined as the speed of degradation/replacement) while it is slower under low light. Collectively, these data suggest that D1 is regulated by an abrupt change in its turnover rate. PsaA protein seems to be more stable, demonstrating that the low levels of PSI observed under high light reflect small changes in the turnover rate. Interestingly, PsaA shows a small increase when cells are treated with chloramphenicol. Considering that this observation implies a balance between synthesis and degradation, this result suggests a slower rate of degradation. The only protease that could be directly affected by chloramphenicol treatment is the single chloroplast protease that contains a chloroplast-encoded subunit, ClpP1. This subunit is involved in the degradation of RpoA, Rps12, and AtpB (Ramundo et al., 2014).

Materials and methods

Strain and culture conditions

The strain used in this study was the WT 1a+ (cell wall-plus) strain derived from 137C. The experiments were performed in filtered HSM (to avoid autoclave-induced precipitate formation), unless otherwise specified. The cultures were all bubbled with ambient air and stirred under fluorescent tubes with the indicated light intensity. *raa1* is a nuclear mutant deficient in *psaA* splicing, and complemented strains have been described (Lefebvre-Legendre

et al., 2014). FUD50 is a chloroplastic mutant carrying an *atpB* deletion (Goldschmidt-Clermont et al., 1991). The cells were harvested in the log phase below 2 × 10^6 cell/mL.

Luciferase strains

The strains expressing luciferase under the control of the *psaA*, *psaB*, and *psaC* 5′UTR were obtained by transformation of the FUD50 strain with a construct containing the *atpB* gene and the different luciferase chimeric genes as described in figure 6 of the MAC1 manuscript. The *psaA lucCP* construct contains 405 bp upstream of the ATG in exon 1, for *psaB* 596 bp upstream of the ATG, and for *psaC* 250 bp.

The transformation was performed by Helium gun as previously described ((Boynton et al., 1988; Neupert et al., 2012). Homoplasmy of the transformants was checked by the lack of the parental FUD50 deletion. PCR gave an amplicon of 1254 bp for the deletion while the wild-type fragment would be 3441 bp long (using *Eco*RI-AtpB-for and *Atp*B-rev4 primers; 35 cycles, 50°C, 1.5 min of elongation).

NBT measurements

NBT (Sigma) was added directly at 50 µM to 2 mL of the culture containing 1 × 10^6 cell/mL, which were then treated with the indicated light level for 1 hour. The reaction mix was then filtered on Whatman TLC grade paper discs and washed once with 1 M HCl and once with 100% methanol. The filters were then dried and scanned.

RNA */probes*

psaA Ex3 (Genomic)	TTAACCTACAGAAATGATACGTG	AAATTTTAGAAGCTCACCGT
psaB (Genomic)	AACTGTTTCCAAAATTTAGC	AATGAGTACATGTGTTGTG
psaC (Genomic)	CCCATTAGCCGTGGTTTTACTCAT	ATTCCCTAATGGACCAAAAGCAGTCATTCAA
psbD (Genomic)	GATGACTATGCACAAAGCAG	ACATTGCGTGTATCTCCAAAA

Luciferase measurements

The different strains were grown under the indicated light conditions until they reached a density of 2 × 10^6 cell/mL. For *psaA* and *psaB* promoters, triplicate samples of 50 µL of each culture was placed in a white 96-well plate on dry ice, the *psaC* culture was concentrated 10-fold before being deposited. Frozen plates were kept at -20°C prior to the measurement. For the measurement, 100 µL of luciferase reagent (potassium phosphate buffer 50 mM pH 7, 150 mM NaCl, protease inhibitor cocktail (Sigma), 2.5 mM ATP, 2.5 mM $MgCl_2$, and 4 mM luciferin) was added to the frozen plate using a multichannel pipette before monitoring luminescence emission kinetics at 28°C in a Biotek Synergy 2 plate reader. The peak value of luminescence was retained for analysis.

Chloramphenicol and protease inhibitor treatments

The experiment was conducted as previously described (Michelet et al., 2011). Briefly, acclimated cultures were supplemented with either 200 µg/mL of chloramphenicol, or with protease inhibitors (1 mM AEBSF [Sigma-Aldrich]) plus two protease inhibitor cocktail tablets for 100 mL culture volume (Complete mini, EDTA-free [Roche]). Samples were harvested at the indicated times following treatment.

GENERAL DISCUSSION

BACKGROUND

The assembly of protein complexes in the chloroplast requires concerted gene expression from both the nucleus and the chloroplast genetic machinery. The nucleus encodes most of the chloroplast proteins that are involved in metabolism, or participate in the assembly and structure of the photosynthetic complexes. Among the nucleus-encoded proteins exported to the chloroplast, many are involved in plastid gene expression, and assist with splicing, maturation, stability and translation of mRNAs. So far, little is known about their role in modulating gene expression in response to environmental changes.

MAC I

The role of MAC1 in *psaC* mRNA expression.

The results presented in this thesis report the characterization of a nuclear mutant of *Chlamydomonas reinhardtii* that is deficient in PSI. This strain, hereafter called *mac1*, was obtained from a random insertional mutagenesis screen and contains only one-fifth the wild-type amounts of PsaA and no detectable PsaC, and has lost the capacity for photoautotrophic growth. This parallels what was reported for a *psaC* deleted strain (Takahashi et al., 1991) in which the whole PSI was rapidly degraded. Further analysis revealed that *mac1* fails to accumulate both the mature *psaC* mRNA and the dicistronic precursor RNA *psaC-petL*, while *petL* is not affected, probably stabilized by independent mechanisms. The presence of wild-type amounts of *petL* also indicates that it is transcribed normally. Similar effects were seen with the deletion of *psbI* that did not affect the presence of the other mature mRNAs transcribed from the same polycistronic unit (Drapier et al., 1998). The transcriptional activity of *psaC* was evaluated in a run-on transcription assay, which revealed that this and other genes appear to be normally transcribed in the *mac1* mutant compared to the WT. This result offers another example that most of the nuclear mutants affected in chloroplast gene expression are not

impaired at the level of their transcription but at subsequent steps (see table 6 in the Introduction). This illustrates a divergence in the regulation of gene expression when compared to the chloroplast ancestor, a cyanobacterium.

Furthermore, in *mac1* other PSI mRNAs are unaffected at the level of their splicing, maturation or stability, but effects on their translation were not investigated. It was demonstrated that the translation or stability of complex subunits in the chloroplast of *C. reinhardtii* can be controlled by the assembly of the complex itself (Choquet et al., 1998; Kuras and Wollman, 1994). The stoichiometric accumulation of the different subunits needed for the accumulation of a complex is thought to be regulated by the presence of unassembled subunits. This feedback regulation known as Control by Epistasy of Synthesis CES acronym was shown to control the expression of the chloroplast-encoded PSI subunits (Wostrikoff et al., 2004). When PsaA is produced in excess of its assembly partner PsaB, unassembled PsaA exerts negative feedback regulation on the translation of its own mRNA. Likewise unassembled PsaC negatively regulates its own translation. CES is also involved in the regulation of the other photosynthetic complexes, such as the b6f complex and PSII. In the case of ATP synthase, overaccumulating AtpB subunit positively regulates AtpA biogenesis while downregulating its own, through effects on translation at the level of the 5'UTR (Drapier et al., 2007). The accumulation of MCA1, which is required for the expression of the *petA* mRNA, increases when the cyt_f is not assembled in the b_6f complex (Boulouis et al., 2011). This behavior was not observed with MAC1, its accumulation is independent of the presence of PsaC in strains where *psaC* is disrupted by the *aadA* cassette (Takahashi et al., 1991)(data not shown).

The sequencing of small RNAs from *C. reinhardtii* revealed that some were present in the chloroplast, due to the protective effect of RNA-binding proteins, producing “footprints” (Loizeau et al., 2014). Similarly plants also produces small RNA in the chloroplast that correspond to protein binding sites (Williams and Barkan, 2003; Ruwe and Schmitz-Linneweber, 2012). While the wild type possesses a 50-nucleotide footprint at the beginning of the *psaC* 5′UTR, the *mac1* strain does not, suggesting that MAC1 has a target in the 5′UTR of *psaC*. In vitro assays confirmed the ability of MAC1 to bind this 50nt track (figure 7 of the manuscript). This mode of action is common in the chloroplast as several key players in mRNA maturation also target the 5'UTR of mRNAs. This is for example the case of MBB1 (Loizeau et al., 2014), MCA1 (Raynaud et al., 2007), MDA1 (Drapier et al., 2002) and numerous others. A firefly luciferase reporter gene under the control of a fragment containing the promoter ant 5'UTR of *psaC* (250bp upstream the start codon) confirmed that a target of MAC1 is the *psaC*

5'UTR.

The *MAC1* gene disrupted in the mutant was cloned by inverse PCR. Genomic fragments corresponding to the whole *MAC1* gene transformed into the *mac1* mutant successfully complemented photoautotrophy. Complementation could be achieved with a large 80 Kb bacterial artificial chromosome (BAC) and with a 6 Kb fragment containing a single open reading frame encoding MAC1. The rescue also restored expression of the luciferase reporter driven by the *psaC* 5′UTR. MAC1 is a protein that is only present in unicellular algae, and is absent in land plants that use CRP1 (a PPR protein) to stabilize the *psaC* and *petA* mRNA (Schmitz-Linneweber et al., 2005). MAC1 was confirmed to be localized in the chloroplast by immunofluorescence and cell fractionation, the latter also showing its presence mainly in the stroma. These result suggest a model where MAC1 binds the 5'UTR of the *psaC* mRNA in the stroma.

BLAST searches with the MAC1 protein sequence revealed homology to MBB1, which promotes the stability of chloroplastic *psbB/T/H* mRNAs (Loizeau et al., 2014) in algae and its homologue HCF107 which stabilizes the 5'UTR of *psbH* and is needed for *psbB* translation in land plants (Felder et al., 2001). Both MAC1 and MBB1 are predicted to contain TPR or HAT repeats. The mutant *mac1* could be rescued with a MAC1 genomic fragment modified to encode a triple HA tag at the C-terminus. The different complemented lines contained various levels of MAC1-HA and showed a parallel accumulation of *psaC* mRNA, but all had wild-type amounts of the PsaC protein. This reveals that *psaC* mRNA is present in apparent excess in the conditions tested, which is compatible with the model that PsaC accumulation is regulated at the level of translation by CES. (Wostrikoff et al., 2004).

The maturation of RNA in the chloroplast depends on specific endonuclease cleavage and on exonuclease degradation from the 5' as well as from the 3'end. (Drager et al., 1996) (Chevalier et al., 2015). Even though 5'-3' degradation is often reported to be a major determinant of the half-life of mRNA, maturation mechanisms of polycistronic RNAs indicated the importance of alternative or partial degradation processes (Barkan et al., 1994; Choquet, 2009). The mature ends were shown to be determined by exonuclease protection, directed by sequence-specific RNA-binding proteins (often from the PPR, OPR, or TPR protein families, see table 6 of the Introduction) or by hairpin structures.(Goldschmidt-Clermont et al., 2008). In order to distinguish between different maturation mechanisms, a polyG tract is often inserted either in the 5' or 3' UTR, to create a structure blocking exonucleases (Drager et al., 1998). The experiments with luciferase reporter expression driven by the *psaC 5'UTR* revealed that the

insertion of a polyG tract does not completely compensate the lack of MAC1 in the mutant. It suggests that the mRNA is still unstable and that either the position of the polyG is not optimal for the re-stabilization, or that mRNA degradation could occur through more complex mechanisms involving endonucleases or 3′–5′ exonucleases that are normally blocked directly or indirectly by MAC1. As the chimeric luciferase transcripts (*psaC:lucCP*) were heterogeneous and could not be reliably detected, it could be argued that MAC1 also plays a role in the *psaC* translation as both the *psaC:lucCP* fusion and the wild type *psaC* gene under the control of the polyG-modified *psaC 5'UTR* did not produce any detectable protein. This role in translation would suggest its transient association within a translation initiation complex containing components such as RBP40 (Schwarz et al., 2007).

The association of MAC1 with other components in a larger machinery was assessed by running crude extracts of soluble proteins on a sucrose sedimentation gradient. This experiment revealed the presence of MAC1 mainly as isolated protein, but also a small amount in a larger complex (figure 3 C of the manuscript). It cannot be excluded that transient or RNA-dependent complexes could have been lost, as the lysis and sedimentation analysis may have disrupted the weaker interactions. It could be speculated that most of MAC1 is acting as an independent protein to stabilize the mRNA and that its role is more likely independent from the translation machinery. This would contrast with the findings with MBB1 (Vaistij et al., 2000a), MCA1 (Boulouis et al., 2011) and NAC2 (Boudreau et al., 2000) that may be involved in a complex coordinating both stability and translation.

In the natural habitat of *C.reinhardti*, iron is thought to often be in low abundance according to the 15 last years of water analyses (Massachusetts Water Resources Authority, 2015). The acclimation of the alga to its environment is a matter of survival, as iron is needed for several key processes such as ROS detoxification, photosynthesis and mitochondrial respiration. During iron starvation, PSI, which contains 12 iron atoms, is preferentially degraded in heterotrophic or mixotrophic conditions. PsaC, which contains 8 of these irons, is drastically affected. The quantity of MAC1 is coordinated with the quantity of *psaC* mRNA, both decreasing in parallel approximately two and four fold respectively. This result, together with the observation that the quantity of *psaC* mRNA parallels MAC1 accumulation in transgenic lines, suggest that MAC1 is a regulator of *psaC* mRNA accumulation and that the targeted degradation of MAC1 could be responsible for the decrease of *psaC* mRNA under iron starvation. In addition, MAC1 was demonstrated to be phosphorylated under normal light, but loses this phosphorylation in low iron. These results suggest a role for MAC in acclimation, but

further investigation is needed. MAC1 phosphorylation is also affected when state 1 or state 2 are induced, the latter showing supernumerary phosphorylation and the disappearance of the non-phosphorylated form. This phosphorylation is independent of Stt7 and is not at the sites identified in published phosphoproteomics data (Wang et al., 2014).

Even if the role of this phosphorylation has not been experimentally elucidated, this is the first case of regulated protein phosphorylation reported at the level of chloroplast mRNA metabolism to my knowledge. Taking all these results together, a model could be built to explain MAC1 activity. I propose that MAC1 accumulation is limiting for *psaC* mRNA accumulation, that phosphorylation enhances the stabilization activity of MAC1 on the *psaC* mRNA but also triggers MAC1 degradation, while the non-phosphorylated protein would be a storage form. This scheme is typical for signaling pathways of all kinds, the active signal should not be stable and consequently time-limited. The response of this phosphorylation to different environmental conditions strongly suggest the existence of regulatory kinases other than STT7 affecting directly the photosynthetic complex subunits. STL1, thought to phosphorylate photosystems core subunits in response to light quality and quantity (Rochaix et al., 2012) is a possible candidate for MAC1 phosphorylation, however no mutants were reported in *C. reinhardtii* to test this hypothesis.

The existence of a factor such as MAC1 involved in the stabilization of the *psaC 5'UTR* is thought to originate from gene duplication and neutral evolution (Barkan and Small, 2014). In this particular case, the paralogy between MAC1 and MBB1 suggest that MAC1 might have derived from a copy of MBB1. This event seems to be relatively recent as the duplication is only found in *Chlorophytae.* RNA binding proteins like MBB1 could have duplicated and one of the copies, MAC1, evolved until its binding activity for *psbB* was lost in favor of *psaC*. The secondary structure originally stabilizing the *psaC* mRNA then drifted little by little as MAC1 binding was enough to guarantee the mRNA stability. This speculation may be supported by the presence of a predicted loose hairpin at the 5'end of the *psaC* mRNA, that may have guaranteed the stability of the mRNA before MAC1 arose. This would also explain the lack of strong interaction with the pre-existing translational machinery on the *psaC 5'UTR* (sucrose gradient experiments, (figure 3 C of the manuscript).

RNA binding proteins of the PPR family bind the target RNA with a correspondence of one repeat for one base, for example PPR10 has 15 repeats that bind 15 bases. Each repeat harbors the key aminoacid responsible for sequence recognition. It was possible to decipher the binding-code of PPR proteins (Barkan et al., 2012). MAC1 protein contains about the same

number of predicted repeats as PPR10 but the footprint at the 5' end of *psaC* appears to be 50 bases long. This discrepancy, together with the fact that MAC1 does not appear to have stable binding-partners prompts me to make several hypotheses. The first would be that the secondary structure of the RNA could pay a role in the binding affinity, but so far, to our knowledge, no other repeat containing protein was shown to have double-stranded RNA as a target. However it should be noted that members of the mTERF family can bind dsDNA (Jiménez-Menéndez et al., 2010) as well as RNA (Hsu et al., 2014). The second hypothesis to explain the lack of the sRNA footprint in *mac1* would be that the secondary structure is possibly responsible for the RNAse-resistant footprint and that the fast degradation of the *psaC* RNA in the mutant prevents its formation . Finally it could be hypothesized that MAC1 has binding partners with which the interaction is too transient or unstable to be clearly observed by sucrose gradient sedimentation experiments.

Acclimation to excess light.

High light affects PS I accumulation and triggers its levels to decrease as was observed in other work (Bonente et al., 2012). This effect is less pronounced in the presence of CO_2 or acetate, and is increased by sparging the culture with N_2. The similar effect between the addition of acetate and the addition of CO_2 may be explained by the fact that the respiration of acetate provides CO_2 needed for phototrophic metabolism (Singh et al., 2014). Similarly, bubbling of nitrogen removes dissolved CO_2 and increases the stress. These observations highlight a link between the availability of electron acceptors and the regulation of photosynthesis through protein stability or biogenesis.

Acclimation to stressing high-light conditions triggers a sequence of events including ROS production, energy quenching, adjustments of protein biogenesis and degradation, and in extreme cases autophagy. The high-light induced autophagy response peaks 6 hours after induction, but goes back to normal after 24h of acclimation (Pérez-Pérez et al., 2012). In the condition used for the experiments presented in this manuscript, ROS production was evaluated after acclimation, and appeared to be below the detection limit. In contrast cells that had been pre-acclimated to medium-light produced a significant amount of ROS after 1 hour of high light treatment.

The up-regulation of PSI appears to be abolished during the night period in cultures acclimated to light/dark cycles. The quantity of PS I stays more or less stable compared to the

amount of protein throughout the dark phase, while an increase can be observed after 3 hours of low light treatment in a culture acclimated to constant high light. The results presented here also reveal that cells appear to be contain less protein in high light, reflecting a difference in cell size.

No effect of high light through *psaA* splicing was observed as PsaA accumulation was comparable in the wild type and in the intron-less *psaA* strain. A minor difference was seen on mRNA accumulation (reported by northern-blot) and on 5'UTR translational activity (reported by luciferase fused to the promoter/5'UTRs of *psaA*, *psaB,* and *psaC*). Chloramphenicol treatment of high-light acclimated cells revealed that PsaA has a slow turn-over rate even under high light, while that of D1 is extremely fast. This suggests that PsaA accumulation in acclimation experiments must be modulated by small changes in its synthesis and or degradation rates, which are difficult to reveal. It also indicates the absence of an autophagy-like response that would have triggered parallel degradation of all thylakoid proteins.

Open questions and perspectives

This study leaves open several questions concerning the modulation of protein synthesis in the chloroplast. It is worth pointing out recent examples concerning regulation of mRNA accumulation and/or translation in response to nitrogen or iron limitation (Wei et al., 2014; Lefebvre-Legendre et al., 2015a). Therefore the questions arise, is there any regulation of MAC1? And does MAC1 regulate *psaC* expression ? With this study, I have contributed to the understanding of the modulation of RNA metabolism, showing, in particular, that MAC1 limits *psaC* mRNA accumulation while conversely, the amounts of *psaC* mRNA and protein do not seem to affect MAC1 accumulation. The mechanism by which MAC1 controls *psaC* mRNA is still unclear, and while we observed that MAC1 binds the 5'UTR in vitro, by analogy with the results obtained with HCF107 in Arabidopsis thaliana (Hammani et al., 2012) and with MBB1 (Loizeau et al., 2014), we cannot exclude a role of the secondary structure predicted at the *psaC* mRNA 5' end in both MAC1 binding and mRNA stabilization. The precise mechanism by which MAC1 stabilizes the *psaC* messenger can be further elucidated by narrowing down the binding site through in vitro assays. This would suggest strategies to engineer the 5' end, removing the hairpin structure while keeping the MAC1 binding site, to investigate the possible role of this structure in the binding affinity.

We have demonstrated that MAC1 is involved in *psaC* RNA stability, but the question

of whether it is also involved in its translation remains unanswered. To solve this problem, a way to re-stabilize the RNA without disturbing the activity of other proteins on the 5′ UTR is needed. To do so, I would propose fusing the PPR10 (*Zea mays*) recognition sequence to the start of the *psaC* 5′UTR of *C. reinhardtii*, and expressing PPR10 (with the transit peptide of PetF or Hsp70B [Tardif et al., 2012]) in the chloroplast or in the nucleus with the proper codon usage. The detailed PPR10 mechanism of action has been described (Prikryl et al., 2011). Its tight binding blocks exonuclease progression but also affects translation by binding to one strand of a hairpin loop, preventing its folding. This secondary structure is thought to mask the ribosome binding site, so that the binding of PPR10 favors accessibility of the ribosome to its binding site. The presence of only the binding sequence of PPR10 in the 5'UTR of *psaC* would be predicted to only ensure stability with no effect on translation, as there will not be the complementary strand forming the translation-blocking loop. This should also retain the native structure of the psaC 5′UTR and preserve mechanisms that affect translation. However it cannot be excluded that the PPR10 protein could respond to regulation in *Z. mays* and that the putative signals could be present as well in *C. reinhardtii* chloroplast and therefore complicate the interpretation of the results. Alternatively the viral *MS2* tag could be fused to the 5'UTR of *psaC* and the MS2 protein expressed as proposed above for PPR10. The binding of the MS2 protein to its RNA target has the advantage that it may re-stabilize the mRNA with no risk of any residual regulatory effect, as the MS2 system was derived from a bacteriophage.

Other precedents suggest that proteins like MAC1 most likely to act through the 5′UTR directly or indirectly, which is confirmed by my experiments with the luciferase reporter. On the other hand, correct 3′UTR maturation is required for translation (Rott et al., 1998). One study suggested that 3'UTRs may not play a major role in translation regulation but can slightly affect the mRNA stability (Barnes et al., 2005). Furthermore, evidence suggested that elements in the coding sequence can influence translation initiation (Chang et al., 2010). It is therefore possible to imagine that MAC1 could play a broader role than initially thought, not only on the 5'UTR, but also in the 3'UTR, or in the coding sequence. The further elucidation of the mode of action of MAC1 could involve the construction of different reporter strains with a longer *psaC* 5′flank and/or 3′flank, because the low level of expression of the luciferase suggests that the promoter used in this work was too short. Using the *gfp* reporter could also be better because the RNA generated with *gfp* chimeric genes seems to be more stable than *luxCP* which gave rise to heterogeneous transcripts (Barnes et al., 2005). The use of a negative selection marker like cytosine deaminase (*crCD*) would be a useful tool to obtain a clean marker-free insertion

(Young and Purton, 2014). This transformation strategy would need first a knock-down of the *psaC* gene with a positive and negative marker substituting the entire gene and 5'UTR, and then the introduction of a chimeric construct where in the original context of the locus the psaC ORF has been replaced by the gfp ORF. Alternatively the use of the crCD-HA-A1 strain (Young and Purton, 2014), in which the crCD gene is inserted downstream of psbH, would allow the seamless insertion of an engineered *psaC* gene at another locus, replacing the negative selection marker.

The 5′UTR of *psaC* hosts a target sequence bound by MAC1 or a partner protein. This sequence is still not precisely identified but chloroplast transformation to generate mutations of the 5′UTR would help mapping it. To define the MBB1 binding site a linker-scan experiment was performed by replacing a sliding six-base window with a restriction site in a series of mutant strains (Loizeau et al., 2014). Interestingly, in the PPR domains, two key amino acids of each repeat were found to be involved in determining the specificity of binding to the respective nucleic acid residue, defining the RNA recognition code of the PPRs (Barkan et al., 2012). Discovery of the MAC1 target sequence could help to find correlations between the bases in the RNA and specific amino-acid residues in the repeats, and thus reveal the recognition code of TPR/HAT repeats.

It is also possible that MAC1 does not bind the mRNA directly. Some of the TPRs in MAC1 could be involved in weak interactions (not detected on sucrose gradients, data not shown) with putative partners, since TPRs were first described as protein-protein interaction domains. In this case, it is possible that one of the MAC1 partners contains another RNA binding domain. The next step could be to identify binding partners via co-immunoprecipitation (co-IP). The co-IP could be done using a HA-tagged MAC1 as the bait bound by anti-HA antibodies covalently coupled to magnetic beads. This technique can be used to co-purify high-affinity partners, but the weak or transient interactions suspected here would need a reversible crosslinking with interacting proteins using molecules like DSP (dithiobis(succinimidyl propionate)) or DTME (dithiobismaleimidoethane) in a method called reverse crosslink immunoprecipitation (Smith et al., 2011). This protocol was designed using human carcinoma cells that may have different permeability to the cross-linkers than *C. reinhardtii,* in particular considering that the reagents would also have to cross the bi-layered chloroplast envelope. Thus, additional optimization of the protocol would be required.

The MAC1 interactome can also be investigated using a yeast double-hybrid screen with a library of *C. reinhardtii* cDNA. This experiment could help to determine partners like it

was done for RAA7 (Lefebvre-Legendre et al., 2015b) and RAA4 (Jacobs et al., 2013). Among the partners that might be identified with this technique, some might have been reported to bind RNA, or to be regulated in response to environmental cues. The results of these experiments might open an area of research on the mode of regulation within such complexes It would thus be interesting to determine whether the MAC1 association in the complex depends on the growth conditions, on its phosphorylation, or the redox status in the chloroplast. The phosphorylation of MAC1 might depend on a kinase that could be found among the interacting partners. The identification of the kinase responsible for MAC1 phosphorylation could be confirmed if corresponding mutants available. As the number of kinases discovered in the chloroplast is limited, it is possible that their substrate specificity is provided through partners that may make the regulation pathway more complex. In the A. thaliana chloroplast, 905 putative kinase substrates were identified (Schönberg and Baginsky, 2015) while only 45 kinases and 21 phosphatases are predicted (Schliebner et al., 2008).

Determination of the exact phosphorylation sites in MAC1 could help to understand their role in its hypothetical regulation. Mapping of the phosphorylation sites by mass spectrometry in a whole protein extract might be difficult because of the low abundance of MAC1. The extract could be enriched using immunoprecipitation techniques with a strain expressing HA-tagged MAC1. The mass spectrometry data should reveal phosphorylated residues of MAC1 that could then be mutated. Mutations to aspartic acid or glutamic acid are commonly used to mimic constitutive phosphorylation of serine and threonine, while alanine is usually used to prevent any phosphorylation. Using such mutants, it would then be possible to verify if phosphorylation affects MAC1 function and if it has a role in its regulation. As the direct binding of MAC1 to RNA remains unclear, the effects of phosphomimetic mutations might be checked at the level of its ability to bind RNA (EMSA with recombinant protein or Yeast triple hybrid) and to bind its eventual partners (yeast two hybrid or co-IP).

MAC1 is approximately 100 KDa in size, and contains 10 cysteins including six that are conserved in Volvox, but none that are conserved in other hypothetical MAC1 orthologous in other species. Thioredoxin regulation of the formation of disulfide bridges by cysteine residues is known to regulate many activities in the chloroplast. The STT7 kinase for example is regulated via disulfide bridges and indirectly affects chloroplast gene expression (Lemeille et al., 2009; Rochaix, 2013). The identification of disulfide bonds and the dependency of their formation according to the growth conditions could help determine whether MAC1 can be regulated through cysteine disulfides. The PEG-maleimid method was used to characterize

disulfide bridge formation in E. coli (Koch et al., 2012). The maleimid moiety reacts with free thiols and the attached PEG molecule retards the migration of the corresponding protein in SDS PAGE gels. The identification of phosphosites and disulfide bridges could also be pursued on the identified partners of MAC1. Several mass spectrometry methods allow the study of disulfide bounds (Tsai et al., 2013) as well as the phosphorylation of protein samples. The effects of stimuli such as iron, sulfur or nitrogen starvation, light intensity or quality, and CO_2 availability could then be investigated on MAC1 partners.

In a more general perspective, MAC1 is only one character among many nuclear players involved in mRNA metabolism and other steps of gene expression. A deeper understanding of long-term acclimation would benefit from the development of new strains with a controllable level of different key components in the photosynthetic chain. Amongst these, the proton gradient could be manipulated by the use of an inducible ATPase. An inducible expression system was developed for the chloroplast using thiamine-regulated *NAC2* expression in the nucleus. The *THI4* riboswitch is able to interfere with intron splicing of *Nac2* leading to the inclusion of an exon containing a premature stop codon in the absence of thiamine. The expression of *NAC2* is required for the stabilization of the *psbD* 5′UTR and expression of the downstream ORF. Hence a vitamin block of *NAC2* expression leads to the loss of the chimeric mRNA target driven by the *psbD* 5'UTR (Ramundo et al., 2014). Similarly, an amiRNA (Molnar et al., 2009) with an inducible promoter such as *THI4* or *NIT*, targeting the expression of *MDA1*, encoding for a factor involved in *atpA* mRNA stabilization, would decrease ATPase function therefore increasing the proton gradient.

Another key component for regulation was demonstrated to be the redox state of the plastoquinone pool. This could be controlled either by the use of inhibitors like DCMU and DBMIB (in low amounts for long term acclimation), or by inducing or repressing plastocyanin or cytochrome c6. A plastocyanin mutant, ac-208, is available and would need to be engineered to express a adjustable amount of PC, for example using the vitamin-repressible system described above (Merchant and Bogorad, 1987). A library of *C. reinhardtii* mutants is under construction in the lab of M. Jonikas (Jinkerson and Jonikas, 2015), but thus far they have not identified any insertion in Cyc6. If a complete knock out is not ideal, their down-regulation could also be obtained by amiRNA silencing using an inducible promoter.

Artificial impairment of the ferredoxin redox balance could be achieved by site-directed mutagenesis affecting its redox potential but would need, for one or several of the 6 ferredoxin genes reported in *C. reinhardtii*, either a knock-out mutant (fdx5 is the only one reported (Yang

et al., 2015)), or an miRNA-driven knock-down. The known crystal structures of the different photosystems could also help in engineering the electron transfer chain, so as to generate strains with impairments in each step, without perturbing the assembly of the respective complexes. This could be done by targeting, in the different proteins, the residues involved in electron transfer and by mutating them to decrease electron transfer efficiency.

ANNEX

Photosynthesis—this is a word that has always directed my professional life. During the few years that I worked on my PhD, I met several people and saw many presentations that offered me the opportunity to discuss my opinions on photosynthesis. My idea about what photosynthesis really is and for what it can be used evolved a lot during this time. I believe that a PhD thesis, in addition to presenting the experimental results, should provide some insight into the subject.

The following paragraph summarizes my vision, which helped me understand where I was in the sea of scientific research and provided me with direction and motivation to pursue the drops of water I might add to this sea.

Photosynthesis in the context of energy crisis

Earth is a confined environment, which receives light as the only external energy source. Like all confined environments, populations grow until they reach a steady state, during which they begin to suffer from a lack of resources. Human beings, *Homo sapiens*, are a relatively new species that have only existed for around 200,000 years, representing around 8000 generations, and today, around 7 billion individuals. Currently, the population growth of our species is thought to be in an exponential phase (figure 1).

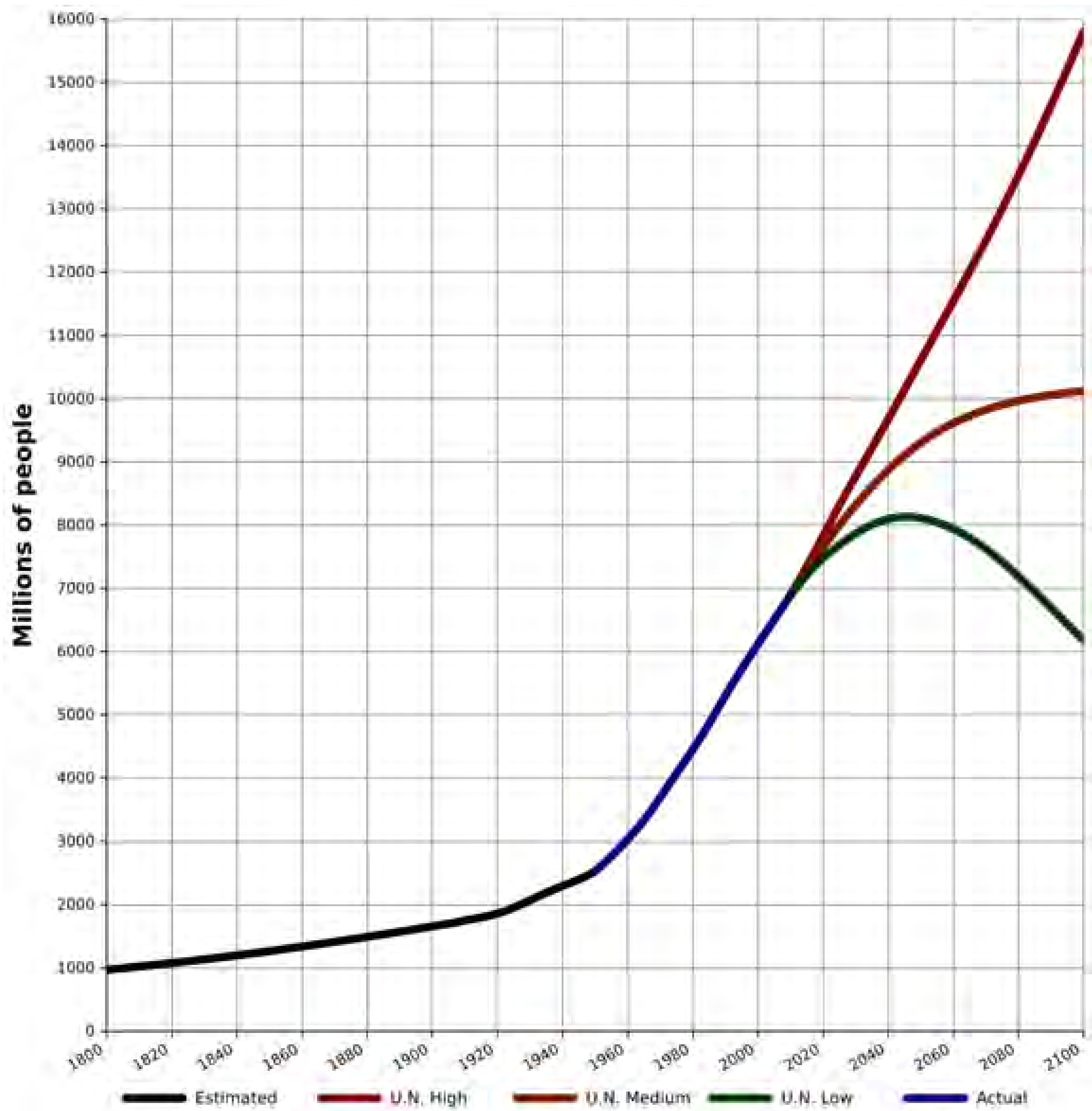


figure 1 (United-Nations, 2013; United-states Census Bureau, 2012)

In the last few decades, we have started to face worldwide limitations of food and energy levels, triggering concerns for the future of human populations. Nowadays, the economic development of countries is decreasing the birth rate, and their energy needs stabilizes. On the other hand, continuous population growth in undeveloped countries leads to a very high demand for food and energy. In both cases, we need to find a way to better utilize available resources, to track waste, and to save and recycle as much as possible.

Therefore, we should now ask, “How do we sustain both industrial and domestic

needs?" If we focus on energy, the first things we should consider is how energy is used and where it is found. According to the Annual Energy Review published by the US Energy Information Administration, the worldwide consumption of energy in 2010 could be broken down as follows: transportation 26%, industrial 52%, residential 14%, and commercial 8%. The total world energy consumption is predicted to increase by 85% until 2040 (+2% per year), mainly due to the rising energy demands of increasing populations and modernization of developing countries. As energy demand are almost stable in developed countries, increased demand in developing countries might be a prerequisite for their development. Limiting energy expenditure is very difficult, even though developed countries are investing in self-sustainability. Therefore, one solution would be to use an energy source that is more widely available.

Not all harvestable sources of energy are known. Among the used ones improvements can be done on limiting the transformation steps often resulting in big losses. Most of the energy available at the surface is derived from the sun light we receive, mainly biomass, including coal, petrol, and natural gas. The sun produces photons from nuclear fusion reactions within its core, hydrogen fusion producing helium and radiation. Such reactions can only occur under very harsh conditions; dozen of millions °C, with a density of around 150 g/cm^3 (The Solar Interior) On Earth, we receive around 1 millionth of the energy produced by the sun (energy emitted by the sun 3.846×10^{26} W (Nasa sun fact sheet, 2000). Energy received on earth $1{,}74\text{X}10^{17}$W(Archer, 2012)), most of which is transformed into heat on the Earth's surface and is reflected by the greenhouse gases (mostly water present in the clouds(NASA - Water Vapor Confirmed as Major Player in Climate Change)). Storage of this radiation occurs mainly as a result of photosynthesis.

Another source of energy available on earth surface is heat from the planet core, and this is a minor resource. Earth's core contributes 0.03% of the surface energy budget (Davies and Davies, 2010). More than half of the core's heat is due to nuclear decay (KamLAND, 2008)(Turcotte and Shubert, 2002), and the other half is derived from heat from the Big Bang. This means that we have a nuclear source of energy under our feet. Until now, only a small amount of this energy has been used via heat pumps and geothermal power stations.

The only non-nuclear derived energy source we have on earth is tidal power, also a minor source of energy. It is believed to originate from the earth rotational energy or the kinetic energy of a collision on earth that created the moon 4.5 billion years ago (Wenshi, 2010). The source of the kinetic energy of particles hitting Earth at this time is not known, but it could have been derived from the Big Bang, an explosion, or other collisions.

Nuclear energy on earth

During antiquity, we started to use nuclear energy from the sun in the form of heat, which triggers the evaporation of water on the Earth's surface providing it with potential energy. This water vapor condenses when it reaches higher and colder air masses, and leads to rainfall that fills rivers and dams. Using water mills, this potential energy is harvested and domesticated, for example, to make flour. On the other hand, because the Earth's surface is not homogenous, the sun does not warm all areas at the same rate, and these differences induce gradients in air mass density, which generates wind. This form of energy has been used in windmills. From the end of the 19th century, these techniques have gradually been modified and used to generate electricity via wind and water turbines.

Today, nuclear energy from the sun is also utilized by burning fossil fuel reserves (including coal, petrol, and natural gas, which all originated from photosynthesis) or biomass (including biogas, wood, oil, and ethanol, which are also derived from photosynthesis) to produce motor force, heat, or electricity. During these processes, there are many intermediate steps, in which energy loss occurs and waste is produced. For example, in the sun, part of the energy produced by nuclear fusion is emitted as light; one billionth of this light is received on Earth. Of this, only a small fraction is utilized by photosynthetic organisms, which use around 10% of this energy to make organic matter (Ruban, 2012). Most of this organic matter is digested by other organisms, and the energy is transformed into heat and mechanical movements etc. The remaining organic matter forms sediments and is partially transformed into gas, petrol, or coal. Some of this buried matter can be harvested through mines or well drilling, which consumes energy. These precious resources are then transported and transformed so they can be used in modern diesel engines, which at best transform 40% of the stored energy into mechanical movement. As a consequence, to obtain good energy production, we need to find a way to decrease intermediaries and increase the efficiency of each step.

Since September 3, 1948 we have been able to make electricity almost directly from nuclear fission. Uranium is mostly used to power the fission reaction, but this is not an unlimited resource. A third of the easily harvestable uranium in mines has been used, and ~70 years' worth of this fuel source remains. In addition, it also generates radioactive waste that has a long half-life. Thorium is a promising material that can produce nuclear energy from fission,

and which produces 200 times more energy per mass unit compared to uranium and is four times more abundant. Thorium generates waste with a short half-life (a few hundred compared to a few hundred thousand years), and it cannot be used to make bombs. However, thorium is at a major disadvantage, because it is lesser known and used than uranium. This means that the price of obtaining purified thorium remains much higher than that of uranium, but as enrichment is easier, the price of thorium will decrease as interest increases.

It is also possible to re-create the fusion reaction that occurs on the sun on Earth. Since the 1950's we have been able to reproduce this reaction, but making it self-sustainable is a major challenge of the 21st century. Deuterium and tritium are abundant elements on earth, and a ton of seawater is estimated to contain as much energy as 400 tons of burning coal (Keith, 2013). As previously noted, the problem of this fusion reaction is that it needs a particular environment, involving extreme temperature and high pressure. Much effort is focused on making this fusion self sustainable on earth. Finding materials that can resist such conditions is almost impossible, the techniques studied involve magnetic confinement, inertial confinement, or magnetized inertial confinement. However, these techniques use a lot of energy and self-sustainability has not yet been reached. For Deuterium–tritium fusion, tritium would be the limiting factor because it is not easy to purify but can be made out of lithium. This reaction needs only 100×10^{6} °C with a density that can be obtained through confinement. For 6 billion people, easily accessible lithium stocks are sufficient to generate 10 kWh/day per person for 1 million years, or ten times more than this if it can be purified from seawater(ITER.org, 2015). To compare, nowadays, power consumption in developed countries is around 150 kWh/day per person. On the other hand, deuterium–deuterium fusion requires a 300°C environment, the isotope is easier to purify and could produce 30,000 kWh/day per person for 60 billion years (Cowley, 2015)

The use of radioactive reactions has received bad press because of the waste generated and the risk of accidents at nuclear plants such as those occurring at Chernobyl and Fukushima. In 2011, we used around 20,000 TWh of electricity worldwide.

There are other possible ways to obtain this energy on Earth, and geothermy, which utilizes radioactive decay in the Earth's core, is one such safe way. The installed geothermal energy capacity is around 65 TWh, of which 20% is electricity according to the International Energy Agency (2010), and production could reach 1000 TWh by 2050.

Another way to obtain energy with fewer intermediaries is by utilizing solar energy via the use of photovoltaic panels. The installed photovoltaic energy capacity is around 110 TWh

worldwide according to the European Photovoltaic Association report (2013), which is predicted to double within the next 10 years according to the predictions. Solar cells used to generate electricity from sun light have an average efficiency of ~10%. Improvements have been made by robotizing the panels to ensure they face the sun throughout the day, thereby increasing the efficiency from 20 to 50% depending on the season (Solar cell efficiency, 2015). Other improvements in light capture have been made to the cell itself. In the laboratory, it is possible to achieve almost 50% of energy transformation (50 mW/cm² with 100 mW/cm² being the average sun irradiance on Earth) but this technology will not be produced commercially for at least another 25 years (Leite et al., 2013).

Photosynthesis to create energy

Photosynthesis is the primary mechanism that enables energy from the sun to be stored. This energy is then transformed into chemical energy via several intermediate steps. The second law of thermodynamics states that the entropy of a closed system never decreases. Considering the solar system as a closed system, each step of the energy conversion process leads to irreversible losses. This means that more energy is fixed during the initial steps of photosynthesis and that this decreases throughout the transformation, and this is what drives us to research ways to harvest energy at the earliest possible stages in this process.

Antenna level/artificial photosynthesis

Natural processes have always been sources of inspiration for innovation. Photosynthesis is not an exception, and our understanding of the mechanism of light absorption and transformation has inspired researches on how to reproduce this process. Artificial photosynthesis, based on pigment chemistry, could help to produce chemical bonds or electricity. Chlorophyll utilizes Mg atoms for charge separation, which is powered by light, from the coupled double bounds in the porphyrin ring. In artificial photosynthesis, chlorophyll is replaced by chelating agents containing conjugated double bounds that mimic the porphyrin moiety of the chlorophyll molecule. In some cases the central metal atom is also modified, and is changed from the classical Mg to Ru, Ir, H_2, or Zn. In other artificial photosynthetic energy transfer systems, chromophores containing aromatic cycles (Hückel) have been used for their particular wavelength absorption, with no need for any metallic atom (Frischmann et al., 2013).

Photosystem level/trimer extractions

The use of natural complexes to drive charge separation on metallic electrodes may also be a reliable way to harvest energy from sun light. In 2012, the production of 81 μW/cm² was achieved in the lab (Mershin et al., 2012) with a total yield of 0.08%, starting from photosystem I trimmers isolated from *Thermosynechococcus elongatus* thylakoids. Those extracted macrocomplexes were air dried on a TiO_2 chip covered by ZnO nanowires, which are thought to be stable for 3 weeks (Kiley et al., 2005). According to Mershin, interest in this method could increase once a straightforward protocol would be developed to enable photosystems to be extracted from crude plant material that is found in backyards and junkyards. A yield of 1 or 2% is needed to obtain a commercially viable product.

PH gradient/nano electrode

Direct harvest of electricity from the electron transfer chain is also possible. The use of nano-electrodes has been proposed (Ryu et al., 2010) in which they are directly inserted into *Chlamydomonas reinhardtii* chloroplasts. More precisely, this would involve one being inserted in the thylakoid membrane and one in the stroma, which would then generate electricity when irradiated. This technique is very efficient and 4 pA has previously been harvested from a single cell, and 5–10 × 10^{-13} W/cell at 100 μmol photons.m-2.s-1 (Ryu et al., 2010), but there are limited possibilities for scaling up.

Final acceptor/direct transfer to electrode

Other teams have been able to extract electricity directly from living cells. For example, *Shewanella oneidensis* MR-1 was submitted to several rounds of positive selection based on its capacity to use an electrode as the final electron acceptor (Tajima et al., 2011). The mutants obtained from this selection process were mainly cell surface mutants with altered polysaccharide metabolism, pilus structure, or secretion pathways. The same species was also used to treat wastewater while generating a current that could be used to produce hydrogen through electrolysis (Wang et al., 2013). The generation of direct current from photosynthetic organisms was attempted using *Chlorella vulgaris*. In a microbial fuel cell, this species was

used as a bio-cathode and wastewater containing natural bacteria was used as a bio-anode; this combination could generate 2.44 μW/cm² from a light emitting 0.3 mW/cm² giving an efficiency of 0.08% (Wu et al., 2013). Understanding the efficiency of energy transfer from the autotroph organism to the electrode is a major challenge in improving the efficiency of such devices. An important factor that affects the efficiency of the system is the material of the electrode itself and its compatibility with the organism to make biofilms. Using *Pseudanabaena limnetica* as an example, the best materials among those tested to make cathodes were stainless steel and indium tin oxide-coated polyethylene (Bombelli et al., 2012)

Pre RUBISCO/Hydrogen

It is possible to obtain energy by harvesting gas directly emitted from the photosynthetic electron transport chain. Under normal growth conditions, cells generate a small excess of reducing power, which is metabolized to make energy reserves for the night. When those organisms face conditions in which the energy received through photosynthesis is too high to be efficiently metabolized, they produce an excess of reducing power.

This excess of reducing power would be turning all the coenzymes into their reduced form, blocking the entire metabolism. A similar problem is encountered when the respiratory chain is blocked, in that the absence of the main electron acceptor, O_2, triggers fermentation, re-oxidation of the coenzymes, and creates fermentative products as waste. The photosynthetic chain faces the same problem, whereby re-oxidation of the coenzyme occurs by using it in anabolism, by the generation of waste products, or by enhancing the synthesis storage compounds (see “Production of organic matter”, “Photosynthesis to produce high value compounds”). One of the waste compounds generated directly from the photosynthetic electron transport chain is di-hydrogen, which is emitted via an anaerobiosis-activated hydrogenase that is considered to be an electron safety valve (Happe et al., 2002; Hemschemeier et al., 2009; Melis and Happe, 2001). This mechanism is found in many green algae, including *Chlamydomonas reinhardtii*, *Chlorella fusca, Scenedesmus obliquus, Chlorococcum littorale,* and *Platymonas subcordiformis* (Das and Veziroglu, 2008; Melis, 2007). This hydrogenase seems to have originated from by horizontal gene transfer from obligate anaerobic bacteria. Linking ferredoxin to hydrogenase has been shown to increase hydrogen production by redirecting electrons to the hydrogenase instead of FNR (Yacoby et al., 2011). Up to 10% (harvested light by photosynthetic complexes versus energy produced

as H2) could be theoretically achieved (Dau and Zaharieva, 2009). The gas produced by this enzyme is purified from the culture gaseous phase and stored in bottles under high pressure. According to initial evaluations, it seems that the compressed hydrogen requires a lot of energy to be stored, which accounts for around 10% of the total stored energy (Leveen, 2003). Great improvements in hydrogen storage have been made with new generation pumps for gas compression, and the creation of metal hybrid systems, ceramics, and liquid organic hydrogen carriers (Wikipedia, 2014).

Post Rubisco/ethanol, glycerol, phytoglycogen, starch, lipids

In several species capable of photosynthesis, lipids act as energy storage compounds. Several higher plants accumulate important amounts of oil, including, palms, sunflowers, olive trees, and colza. As the price of petrol increases, these oils are being used more to make the so called biodiesel, which can fuel cars and buses, etc. Fuel generation in this way involves about 40 Mha of land (2015) and competes with the 1600 Mha of crops worldwide (European commission, 2012). The best yields are achieved by palm, which generates around 600 L/m^2/year (Hill et al., 2010). Oil from microalgae has been reported to generate 10–30 times better yields than those obtained from oil palm (5000–15,000 L/m^2/year) depending on the cultivation mode (Chisti, 2007) and the species. *Schizochytrium* sp. *HX-308* (CCTCC M 209059) can produce up to 70% of its dry weight in lipids (Ren et al., 2014).

Extraction of oil from algae is presently more expensive than extraction from crop plants. This process involves the first step of filtration, centrifugation, flotation, or flocculation of the culture media. The techniques used and the efficiency of the process vary according to the species, and as a consequence, the price fluctuates from one to the other. The second step of the extraction is purification of the oil itself, which can be done using mechanical techniques such as presses and ultrasonication. Chemical extraction is also possible but involves treatments with toxic solvents or expensive supercritical CO_2. A new cost-effective method was patented a few years ago, developed by OriginOil (Eckelberry et al., 2010). This method involves the use of CO_2 combined with a magnetic field. Advances have also been made at the harvesting level via bio-flocculation (Salim et al., 2011), auto-flocculation (González-Fernández and Ballesteros, 2013), and induced aggregation (Arakaki et al., 2013). The algae oil industry could be as profitable as the palm oil industry, and most of the costs depend upon the processing and lipid production rate (Torres et al., 2013). Genetic engineering has become

an important player in the improvement of both harvests and production rate.
Other fuels can be generated from photosynthesis. The most popular and common is ethanol. Direct production of ethanol has been demonstrated from *Synechococcus sp.* strain *PCC 7942* (Deng and Coleman, 1999) but with very low yields. A pilot experiment led to the generation of ~23 µg/L, which was obtained from culture media 5 days into the exponential growth phase. According to those authors, competing pathways might be the limiting factor rather than the heterologous levels of pyruvate dehydrogenase complex and alcohol dehydrogenase. Improvements of strains to increase ethanol production have to take into account its toxicity as only a few species can resist levels of ethanol as high as 150 g/L. Other fuels can be produced with much higher yields, but with wider uses; these are discussed in the section "Photosynthesis to produce high value compounds."

Global growth/biomass

Photosynthesis-derived biomass can be efficiently transformed to heat without requiring particularly expensive or massive technical installations. Biomass is an easy accessible resource that can be obtained from forests, crops, wastewater, and industrial waste. Burning is a common way to utilize biomass as a heat source, but other methods also exist. Biotransformation of biomass in fermenters is one such strategy that is gaining interest. For example, the digestion of heterogeneous raw material by microorganisms can produce methane and ethanol through anaerobic fermentation. The city of Linköping in Sweden has already demonstrated that tons of waste can be treated to produce methane, fertilizers, and ethanol. Production from a waste-treating factory covers 56% of the local heat demand, fuels 6% of all cars, and all public transport (ISWA2013).

The range of compounds produced depends on the microorganisms used and the chemical nature of the substrate. Ethanol for example, is mainly produced from carbohydrate-rich biomass (starch and sugars) that originates from coarse grains and sugarcane (OECD-FAO, 2013). Furthermore, biomass processing by microorganisms can directly generate electricity via the microbial fuel cell (Wu et al., 2013). This method efficiently extracts electrons from diverse catabolic pathways, but it requires high levels of maintenance and generates a very low power density (Rosenbaum et al., 2005; Wu et al., 2013). A mixed type microbial fuel cell prototype was built and operated with a *Chlorella vulgaris* bio-cathode to generate electricity (Wu et al., 2013).

A recently developed technique optimizes biomass and lipid production in photosynthetic microorganisms (Komolafe et al., 2014). Increasing growth yields of plants by engineering their metabolism could have multiple advantages, including the creation of additional biomass to relieve famine whilst having a limited effect on traditional farming (Yoon et al., 2013). Engineering such as this was performed in *Arabidopsis thaliana* and involved modification of the Rubisco activase enzyme (Kurek et al., 2007). The thermostability enhancement of this maturation factor essentially eliminated the phenomenon of decreased CO_2 uptake which usually follows heat stress. This method also allows normal fecundity during heat stress.

Challenge of Rubisco C3 C4

In C3 metabolism, Rubisco's environment is low in CO_2, leading to a competitive effect of oxygen that consequently forms byproducts that need to be recycled. This process is called photorespiration and accounts for losses of ~25% of fixed CO_2 (Whitney et al., 2011) and provides a lead for improvements in biomass production.

Structural characterization of several Rubisco orthologs might help us to understand the basis of these catalytical differences, which might also play an important role in carboxylation. Miller et al. (2013) reported that it was possible to use site directed mutagenesis to screen for modifications in the characteristics of the enzyme in *Synechococcus*. The characterization of two different Rubisco isoforms from two commonly cultivated C3 species revealed a 2-fold difference in the Vm and Km constants (O'Donnelly et al., 2014).

The low internal CO_2 level surrounding Rubisco can explain the low efficiency of carbon fixation, and several species have developed mechanisms to increase the local CO_2 content. This process is known as the carbon concentrating mechanism (CCM) and occurs in nearly all algae and in many land plants. In higher land plants, the CCM is known as C4 and CAM and requires separation of CO_2 capture and its use in the Calvin–Benson–Bassham cycle (hereafter called the CBB cycle). C4 and CAM differ in the organization of CO_2 trapping, being resolved through space or time, respectively. In particular, C4 plants are capable of fixing a greater quantity of CO_2 than C3 plants, at 550 ppm CO_2 and 35°C, 450 ppm at 30°C, and 350 ppm at 25°C (Ehleringer et al., 1997) (the current level of 400 ppm, has not been exceed over the past 30 million years). Moreover, C4 consumes five ATP molecules per CO_2 instead of the three consumed by C3 plants, which gives this process a weakness under low light and shaded

environments when light-generated ATP becomes a limiting factor. Rice is the main food source for almost half of the world's population, with 90% of it being produced in Asia. The light, temperature, and humidity conditions required for cultivated species mean the growing area is restricted to tropical and subtropical areas. To increase production yields and to decrease the environmental requirements, several research teams are trying to introduce a C4 pathway into rice. Attempts have been made to create C4 rice at a single cell level (von Caemmerer et al., 2012), but the team realized the need for additional regulations (Langdale, 2011). Phosphorylation of PEP is an important step in the modulation of C4 metabolism and is one of the first steps to be engineered. On the other hand, the specific localization and/or activation pattern of Rubisco is an important feature of CCM-type photosynthesis and is hard to reproduce.

The success of experiments attempting to introduce CCM into a non-CCM plant depends on finding a model that is best adapted to the new host. Higher plants present an important source of diversity, but photoautotroph unicellular organisms are even more diverse. Rubisco from cyanobacteria was successfully expressed in tobacco and led to greater carboxylase activity than was observed in WT tobacco Rubisco (Lin et al., 2014); however, this technology needs engineering on the expression regulations of diverse transporters to be really outcompeting.

Photosynthesis to produce high value compounds

The production of high value compounds from living organisms has been known since the Neolithic age with the discovery of agriculture and fermentation (wine traces found in jars in "Hajji Firuz Tepe"). Since then, living organisms have been domesticated to produce more and more complex molecules, such as drugs. In the 1970's, Stanley Cohen and Herbert Boyer succeeded in performing the first transgenesis and greatly improved the possibility of producing high-value compounds from living organisms. A few years later, human insulin was produced using bacteria, which replaced the chemically synthetized and pork purified insulins. In addition, it was cheaper, unlimited, and genetically identical to insulin produced by humans.

The production of such compounds by living organisms greatly simplifies their synthesis, and offers the possibility of producing a broad range of molecules ranging from simple metabolites to large proteins that contrast with chemistry limited to small molecules.

This process also offers self-sufficiency of petrol-derived compounds, and minimize price fluctuations as the reactions can easily be fed with minerals and organic matter. Another advantage of using bioderived compounds is that the waste generated is environmentally friendly and can be reused to produce biogas and fertilizers. This contrasts with the chemical synthesis of solvents and toxic compounds that are hard to recover. Furthermore, from an industrial point of view, chemistry needs extremely complex machines and often requires high temperatures and pressures, making the whole process hard to upscale. On the contrary, the use of biological systems is easier to upscale and is both cheaper and safer. However, one drawback of biological systems is that the product of interest is part of a complex mixture that makes purification less efficient.

Photosynthesis based economy

Photosynthesis is one of the most efficient and sustainable processes we know of in nature, as it only needs CO_2, water, and is powered by light from the sun. This is the power input step in the carbon cycle.

From an economic point of view, the use of photosynthesis to produce high value compounds is viable even in the short-term as it mainly uses energy from the sun. Some external energy is still needed to cultivate and harvest the autotrophic organism, as well as to purify the produced compound.

Species selection plays a major role in the economic sustainability of a production system. Depending on the nature of the compound produced, the growth milieu, its mineral and organic composition, the temperature and irradiance, and its reaction to different stresses, the use of one species might be preferred over another. The composition of biomass also affects the value of the produced waste, as does the ease of extracting the product of interest.

An increasing number of products of interests are dependent on genetically engineered organisms. Of course, the ability to genetically modify a species plays a major role in its selection, but it also involves particular attention to laws and regulations. The rules applicable to a certain type of culture should also be considered during species and culture-type selection.

Photosynthetic microorganisms are notably interesting in several ways. They can grow in open environments allowing large-scale production, and in completely closed environments under partially or completely controlled conditions. This is ideal for genetically modified organisms, and decreases water and nutrient losses that usually occur due to evaporation or

draining. As microorganisms grow in liquid, a co-culture can also be set up; therefore, allowing the capacities of different species to complement each other. For example, nitrogen-fixing cyanobacteria can complement a culture media that is deficient in nitrogen, and nitrogen constitutes a major cost of fertilizers. One drawback is that it is harder to harvest microorganisms than plant cultures, but it provides a biomass that is easier to treat.

In the last decades, an increasing number of high-value compounds have been purified from different photosynthetic organisms. In plants, secondary metabolites have been extracted and used as drugs. It is estimated that around 80% of the 30,000 natural pharmaceutical products are from plants (Rao and Ravishankar, 2002; Rischer et al., 2013), many of which are still extracted because the complexity of the active compound makes them difficult to synthetize.

Molecular farming is now used to produce many compounds that are utilized to make clinical advances. Next generation vaccines that confer protection against diseases without the need for injections (Streatfield and Howard, 2003), and hemoglobin and collagen that are used to synthesize in vitro tissues (Makhzoum et al., 2013) are a few examples of innovations being made in the field.

In unicellular algae, hemagglutinin, vitamins, PUFAs, antioxidants, glycerol, transgenic proteins (Mayfield et al., 2007; Rasala et al., 2010), toxic fatty acids (Skjanes et al., 2013), poly-3-hydroxybutyrate (PHB) (Chaogang et al., 2010), in chloro vaccine against plasmodium with fused cholera toxin adjuvant (Gregory et al., 2013) have been successfully produced.

Ethylene is a major precursor derived from petrol that is used in in the chemical industry. Its derivatives are used for the synthesis of numerous polymers. This molecule has been produced from cyanobacteria (Takahama et al., 2003; Skjanes et al., 2013). More recently, the production of isobutanol and isobutyraldehyde, which can be used to produce biofuels, has been achieved in *Synechococcus elongatus* PCC 7942 (Atsumi et al., 2009). Isoprene, which is a chemical precursor of several rubber type polymers and is usually synthetized from petrol, was produced by a modified Synechocystis (Lindberg et al., 2010)

The role of photosynthesis is becoming more and more important in the synthesis of high-value compounds, but production yields depend on the ability of the host organism to withstand energy loss triggered by the redirection of metabolites.

This process is very sensitive to saturation as excess electrons produce reactive oxygen species (ROS), which are extremely harmful to the cell. For more than 2 billion years, this process evolved in parallel with other stresses to become tightly regulated and now has

high plasticity, and provides optimal performance in a given environment, despite non-maximal yields. As a consequence, the engineering of photosynthetic organisms is doomed to fail if we do not endeavor to understand the deep regulation of photosynthetic processes.

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