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# The Role of nitric oxide in the remodeling of the photosynthetic apparatus under abiotic stress in *Chlamydomonas reinhardtii*

Marcello de Mia

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Sciences du Végétal : du Gène à l'Ecosystème

Spécialité de doctorat : Biologie

Par

**Marcello De Mia**

The role of nitric oxide in the remodeling of the photosynthetic apparatus under abiotic stress

in *Chlamydomonas reinhardtii*

**Thèse présentée et soutenue à Paris, le 15 décembre 2017**

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## Abbreviations

### A

AG	amino guanidine
ASC	ascorbate
APS	ammonium persulfate
ATP	adenosine TriPhosphate

### B

BCA	bicinchoninic acid
BPGA	1,3-bisphosphoglycerate
BST	biotin switch technique

### C

cPTIO	2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
cyt $b_6f$	cytochrome $b_6f$

### D

DEANO	diethylamine NONOate diethylammonium salt
DFMO	DL- $\alpha$ -Difluoromethylornithine
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DMSO	dimethyl sulfoxide
DTT	dithiothreitol

### E

ECL	Enhanced ChemiLuminescence
EDTA	ethylenediaminetetraacetic acid

### F

<b>FBA</b>	fructose-1,6-bisphosphate aldolase
FBPase	fructose-1,6-bisphosphate phosphatase
Fd	ferredoxin
FNR	ferredoxin-NADP reductase
FTR	ferredoxin-thioredoxin reductase

### G

G3P	glyceraldehyde-3-phosphate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GRX	glutaredoxin
GSH	reduced glutathione
GSNO	nitroso-glutathione
GSSG	oxidized glutathione
GST	glutathione S-transferase

### H

HPDP-Biotin	N-[6-(biotinamido)hexyl]-3'-(2'-Pyridyldithio)-propionamide
HPLC	High Performance Liquid Chromatography

**I**

IAA iodoacetate  
IAM iodoacetamide

**L**

L-NAME L-N<sup>G</sup>-Nitroarginine methyl ester  
LHC light harvesting complex

**M**

MALDI-TOF Matrix Assisted Laser Desorption/Ionisation – Time Of Flight

**N**

NADP Nicotinamide Adenine Dinucleotide Phosphate  
NADP-MDH NADP malate dehydrogenase  
NEM N-Ethylmaleimide  
NTR NADPH-Thioredoxin Reductase

**O**

O<sub>2</sub> dioxygen  
O<sub>2</sub><sup>•-</sup> superoxide radical  
<sup>•</sup>OH hydroxyl radical  
<sup>1</sup>O<sub>2</sub> singlet oxygen  
ONOO<sup>-</sup> peroxyntirite

**P**

PC plastocyanin  
PGA 3-phosphoglycerate  
PGK 3-phosphoglycerate kinase  
PQ<sup>+</sup> oxidized plastoquinone  
PQH<sub>2</sub> reduced plastoquinone  
PRK phosphoribulokinase  
PRX peroxiredoxin  
PSI photosystem I  
PSII photosystem II

**R**

RCA rubisco activase  
RNS reactive Nitrogen Species  
ROS reactive Oxygen Species  
RPE ribulose 5-phosphate epimerase  
RPI ribulose 5-phosphate isomerase  
Rubisco ribulose-1,5-bisphosphate carboxylase/oxygenase

**S**

SBPase sedoheptulose-1,7-bisphosphate phosphatase  
SDS sodium Dodecyl Sulfate  
SDS-PAGE SDS - polyacrylamide gel electrophoresis  
SNAP S-Nitroso-N-acetyl-DL-penicillamine  
SNP sodium nitroprusside dihydrate

**T**

TAP	Tris Acetate Phosphate
TE	Tris EDTA
TK	Transketolase
TEMED	N,N,N',N'-tetramethylethylenediamine
TPI	triosephosphate isomerase
Tris	tris (hydroxymethyl)-aminomethane
TRX	thioredoxin

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# I Introduction en Français

## Photosynthèse

La photosynthèse oxygénique est le seul processus dans la nature capable de convertir l'énergie solaire en énergie électrochimique, réalisant une réaction nette où 6 molécules de  $\text{CO}_2$  sont converties en une molécule de glucose, avec une libération concomitante de 6  $\text{O}_2$ . Les photoautotrophes oxygéniques appartiennent aussi bien à la lignée procaryote qu'à la lignée eucaryote. Parmi les premiers, nous pouvons citer les cyanobactéries, considérées comme le premier groupe de photoautotrophes oxygéniques; et de nombreux eucaryotes appartenant aux groupes des algues et des plantes terrestres. Tous ces organismes partagent des structures et des architectures communes au niveau des systèmes photosynthétiques, même si chaque groupe possède des régulations spécifiques et présente des différences au niveau métabolique, avec une grande plasticité en particulier chez les algues vertes. C'est l'une des raisons pour lesquelles les algues sont utilisées comme organismes modèles.

Le chloroplaste est l'organite eucaryote qui effectue la photosynthèse. Cet organite dérive d'une ancienne cyanobactérie, incorporée par un hôte eucaryote il y a plus d'un milliard d'années dans un événement endosymbiotique qui a permis l'apparition des glaucophytes, des algues rouges, des algues vertes et des plantes. Les réactions photosynthétiques, présentes dans le chloroplaste, sont spatialement cloisonnées et sont divisées en une phase photochimique, se produisant dans les membranes des thylakoïdes, et une phase biosynthétique (ou phase de fixation du  $\text{CO}_2$ ), se produisant dans le compartiment stromal.

La phase photochimique consiste en une série de réactions redox entre les complexes photosynthétiques et assure le transfert des électrons et la translocation des protons à travers les membranes des thylakoïdes. Les électrons sont transférés de l'eau au  $\text{NADP}^+$ , suivant un flux d'électrons linéaire. Dans le même temps, les protons sont déplacés du stroma au lumen, créant un  $\Delta\text{pH}$  qui conduit à la synthèse de l'ATP.

L'ATP et le NADPH générés au cours de la phase photochimique sont injectés dans le métabolisme cellulaire et alimentent notamment la phase biosynthétique de la photosynthèse. La fixation du  $\text{CO}_2$  se produit dans le stroma et permet l'insertion de  $\text{CO}_2$  dans des squelettes carbonés utilisés par la suite pour la biosynthèse de

l'amidon et du saccharose. La voie de réduction du CO<sub>2</sub> en sucres phosphates est hautement conservée et connue sous le nom de cycle réductif des pentoses phosphate réductrice (RPP) ou cycle de Calvin-Benson (CBC). Le cycle peut être divisé en 3 phases séquentielles appelées Carboxylation, Réduction, Régénération et est réalisé par 11 enzymes différentes catalysant 13 réactions.

#### Régulation de la photosynthèse

Plusieurs mécanismes existent pour contrôler le taux d'excitation des photosystèmes et l'allocation des électrons, rendant le système hautement dynamique et adaptable à des conditions environnementales variables. Les mécanismes les plus étudiés comprennent la désactivation non photochimique (Non Photochemical Quenching, NPQ), agissant au niveau du PSII; le transfert d'électrons cyclique autour du PSI (CEF); l'activation de voies alternatives d'électrons drainant les électrons vers d'autres puits; et la modulation de la taille des antennes collectrices. Au total, ces mécanismes permettent de libérer l'excès d'énergie du système, évitant ainsi des dommages irréversibles.

La régulation du CBC est certainement le mécanisme de régulation photosynthétique le plus étudié. C'est en effet la première cible dans de nombreuses conditions environnementales et surtout en conditions de stress. Cependant, sa régulation est strictement interconnectée à la phase photochimique et se produit à plusieurs niveaux (expression génique, modifications post-traductionnelles des protéines et dégradation des protéines). Néanmoins, le mécanisme régulateur principal est dépendant de la lumière, avec 4 enzymes (PRK, GAPDH, FBPase, SBPase) présentant une activité faible à l'obscurité et une activité élevée à la lumière. Cette régulation est opérée par le système ferrédoxine / thiorédoxine (Fd / TRX). A côté de ce système régulateur principal, les enzymes du CBC peuvent également être régulées par un mécanisme redox alternatif incluant des modifications redox post traductionnelles, se produisant au niveau des résidus cystéine et induites par les espèces réactives de l'oxygène (ROS) et de l'azote (RNS), en condition basale ainsi qu'en condition de stress.

#### Stress et photosynthèse

La photosynthèse est responsable de la plus grande partie de la production d'énergie et de squelette carboné. Par conséquent, tous les stress affectant le métabolisme

cellulaire et la consommation d'énergie ont un effet direct ou indirect sur cette voie. Les effets néfastes découlent du déséquilibre entre la fixation du CO<sub>2</sub> et le transport des électrons, ce qui entraîne une réduction excessive de la chaîne de transfert d'électrons photosynthétique et un blocage du transfert d'électrons, qui aboutit à une production subséquente de ROS et à un stress oxydatif. L'équilibre redox chloroplastique est maintenu efficacement par les mécanismes de régulation déjà décrits, mais lorsque les conditions de stress dépassent la capacité d'acclimatation des cellules, les photo-dommages prennent le dessus, induisant la dégradation des composants photosynthétiques, le blocage de la fixation du CO<sub>2</sub> et l'activation des voies apoptotiques. Les principaux stress abiotiques pour les photoautotrophes oxygénés sont: la carence en eau; le stress thermique / froid; l'irradiation excessive ou insuffisante de la lumière; les carences nutritives; les polluants du sol (par exemple les métaux lourds) et le stress salin.

#### Carences en nutriments

Le carbone, l'azote, le phosphore et le soufre sont considérés comme des macronutriments, les briques de construction pour toutes les biomolécules comme les protéines, les acides nucléiques, les carbohydrates et les lipides, requis dans à une concentration de l'ordre du millimolaire dans le milieu. Lorsque *Chlamydomonas reinhardtii* subit des limitations en macronutriments, des réponses générales (communes à toutes les limitations) et des réponses spécifiques au nutriment limitant sont activées afin d'atteindre l'acclimatation. La réponse commence généralement par un arrêt du cycle cellulaire, la mise en place de mécanismes permettant l'acquisition de sources alternatives externes et une mobilisation accrue des ressources internes. Ce sont des caractéristiques communes de toutes les limitations, même si chaque type de carence en nutriment active ses propres voies de régulation et possède ses régulateurs spécifiques. Au cours de cette phase initiale, les protéines responsables de l'importation de sources externes et de la dégradation des réserves internes sont exprimées. De manière concomitante, la teneur totale en ARNm codant pour les protéines impliquées dans les voies anaboliques chute considérablement. Si la limitation persiste, les cellules mettent en œuvre des mécanismes de sauvegarde et de recyclage où les protéines essentielles sont remplacées par des isoformes alternatives avec une composition en acides aminés différente, afin de réduire les besoins pour l'élément manquant. Les protéines

non essentielles, riches en acide aminé contenant le nutriment limitant, sont sélectivement dégradées et leurs composants sont recyclés.

Certaines limitations, comme les carences en azote et en soufre, montrent des caractéristiques ayant un intérêt particulier pour des applications biotechnologiques, en particulier si elles sont appliquées à des espèces de cyanobactéries et de microalgues. En effet, ces organismes, sous l'effet de la carence, induisent une réorganisation (ou un remodelage) de la machinerie photosynthétique d'une manière fortement régulée. Le blocage de la photosynthèse provoque alors un basculement vers un métabolisme fermentaire ou respiratoire qui facilite l'accumulation de carbone réduit. Cette stratégie est censée minimiser les photo-dommages, induisant la redirection des électrons vers d'autres puits.

### Fluctuations de lumière

Dans la nature, la qualité et la quantité de lumière changent continuellement sur une échelle de temps variant entre des fractions de secondes et des jours. Ainsi, les organismes photosynthétiques ont développé plusieurs réponses pour faire face à ces changements et s'acclimater. Ces réponses peuvent être classées en réponses à court et à long terme qui coopèrent pour affiner la capacité de récolte de la lumière et l'utilisation de l'énergie. Si les changements de lumière se produisent sur des temps courts, non compatibles avec une régulation de la synthèse ou de la dégradation des protéines, les cellules répondent par un mécanisme rapide (NPQ et CEF). Cependant, la lumière peut également changer sur une échelle à long terme (heures ou jours) et, dans une telle situation, l'acclimatation se produit par le remodelage efficace de l'appareil photosynthétique, notamment par la modulation de la taille de l'antenne. Tous les organismes photosynthétiques montrent la même stratégie en réponse aux fluctuations de la lumière à long terme. Sous une lumière faible, les antennes sont plus grandes pour conduire autant d'énergie que possible vers les centres réactionnels des photosystèmes. Inversement, sous une lumière intense, la taille de l'antenne est réduite pour minimiser les effets néfastes de la surexcitation. Les stratégies pour atteindre cette acclimatation sont multiples et varient selon les espèces. Un acteur clé dans ces processus adaptatifs est le système d'antenne photosynthétique LHCI.

## La nature du signal régissant les réponses environnementales

L'acclimatation aux stress environnementaux et / ou aux conditions changeantes, ainsi que l'ajustement métabolique aux conditions physiologiques et de développement, nécessite une coordination précise entre l'expression des gènes nucléaires et plastidiaux. Par conséquent, une ou plusieurs voies de signalisation reliant ces organites doivent exister pour accomplir cette tâche.

Nous nous référons communément au terme de "signalisation rétrograde" pour décrire la stratégie assurant l'échange d'informations entre les plastes et / ou les mitochondries avec le noyau. Le signal rétrograde du plaste est responsable du contrôle opérationnel qui synchronise le métabolisme cellulaire à l'état développemental et physiologique du plaste. Le contrôle opérationnel est crucial pour les réponses au stress et, grâce aux progrès de la génétique, au profilage des métabolites et à la bioinformatique; nous avons maintenant une connaissance plus approfondie de cette signalisation dans les organismes photosynthétiques. Même si plusieurs messagers et voies ont été proposés ces dernières années, aucun d'entre eux n'a été démontré de manière convaincante *in vivo*. Les meilleurs candidats, proposés au cours de la dernière décennie, agissent très probablement en parallèle, formant un réseau complexe de signaux. Ils comprennent les tétrapyrroles; l'état redox du pool de plastoquinone; les hèmes; les phosphonucléotides (PAP); les précurseurs / produits de dégradation du  $\beta$ -carotène ou les ROS / RNS produits dans le plaste. A côté de ces messagers putatifs, l'oxyde nitrique (NO) est un autre acteur clé dans les voies de signalisation, chez les animaux et les plantes, dans des conditions physiologiques aussi bien que lors d'un stress. Le NO est produite dans toutes les cellules et son implication dans la signalisation mitochondriale rétrograde a été établie dans les systèmes nerveux animaux. Compte tenu de sa nature paracrine, c'est un bon candidat pour la signalisation plastid rétrograde ainsi.

Le NO agit principalement en induisant des RPTM dans tous les organismes et est capable de moduler l'activité enzymatique, la localisation et l'interaction des protéines ainsi que l'activation de nouvelles fonctions dites « moonlighting ».

## II Introduction

### II.1 Photosynthesis

Oxygenic photosynthesis is the only process in nature capable to convert solar energy into electrochemical energy, performing a net reaction where 6 molecules of CO<sub>2</sub>, taken from atmosphere, are converted into a molecule of glucose, with concomitant release of 6 O<sub>2</sub> (Fig.1A) (Minagawa *et al.* 2015). Photosynthesis contributed strongly to make the planet Earth as we know it nowadays, since the entire biomass present on the planet has been fixed by photosynthetic organisms since the first appearance of this peculiar metabolism.

Oxygenic photoautotrophs belong to the prokaryote as well as to the eukaryote lineage. Among the former we can cite cyanobacteria, believed to be the first group of oxygenic photoautotrophs; and many eukaryotes belonging to the algae and land plant groups. All these organisms share common structures and architectures at the level of photosynthetic systems, even though each group possesses peculiar regulatory features and differences at metabolic level, with a great plasticity especially in green algae. This is one of the reasons why algae are used as model organisms.

#### II.1.1 The Chloroplast and its structure

The chloroplast (Fig.1B) is the eukaryotic organelle that performs photosynthesis. This organelle derives from an ancient cyanobacterium, incorporated by a eukaryotic host more than 1 billion years ago in an endosymbiotic event that gave rise to glaucophytes, red algae, green algae and plants. Therefore, the chloroplast genome exhibits many prokaryotic features even though, during evolution, it has undergone a dramatic reduction in size, mainly as a result of a huge gene loss and a large-scale gene transfer towards the nuclear genome. Thus, the genome of modern chloroplasts (plastomes) contains around 100 genes, most of which encode components of the organelle's gene expression machinery and the photosynthetic apparatus (Checchetto *et al.* 2013; Jensen *et al.* 2014). The plastome of land plants varies considerably in size among species, ranging from 107 kb (*Cathaya argyrophylla*) to 2018 kb (*Pelargonium*) (Daniell *et al.* 2016). The chloroplast genome of *C. reinhardtii* is 204 kb in size and contains 109 genes (Maul *et al.* 2002). The photosynthesis-

related genes comprise genes for subunits of the major multiprotein complexes in the thylakoid membrane that participate in photosynthetic electron transfer and ATP generation (photosystem II, cytochrome  $b_6/f$  complex, photosystem I, ATP synthase). The set of genes participating in the chloroplast gene expression machineries encode components of the transcriptional apparatus (subunits of the plastid-encoded RNA polymerase, PEP) and the translational machinery (rRNAs, tRNAs, ribosomal proteins) (Daniell *et al.* 2016).

The chloroplast is delimited by an envelope formed by two distinct membranes, the outer- and inner-membrane, separated by an inter-membrane space. On the inside, we observe a complex system of membrane discs with a diameter of about 300-600 nm, the thylakoids. Thylakoids are surrounded by an amorphous matrix, called stroma. Each disc delimitates an internal compartment called “the lumen”. The thylakoid architecture is highly dynamic and interconnected. Two macro domains can be distinguished: the “grana”, constituted by stacked thylakoids and the “stroma lamellae”, formed by unstacked thylakoid discs (Jensen *et al.* 2014).

Chloroplasts are crucial for cell metabolism. Indeed, besides photosynthesis, they are also responsible for nitrate and sulfate assimilation, synthesis of amino acids and fatty acids, as well as chlorophyll, carotenoids and Hemes (Jensen *et al.* 2014). However, the main function remains the photosynthetic metabolism, controlled in close synergy with the nucleus (Minagawa *et al.* 2015).

In the following sections the reader will be driven into the photosynthetic machinery, its function and its regulation in response to external cues.

## **II.1.2 Photosynthetic reactions and their compartmentation**

The photosynthetic reactions, occurring in the chloroplast, are spatially partitioned and are divided into the Photochemical phase, occurring in thylakoid membranes, and the biosynthetic phase (or  $\text{CO}_2$  fixation phase), occurring in the stromal compartment (Fig.1 C-D) (Eberhard *et al.* 2008).

### *II.1.2.a Photochemical phase*

The photochemical phase consists of a series of redox reactions between photosynthetic complexes and ensures the transfer of electrons and the translocation of protons across the thylakoid membranes. The electrons are transferred from water to  $\text{NADP}^+$ , following a linear electron flow (LEF) well described in the “Z-scheme”

(Fig. 2) of photosynthesis (Hill *et al.* 1960). At the same time, protons are relocated from the stroma to the lumen, creating a  $\Delta pH$  that drives ATP synthesis.

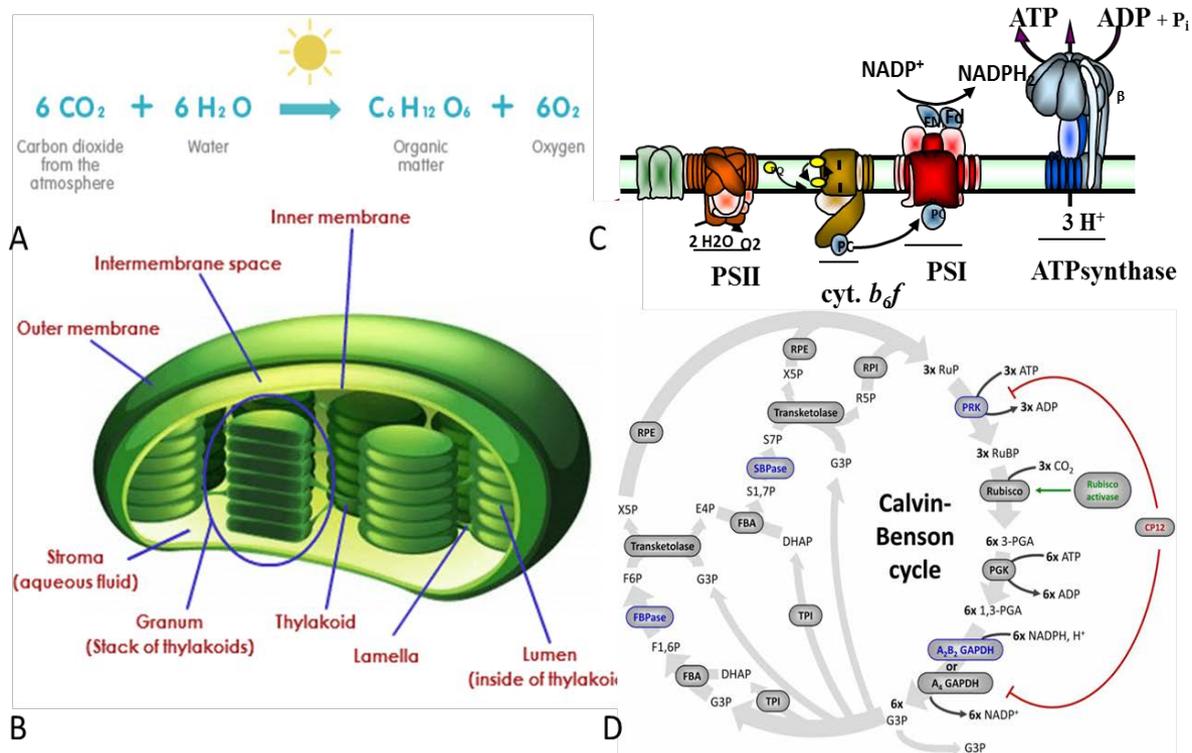


Figure 1: The photosynthetic metabolism in eukaryotes. A) The net reaction releasing  $\text{O}_2$  while producing sugars; B) The chloroplast organelle, where the photosynthetic metabolism takes place; C) The Electron Transport Chain (ETC) responsible for the “Photochemical Phase” in thylakoid membranes; D) The Calvin-Benson cycle (CBC) responsible for  $\text{CO}_2$  fixation in the stroma of chloroplasts

Photosynthetically produced NADPH and ATP molecules are used for  $\text{CO}_2$  assimilation in the biosynthetic phase (Mitchell 1966; Ku *et al.* 1983; Foyer *et al.* 2012; Minagawa *et al.* 2015).

The machinery embedded in the thylakoid membrane is commonly called electron transport chain (ETC) and is formed by 4 multimeric protein complexes, encoded by the plastome and the nuclear genome (Fig.1C) (Minagawa *et al.* 2015). The main complexes are photosystem II and photosystem I (PSII and PSI), chlorophyll-associated multimeric complexes responsible for the charge separation that drives electron flow using light energy. PSII and PSI contain few hundreds of pigments and are structured in a core domain, containing the reaction center (RC) and cofactors, surrounded by light harvesting complexes called antennae, capable of increasing the effective absorption cross-section of the system (Croce *et al.* 2014).

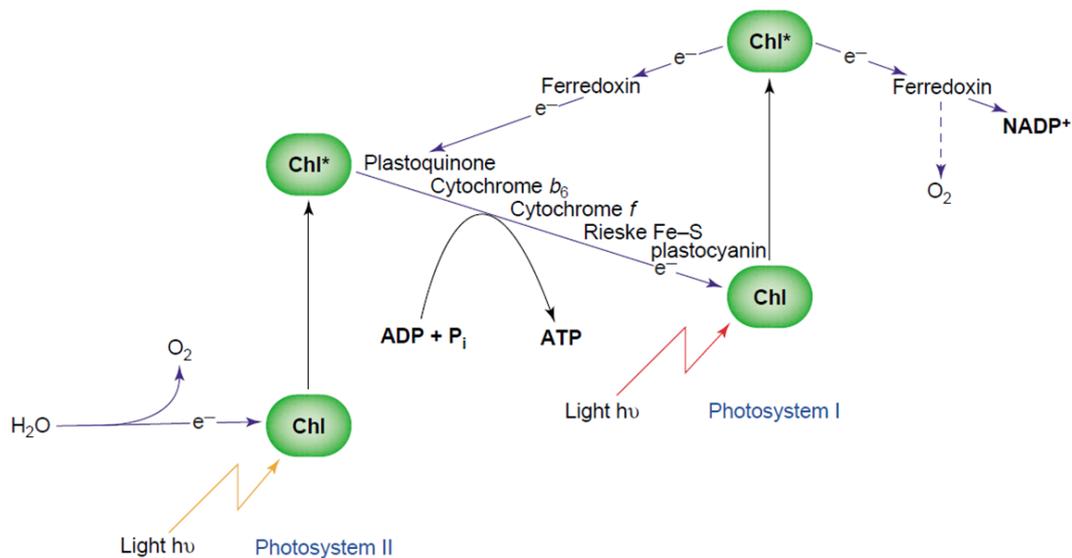


Figure 2: The Z-scheme for photosynthetic electron transfer as envisaged by Hill and Bendall (1960). Adapted from (Allen 2004).

Each photosystem possesses its particular pigments and cofactors but they are extremely well conserved in all oxygenic organisms.

PSII and PSI are working in series since they are interconnected via the membrane-bound cytochrome- $b_6f$  complex and the mobile electron carriers plastoquinone (PQ) and plastocyanin (PC).

The PSII is associated with the so called light-harvesting complex II proteins (LHCII), to constitute a large super-complex with more than 30 subunits. Light energy captured by LHCII is transferred to the central core complex of PSII, P680, where it is used to perform charge separation and drive electrons from water to plastoquinone (PQ) with simultaneous injection of  $H^+$  into the lumen (Cardona *et al.* 2012; Tikhonov 2014).

The cytochrome  $b_6f$  complex is composed by eight polypeptide subunits, including four major subunits involved in electron transport: the Rieske iron-sulfur protein, the *cyt- $b_6$*  and *cyt- $f$*  proteins, and subunit IV. It is responsible for the electron transfer between  $PQH_2$  and PC, activating the so called “Q-cycle” with concomitant pumping of  $H^+$  into the lumen (Stroebel *et al.* 2003; Cramer *et al.* 2006; Tikhonov 2014).

The PSI possesses its own light-harvesting complex (LHCI) and forms a super-complex with nearly 20 subunits. PSI collects light energy towards its reaction center, P700, where charge separation takes place and drives electrons from plastocyanin to

Ferredoxin (Fd), the final acceptor that will distribute them to several possible acceptor among which NADP<sup>+</sup> (Chitnis 2001; Berthold *et al.* 2012).

The last multimeric complex involved in the process is the ATP-synthase, belonging to the family of F-type ATPases. It is composed of two distinct domains: CF<sub>1</sub>, the hydrophilic head group, and CF<sub>0</sub>, the transmembrane ring (or rotor). ATP-synthase uses the ΔpH generated by the ETC to produce ATP and to provide energy for cell metabolism (Mitchell 1961; Bottcher *et al.* 2000; Richter *et al.* 2005).

#### *II.1.2.b Biosynthetic phase (CO<sub>2</sub> fixation)*

ATP and NADPH generated in the photochemical phase are injected in the cell metabolism and, notably, feed the biosynthetic phase of photosynthesis. The CO<sub>2</sub> fixation occurs in the stroma and allows the insertion of CO<sub>2</sub> in carbon skeletons subsequently used for starch and sucrose biosynthesis (Buchanan 1991). From the simplest prokaryotic cyanobacteria to the most complicated land plants, the pathway for the reduction of CO<sub>2</sub> into sugar phosphates is identical and known as reductive pentose phosphate pathway (RPP) or Calvin-Benson cycle (CBC) (Fig.1D) (Bassham *et al.* 1950). The cycle can be divided in 3 sequential phases called Carboxylation, Reduction, and Regeneration. It is accomplished by 11 different enzymes catalyzing 13 reactions (Fig. 1D). The first phase is initiated by the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) which catalyzes the carboxylation of the CO<sub>2</sub> acceptor molecule, ribulose- 1,5-bisphosphate (RuBP). The two molecules of 3-phosphoglycerate (3-PGA) formed by this reaction are then utilized to form the triose phosphates, glyceraldehyde phosphate (G-3-P) and dihydroxyacetone phosphate (DHAP) in the reductive phase, via two enzymes that consume ATP and NADPH: phosphoglycerate kinase (PGK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), respectively. The regenerative phase of the cycle involves a series of reactions that convert a portion of the triose phosphates into the CO<sub>2</sub> acceptor molecule RuBP, and require the sequential action of 9 enzymes, namely triose phosphate isomerase (TPI); fructose-1,6-bisphosphate aldolase (FBA); fructose-1,6-bisphosphatase (FBPase); transketolase (TK); sedoheptulose-1,7-bisphosphatase (SBPase); ribulose-5-phosphate 3-epimerase (RPE); ribose-5-phosphate isomerase (RPI) and phosphoribulokinase (PRK). Thus, a considerable amount of the triose phosphates produced in the cycle remains within the cycle to regenerate RuBP and, only a portion of glyceraldehyde 3-phosphate (1 out of 6

trioses), not used to sustain the cycle, constitutes the net result of CO<sub>2</sub> fixation. However, net carbon fixation is sufficient to synthesize sucrose and starch and to ensure growth and development of the organism (Buchanan 1991; Raines 2003; Michelet *et al.* 2013). It is important to note that the CBC supplies intermediates to an array of other pathways in the chloroplast, including the shikimate pathway for the biosynthesis of amino acids and lignin, isoprenoid biosynthesis and precursors for nucleotide metabolism and cell wall synthesis (Lichtenthaler 1999).

## **II.2 Regulation of photosynthesis**

All metabolic processes occurring in the chloroplasts are sustained by ATP and NADPH generated by the photosynthetic machinery. Among these pathways we can cite nitrate and sulfate metabolism, lipid, amino acids and pigment synthesis (Eberhard *et al.* 2008; Michelet *et al.* 2013). Thus, being pivotal for cell metabolism and survival, photosynthesis must be always kept under tight control in all environmental conditions, in order to maintain the balance between metabolic requirements and NADPH/ATP availability. Indeed, any imbalance can lead to damages of the photosynthetic machinery, especially via production of reactive oxygen species (ROS). In order to respond to fluctuating light intensities and temperatures, CO<sub>2</sub>, water and other nutrients availability, light reactions and CO<sub>2</sub> fixation must be always adjusted, to get the maximum efficiency in photon usage and carbon assimilation (Minagawa *et al.* 2015).

This adjustment between electron/proton fluxes and CO<sub>2</sub> fixation acquires a capital importance under stress conditions, where CBC activity is impaired. To ensure photosynthesis regulation, cells can activate a plethora of different responses at the level of thylakoid complexes or CO<sub>2</sub> fixation enzymes. The mechanisms are multiple and interconnected, going from gene-expression and post-translational gene-regulation, to post-translational modification and degradation of proteins (Eberhard *et al.* 2008; Michelet *et al.* 2013; Minagawa *et al.* 2015).

In the following sections, an overview of the main approaches used to investigate the photosynthetic function will be provided, together with a description of the most studied regulatory mechanisms. For the purpose of this thesis, the focus will be put on protein post-translational modifications and degradation.

## II.2.1 Study of photosynthetic function and regulation: a biophysical approach

Today, after 60 years of functional and genetic studies, the overall picture describing photosynthesis correlates with the early proposal of a Z scheme (Hill *et al.* 1960). However, currently the view is broader and considers photosynthetic systems as flexible molecular machines capable of rapid adaptation to fluctuating environmental conditions. Dynamic adaptation can be observed and described using multi-disciplinary approaches including genetics and molecular biology, protein crystallography, time-resolved spectroscopy and fluorescence measurements on living organisms, together with bioinformatics for integration of changing functionalities with changes in transcriptome, proteome, and metabolome. All these techniques allow the analysis of photosynthetic function *in vivo* under precisely regulated growth conditions or *in situ*, in natural environments.

Among the different approaches currently in use, biophysical analysis of photosynthetic function is crucial for the understanding of the light energy conversion. Indeed the measurements are not destructive, highly sensitive, and allow observation of the function in an entire natural system (Eberhard *et al.* 2008). Among the great number of biophysical methods that can be applied to photosynthesis, fluorescence spectroscopy is of great importance and will be described below.

The fluorescence is a physical property of chlorophyll (chl) moieties embedded in the thylakoid complexes (Fig. 3A). Once chlorophyll absorbs light energy, it gets excited and there are 3 possible ways to release the excess of energy: photochemistry (charge separation and electron transport to drive photosynthesis); heat dissipation or fluorescence emission. These three mechanisms occur in competition, such that any increase in the efficiency of one will result in a decrease in the yield of the other two (Maxwell *et al.* 2000).

From a mechanistic point of view, changes in fluorescence yield are linked to the redox state of electron carriers. The reduction of electron acceptors downstream of PSII (namely PQ and  $Q_A$ ) is responsible for fluorescence augmentation blocking temporary reaction centers in a “closed” state ( $Q_A^-$ ), with a decrease in photochemistry efficiency and augmented fluorescence emission (Maxwell *et al.* 2000). Thus, fluorescence yield measures can give information on the redox state of PSII ( $Q_A/Q_A^-$  ratio), a precise picture of light utilization and dissipation rates.

This approach can be efficiently used to screen for photosynthetic mutants, since the fluorescence signature is characteristic for each dysfunction in the photosynthetic machinery (Fig. 3C). The power of this approach relies on the possibility to calculate several parameters from fluorescence curves. These parameters can be easily compared between strains or under different growth conditions, allowing a real-time quantification of photosynthetic efficiency (Fig. 3B).

A great number of mutants defective for photosynthesis-related processes have been identified over the years with fluorescence-based screens. Among them there are strains defective for electron transport or light phase regulation (e.g. NPQ; light-acclimation or Cyclic electron flow) or CBC regulation (e.g. Photorespiration or Carboxylation)(Miles 1980; Genty *et al.* 1990; Matsuda *et al.* 1996; Walters *et al.* 2003).

These screenings, coupled with genetic and biochemical tools allowed the discovery of complex gene circuits and post translational mechanisms, often interconnected, responsible for photosynthesis regulation (Eberhard *et al.* 2008).

## **II.2.2 Photochemical phase regulation**

Several mechanisms exist to control the photosystems excitation rate and electron allocation, making the system highly dynamic and adaptable to varying environmental conditions. The most studied mechanisms include Non Photochemical Quenching (NPQ), acting at the level of PSII; cyclic electron flow around PSI; the activation of alternative electron pathways draining electrons towards other sinks; and antenna size modulation (Eberhard *et al.* 2008; Foyer *et al.* 2012; Croce *et al.* 2014; Minagawa *et al.* 2015). Altogether, these mechanisms operate to release the excess of energy from the system, avoiding irreversible damage.

### *II.2.2.a Non Photochemical Quenching*

NPQ refers to the mechanisms that allow decreasing the fluorescence yield of PSII, without triggering any photochemical process. It has 3 major components, acting through distinct mechanisms: (i) energy dependent quenching (qE); (ii) state-transition quenching (qT); (iii) photo-inhibition quenching (qI) (Horton *et al.* 1996).

The qE (Fig. 4A) is the major component in plants and is activated within few minutes (1-5) after exposure to light. It is  $\Delta$ pH-dependent and takes place as a result of over acidification of the lumen (a situation occurring under excess light conditions).

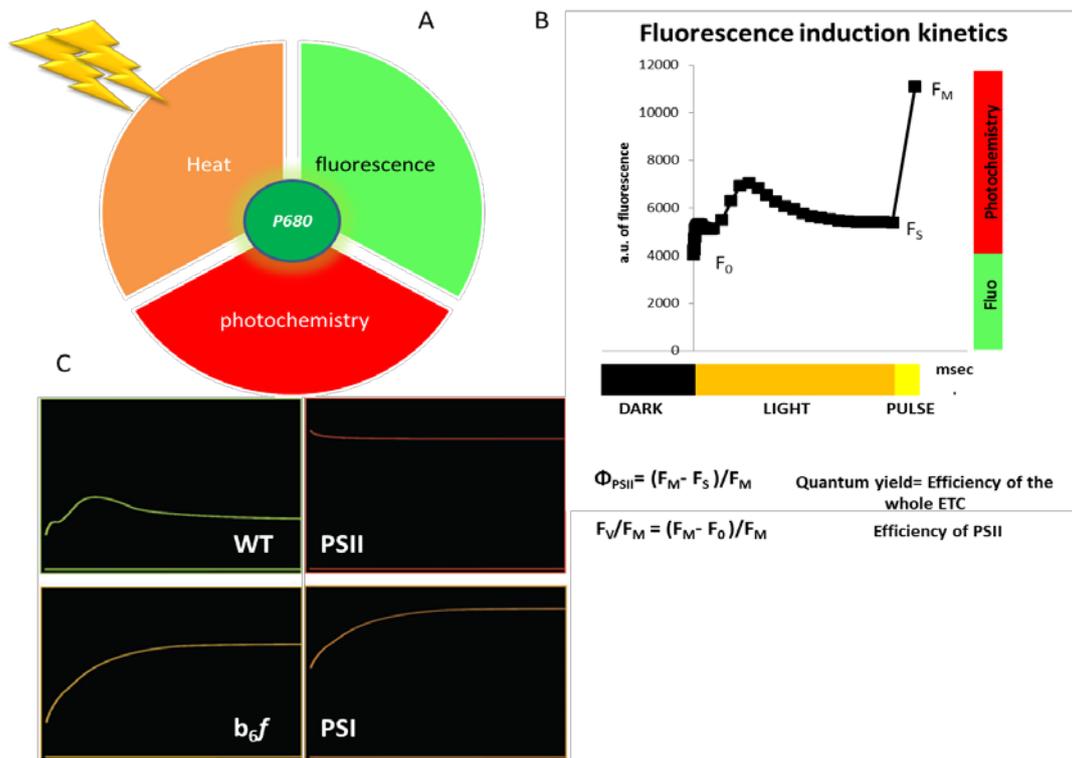


Figure 3: The use of chlorophyll fluorescence in the study of photosynthesis. A) Fluorescence is a physical property of Chl and it is in competition with photochemistry and heat dissipation. B) A typical induction curve for a wild type in optimal growth conditions.  $F_0$ = fluorescence of dark-adapted cells;  $F_s$ = Stationary fluorescence, when the system is working;  $F_M$ = Maximal fluorescence measured after a saturating light pulse. C) Characteristic fluorescence signatures of mutants K.O. for the major photosynthetic complexes.

The decrease in lumenal pH causes conformational changes in the PSII associated antenna, most probably through protonation of surface residues within the lumen. These changes are then transmitted to neighboring pigments, affecting their photo-physical properties and leading to deactivation of over-excited chlorophyll-b ( $^1\text{Chl-b}$ , singlet). The deactivation occurs through the generation of a chl-zeaxanthin dimer that transfers energy away from the reaction center. Zeaxanthin is a carotenoid located in the proximal LHCII antenna and to be effective it requires activation via a thylakoid-bound deepoxidase, responsible for the so called “*xanthophyll cycle*”, where the precursor violaxanthin is converted into the active form zeaxanthin (Wraight *et al.* 1970; Briantais *et al.* 1979; Pfundel *et al.* 1993; Walters *et al.* 1994; Horton *et al.* 1996; Ruban *et al.* 2007; Eberhard *et al.* 2008). Beside the xanthophyll cycle, additional regulators play a crucial role in qE activation: PsbS in land plants

and Lhcsr3 in *Chlamydomonas* (Petroutsos *et al.* 2016). These regulators, constitutively expressed in land plants and UV-induced in algae, are able to enhance the qE response even though the molecular mechanism is still unresolved so far (Peers *et al.* 2009; Tokutsu *et al.* 2013).

qE is present in most, if not all, photosynthetic organisms and its physiological relevance is supported by the phenotype of mutants defective for the xanthophyll cycle, PsbS or Lhcsr3, that show reduced fitness under naturally fluctuating light conditions (Kulheim *et al.* 2002; Petroutsos *et al.* 2016).

The state transition or qT (Fig. 4B), is the major component of NPQ in green algae such as *Chlamydomonas reinhardtii*, especially under non-saturating light conditions (Bonaventura *et al.* 1969; Murata 1969; Wollman 2001). The process balances the light absorption capacity between the two photosystems in response to light quality changes. The qT is slower than qE, being dependent on the redox state of the plastoquinone pool (PQ-pool), rather than on the electron transport rate (Eberhard *et al.* 2008; Minagawa *et al.* 2015). It allows energy redistribution via the reversible association of LHCII antenna, either with PSII (in state 1) or with PSI (in state 2) (Allen 1992; Wollman 2001; Turkina *et al.* 2006). This differential association is triggered by phosphorylation of the antenna through the membrane-bound Serine-Threonine kinase STT7 (Depege *et al.* 2003; Turkina *et al.* 2006). Thus, excessive stimulation of PSII leads to over-reduction of the plastoquinone pool, binding of a PQH<sub>2</sub> molecule to the Q<sub>0</sub> site of cytochrome b<sub>6</sub>f, activation of STT7, phosphorylation of LHCII antennae and their displacement from PSII towards PSI (state 2). Conversely, the oxidation of the plastoquinone pool by excess stimulation of PSI reverses this process (state 1) stimulating the de-phosphorylation of the antennas (Bellafiore *et al.* 2005; Turkina *et al.* 2006). In green algae ~80% of LHCII are involved in this displacement and the qT acquires a predominant role in energy dissipation together with the cyclic electron flow (CEF). However, the interconnection between CEF and qT is still a matter of debate and the most recent studies rather support their independency (Finazzi *et al.* 2004; Takahashi *et al.* 2013).

The ql (Fig. 4C) occurs under high light, when photo-damage and degradation of photosynthetic complexes occur. It is a well conserved mechanism, observed in cyanobacteria, algae and land plants (Nixon *et al.* 2010; Komenda *et al.* 2012; Jarvi *et al.* 2015). It involves, the proteolytic degradation and the replacement of photo-damaged PSII reaction centers (RC), with a reversible disassembly and inactivation

of the complexes. PSII photo inactivation is strictly linked to reactive oxygen species (ROS), normally produced in oxygenic atmosphere, especially under high light conditions, when the ETC is over reduced (Aro *et al.* 1993; Eberhard *et al.* 2008). In normal conditions, the neo-synthesis of the RC efficiently counterbalances the degradation process avoiding irreversible damage. Nevertheless, there are stress conditions (*e.g.* high light and nutrient limitations) where this balance is not achieved and the net result is PSII-RC degradation. The PSII repair cycle requires a lateral migration of RC from grana towards stroma lamellae and is phosphorylation dependent. The target is the D1 core protein, which dissociates from the RC, migrates in the lamellae, and is degraded by DEG-P2 and FTS-H plastidial proteases (Adam *et al.* 2001; Antao *et al.* 2005; Aro *et al.* 2005; Adam *et al.* 2006; Eberhard *et al.* 2008; Kato *et al.* 2012).

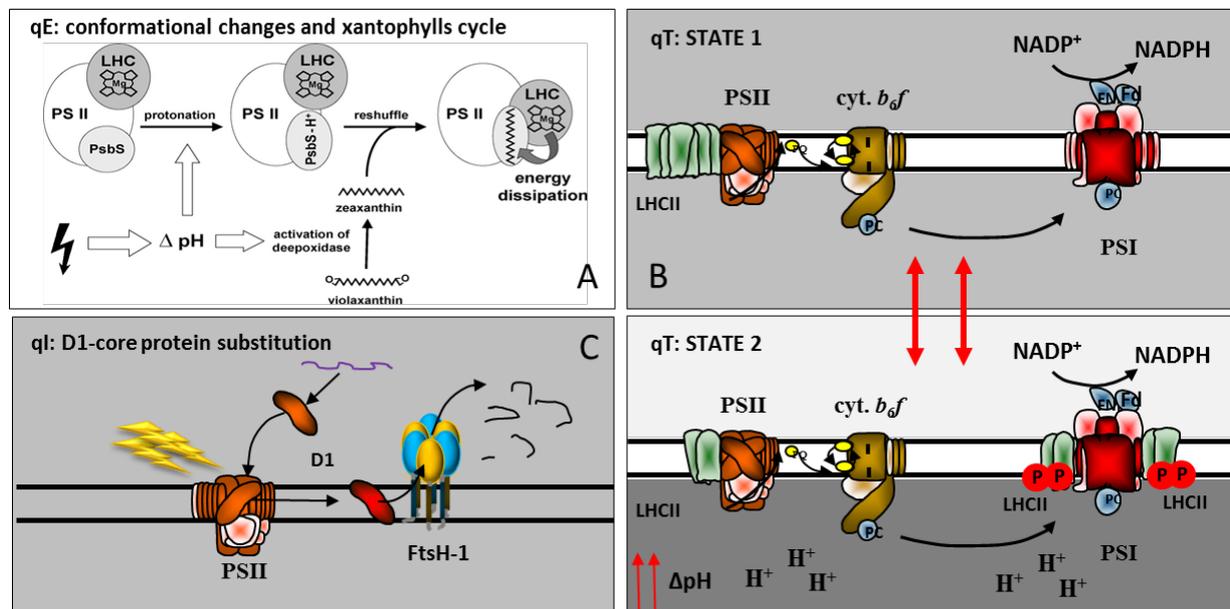


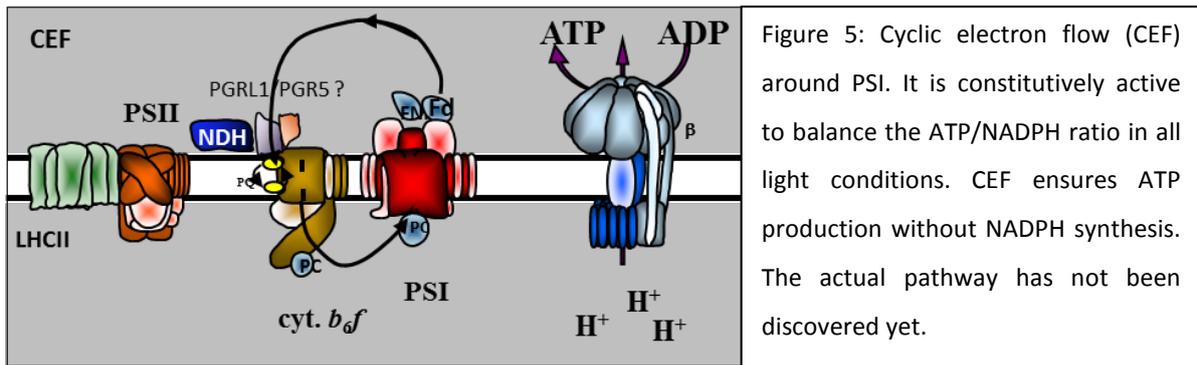
Figure 4: NON PHOTOCHEMICAL QUENCHING. A) qE component, heat dissipation triggered by  $^1\text{Chl-b}$  and performed by carotenoids and conformational changes in antenna. B) qT component, State transitions triggered by  $\Delta\text{pH}$ -dependent phosphorylation of LHCII. C) qI component, degradation of damaged D1-core protein and parallel co-translational substitution.

### II.2.2.b Cyclic electron flow around PSI

The cyclic electron flow (CEF) around PSI (Fig. 5) was demonstrated initially by Arnon and coworkers in the late 50's (Arnon 1959). In this cyclic mode, electrons are reinserted from Ferredoxin (Fd), the PSI acceptor, into the PQ-pool and then, via cytochrome  $b_6/f$ , they flow back into PSI. This cyclic flow generates  $\Delta\text{pH}$  via the Q-cycle of cytochrome  $b_6/f$  but does not allow reduction of  $\text{NADP}^+$ , thus adjusting the

ratio ATP/NADPH (Joliot *et al.* 2006). Unlike the NPQ, CEF seems to be constitutively active in all conditions (DalCorso *et al.* 2008). Nevertheless, stresses that decrease carbon fixation capability enhance the CEF rate. This is because the system tries to avoid photo-damage when NADPH, the major sink for electrons, cannot be consumed by the primary metabolism and starts to accumulate. Thus, CEF can be used by cells when primary metabolism is working slowly and some researchers support the hypothesis that it can modulate also the light usage via  $\Delta$ pH-dependent NPQ (Johnson 2005; Minagawa *et al.* 2015). The electron pathway of CEF is still under debate. Two major routes have been proposed. One involves the plastidial NAD(P)H-plastoquinone reductase (NDH); the other is dependent on an as-yet unidentified ferredoxin-quinone reductase (FQR) activity. To date, there are no biochemical evidence demonstrating the existence of an “FQR-enzyme” but there are two major hypothesis: the first claims the existence of a super complex involving PSI; Fd; FNR; cyt. $b_6f$ ; PC; in association with PGR5 and PGRL1 proteins (Nandha *et al.* 2007; DalCorso *et al.* 2008). The second hypothesis links FQR activity directly to the cytochrome  $b_6f$  complex, and in particular to the  $c'$ -heme (Stroebele *et al.* 2003; Joliot *et al.* 2004).

Beside these two major hypotheses there is a third one, not implying a permanent association between protein complexes, but rather taking advantage of the structural model of thylakoid organization proposed by Albertsson and colleagues (Albertsson 1995; Albertsson 2001). In these models CEF and LEF (linear flow) are spatially separated in “micro-domains”. These domains have different protein composition. Indeed, a large fraction of PSI centers are at the margin and the ends of the grana stacks, close to the appressed region where PSII and cytochrome  $b_6f$  are localized. In these regions protein complexes would be preferentially involved in the linear pathway (LEF). The other fraction of the PSI centers is localized in the stroma lamellae, together with a fraction of cytochrome  $b_6f$ , and would be preferentially involved in the cyclic pathway. Thus, the segregation of PSI and PSII centers in different membrane regions provides a rationale for both a linear and a cyclic electron flow taking place in the photosynthetic process (Albertsson 2001; Joliot *et al.* 2004; Johnson 2005).



### 11.2.2.c Alternative electron sinks

As already mentioned, ATP/NADPH and metabolic requirements must be balanced in all conditions. Therefore, a certain metabolic plasticity is required in order to modulate energy utilization. In general, all external cues that decrease CBC activity lead to full reduction of the photosynthetic electron carriers, since the excess of electrons cannot be injected into the metabolism. Thus, not just energy absorption, but also energy utilization can be tuned to maximize carbon fixation to avoid damages. An effective strategy to release the pressure from photosystems is the redirection of electrons towards alternative sinks. Through this metabolic flexibility a sensitive portion of the LEF is used to reduce other acceptors, sustaining alternative metabolic pathways (Laisk *et al.* 2007). Alternative flows account for up to 60% of the electrons generated by the water splitting complex and include photorespiration, Mehler reaction, malate shuttle, chlororespiration, as well as other acceptors like flavodiiron proteins (Fig. 6) (Asada 1999; Ort *et al.* 2002; Kramer *et al.* 2004; Laisk *et al.* 2007; Bailey *et al.* 2008; Cardol *et al.* 2008; Eberhard *et al.* 2008; Gerotto *et al.* 2016; Alric *et al.* 2017).

Photorespiration operates alongside carbon assimilation, having a major impact on cellular metabolism, particularly under high light, high temperatures, CO<sub>2</sub> and water deficits (Foyer *et al.* 2009). It derives from the oxygenation activity of Rubisco (Wingler *et al.* 2000) that uses ATP and reducing equivalents to convert Ribulose biphosphate (RuBP) into the CBC intermediate glycerate-3-phosphate (G3-P) and the toxic by-product 2-phosphoglycolate (2PGA) that is then detoxified in peroxisomes and mitochondria in an energy consuming pathway, with net release of CO<sub>2</sub> and NH<sub>3</sub> (Foyer *et al.* 2009). In the last years, photorespiration has been considered not only as a wasteful process but also as a protective mechanism that provides an energy sink preventing over-reduction of the ETC, especially under conditions of limited CO<sub>2</sub>

concentration. Furthermore, photorespiration provides metabolites for other metabolic processes, such as glycine for the synthesis of glutathione (Ort *et al.* 2002; Eberhard *et al.* 2008). The importance of photorespiration in the control of photosynthetic metabolism is demonstrated by the fact that several attempts to engineer Rubisco, in order to reduce its oxygenase activity, failed to enhance the overall photosynthetic fitness, or have given controversial results (John Andrews *et al.* 2003; Khan 2007; Xin *et al.* 2015; Basler *et al.* 2016), but the mutation of other enzymes involved in this pathway leads to severe phenotypes as well (often the plants are viable only under high-CO<sub>2</sub> conditions) (Dellero *et al.* 2016).

The Mehler reaction is important in the dark–light induction phase or during CO<sub>2</sub>-limited photosynthesis (Asada 2006; Krieger-Liszkay *et al.* 2011). The reaction corresponds to direct reduction of dioxygen at the acceptor side of PSI with consequent production of superoxide anions (O<sub>2</sub><sup>•-</sup>), a highly reactive and harmful ROS. Superoxide is then promptly dismutated into hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) by chloroplastic superoxide-dismutase (SOD), located in the vicinity of PSI. The dismutation is the first step of a process commonly called “water-water cycle” which, besides SOD, requires the action of enzymes allowing detoxification of H<sub>2</sub>O<sub>2</sub>: ascorbate peroxidase (APX) coupled to dehydroascorbate reductase (DHAR) and glutathione reductase (GR), glutaredoxins/peroxiredoxins (GRX/PRX), glutathione-S-transferases (GST) and catalase (CAT) (Fig. 6) (Foyer *et al.* 2011). The final result of the water-water cycle is the reduction of O<sub>2</sub><sup>•-</sup> into H<sub>2</sub>O, with net electron transport from water to water. It is commonly accepted that the water-water cycle fulfills two functions especially important under redox-stress conditions: photo-protection and ATP/NADPH ratio balancing (Badger *et al.* 2000; Forti *et al.* 2005; Asada 2006; Eberhard *et al.* 2008; Foyer *et al.* 2011).

The “Malate Valve” is a shuttle capable of exporting reducing power (NADPH) from the chloroplast toward cytosol and mitochondria, when the NADPH to NADP<sup>+</sup> ratio is high, thus poising the NADPH to ATP ratio required for optimal carbon reduction in the light (Scheibe 1987; Laisk *et al.* 2007). In chloroplasts the NADPH in excess, generated by the ETC, can be used to reduce oxaloacetate (OAA) to malate by malate dehydrogenase (MDH); the malate/OAA transporter then shuttles the substrates between the chloroplast and the cytosol where the malate is oxidized to OAA by the cytosolic NAD<sup>+</sup>, or is transferred into the mitochondrion to be further metabolized.

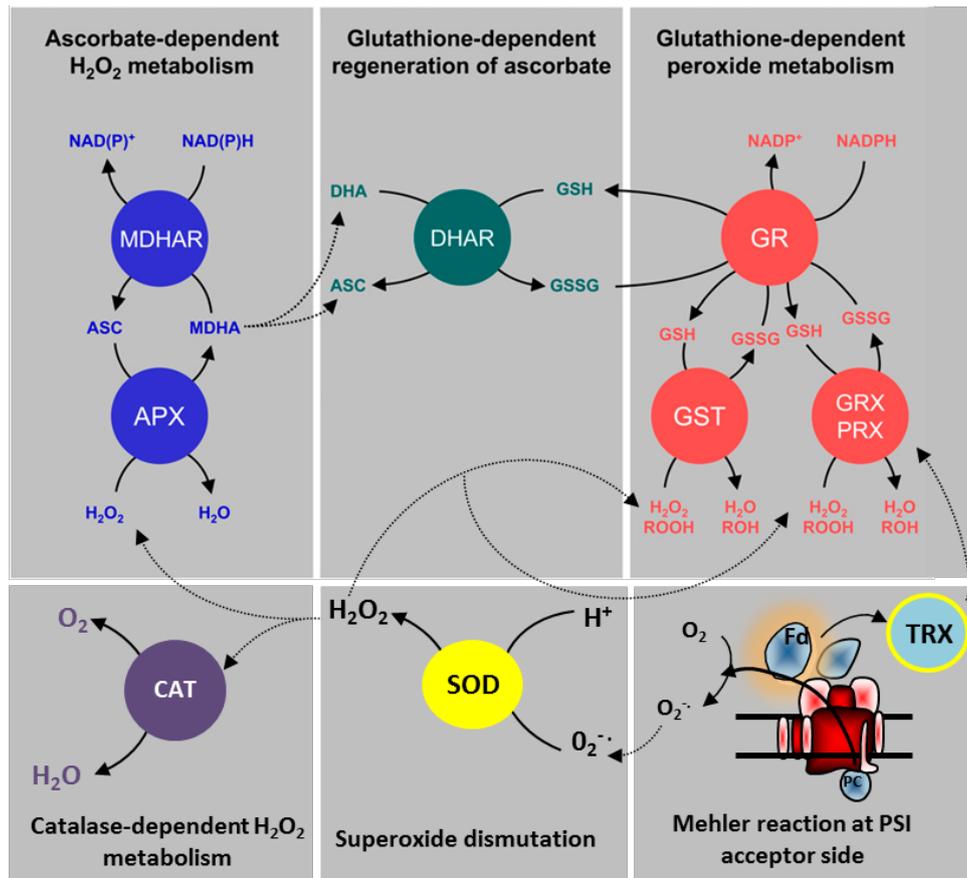


Figure 6: The « water-water cycle » and the antioxidant system involved in ROS removal at the level of PSI. APX= ascorbate peroxidase; MDHAR= monodehydroascorbate reductase; DHAR= dehydroascorbate reductase; GR= glutathione reductase; GST= Glutathione S-transferase; GRX= Glutaredoxins; PRX= peroxiredoxins; SOD= superoxide dismutase; CAT= catalase; TRX= Thioredoxin (Modified from Fover & Noctor 2011).

Although the actual metabolic consequences are still a matter of debate, the physiological role of the malate valve as a sink for photosynthetic electrons is universally accepted and, based on the study of Laisk *et al.* (2007), its contribution can represent up to 10% of the overall electron flux generated by PSII-driven water oxidation.

Chlororespiration was discovered recently in prokaryotic and eukaryotic marine phytoplankton. The reaction reroutes PSII-generated electrons into a water-water cycle upstream to the cytochrome *b<sub>6</sub>f* (Bailey *et al.* 2008; Cardol *et al.* 2008). This process is catalyzed by two enzymes associated with thylakoid membranes: NAD(P)H dehydrogenase (called NDH in land plants and NDA2 in most unicellular green algae) and Plastid-terminal oxidase (PTOX). These enzymes transfer electrons from NADPH (or Fd) to the PQ-pool first and finally to dioxygen, which is reduced to water (Feilke *et al.* 2014; Nawrocki *et al.* 2015). The physiological role(s) of

chlororespiration and the interplay with the photosynthetic function remain under debate. Even though many studies have suggested a role in photo-protection, this option does not seem realistic because the electron flow is too low to have an effect on the ETC (Nawrocki *et al.* 2015).

Another photo-protective strategy involves a class of recently discovered proteins: the flavodiiron proteins (FLVs). These peptides are located in the proximity of PSII and/or PSI and are composed of three domains: an N-terminal  $\beta$ -lactamase-like domain, a flavodoxin-like domain and a C-terminal NAD(P)H-flavin reductase-like domain. FLVs have been found in Archea and anaerobic bacteria, where they possess the first two domains and are able to reduce  $O_2$  and NO (Allahverdiyeva *et al.* 2015). More recently it has been demonstrated that cyanobacteria, *Chlamydomonas*, bryophyte and gymnosperms contain four flavodiiron isoforms with the additional C-terminal domain. FLVs assemble, 2 by 2, to form heterodimers called FLV-1/3 and FLV-2/4. FLV-1/3 is able to reduce  $O_2$  to water using NADPH as electron donor. It is believed to protect PSI from light stress, especially at the onset of illumination or under fluctuating light conditions (Allahverdiyeva *et al.* 2013; Gerotto *et al.* 2016). FLV-2/4 instead, has been shown to be active in photo-protection of PSII (Zhang *et al.* 2012; Allahverdiyeva *et al.* 2015).

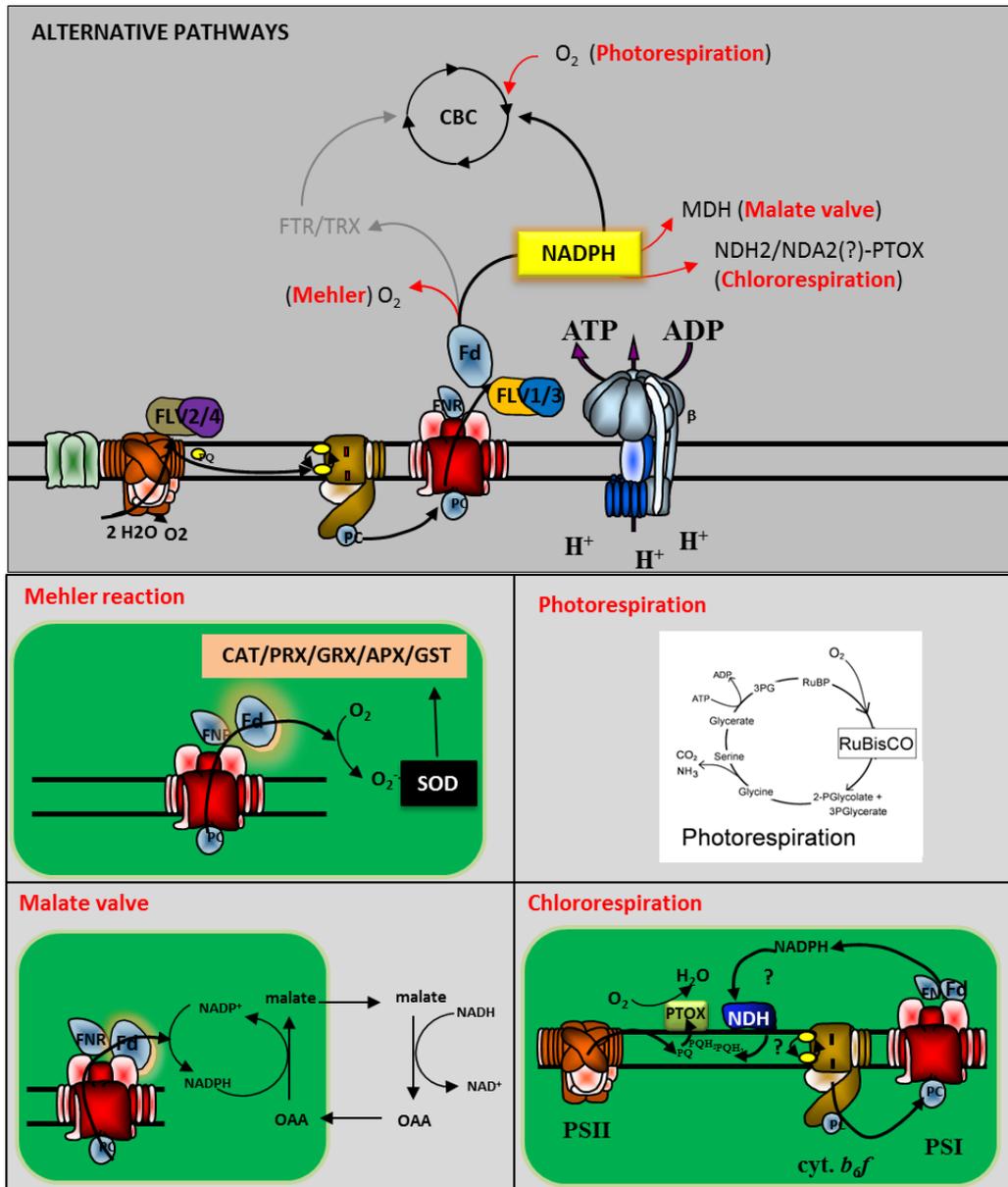


Figure 7: ALTERNATIVE ELECTRON SINKS activated when the  $\text{CO}_2$  fixation is limited by external factors. The branching point is the acceptor side of PSI. Oxygen, NDH2 or NDA2, as well as MDH can receive electrons directly from Fd or through NADPH activating, respectively, Mehler reaction; Chlororespiration or the Malate valve. Photorespiration is enhanced under  $\text{CO}_2$  limiting conditions and starts with oxygenation of RUBP. Beside these sinks, Flavodiiron proteins can contribute to the photoprotection draining out the electrons directly from PSI/PSII.

## II.2.3 The regulation of the Calvin-Benson cycle

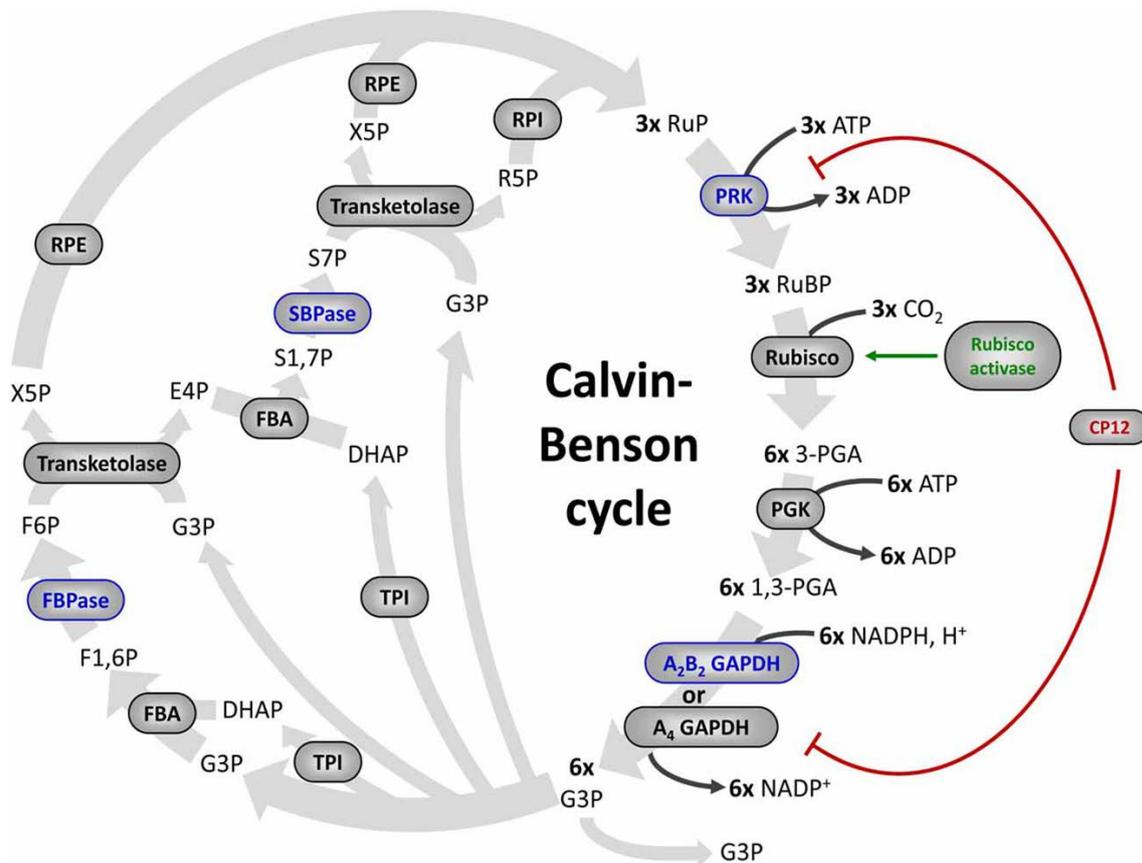


Figure 8: A scheme showing the Calvin-Benson cycle with all the enzymes involved. In blue are highlighted enzymes target for light-dependent redox regulation. In red and green two other proteins, involved in light-dependent regulation of CBC. The other enzymes are target of additional Redox Post Translational Modifications (RPTMs). From Michelet et al., (2013).

Regulation of the CBC (Fig.8) is certainly one of the most studied mechanisms of photosynthetic regulation. It is indeed the first target under many environmental conditions and especially under stress. However, its regulation is strictly interconnected with Photochemical phase regulations and, as them, its modulation occurs at many levels (gene expression, protein post-translational modifications and protein degradation), but for the scope of this work I will describe only the post-translational regulations.

After the discovery of the pathway by Bashaam et al. (1950), all the enzymes of the cycle have been purified and characterized from diverse sources including plants, algae and cyanobacteria, and this allowed a deep understanding of regulatory mechanisms. Important steps forward were done in the late 60s and 70s, when the

activity of 4 enzymes (PRK; GAPDH; FBPase; SBPase) was shown to be regulated by light, with a low-activity state in the dark and a high-activity state in the light (Schürmann *et al.* 2008; Buchanan *et al.* 2012; Michelet *et al.* 2013). This regulation is operated by the ferredoxin/thioredoxin (Fd/TRX) system that links the activity of CBC enzymes to the redox state of the photosynthetic ETC. Once reduced in the light, TRXs are able to reduce regulatory disulfides present in their target enzymes. This reduction induces a conformational change of the target, converting the protein from a low activity to a high activity conformation. This type of redox regulation is not limited to the photosynthetic metabolism, but is involved in numerous fundamental cellular processes including central metabolic pathways. Besides TRX dependent reduction of disulfide bonds, other types of reversible redox post-translational modifications (RPTMs) of cysteine residues, such as glutathionylation or nitrosylation, have emerged as important mechanisms of enzymatic regulation and metabolic plasticity. RPTMs can modulate protein activity, stability, localization, function or oligomerization (Foyer *et al.* 2009; Buchanan *et al.* 2012; Zaffagnini *et al.* 2012; Zaffagnini *et al.* 2012). The redox regulation of the CBC is achieved through multiple RPTMs including light dependent activation by the Fd/TRX system.

#### *II.2.3.a The thioredoxin-dependent regulation*

The Ferredoxin/Thioredoxin system was discovered by Buchanan and colleagues in the 60's and 70's as responsible for the light-dependent regulation of CO<sub>2</sub> fixation (Buchanan *et al.* 1967; Buchanan *et al.* 1971; Buchanan 1991). The system is composed of three types of soluble proteins, located in the stroma of chloroplasts, in close connection with thylakoid membranes: Ferredoxin (Fd), Ferredoxin/Thioredoxin reductase (FTR) and thioredoxin (TRX) (Fig.9) (Buchanan 1991; Buchanan *et al.* 2012). Fd is the final acceptor of the ETC located downstream of PSI. FTR is a thin and flat [4Fe-4S]-enzyme interacting with Fd on one side and TRX on the other side (Dai *et al.* 2007). TRXs belong to a multigenic family, composed of small (~12 kDa) disulfide-containing redox proteins, ubiquitous in all kingdoms and localized in cytoplasm/nucleus, chloroplasts, mitochondria, flagella or in the secretory pathway (Buchanan *et al.* 2012). Their active site is composed of a Cys-Gly-Pro-Cys sequence highly conserved throughout evolution (Koharyova *et al.* 2008).

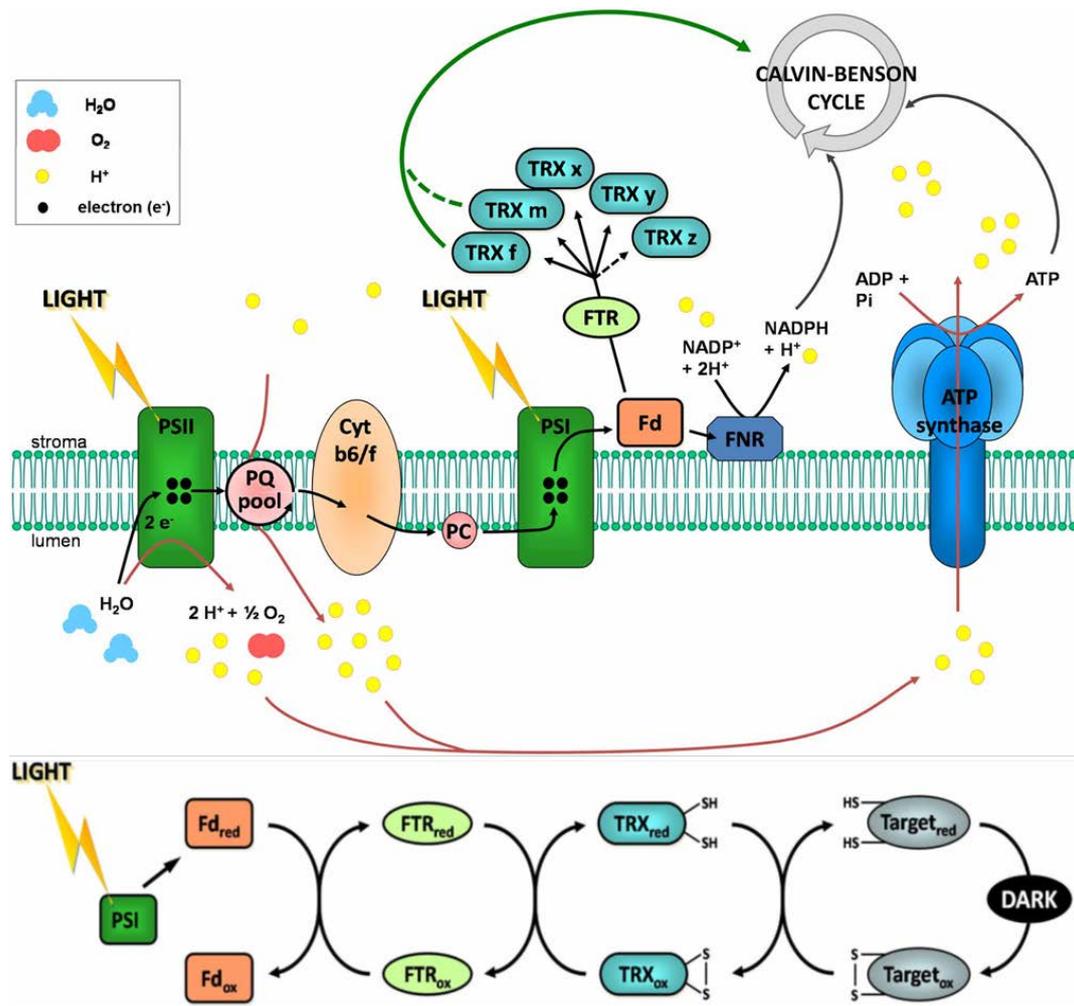


Figure 9: The Fd/TRX system and its action mechanism in the dark to light transition. (Adapted from Michelet *et al.*, 2013)

The TRX enzyme exists in its oxidized form (Trx-S<sub>2</sub>), where two cysteine residues are linked by an intramolecular disulfide-bond (-SS-), or in the reduced form (Trx-(SH)<sub>2</sub>), containing two thiol groups able to reduce exposed disulfides (Holmgren 1985; Michelet *et al.* 2013). Once reduced, TRXs activate their targets through a mechanism known as “thiol-disulfide exchange” (Fig.10), involving the formation of a transient mixed disulfide intermediate between the target and TRX. The reaction is reversible and TRX may either break or form disulfides depending on the redox potential of its substrate (Koharyova *et al.* 2008; Michelet *et al.* 2013).

Thus, CBC enzymes are oxidized (-SS-) and have a low activity conformation in darkness. Upon illumination, the ETC reduces Fd, thereby allowing NADP<sup>+</sup> reduction by Fd-NADP-Reductase (FNR).

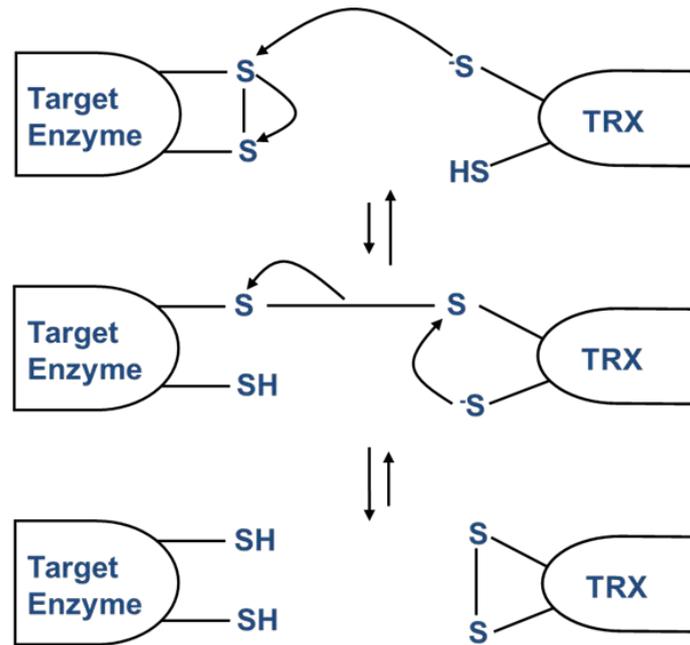


Figure 10: Catalytic mechanism of thioredoxins

Photoreduced Fd also allows reduction of chloroplastic TRXs by FTR. Consequently reduced thioredoxins are capable of reducing the 4 CBC targets, initiating the CO<sub>2</sub> fixation process (Michelet *et al.* 2013). This regulatory pathway is strongly integrated with regulations operating on the photochemical phase and acts in concert with other modulation processes, to adjust NADPH/ATP consumption with the electron transport rate. Thus, the acceptor side of PSI constitutes the “central hub” of the photosynthetic regulation. According to the redox state of the thylakoids, cells modify either the energy absorption or the energy utilization by the metabolism.

There are 5 highly conserved types of chloroplastic thioredoxins: *f*; *m*; *y*; *x*; *z*. Land plants possess several isoforms of each type: 2 TRXf, 4 TRXm, 1 TRXx, 2 TRXy, 1 TRXz in Arabidopsis; 1 TRXf, 8 TRXm, 1 TRXx, 2 TRXy, 1 TRXz in poplar. This variability cannot be observed in unicellular eukaryotic algae, *Chlamydomonas reinhardtii* indeed possesses 2 TRXf, 1 TRXm, 1 TRXx, 1TRXy and 1 TRXz, while *Ostreococcus lucimarinus* 2 TRXf, 1 TRXm, 1 TRXx no TRXy and 1 TRXz. (Lemaire *et al.* 2007; Michelet *et al.* 2013). TRXx and TRXy appear to be reduced by FTR, they were found to be the most efficient TRXs for the reduction of PRXs, GPXs and MSRs and thus they are believed to play a major role as antioxidants rather than in photosynthesis regulation (Michelet *et al.* 2013). TRXz has been recently

characterized as a subunit of the plastid encoded RNA polymerase and plays an important role in chloroplast transcription and chloroplast development (Arsova *et al.* 2010; Schroter *et al.* 2010). In addition to this role, TRXz was suggested to be redox active although its mode of reduction remains controversial (Chibani *et al.* 2011; Bohrer *et al.* 2012). Conversely, TRXf and TRXm are reduced by FTR and are clearly dedicated to the regulation of the CBC, although TRXf plays a more prominent role. Indeed, all TRX-dependent enzymes of the CBC are exclusively or preferentially reduced by TRXf. TRXm was historically considered has a regulator of other chloroplastic processes, such as the transfer of reducing equivalents between stroma and lumen, or the regulation of proteins involved in electron transfer (Motohashi *et al.* 2006; Motohashi *et al.* 2010; Courteille *et al.* 2013). However, more recently its role has been revisited, since Okegawa and colleagues (Okegawa *et al.* 2015) demonstrated how the deficiency of TRXm has a severe impact on photosynthetic CO<sub>2</sub> assimilation and on the reduction state of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase, suggesting a prominent role in photosynthesis regulation.

### *II.2.3.b Insight into the trx-dependent regulation*

The 4 light-regulated enzymes GAPDH, PRK, FBPase and SBPase, are the classical examples of TRX-mediated thiol-disulfide exchange and have been extensively characterized over the last decades.

GAPDH was the first CBC enzyme reported to be activated in the light by reduced TRX (Wolosiuk *et al.* 1978). Two genes for GAPDH exist: *gapA* and *gapB*. They encode two different isoforms that assemble in A<sub>4</sub>-GAPDH or A<sub>2</sub>B<sub>2</sub>-GAPDH tetramers. Land plants contain both isoforms while other photoautotrophs possess only A<sub>4</sub>-GAPDH. These two tetramers are differently regulated. Indeed, GAPA and GAPB subunits differ between each other for a C-terminal extension (CTE), carrying the cysteines (Cys349 and Cys358) target of TRX-f and present only in GAPB (Baalmann *et al.* 1996; Sparla *et al.* 2002). The oxidation of A<sub>2</sub>B<sub>2</sub>-GAPDH modifies the CTE conformation, leading to a hairpin in proximity of the coenzyme-binding site, with consequent repression of NADPH-dependent activity (Fermani *et al.* 2007). Conversely, A<sub>4</sub>-GAPDH, the only isoform present in cyanobacteria, green and red algae, is not TRX-regulated *per se* but acquires this regulation through the interaction with the TRX-regulated proteins CP12 and PRK, forming a multimeric complex where

the small CP12 behaves as the CTE domain of A<sub>2</sub>B<sub>2</sub>-GAPDH (Petersen *et al.* 2006; Trost *et al.* 2006). The supramolecular complex formation is promoted by the oxidation of CP12. When oxidized, it forms a binary complex with A<sub>4</sub>-GAPDH and ultimately, a ternary complex with the oxidized PRK dimer.

PRK dimers interact with the binary complex CP12-GAPDH after the oxidation of Cys243 and Cys249, two additional residues well conserved across the eukaryotic PRKs (Thieulin-Pardo *et al.* 2015). Even though PRK has been described as a component of this ternary complex, its light activation was initially reported as a classical thiol-disulfide exchange at the level of Cys16 and Cys55, the residues largely conserved in the eukaryotic and cyanobacterial PRKs. The residues forming the disulfide are located in the catalytic site of each PRK monomer and are target of TRX-f and m (even though TRX-m is less efficient) (Brandes *et al.* 1996; Michelet *et al.* 2013). In land plants both regulatory strategies co-exist, suggesting a fine regulation in response to redox potential and therefore to light intensity. However, the latter regulation is not present in all photosynthetic organisms: Rhodophytes, Cryptophytes and Heterokonts display very weak or non-existent redox regulation via Cys16 and Cys55, despite the presence of both residues (Thieulin-Pardo *et al.* 2015). The two phosphatases involved in CBC, FBPase and SBPase, were analyzed in different organisms in the attempt to dissect the molecular mechanism of their redox-regulation. In *Physcomitrella patens*, they display ~25% sequence identity at the amino acid level but the homology at the level of their 3D architecture is much higher, although they have different evolutionary histories (Gutle *et al.* 2016). Both enzymes possess a single disulfide, highly conserved among species. FBPase forms a disulfide between Cys153 and Cys178 in pea, or Cys224–Cys241 in *P. patens*. SBPase is regulated at the level of Cys52 and Cys57 in wheat, or Cys120 and Cys125 in *P. patens* (Michelet *et al.* 2013; Gutle *et al.* 2016).

The light-dependent activation of FBPase was initially reported in *Chlorella* (Pedersen *et al.* 1966). Oxidized FBPase has a basal activity (20–30%) and becomes fully activated upon disulfide reduction which is strictly dependent on TRX-f, with other TRX types being inefficient (Jacquot *et al.* 1995; Jacquot *et al.* 1997). In *P. patens*, TRX-f and TRX-m are both able to reduce the disulfide but the former is much more efficient (Gutle *et al.* 2016). The enzyme is a homotetramer present in two isoforms: a cytosolic FBPase which participates in gluconeogenesis and is not redox regulated, and a TRX-dependent chloroplastic isoform (Michelet *et al.* 2013;

Gutle *et al.* 2016). The disulfide formation forces a loop, connecting two antiparallel beta strands, to slide in toward the active site, thereby disrupting the binding sites for the catalytic  $Mg^{2+}$  cations (Chiadmi *et al.* 1999).

SBPase is a homodimer, unique to the CBC and without cytosolic counterpart (Gutle *et al.* 2016). It can be found in all photosynthetic eukaryotes but not in cyanobacteria, which encode a bifunctional FBPase possessing also SBPase activity (Tamoi *et al.* 1996). As in the case of GAPDH and FBPase, the light dependent activation of SBPase was initially reported in *Chlorella* (Pedersen *et al.* 1966; Bassham 1971; Michelet *et al.* 2013). However, the biochemical characterization was performed on the wheat enzyme leading to disentangle the molecular mechanism of regulation (Dunford *et al.* 1998). The oxidized SBPase is completely inactive in darkness and its reactivation absolutely requires the TRX-f-mediated light activation. SBPase in *P. patens* requires high levels of TRX-f if compared with FBPase and the activation from TRX-m is marginal. These results suggest SBP as a bottleneck in the cycle, thus making it a limiting factor for plant productivity together with Rubisco (Tamoi *et al.* 2006; Gutle *et al.* 2016). The differences in FBPase versus SBPase activation kinetics may be linked to the fact that FBPase is also involved in starch synthesis while SBP is not. Thus, the cells require a strategy to separate fine control of starch synthesis from the Calvin–Benson cycle (Gutle *et al.* 2016).

Even though Rubisco is the crucial enzyme involved in biomass production via carbon assimilation, its redox regulation has only been demonstrated around 20 years ago (Zhang *et al.* 1999; Zhang *et al.* 2001). In land plants and green algae Rubisco is composed of 8 large subunits, encoded in the plastome by the *rbcL* gene, and 8 small subunits, encoded by multiple *rbcS* genes in the nucleus. Two L monomers assemble head-to-tail to form an  $L_2$  dimer, with two catalytic sites at the L-L interface. Four  $L_2$  dimers interact together to form an octamer, which finally interacts with 8 small subunits in a  $(L_2)_4S_8$  structure (Whitney *et al.* 2011). The abundance of Rubisco can be explained by the fact that it is a slow and highly aspecific enzyme, considered as the main bottleneck in the entire pathway. Its regulation is known to be light dependent but is more complex than the other 4 enzymes described above. Rubisco is not directly targeted by Trx-f, but requires a protein, called Rubisco Activase (RCA), which is the actual Trx-f target (Zhang *et al.* 1999). The catalytic site of Rubisco indeed, can be occupied by different sugars, naturally produced, which act as inhibitors: 2-carboxy-D-arabinitol-1-phosphate

(CA1P); xylulose-1,5-bisphosphate (which can be produced after a misfired carboxylation reaction); and RuBP itself (Pearce 2006; Parry *et al.* 2008). Thus, RCA uses the energy of ATP to remove these sugars from the active site. Its ATPase activity can be in turn modulated by Trx-f at the level of two cysteines, Cys392 and Cys411, through the classical thiol-disulfide exchange (Zhang *et al.* 1999; Zhang *et al.* 2001). ATP hydrolysis promotes the interaction of the C-terminal domain of RCA with the region comprised between residues 89-94 of the L-subunit of Rubisco, with consequent release of sugar from the active site (Portis *et al.* 2008). At this point, Rubisco should be *preactivated* via the reaction of CO<sub>2</sub> with a conserved Lys201 which leads to the formation of a carbamate, stabilized with a Mg<sup>2+</sup> cation, capable to carboxylate RuBP (Andersson *et al.* 2008).

An additional enzyme of the CBC with a low activity in the dark and a TRX-dependent activation in the light, in green algae but not in land plants, is the PGK (Morisse *et al.* 2014). PGK exist in two isoforms, one chloroplastic participating in the CBC and one cytosolic, present in most organisms and participating in the glycolytic pathway. The two isoforms show high sequence similarity, suggesting that the molecular structure of the enzyme might be strongly conserved among several organisms. PGK is a monomeric enzyme composed of two domains linked by an  $\alpha$ -helix with the N-terminal domain containing the substrate binding site, whereas the ATP cofactor binding site is placed in the C-terminal domain. After substrate binding, a conformational change brings the two domains in close proximity, generating the so-called “closed conformation” that ensures catalytic reactions. PGK from *C. reinhardtii* can be regulated by TRX-f but not TRX-m. The regulation involves the not strictly conserved Cys227 and Cys361 and the formation of the disulfide was proposed to impose structural constraints in the C-terminal domain of the enzyme that may lower its catalytic efficiency (Morisse *et al.* 2014).

Although the CBC can be considered as the paradigm of redox regulation, the improvement of proteomic approaches allowed a deeper understanding of the TRX-interactome (or thioredoxome), revealing how other cellular processes, beside CO<sub>2</sub> fixation, might be regulated by TRXs (Koharyova *et al.* 2008; Buchanan *et al.* 2012; Perez-Perez *et al.* 2017). Some examples of alternative targets are malate dehydrogenase; Acetyl-CoA carboxylase; ADP-glucose pyrophosphorylase; glucan:water dikinase; beta-amylase; glucose-6-phosphate dehydrogenase; peroxiredoxins; and FtsH plastidial protease (Wenderoth *et al.* 1997; Ballicora *et al.*

2000; Geigenberger *et al.* 2005; Mikkelsen *et al.* 2005; Sparla *et al.* 2006; Lemaire *et al.* 2007; Nee *et al.* 2009; Lindahl *et al.* 2011; Sevilla *et al.* 2015; Wang *et al.* 2017). Even though these studies have investigated single proteins, most recently our team has made a major step forward in the understanding of the thioredoxome, obtaining a comprehensive overview of TRX-targets in *Chlamydomonas* (Perez-Perez *et al.* 2017). In this study indeed, more than 1000 proteins have been identified. Around 30% of these targets are involved in cell metabolism (e.g. starch, lipids, amino acids, ATP-synthesis); another 22% is implicated in genetic information processing (e.g. DNA-transcription/replication/repair and protein metabolism); more than 8% is involved in cell motility and transport and another 8% can be divided between stress response, redox homeostasis and environmental information processing. Moreover, 30% of them are not yet annotated. All these data support the idea of a central role for redox regulation in the cell at many levels.

### *II.2.3.c Redox post-translational modifications (RPTMs)*

The progresses achieved in the proteomic field in the last 20 years led also to identify new redox mechanisms regulating many pathways, among which the CBC. They seem to operate in parallel (or on top) of the Fd/TRX system, through diverse reversible RPTMs among which glutathionylation and nitrosylation have been the most studied (Fig. 8). These RPTMs are triggered by ROS and/or RNS molecules, side products of the oxygenic metabolism in mitochondria and chloroplast, (Michelet *et al.* 2013). These species are normally kept under control by the antioxidant system composed of Glutathione (GSH) and Ascorbate (ASC); Ascorbate peroxidase (APX); Peroxiredoxins (PRXs); Thioredoxins (TRXs); Glutaredoxins (GRXs); Glutathione Reductase (GR); Glutathione S-Transferase (GST); Ascorbate Reductase (MDHAR/DHAR); Nitroso-glutathione Reductase (GSNOR); Superoxide Dismutase (SOD); Catalase (CAT) and diverse peroxidases (Foyer *et al.* 2013). Thus, the rate of RPTMs depends on the rate of formation of reactive species and the rate of scavenging activity by antioxidant system. However, in some cases the RPTMs are independent from this balance and some proteins can be found constitutively modified also in basal conditions, not only in case of redox perturbation. In the latter case, redox modifications can be linked to the local concentration of ROS/RNS and/or to the specific production pathway, often not elucidated. Therefore the current

idea is that the RPTMs are a powerful tool the cells use to regulate metabolism and signaling, and this is true for all organisms, under normal or stress conditions.

RPTMs occur on specific, reactive, cysteines that can undergo different states of oxidation including protein disulfide bonds (intra-or intermolecular), thiol oxidation to sulfenic ( $-SOH$ ), sulfinic ( $-SO_2H$ ) and sulfonic ( $-SO_3H$ ) acids, S-glutathionylation (SSG), S-sulfhydration (SSH) or S-nitrosylation (SNO). The oxidation is induced by radical molecules as superoxide ( $O_2^{\cdot-}$ ) or singlet oxygen ( $^1O_2$ ), produced respectively by PSI and PSII under stress conditions (e.g. high light or CBC limitation). Since they are not long lasting, the best oxidative agent in most conditions is hydrogen peroxide ( $H_2O_2$ ), the most stable ROS produced in peroxisomes, in chloroplast and mitochondria by the SOD enzyme (Zaffagnini *et al.* 2012; Michelet *et al.* 2013).

The sulfenic acid ( $-SOH$ ) is the only reversible form and, in normal redox conditions, its reversibility is ensured by the second major RPTM: glutathionylation. The reaction is carried out by reduced glutathione (GSH), a tripeptide composed by  $\gamma$ -L-Glutamyl-L-cysteine-glycine and considered as the major intracellular redox buffer (Foyer *et al.* 2011). The reaction corresponds to the formation of a mixed disulfide bond between a GSH molecule and a protein cysteine ( $-S-SG$ ). The glutathionylated target can then be deglutathionylated by reduced glutaredoxin ( $GRX-S^-$ ), small oxidoreductase belonging to the superfamily of TRXs. After the deglutathionylation event, GRX becomes itself glutathionylated ( $GRX-SSG$ ) and is reduced back by GSH. This process has been described as the Glutathionylation/Deglutathionylation-cycle (G/D-cycle) (Zaffagnini *et al.* 2012) and may be envisioned as either a mechanism of protection against over-oxidation, ROS scavenging and/or redox signaling (Michelet *et al.* 2008; Zaffagnini *et al.* 2012; Zaffagnini *et al.* 2013). However, it can also serve as a trigger for alternative functions (e.g. *moonlighting functions*) or for enzymatic activity modulation. In any case, its rate increases dramatically under redox stress conditions (Zaffagnini *et al.* 2012). -

Another crucial RPTM is nitrosylation, triggered by NO or its higher oxides  $N_2O_3$  and  $NO_2^-$ , either under stress or physiological conditions. This reaction corresponds to the formation of a protein nitrosothiol ( $-SNO$ ) with consequent effects on stability, localization, oligomerization and activity of the target (Zaffagnini *et al.* 2016). Nitrosylation, as glutathionylation, is a reversible modification that can be controlled by two distinct systems: the TRX-system (NADPH/NADPH-Thioredoxin-

Reductase/TRX) or the GSH/GSNOR system. They act on different targets and can be considered as complementary systems (Zaffagnini *et al.* 2016).

Glutathionylation and Nitrosylation have been recently shown to be crucial reactions for signaling and regulation inside the chloroplast (Zaffagnini *et al.* 2012; Zaffagnini *et al.* 2016). Interesting is the discovery that all 11 enzymes of the CBC are potential targets of nitrosylation and glutathionylation (Zaffagnini *et al.* 2012; Morisse *et al.* 2014). But the striking discovery is the fact that the two modifications can have diverse consequences on the enzymatic activity, pointing for a different role in signaling and/or in metabolism regulation. For example, cytosolic GAPDH and TPI have both been demonstrated as target for glutathionylation and nitrosylation in *C. reinhardtii* (Zaffagnini *et al.* 2007; Zaffagnini *et al.* 2013; Zaffagnini *et al.* 2013; Zaffagnini *et al.* 2014), but if in the former enzyme these modification completely inhibit the activity, in the latter their effect is minor, suggesting a different physiological role.

Altogether, these studies suggest the existence of a complex network of interconnected RPTMs (Fig. 11) acting on top or in parallel with TRX-regulation. The contribution of redox modifications can be observed either in short- or long-term acclimation responses, enabling metabolic readjustments to maintain cellular homeostasis (Scheibe *et al.* 2012). This network not necessarily regulates the pathway activity, rather it can affect a minor pool of the total protein (especially for abundant proteins such as those involved in the CBC), activating signaling circuits or triggering *moonlighting* functions, new functions unrelated to the main metabolic role of the enzyme (Zaffagnini *et al.* 2013).

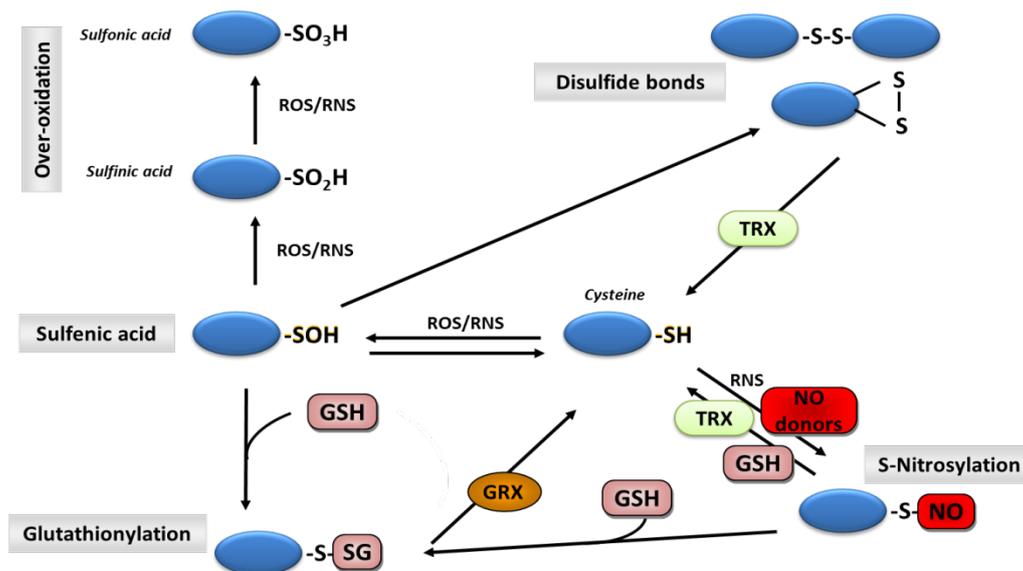


Figure 11: Main thiol-based redox PTMs and their reduction pathways

### II.3 Stress and photosynthesis: acclimation to avoid photodamage

Photosynthesis is responsible for most of energy and carbon skeleton production. Therefore, all stresses affecting cell metabolism and energy usage have a direct or indirect effect on this pathway. Detrimental effects derive from the imbalance between CO<sub>2</sub> fixation and electron transport, which leads to over-reduction of the ETC and block of electron transfer, with subsequent ROS production and oxidative stress (Laloi *et al.* 2015). The chloroplast redox balance is maintained efficiently by the regulatory mechanisms already described, but when the stressful cues overwhelm the acclimation capability of the cells, photodamage takes over inducing degradation of photosynthetic components, block of CO<sub>2</sub> fixation and activation of pro-apoptotic pathways in the worst cases.

The major abiotic stresses for oxygenic photoautotrophs are: water shortage; heat/cold stress; excessive or insufficient light irradiation; nutrient starvations; soil pollutants (*e.g.* heavy metals) and salt stress (Grossman 2000; Irihimovitch *et al.* 2008; Amtmann *et al.* 2009; Hansch *et al.* 2009; Watanabe *et al.* 2010; Yang *et al.* 2010; Merchant *et al.* 2012; Obata *et al.* 2012; Claeys *et al.* 2013; Golldack *et al.* 2014; Laloi *et al.* 2015; Baurle 2016; Koroban *et al.* 2016). In this work, two of these major stresses will be discussed, nutrient starvations and light irradiance fluctuations, with particular interest for their effects on the organization of the photosynthetic machinery in *Chlamydomonas reinhardtii*.

### II.3.1 Nutrient Starvations

Carbon, Nitrogen, Phosphorus and Sulfur are considered as macronutrients, the building bricks for all biomolecules as proteins, nucleic acids, carbohydrates, and lipids, required in millimolar range in the medium. In the wild, and especially in open oceans, such elements are limiting and their shortage has a dramatic impact on cell fitness and biomass production. Thus, photosynthetic organisms have evolved complex mechanisms to deal with macronutrient deprivations, adjusting their chemical composition, their metabolism and physiology, to respond efficiently to the stress, at least for a certain time span. If the limitation lasts for too long, pro-apoptotic mechanisms are activated.

When *Chlamydomonas reinhardtii* experiences nutrient limitations, general responses (common to all limitations) and nutrient-specific responses are activated, acting either simultaneously or sequentially to reach acclimation (Merchant *et al.* 2012). Acclimation typically begins with cell cycle arrest, increased commitment for alternative sources acquisition, and enhanced internal resources mobilization (Grossman 2000). These are common features of all limitations, even if each nutrient deprivation activates its own regulatory pathways and possesses its particular regulators. During this initial phase, proteins responsible for import of external sources and for degradation of internal reserves are expressed. For example during sulfur limitation *Chlamydomonas* synthesizes extra-cellular sulfatases that cleave sulfate from aromatic carbon compounds in the environment (de Hostos *et al.* 1988). Phosphorus limitation induces the synthesis of extra-cellular phosphatases and RNAses (Löffler *et al.* 1993; Bariola *et al.* 1994). An extra-cellular amino acid oxidase is synthesized in response to nitrogen limitation (Vallon *et al.* 1993). Concomitantly, total mRNA content encoding proteins involved in anabolic pathways dramatically drops (Merchant *et al.* 2012). If the limitation persists, cells implement sparing and recycling mechanisms where essential proteins are replaced by alternative isoforms with different amino acid composition, in order to reduce requirement for the lacking element. Non-essential proteins, rich in amino acid containing the limiting nutrient, are selectively degraded and their components are recycled (Merchant *et al.* 2012). Some limitations, as nitrogen or sulfur starvations, show characteristic features of particular interest for biotechnological purposes, especially if applied to cyanobacteria and microalgal species (Hejazi *et al.* 2004; Gillet 2015). These organisms indeed,

under starvation induce reorganization (or remodeling) of the photosynthetic machinery in a highly regulated fashion. The block of photosynthesis then causes a switch toward a fermentative or respiratory metabolism which facilitates the accumulation of reduced carbon. This strategy is believed to minimize photodamage, inducing the redirection of electrons towards alternative sinks (Bulte *et al.* 1992; Wykoff *et al.* 1998; Grossman 2000; Wei *et al.* 2014). For the scope of this thesis, nitrogen and sulfur starvations, and their peculiar effects on the photosynthetic apparatus of *Chlamydomonas reinhardtii* will be described.

### **II.3.2 Nitrogen and Sulfur Starvations: some similarities**

Nitrogen is one of the macronutrients necessary to synthesize amino acids and nucleic acids, and plays a pivotal role in the cell metabolism. It has been known since the late '60s that nitrogen deprivation promotes gametogenesis in *Chlamydomonas* (Harris, 2008). Indeed incubating vegetative cells in a nitrogen-depleted medium can trigger the differentiation into gametes, which fuse to form diploid zygotes, extremely resistant to adverse environmental conditions. After the observation of this first landmark, several other studies were carried out late in the '70s, describing the main effects of nitrogen deprivation on cell morphology and physiology. Upon limitation, *Chlamydomonas* was shown to degrade plastidial and nuclear-encoded ribosomes, to present major alteration in the endoplasmic reticulum envelope and in chloroplast morphology, with the concomitant photosynthetic inactivation. At the same time, a great accumulation of starch was described (Cavalier-Smith 1975; Martin *et al.* 1975). Moreover, when starved for nitrogen, *Chlamydomonas reinhardtii* increases in cell volume and accumulates great amounts of lipid bodies (composed of triacylglycerol lipids TAGs) deriving from degradation of internal cell components (Rodolfi *et al.* 2009; Schmollinger *et al.* 2014). Over accumulation of starch and TAGs can be exploited for biotechnological purposes, with a proper metabolic engineering strategy, in order to produce biofuels or other valuable molecules (Wen *et al.* 2003; Hejazi *et al.* 2004; Aro 2016; Zhu *et al.* 2016).

In the early 90's the attention of the research community turned on into the understanding of photosynthetic inhibition processes. It appeared that the decrease in photosynthetic efficiency was achieved through an effective remodeling of the photosynthetic apparatus. Bulté and Wollman in 1992 have demonstrated that nitrogen starvation leads to photosynthetic inactivation through the selective loss of

the cytochrome *b<sub>6</sub>f* complex and, recently, this mechanism has been extensively described (Bulte *et al.* 1992; Wei *et al.* 2014). The loss of cytochrome *b<sub>6</sub>f* is not linked to photodamage or aspecific degradation since it is independent of light. Moreover, cells grown in photoautotrophic conditions do not lose the complex, meaning that the degradation only occurs if photosynthesis is dispensable (*e.g.* in the presence of acetate). When the loss of cytochrome *b<sub>6</sub>f* is observed, it correlates with Rubisco degradation, the most abundant protein and one of the main sinks of nitrogen, and with increased O<sub>2</sub> consumption, pointing to a general inhibition of photosynthesis in favor of respiration. At the same time, chlororespiratory enzymes (PTOX and NDA2) are induced suggesting an attempt of protecting the PSII in such conditions (Wei *et al.* 2014). Some data indicate that the remodeling process is tightly regulated and not just an aspecific degradation resulting from oxidative damage. Indeed, the inactivation of photosynthesis correlates with significant production of nitric oxide (NO) slightly before the degradation of cytochrome *b<sub>6</sub>f* and Rubisco. Moreover, NO-donors and scavengers influence the kinetics of photosynthetic inactivation and protein degradation, pointing for a direct involvement of NO in this process (Wei *et al.* 2014). Two crucial aspects are still poorly understood: the production of nitric oxide and the molecular mechanism through which it acts.

Another key macro nutrient is certainly sulfur, which is required to synthesize cysteine and methionine, glutathione and its derivatives, as well as sulfolipids and Fe/S clusters, components crucial for the cell functions. The focus on sulfur deprivation has risen in the last 30 years, driven especially by the possible biotechnological applications for alternative energy sources production. The response to sulfur starvation in microalgae shows many similarities with the response to nitrogen limitation. For example, *C. reinhardtii* cells change drastically their metabolism, blocking photosynthesis through thylakoid remodeling, arresting cell division and accumulating reduced carbon as TAGs in lipid bodies (Zhang *et al.* 2004; Sato *et al.* 2014; Yang *et al.* 2015). The composition of membrane changes as well. For instance, sulfoquinovosyl diacylglycerol lipids (SQDGs), sulfolipids important for the stabilization of PSII and other thylakoid proteins in cyanobacteria and *Chlamydomonas* (Sato *et al.* 1995; Minoda *et al.* 2003; Aoki *et al.* 2004), are massively degraded after 6 hours from the onset of the starvation. This degradation is believed to serve as a scavenging strategy to redistribute sulfur to the protein fraction (Sugimoto *et al.* 2007; Yang *et al.* 2015). Sulfur starvation also has a severe

impact on redox homeostasis. The green alga *Chlorella sorokiniana* shows a transient burst in H<sub>2</sub>O<sub>2</sub>, which correlates with augmented activity of SOD and APX (Salbitani *et al.* 2015), and also a strong decrease in the intracellular content of cysteine and glutathione. The red alga *Galdieria phlegrea* shows the same decrease under autotrophy, whereas under heterotrophy the glutathione content rather increases (Carfagna *et al.* 2016).

As mentioned above, the decrease in photosynthetic efficiency upon sulfur starvation is achieved through massive thylakoid reorganization and photosynthetic complex degradation, and this is one of the main similarities between nitrogen and sulfur starvation. However, besides these similarities, it looks like under sulfur limitation, photosynthetic inhibition occurs through different targets, inducing different outcomes. Indeed, the photosynthetic quantum yield decrease has been described as mainly due to inactivation and degradation of PSII, which was identified as the main target.

PSII inactivation has been deeply characterized since it is crucial to induce H<sub>2</sub> production, the most striking response activated under sulfur starvation (Wykoff *et al.* 1998; Grossman 2000; Zhang *et al.* 2002; Zhang *et al.* 2004; Hemschemeier *et al.* 2008; Burgess *et al.* 2011). Hydrogen production requires the block of the photosynthetic ETC at the level of PSII to arrest O<sub>2</sub> evolution. This arrest is an indispensable pre-requisite for the activation of hydrogenase (HYD1), the H<sub>2</sub>-producing enzyme localized in the vicinity of PSI and extremely sensitive to oxygen (Wykoff *et al.* 1998). In experimental conditions hypoxia can be reached by starving the cells for sulfur in sealed flasks. In these conditions PSII inactivation is counteracted by mitochondrial respiration which consumes O<sub>2</sub>. Thus, when the partial oxygen pressure falls below the threshold required for HYD1 activation, H<sub>2</sub> production begins and lasts for several days (Zhang *et al.* 2002; Burgess *et al.* 2011). Two hypotheses were formulated to explain the source of electrons which are injected into the ETC and sustain H<sub>2</sub> evolution. For instance it was postulated that electrons are provided by the activity of the oxygen evolving complex (Kosourov *et al.* 2003). Moreover, it was also proposed that they derive by the degradation of internal reserves (e.g. starch or proteins) (Melis *et al.* 2000). Most probably, both activities are required in distinct moments. Indeed, Hemschemeier and colleagues (Hemschemeier *et al.* 2008) have shown how, during the first 24 hours of starvation, PSII-driven O<sub>2</sub> evolution provides electrons for starch synthesis. Consequently,

starch reserves are degraded releasing electrons for the non-photochemical reduction of the PQ-pool, from which they are then transferred to the HYD1 via the ETC.

Despite the efforts made so far, the effects of sulfur starvation on the photosynthetic complexes are still poorly characterized at the molecular level, and little is known about the degradation mechanisms. Even more, no investigations have been carried out regarding the possible messenger involved in response activation. In all cases, besides PSII inactivation, preliminary data from the group of Francis-André Wollman (Malnoe *et al.* 2014) suggested a light-independent loss of cytochrome *b<sub>6</sub>f*, albeit with a slightly different kinetics compared to nitrogen starvation. This prompted us to investigate these aspects during the course of this work.

### **II.3.3 Light fluctuations and long term acclimation**

In nature, light quality and light quantity are continuously changing on a time scale varying between fractions of seconds to days. Thus, photosynthetic organisms have evolved several responses to face these changes and acclimate. These responses can be classified in short-term and long-term responses that cooperate to fine tune the light-harvesting capability and energy utilization (Eberhard *et al.* 2008; Croce *et al.* 2014; Rochaix 2014). If light changes occur on a short-term scale, not suitable for protein synthesis or degradation, the cells respond with fast mechanism (NPQ and CEF) generally acting at the level of pigment-pigment interactions, achieved by conformational changes of the proteins; through the balance of light absorption between PSII and PSI; or via TRX-dependent CBC modulation. These fast responses have been described above (section I.2.2 and II.2.3a/b).

However, light can also change in a long term scale (hours or days) and, in such a situation, the acclimation occurs through the effective remodeling of the photosynthetic apparatus, especially through the modulation of the antenna size. This remodeling is achieved through gene expression regulation (at transcriptional, post-transcriptional, and translational level), post-translational regulation and degradation of the LHCs (Croce *et al.* 2014; Rochaix 2014). All photosynthetic organisms show the same strategy in response to long-term light fluctuations. Under low light the antennas are bigger to drive as much energy as possible towards reaction centers. Conversely, under high light the antenna size is reduced to minimize harmful effects of over excitation (Rochaix 2014). The strategies to reach this acclimation are

multiple and vary among species. A key player in these adaptive processes is the LHCII photosynthetic antenna system, while LHCS of PSI seems to be less affected by these light fluctuations. Indeed, antenna systems of PSII and PSI are regulated in different ways. For example in Arabidopsis plants transferred from low to high light, the PSII antenna size is greatly diminished, whereas the stoichiometry of all LHCA antenna proteins of PSI is unchanged. Despite this stability in antenna stoichiometry, PSI light harvesting function is regulated through the control of PSI/PSII ratio, achieved via the state transitions, in response to differential excitation of the two photosystems (Ballottari *et al.* 2007).

Several factors are involved in integrating and assembling the LHC proteins in the thylakoid membranes. The chloroplast signal recognition particle (SRP), which interacts post-translationally with LHC substrates, forms a transient complex to keep the hydrophobic LHC proteins soluble in the stroma during their migration to the thylakoid membranes (Schuenemann *et al.* 1998; Klimyuk *et al.* 1999). Alb3, which is well conserved in land plants and algae, is required for the assembly of the light harvesting system. Loss of this protein in Arabidopsis leads to an albino phenotype (Sundberg *et al.* 1997). In Chlamydomonas, two nucleus-encoded proteins, Alb3.1 and Alb3.2, are involved in LHCII assembly. Loss of Alb3.1 leads to the specific depletion of LHCII and to a decrease of PSII (Bellafiore *et al.* 2002). Alb3.2 appears to play an essential role in thylakoid membrane assembly, and a mild decrease of this protein is lethal (Gohre *et al.* 2006). Another important factor in Chlamydomonas is Tla1, a nuclear encoded protein, localized on the chloroplast envelope, which acts as a regulator of chlorophyll content and chlorophyll antenna size. Overexpression or repression of Tla1 leads to a larger or smaller antenna respectively (Mitra *et al.* 2010; Mitra *et al.* 2012).

#### **II.4 The nature of the signal governing environmental responses**

The acclimation to environmental stresses and/or changing conditions, as well as the metabolic adjustment to physiological and developmental conditions, requires a precise coordination between nuclear and plastidial gene expression. Therefore one or more signaling pathways connecting these organelles must exist to fulfill the task. We commonly refer to “retrograde signaling” to describe the strategy ensuring the exchange of information between plastid and/or mitochondria with the nucleus

(Kleine *et al.* 2016). The plastid-derived retrograde signal is responsible for the operational control that synchronizes cellular metabolism to developmental and physiological status of the plastid.

The operational control is crucial for stress responses and, thanks to the improvements in genetics, metabolite profiling and bioinformatics; we now have a deeper knowledge of this signaling in photosynthetic organisms. Even though several messengers and pathways have been proposed in the last years, none of them has been convincingly demonstrated *in vivo* (Pfannschmidt 2010).

The best candidates, proposed along the last decade, are most probably acting in parallel, forming a complex network of signals. They include tetrapyrroles; PQ-pool redox state; Hemes; phosphonucleotide (PAP);  $\beta$ -carotene precursors/degradation products; ROS/RNS produced in the plastid (Kleine *et al.* 2016).

Beside these putative messengers, nitric oxide (NO) is another key player in signaling pathways, either in animals and plants, under physiological conditions as well as under stress. Produced in all cells, its involvement in mitochondria retrograde signaling has been established in animal nervous systems (Suvarna *et al.* 2016). Given its paracrine nature, it is a good candidate for plastid retrograde signaling as well.

NO acts mainly inducing RPTMs in all organisms. My thesis work is focused on the role of NO in stress response. This led me to participate in the writing of a review article, published in 2016 in the journal *Biochimica et Biophysica Acta* (BBA), which details NO production and activity with particular interest for intracellular nitrosylation targets and the physiological roles known so far in photosynthetic organisms. This article is presented as "Article 1" in this manuscript.

## **II.5 Objectives of the work**

My thesis work has been focused on the study of nitric oxide involvement in the regulation of photosynthesis under sulfur starvation and upon long-term light fluctuations in the unicellular photosynthetic organism *Chlamydomonas reinhardtii*. The global aim was the understanding of the molecular mechanism through which NO governs the remodeling of the photosynthetic apparatus.

### **II.5.1 *Chlamydomonas reinhardtii* as a model organism**

*Chlamydomonas reinhardtii* is a photosynthetic unicellular eukaryotic green alga, measuring about 10 micrometers, widely distributed over the world, in soil and fresh water. It has a cell wall, contains a single large chloroplast (over half of the volume of the cell), an eyespot that is responsible for light sensing during phototaxis and two anterior flagella that allow cellular motility (Fig. 12). *Chlamydomonas* is an important model organism to study numerous biological processes, including photosynthesis, membrane biogenesis, cell motility, phototropism, circadian rhythm, gametogenesis and bioenergy (Rochaix 1995; Grossman 2000; Dent *et al.* 2001; Matsuo *et al.* 2011; Mussnug 2015). In addition, it is an organism really easy to handle compared to higher plants. The green alga requires very little space for growth and has a short generation time. Moreover, the nuclear, chloroplastic and mitochondrial genomes of *Chlamydomonas* have been sequenced and are well annotated (Merchant *et al.* 2007). Comparative phylogenomic analyses, revealed a highly conserved photosynthetic apparatus between the alga and higher plants, thereby confirming the interest to use *Chlamydomonas* as a model system to study photosynthesis. One other major advantage is that *Chlamydomonas* is a facultative phototrophic organism since it has the ability to grow either photoautotrophically or heterotrophically using acetate as a reduced carbon source. Moreover, in the dark, *Chlamydomonas* is able to synthesize chlorophyll and to assemble its photosynthetic apparatus. These features allowed isolation of viable mutants that are unable to perform photosynthesis and/or chloroplast biogenesis (Dent *et al.* 2001)

*Chlamydomonas* possesses several characteristics that make it an excellent organism for basic genetic analysis. It is mainly haploid and gametes have two different mating types: mt (+) and mt (-), allowing fusion to form a diploid zygote surrounded by a durable coat to protect against harsh environmental conditions. The

progeny of meiosis from diploid zygotes can be characterized through tetrad analysis. In addition, new genes can be introduced into these genomes through different techniques such as electroporation or agitation with glass beads (Grossman 2000) or applying new techniques such as CRISPR/CAS9, amiRNA and 2A peptides (Rasala *et al.* 2012; Greiner *et al.* 2017; Wang *et al.* 2017). Even more, chloroplast transformation can be achieved taking advantage of the active homologous recombination. It is possible to perform site-directed mutagenesis by particle bombardment, thus generating k.o. mutants (Dent *et al.* 2001). By contrast, nuclear transformation occurs by random insertion of recombinant DNA (as it does in *Arabidopsis*). Nuclear transformation can therefore be used as an insertional mutagenesis method with tagging of the interrupted gene. *Chlamydomonas* is unicellular, a feature precluding interference with cell differentiation and tissue specificity and allowing growth in liquid and solid media under different conditions. Its culture conditions are easily controlled, especially in the case of biotic, abiotic or chemical treatments and the response to treatments is rapid (Rolland *et al.* 2009).

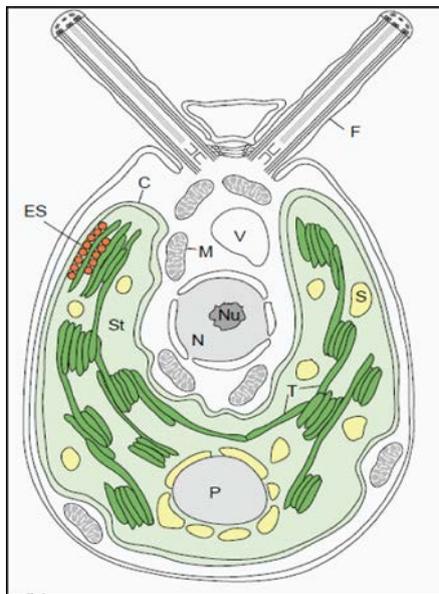


Figure 12: The morphology of *Chlamydomonas reinhardtii*

Schematic representation of *Chlamydomonas* cell, showing nucleus (N), nucleolus (Nu), chloroplast (C), thylakoids (T), starch grains (S), pyrenoid (P), stroma (St), eyespot (Es), flagella (F), vacuoles(V), mitochondria (M). Image acquired from (Dent *et al.* 2001).

## II.5.2 Background

In the last decades, *Chlamydomonas reinhardtii* has emerged as a powerful model for studying a great number of stress conditions such as nutrient limitations, heat shock or light stress (Mendez-Alvarez *et al.* 1999; Grossman 2000; Erickson *et al.* 2015; Schroda *et al.* 2015). This is because it possesses a great metabolic plasticity that allows resisting efficiently to harsh environmental conditions.

In the last 30 years, several studies demonstrated that plants and algae produce NO. A major nitrite-dependent NO production has been reported in *Chlamydomonas*, both through nitrate reductase-dependent (Sakihama *et al.* 2002; de Montaigu *et al.* 2010; Chamizo-Ampudia *et al.* 2016) and independent pathways (Wei *et al.* 2014). In addition, arginine-dependent synthesis has been also found in land plants, even though the components of the pathway remain elusive and no orthologues of animal nitric oxide synthases (NOS) have been identified. This pathway was demonstrated in many studies in which plant extracts were found to produce NO and citrulline from arginine and this activity can be inhibited by classic NOS inhibitors (arginine analogues) (Cueto *et al.* 1996; Ninnemann *et al.* 1996; Barroso *et al.* 1999) Wendehenne *et al.*, 2001). In addition, these inhibitors block some NO-mediated responses such as ABA-induced stomatal closure (Wendehenne *et al.* 2001). The enzyme responsible for this activity has not yet been identified. Nevertheless it is now well-established that in numerous photosynthetic organisms, including *Chlamydomonas*, NO production is enhanced under abiotic stress (Zaffagnini *et al.* 2016).

In *Chlamydomonas*, functional studies have established a role for NO during the hypoxic response, which is important for the cells to adapt to and grow under conditions of O<sub>2</sub> limitation (Hemschemeier *et al.* 2013), in the acclimation of the alga to growth on ammonium (de Montaigu *et al.* 2010), to copper stress (Zhang *et al.* 2008), or in the response to nitrogen starvation (Wei *et al.* 2014). All these data suggest the existence of NO-based signaling pathways in *Chlamydomonas*, even though the molecular mechanisms are mostly unknown. An important step forward in the understanding of NO action in *Chlamydomonas* has been recently made, with the identification of almost 500 nitrosylated proteins under nitrosative stress by the group of S. Lemaire (Morisse *et al.* 2014). Among these targets, all cytochrome b<sub>6</sub>f subunits, Rubisco, FtsH and ClpP (the main thylakoid proteases) have been identified. Cytochrome b<sub>6</sub>f and Rubisco, are targeted for degradation under nitrogen starvation and few indications suggest the same fate under sulfur starvation. Moreover, FtsH and ClpP are required for the turnover of these proteins under nitrogen starvation. Therefore it is reasonable to speculate a link between NO production, protease activity and thylakoid remodeling under sulfur starvation as well. The scope of my work covers the involvement of nitric oxide in the signaling that leads cells to respond to sulfur starvation and to long term light fluctuations. The

major focus of this thesis is on the molecular mechanisms through which NO acts in *Chlamydomonas*.

### **II.5.3 Major Goals**

#### *II.5.3.a The role of nitric oxide in the remodeling of the photosynthetic apparatus under sulfur starvation in *Chlamydomonas reinhardtii**

Despite the numerous studies carried out on sulfur starvation in *Chlamydomonas*, at the moment we are still lacking a detailed biochemical characterization of the phenomenon. Moreover, we have very few information regarding the signal involved in the response to sulfur limitation and the molecular mechanism through which the signal acts is unknown. In this work we carried out an extensive characterization of sulfur starvation in different growth and light conditions. We also wanted to identify the signaling molecule involved in the response, trying to shed light on the molecular mechanism through which the signal is transmitted.

#### *II.5.3.b The role of nitric oxide and nitrosylation in the modulation of antenna size upon light fluctuations in *Chlamydomonas reinhardtii**

In *Chlamydomonas*, a peculiar cytoplasmic protein, NAB1, regulates antenna size at the translational level. NAB1 acts as a cytoplasmic repressor of LHCBM translation by binding specific LHCBM mRNAs and sequestering them in silent ribonucleoprotein complexes (Wobbe *et al.* 2009). The existence of a redox regulatory mechanism, governing the activity of the NAB1 repressor, has been recently demonstrated (Wobbe *et al.* 2009). In this work we wanted to find out which kind of redox modification occurs on the regulatory cysteine, and how it is regulated *in vivo* and *in vitro*.

### **III Materials and Methods**

Here are provided all steps and chemical information required for performing the experiments described in this manuscript, in addition to materials and methods sections of published papers or submitted manuscripts.

#### **III.1 Cell cultures and stress treatments**

##### **III.1.1 Chlamydomonas strains and cultures conditions**

*Chlamydomonas reinhardtii* S24 (*nit1<sup>-</sup>/nit2<sup>-</sup>*, mt<sup>-</sup>) CC5100 strain (Gallaher *et al.* 2015), exhibiting normal photosynthetic properties, was used for the characterization of sulfur starvation as well as for the drug treatments, microscopy observations and proteomic analyses. Knock-out mutants for FtsH1 (Malnoe *et al.* 2014) and ClpP (Majeran *et al.* 2000) proteases were used for complementary experiments.

To characterize the regulation of NAB1 we have used a complemented (WT-like) line constructed transforming *nab1* K.O. (*stm3*) (Mussnug *et al.* 2005) with a WT-NAB1 isoform under the control of a constitutively active *PsaD* promoter (Wobbe *et al.* 2009). Strains complemented with NAB1 cysteine mutants C181S and C226S were also used.

##### **III.1.2 Culture Media**

Pre-cultures were grown in mixotrophic Tris acetate phosphate medium (TAP) (pH 7.2) (Rochaix *et al.* 1988; Harris 1989) at 25°C, either on 1.6% agar plates or in liquid cultures kept continuously under agitation using an Infors CH-4103 agitator. For sulfur starvation two different media were used: TAP-S, mixotrophic TAP depleted in sulfate; Minimal Medium-S, autotrophic (without acetate) medium depleted in sulfate.

##### **III.1.3 Culture preparation for stresses**

All strains were grown on a shaker at 140 rpm, under continuous light, at 25°C, before being diluted in fresh liquid TAP medium at a cell density between 0.5 and 1.10<sup>6</sup> cells/ml. After overnight growth the cell density reached was 3.10<sup>6</sup> to 5.10<sup>6</sup> cells/ml. Final cultures were then used to carry out the experiments. Cell density was determined using Malassez hemocytometer or a Beckman cell counter.

### III.1.4 Sulfur starvation

For sulfur starvation experiments, TAP-pregrown cells (mid log phase) were centrifuged at 3000g for 5 min at room temperature, washed once in sulfur-free medium, resuspended at  $2 \cdot 10^6$  cells/mL in sulfur-free medium provided or not with acetate (respectively named TAP-S and Minimum Medium-S). Cultures were kept on a rotary shaker with vigorous aeration (210 rpm) for 72 hours at least, under 5 to 15  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ , for low light, 120 to 150  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  for high light, or in darkness.

### III.1.5 NO-donor /scavenger or NO-synthesis inhibitors

WT-S24 cells (150 mL), at a concentration of  $2 \times 10^6$  cells  $\text{mL}^{-1}$ , were transferred in Sulfur-free medium (TAP-S) for 24 h in a 500 mL Erlenmeyer flask. Later, equal volumes of the initial culture were divided in different 250 mL Erlenmeyer flasks, one flask for each incubation, namely control; Carboxy-PTIO potassium salt (cPTIO); sodium nitroprusside (SNP), S-Nitroso-N-acetyl-DL-penicillamine (SNAP), SNP + cPTIO; SNAP + cPTIO. Drugs were added immediately after the subdivisions, to reach the following concentrations: cPTIO 0.2 mM; SNP 0.5 mM; SNAP 500  $\mu\text{M}$ ; same concentration when drugs were added together. Every 2 hours samples were taken and dark-adapted under strong agitation for 30 min in open Erlenmeyer flasks. Fluorescence was recorded as described below and photosynthetic parameters were calculated (see Fluorescence measurements).

Treatments with NO-synthesis inhibitors were performed on WT-S24 cells (150 mL), at a concentration of  $2 \times 10^6$  cells  $\text{mL}^{-1}$ . Cells were transferred in Sulfur-free medium (TAP-S) for 24 h in a 500 mL Erlenmeyer flask. Later, equal volumes of the initial culture were divided in different 250 mL Erlenmeyer flasks, one flask for each incubation, namely control; L-N<sup>G</sup>-Nitroarginine methyl ester (L-NAME); Amino guanidine (AG); DL- $\alpha$ -Difluoromethylornithine (DFMO); tungstate. Drugs were added immediately after the subdivisions (T24) and after one day (T48), to reach the final concentration of 2 mM for each compound. Fluorescence was recorded as described below and photosynthetic parameters were calculated.

### III.1.6 Nitrosative treatments on recombinant proteins

Nitrosative treatment was performed by addition of 2 mM nitrosoglutathione (GSNO), S-Nitroso-N-acetyl-DL-penicillamine (SNAP), Diethylamine NONOate

diethylammonium salt (DEANONOate) for 20 min in a Thermomixer comfort (Eppendorf AG, Hamburg, Germany) using *interval* mixing mode (every 2 min, the plate was shaken for 2 min and 30 s at 1100 rpm). GSNO concentration in the stock solution was determined spectrophotometrically using its molar extinction coefficient ( $\epsilon_M=920 \text{ M}^{-1}.\text{cm}^{-1}$  at 335 nm). After nitrosative treatment, proteins were precipitated with two volumes of 100% cold acetone for at least 20 min, pelleted by centrifugation at 15000 rpm for 10 min. The pellet was resuspended in a specific buffer depending on the experiment as detailed below (see section BST).

## **III.2 Methods related to proteins**

### **III.2.1 Protein preparation, separation and immunoblot analysis**

Protein extraction and immunoblot analyses were performed as described in (Kuras *et al.* 1994). Cell extracts were loaded on an equal chlorophyll basis (3.5/4  $\mu\text{g}$  per lane) and, for each experiment shown; at least three biological replicates were performed. Protein detection was performed with ECL in a Chemidoc<sup>TM</sup> XRS+ System scan for membranes (BioRad®), using ImageLab (v.3.0) software. Primary antibodies were diluted as described in Wei *et al.*, 2014 and Malnoe *et al.*, 2014. All the antibodies were revealed by a horseradish peroxidase-conjugated antibody against rabbit IgG (Promega®). Antibodies against D1, D2, PsaA, SLT2, LHCII were purchased from Agrisera®. Rubisco antibodies were kindly provided by the group of S. Whitney, Australia. Other antibodies were described previously: Cytochrome *b<sub>6</sub>f* subunits (Kuras *et al.* 1994),  $\beta$  subunit of ATP-synthase (Drapier *et al.* 1992), PTOX2 (Houille-Vernes *et al.* 2011), NDA2 (Desplats *et al.* 2009), ClpP and FtsH1 (Malnoe *et al.* 2014; Wei *et al.* 2014)

### **III.2.2 Bicinchoninic acid (BCA) method**

The BCA method is based on cupric ion ( $\text{Cu}^{2+}$ ) reduction by peptide bond and specific amino acids such as cysteine, tyrosine and tryptophan at alkaline pH. The cuprous ion ( $\text{Cu}^{1+}$ ) generated is forming a purple colored complex with two molecules of BCA. The complex  $\text{Cu}^{1+}$ -BCA can be detected spectrophotometrically at 562 nm, allowing the determination of protein concentration after comparison with a standard

curve obtained in the presence of increasing concentrations of bovine serum albumin (BSA).

### III.2.3 Identification and analysis of nitrosylated proteins in whole extracts

The S-nitrosylation state was analyzed by the Biotin Switch Technique (BST, see below), after treatment (15 min) with 1 mM of GSNO, DEANONOate or SNAP, in the case of recombinant proteins, or at T0 and T30 after the onset of S-starvation when analyzing cell extracts.

#### III.2.3.a Biotin Switch Technique (BST)

The BST (Fig. 13) is a multistep approach for the identification of nitrosylated proteins in a complex extract, or for the detection of nitrosylated residues on purified proteins. Here a modified protocol, developed for the analysis of membrane protein and soluble proteins in parallel will be described.

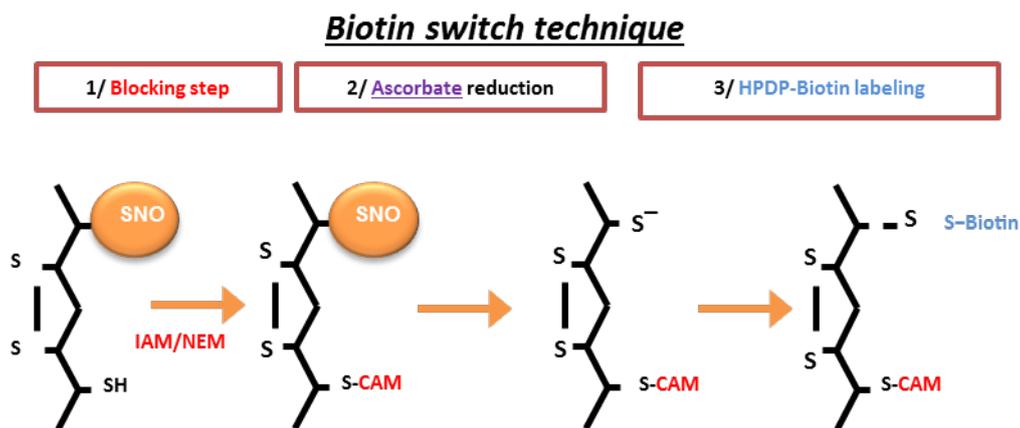


Figure 13: schematic representation of the biotin switch technique. For simplicity reduction and labeling are separated even though they are performed together in the same reaction tube.

#### III.2.3.b Workflow

1. Protein extraction from *Chlamydomonas* cells
2. Irreversible blocking of free thiols: ALKYLATION I in native conditions; ALKYLATION II under denaturing conditions.
3. 2x acetone precipitation.
4. Selective reduction of -SNO to thiols via chemical ascorbate reduction.
5. Biotin-labeling of the nascent thiols.

6. Acetone precipitation.
7. Detection of the biotin-labeled proteins (SDS-PAGE, immunoblot).
8. Purification of biotin-tagged proteins/peptides by affinity column (with streptavidin beads).
9. Analysis and identification by MS.

#### *III.2.3.c Buffers and reagents preparation*

The buffer used for the entire experiment (TEN) is composed of 50 mM Tris-HCl, pH 7.9, 1 mM EDTA, 100 mM NaCl and can be stored for long time at 4°C. From this master buffer, the single step buffers must be prepared.

The Lysis/Alkylation I step is performed in TEN, 20 mM IAM, 20 mM NEM, Protease inhibitor (SigmaFast EDTA Free; Roche, Germany).

The Alkylation II is performed in TEN, 1% SDS (2% for membrane protein fraction) (TENS).

The Reduction/labeling step is performed in TENS + 40 mM ascorbate and 0.2 mM HPDP-Biotin (Pierce). **Reagents must be freshly prepared and stored for no longer than 24h** on ice and in darkness. A stock solution of 200 mM IAM and 200 mM NEM is prepared in distilled sterile H<sub>2</sub>O. Warm and shake the thiol-blocking solutions for several minutes at 25°C until complete dissolution. A stock solution of 1 M sodium ascorbate is prepared in distilled sterile H<sub>2</sub>O and 20 mM HPDP-Biotin is prepared in DMSO.

To prevent sample loss during the acetone precipitation steps, the experiments should be performed with tubes that are made of polypropylene rather than polycarbonate or other acetone-soluble polymers.

#### *III.2.3.d Cell preparation, protein extraction and alkylation I*

Cell cultures are performed as described in section (II.1.3/II.1.4). Cells are then broken by three freeze-thaw cycles in ice-cold Lysis/Alkylation I buffer, to allow free thiols blocking during the lysis procedure in native conditions (see below for details).

It is critical to **AVOID REDUCTANTS (e.g. DTTred, 2-mercaptoethanol** or other reducing compounds) in the lysis buffer. To minimize photo-decomposition of nitrosothiols (SNO), **cell extraction has to be performed on ice** and **PREVENTED FROM DIRECT ILLUMINATION** until completion of the biotin labeling.

After protein extraction, samples are homogenized in a cold room with the help of a potter and ultracentrifuged at 80.000 rpm for 20 min at 4°C. Then, the supernatant is collected in a new tube and 1% SDS is added.

The pellet is washed twice in ice-cold Lysis/Alkylation I buffer and 2% SDS is added to help the resuspension, in a cold room with the help of a potter.

#### *III.2.3.e Irreversible free thiol blocking*

Some protein thiols cannot be blocked either by increasing the concentration of the alkylating agents (*i.e.* NEM or IAM) or by increasing the duration of the incubation.

Once again **to maximize the efficiency of the thiol blocking**, the alkylation is performed in two steps: the first in native conditions, directly with the lysis step (described above), the second in the presence of 1-2% SDS.

- The second alkylation is performed in the presence of SDS, into a 2 ml capacity polypropylene tube, at 25°C in the dark for 1 h, with frequent vortexing in a Thermomixer comfort (Eppendorf AG, Hamburg, Germany) using *interval* mixing mode (every 2 min, the plate was shaken for 2 min and 30 sec at 1100 rpm).
- Proteins are precipitated by addition of two volumes of 100% cold acetone. After incubation for at least 20 min at -20°C, samples are centrifuged for 10 min at 14000 rpm at 4°C.
- The clear supernatant is discarded and the protein pellet is resuspended in TENS buffer before the addition of two more volumes of 100 % cold acetone for a second precipitation that ensures the complete removal of NEM and IAM.
- Samples are resuspended in TENS buffer and protein concentration is assessed with BCA assay. Final concentration must be adjusted to max 1.5 mg/ml before the next step (keep samples in darkness and on ice).

#### *III.2.3.f Specific detection of S-nitrosylated thiols*

- Alkylated protein samples (prepared at max 1.5 mg/ml) are incubated in TENS + 40 mM sodium ascorbate + 0.2 mM HPDP-Biotin (Pierce) for 30 min at 25°C in a Thermomixer Comfort using *interval* mixing mode (every 2min, the plate was shaken for 2 min 30 sec at 1100 RPM) and prevented from

light.

- Proteins are precipitated by addition of two volumes of 100% cold acetone. After incubation for at least 20 min at -20°C, samples are centrifuged for 10 min at 14000 rpm at 4°C.
- The clear supernatant is discarded and the protein pellet is resuspended in TENS buffer.

An ascorbate-free control is necessary to verify the ascorbate dependency of the biotin labeling. A biotinylation control can be performed by omitting HPDP-Biotin to confirm the specificity of the anti-biotin antibody.

#### *III.2.3.g Detection of biotin-labeled proteins by immunoblot*

- Separate the biotinylated proteins by SDS-PAGE gel containing 12% of acrylamide under non-reducing conditions (Laemmli 1970)(boiling the sample for 1 min at 95°C prior to SDS-PAGE gel loading).
- Transfer onto 0.2 µm nitrocellulose membrane (Towbin *et al.* 1979) by liquid transfer system according to manufacturer's instructions (Bio-Rad, Mini PROTEAN® Tetra Gel, ref: 2895)
- Detect the biotin-labeled proteins by chemiluminescence, using mouse monoclonal anti-biotin antibody (Sigma-Aldrich; dilution 1:10000) as primary antibody, and monoclonal peroxidase-conjugated anti-mouse antibody (Sigma-Aldrich dilution 1:10000)

### **III.3 Methods for fluorescence measurements and microscopy**

#### **III.3.1 Fluorescence Measurements**

Liquid cultures were dark-adapted under strong agitation for 30 min in open Erlenmeyer flasks and fluorescence was then recorded using a home-built fluorimeter. Measurements were carried out for a time-span of 2.5 s, with a final saturating pulse of actinic light. The following parameters were recorded:  $F_0$ , fluorescence yield of dark adapted cells;  $F_m$ , the fluorescence yield of the same cells after a saturating actinic pulse;  $F_s$ , the fluorescence yield of cells in the actinic light

( $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) before the final pulse. Quantum yield and  $F_V/F_M$  were calculated as follows:  $\Phi_{\text{PSII}} = (F_M - F_s)/F_M$ ;  $F_V/F_M = (F_M - F_0)/F_M$ .

### III.3.2 Fluorescence Microscopy

Cells, starved for sulfur for the indicated times, were incubated 1 hour in the presence of DAF-FM DA  $5 \mu\text{M}$ , washed and concentrated 10 times by centrifugation at 3000 rpm, 5 min,  $4^\circ\text{C}$ , in sulfur-depleted medium and imaged rapidly at room temperature with a Zeiss® Axio Observer Z1 microscope equipped with a PA 63x/1.4 oil objective. Excitation was performed simultaneously for Chlorophyll and DAF-FM DA using a blue led coupled with a cut-off filter at 470 nm. Emission was recorded separately for DAF-FM DA (595 nm) and Chlorophyll (650 nm), in order to separate the signals arising from NO sensor and endogenous chlorophyll. Images were obtained either with single pictures or with 2 x 2 tile scanning. Images were collected and treated with the ZEN 2011 (Zeiss®) software.

## IV Results

### IV.1 The role of Nitric Oxide in the remodeling of the photosynthetic apparatus under Sulfur starvation in *Chlamydomonas reinhardtii*.

The response to nutrient limitation in *Chlamydomonas reinhardtii* leads to a well-regulated inactivation of photosynthesis. This inactivation is crucial for the metabolic switches described in the literature and seems to be crucial for the exploitation of this microalga as a source of green and alternative energy. The work presented in ARTICLE 2 focuses on the study of sulfur starvation effects at the level of the photosynthetic apparatus. In the first part we characterize the phenomenon, then we identify the signal involved in this process and at the end we design an approach to gain information about the molecular mechanisms. All the experiments shown have been performed by me, using the same set up developed for the study of nitrogen starvation in our team (Wei *et al.*, 2014). The aim of the work is the comparison between nitrogen and sulfur starvation responses with a particular interest for the role of nitric oxide.

The manuscript entitled “The role of nitric oxide in the remodeling of the photosynthetic apparatus under sulfur starvation in *Chlamydomonas reinhardtii*” by *Marcello De Mia, Stéphane D. Lemaire, Yves Choquet and Francis-André Wollman* is presented in its final form ready for submission.

**Nitric oxide remodels the photosynthetic apparatus under sulfur starvation in  
*Chlamydomonas reinhardtii***

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## ABSTRACT

Here we report a detailed characterization of sulfur starvation under heterotrophy and photo-autotrophy in *Chlamydomonas reinhardtii*. Photosynthetic inactivation under low light and darkness is achieved through specific degradation of RuBisCO and cytochrome  $b_6f$  and occurs only in the presence of reduced carbon in the medium. The process may be regulated by nitric oxide, which has been shown to be produced, after 24 hours from the onset of starvation, with NO-sensitive fluorescence probes visualized by confocal microscopy. We provide pharmacological evidence that intracellular NO production governs this degradation pathway: addition of a NO scavenger or NO synthesis inhibitors, as well as NO donors decrease and increase, respectively, the rate of cytochrome  $b_6f$  degradation. Taken together, these data suggest an active role for NO in the remodeling of thylakoid complexes upon sulfur starvation.

## **INTRODUCTION**

In the wild, growth of photosynthetic autotrophs is often limited by nutrient availability. Macronutrients as Nitrogen, Sulfur, Phosphorus and Carbon, the building bricks for all biomolecules, are in suboptimal concentration in many ecosystems. Organisms have evolved a variety of responses to adjust their metabolism to nutrient limitation, resulting in their acclimation to the new environmental conditions. These responses can be divided into general or nutrient-specific responses (reviewed in (Grossman 2000; Merchant *et al.* 2012; Obata *et al.* 2012). They encompass cell division arrest, gene expression changes, down regulation of anabolic pathways, increase uptake from alternative sources and activation of scavenging processes. In several instances, proteins rich in the lacking element are replaced by alternative isoforms with a more appropriate amino acid composition.

In the green alga *Chlamydomonas reinhardtii*, the available genetic tools proved powerful enough (Grossman *et al.* 2007) to study in details ubiquitous stress responses as well as specific responses to nutrient limitations which consistently encompass expression of scavenging enzymes for the missing nutrient and selective degradation processes (Merchant *et al.* 2012). For instance, when facing phosphorus starvation, cells attempt to recover phosphate by degrading a fraction of the polyploid chloroplast chromosome and secreting phosphatases that scavenge phosphate from the surrounding environment (Quisel *et al.* 1996; Wykoff *et al.* 1998; Yehudai-Resheff *et al.* 2007). Phosphorus starvation also leads to an increased susceptibility to photoinhibition, which targets primarily PSII (Malnoe *et al.* 2014). Under nitrogen starvation, *C. reinhardtii* also degrades a fraction of its polyploid chloroplast chromosome (Sears *et al.* 1980) and expresses an L-amino acid oxidase (LAO1) as a scavenging enzyme in the periplasm (Vallon *et al.* 1993). In addition, cells undergo a transition from a vegetative to a gamete state together with a replacement of their ribosomes by other isoforms which are more error-prone (Picard-Bennoun *et al.* 1985). Other changes upon nitrogen starvation encompass storage of reduced carbon as starch and triacylglycerol lipids (TAGs) (Yang *et al.* 2015), and a block of photosynthesis due to a specific degradation of cytochrome *b<sub>6</sub>f* and RuBisCO (Wei *et al.* 2014).

When starved for sulfur, *C. reinhardtii* synthesizes an extracellular arylsulfatase (ARS) as a scavenging enzyme (Schreiner *et al.* 1975). It accumulates

TAGs, degrades sulfoquinovosyl diacylglycerol (SQDG) (Sugimoto *et al.* 2007; Yang *et al.* 2015) and down regulates photosynthesis, a condition that can lead, under specific growth conditions, to hydrogen evolution (Wykoff *et al.* 1998; Ghirardi *et al.* 2000; Melis *et al.* 2000; Zhang *et al.* 2002; Antal *et al.* 2003; Forestier *et al.* 2003; Kosourov *et al.* 2003; Hemschemeier *et al.* 2008; Grossman *et al.* 2011). However, there are conflicting reports as to the site at which photosynthesis is actually inhibited upon sulfur starvation: it has been mainly attributed to a PSII photodestruction (Wykoff, 1998 #13277) but Malnoe *et al.* (2014) reported that this photoinhibitory effect was indirect, being the result of a downstream block in photosynthetic electron transfer at the level of cyt.  $b_6f$  complexes. The selective degradation of cyt.  $b_6f$  complex in absence of sulfur was thus reminiscent of a similar process previously observed in absence of nitrogen sources (Bulte *et al.* 1992). These two macronutrients contribute to widely different metabolic pathways that nevertheless overlap in some instances like the biosynthesis of sulfur amino-acids or glutathione. Sulfur is critical for the metabolism of amino acids, lipids, glutathione and other metabolites. In *C. reinhardtii*, its assimilation is regulated by the transmembrane polypeptide SAC1 (for sulfur acclimation 1), a transporter-like protein induced upon sulfur limitation, and by the kinase SAC3, activated through phosphorylation (Zhang *et al.* 2004; Pollock *et al.* 2005). The sulfate anion ( $\text{SO}_4^{-2}$ ) is the preferred source of S and is imported by a number of transporters, some of which, as SLT2, a sodium/sulfate co-transporter, are only expressed upon sulfur shortage. Once inside, it must be activated by the enzyme ATP sulfurylase in order to be assimilated. The activated form of  $\text{SO}_4^{-2}$ , Adenosine phosphosulfate (APS), can serve as a substrate for  $\text{SO}_4^{-2}$  reduction or can be further phosphorylated by APS kinase to give 3'-phosphoadenosine phosphosulfate (PAPS), used by sulfotransferases to catalyze the sulfation of various cellular substrates (Pollock *et al.* 2005). In the pathway leading to  $\text{SO}_4^{-2}$  reduction, the S of APS is reduced to  $\text{SO}_2^{-2}$ , which is further reduced to sulfide by sulfite reductase in the chloroplast, with final incorporation into cysteine and methionine (Leustek *et al.* 1999).

Given the similarities shared by nitrogen and sulfur starvation responses, it is tempting to imagine that analogous signaling pathways are at work. However, we need to get a better understanding of the mechanisms leading to photosynthesis inactivation, notably the identification of the signaling molecule(s) and of the metabolites from which they are produced.

Here, we characterized extensively the effects of sulfur starvation on the photosynthetic apparatus of *C. reinhardtii*, under the same growth (heterotrophy/ photoautotrophy) and light conditions, that we used previously to study nitrogen starvation (Wei *et al.* 2014). By comparing the responses to the two starvation using the same approaches we aimed to gain information about the signal involved in the remodeling of the photosynthetic apparatus. Indeed we provide overwhelming evidence that NO plays a key role in the photosynthetic response to sulfur starvation, most likely originating from a mobilization of the glutathione pool for delivering sulfur.

## **MATERIALS AND METHODS**

### **Strains, Media, Culture conditions, Chemicals**

*C. reinhardtii* wild-type S24 (CC-5100, (Gallaher *et al.* 2015), *ftsH1-1* (Malnoe *et al.* 2014) and *ClpP<sub>ATT</sub>* (Majeran *et al.* 2000) mutants were grown on a shaker at 140 rpm, under continuous light (5 to 15  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for low light; 120  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for high light), or in darkness, at 25°C. Pre-cultures were grown in TAP (Tris-acetate\_phosphate) medium (pH 7.2) (Harris 1989). For sulfur starvation experiments, cells were pregrown in TAP to reach a concentration of  $2 \times 10^6$  cells.mL<sup>-1</sup> (mid log phase). Cells were then centrifuged at 3000g for 5 min at room temperature, washed once in sulfur-free medium, resuspended at  $2 \times 10^6$  cells.mL<sup>-1</sup> in sulfur-free medium provided (TAP) or not (Minimum Medium) with acetate (respectively named TAP-S and MM-S), and kept on a rotary shaker with vigorous aeration (210 rpm). Carboxy-PTIO potassium salt (cPTIO); sodium nitroprusside (SNP), S-Nitroso-N-acetyl-DL-penicillamine (SNAP), Diethylamine NONOate diethylammonium salt (DEANONOate), 4-amino-5-methylamino-2',7'-difluoro-fluorescein diacetate (DAF-FM DA), DL- $\alpha$ -Difluoromethylornithine hydrochloride (DFMO), N $\omega$ -Nitro-L-arginine methyl ester (L-NAME), Amino guanidine (AG) and Tungstate were all purchased from Sigma-Aldrich®. Fresh solutions were prepared before each experiment and were kept on ice and in darkness for no longer than 6 hours.

### **Protein Preparation, separation and analysis**

Protein extraction and immunoblot analyses were performed as described in (Kuras *et al.* 1994). Cell extracts were loaded on an equal chlorophyll basis (3.5  $\mu\text{g}$  per lane) and, for each experiment shown, at least three biological replicates were performed. Protein detection was performed with ECL in a Chemidoc™ XRS+ System scan for membranes (BioRad®), using ImageLab (v.3.0) software. Primary antibodies were diluted as in (Wei *et al.*, 2014) and (Malnoe *et al.*, 2014). All the antibodies were revealed by a horseradish peroxidase-conjugated antibody against rabbit IgG (Promega®). Antibodies against D1, D2, PsaA, SLT2, and LHCII were purchased from Agrisera®. RuBisCO antibodies were kindly provided by the group of S. Whitney, Australia. Other antibodies were described previously: Cyt. *b<sub>6</sub>f* subunits

(Kuras *et al.* 1994),  $\beta$  subunit of ATP-synthase (Drapier *et al.* 1992), PTOX2 (Houille-Vernes *et al.* 2011), NDA2 (Desplats *et al.* 2009).

## Fluorescence Measurements

Liquid cultures were dark-adapted under strong agitation for 30 min in open Erlenmeyer flasks and fluorescence was then recorded using a home-built fluorimeter. Measurements were carried out for a time-span of 2.5 s, with a final pulse of saturating actinic light. The following parameters were recorded:  $F_0$ , fluorescence yield of dark adapted cells;  $F_M$ , the fluorescence yield of the same cells after the saturating actinic pulse;  $F_S$ , the steady state fluorescence yield reached under a continuous illumination ( $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ), recorded before the saturating light pulse. Quantum yield and  $F_V/F_M$  were calculated as follows:  $\Phi_{\text{PSII}} = (F_M - F_S)/F_M$ ;  $F_V/F_M = (F_M - F_0)/F_M$ .

## Fluorescence Microscopy

Aliquots (15 mL) of cultures starved for the indicated times, were incubated 1 h in the presence of DAF-FM DA  $5 \mu\text{M}$ , washed and concentrated 10 times by centrifugation at  $3000g$ , 5 min,  $4^\circ \text{C}$ , in sulfur-depleted medium and imaged rapidly at room temperature with a Zeiss® Axio Observer Z1 microscope equipped with a PA 63x/1.4 oil objective. Excitation was performed simultaneously for Chlorophyll and DAF-FM DA using a blue led coupled with a cut-off filter at 470 nm. Emission was recorded separately for DAF-FM DA (595 nm) and chlorophyll (650 nm), in order to separate the signals arising from the NO sensor or from endogenous chlorophyll. Images were obtained either with single pictures or with  $2 \times 2$  tile scanning. Images were collected and treated with the ZEN 2011 (Zeiss®) software. The specificity of DAF-FM for NO was tested using DEANONOate and cPTIO.

## NO donors and NO-scavengers

WT-S24 cells (150 mL), at a concentration of  $2 \times 10^6 \text{ cells}\cdot\text{mL}^{-1}$ , were transferred in Sulfur-free medium (TAP-S) for 24 h in a 500 mL Erlenmeyer flask. Equal volumes of the original culture were divided in 250 mL Erlenmeyer flasks, one flask for each incubation (namely control; cPTIO; SNP; SNAP; SNP + cPTIO; SNAP + cPTIO). Drugs were added immediately after sampling (T24) and after 2 hours incubation (T26), to reach the following concentrations: cPTIO 0.2 mM; SNP 0.5 mM; SNAP 0.5

mM; the same concentrations were used when different were added simultaneously in mixtures. Samples were taken every 2 hours, then dark-adapted under strong agitation for 30 min in open Erlenmeyer flasks. Photosynthetic parameters were calculated from the fluorescence records as described above (see Fluorescence measurements).

### **NO-synthesis inhibitors**

Treatment of WT-S24 cells starved in sulfur, was as above. A series of 250 mL Erlenmeyer flasks, encompassed a control; L-N<sup>G</sup>-Nitroarginine methyl ester (L-NAME); Amino guanidine (AG); DL- $\alpha$ -Difluoromethylornithine (DFMO); tungstate. Drugs were added immediately after sampling (T24) and after one day (T48), to reach a final concentration of 2 mM for each compound. Fluorescence parameters were recorded as described above.

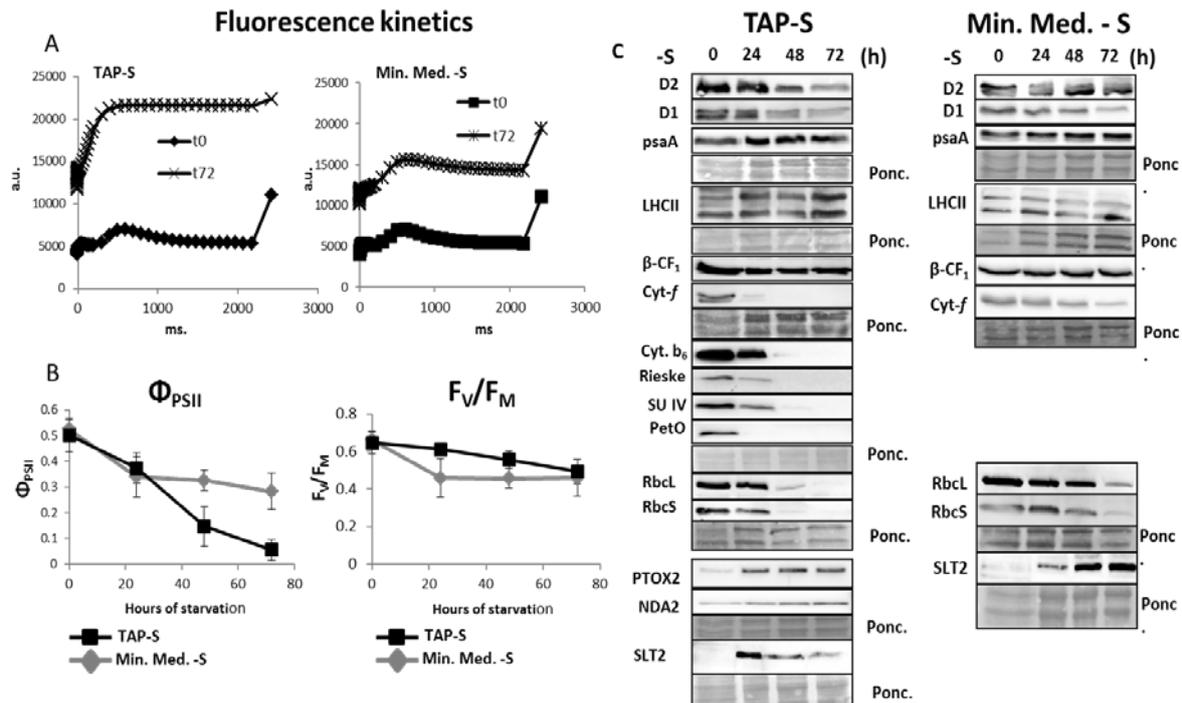
## RESULTS

### **Sulfur starvation induces inactivation of photosynthesis through specific degradation of cytochrome *b<sub>6</sub>f* and RuBisCO.**

Fig1 shows the major photosynthetic changes undergone by the photosynthetic apparatus of WT-S24, a *C. reinhardtii* strain which is wild type for photosynthesis, when starved of sulfur for 72 hours under low light ( $5\text{-}10\ \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ ) in heterotrophic conditions. A dramatic decrease in photosynthetic efficiency ( $\Phi\text{PSII}$ ) is observed (Fig 1A, black line) but this inactivation does not occur at the level of PSII, as demonstrated by the limited change in  $F_V/F_M$  (Fig 1B, black line). Consistent with the fluorescence data, PSII accumulation, represented by its core proteins D1 and D2, does not significantly decrease in these experimental conditions (Fig 1C, left panels). By contrast, cyt. *b<sub>6</sub>f* and RuBisCO appeared as the main targets of photosynthesis inactivation, as their immunodetection vanished after 24 to 48 hours of sulfur starvation. The loss of cyt. *b<sub>6</sub>f* is consistent with the drop in  $\Phi\text{PSII}$  recorded in our fluorescence analysis. The immunoblot analysis also revealed an increased accumulation of the two major chlororespiratory enzymes NDA2 and PTOX2, as previously reported for cells starved of nitrogen (Wei et al. 2014).

When starved for sulfur in minimal medium (Min. Med.-S), where survival strictly depends on photosynthesis, WT-S24 showed a more limited loss in photosynthesis, as demonstrated by the less pronounced decrease in  $\Phi\text{PSII}$  over 72 hours of starvation (Fig 1A). Analysis of the content in photosynthetic proteins revealed that both cyt. *b<sub>6</sub>f* and RuBisCO were retained much longer even though sulfur starvation is still sensed by the cells, as demonstrated by the induction of the SLT2 transporter (Fig 1C).

The above results show a striking similarity with those obtained during nitrogen starvation (Wei et al. 2014), where a similar remodeling of the photosynthetic apparatus was observed. To further pursue the comparison between sulfur and nitrogen starvation, we analyzed the impact of sulfur starvation in darkness and at higher light intensity, to determine whether the remodeling of thylakoid proteins is nonspecific, *i.e.* induced by light and linked to photo-damage events, or is a specific and tightly regulated process independent from light.



**Fig. 1: Effects of sulfur deprivation on the photosynthetic apparatus of *Chlamydomonas* kept in low light  $15\mu\text{E m}^{-2} \text{s}^{-1}$  in presence or absence of acetate.**

**(A)** Kinetics of fluorescence induction of dark adapted (30 min) WT-S24 cells in TAP-S and Min. Med. -S, recorded at  $t=0\text{h}$  and  $t=72\text{h}$  a.u.= arbitrary units.

**(B) LEFT:**  $\Phi_{PSII}$  (quantum yield) calculated as  $(F_M - F_S)/F_M$ . This parameter shows the efficiency of the entire Electron transport chain. **RIGHT**  $F_V/F_M$  calculated as  $(F_M - F_0)/F_M$ . This parameter gives information about the PSII efficiency. Black/squared line: heterotrophic medium (with acetate); gray/circled line: photo-autotrophic medium (without acetate). Mean of seven independent experiments  $\pm$  SD.

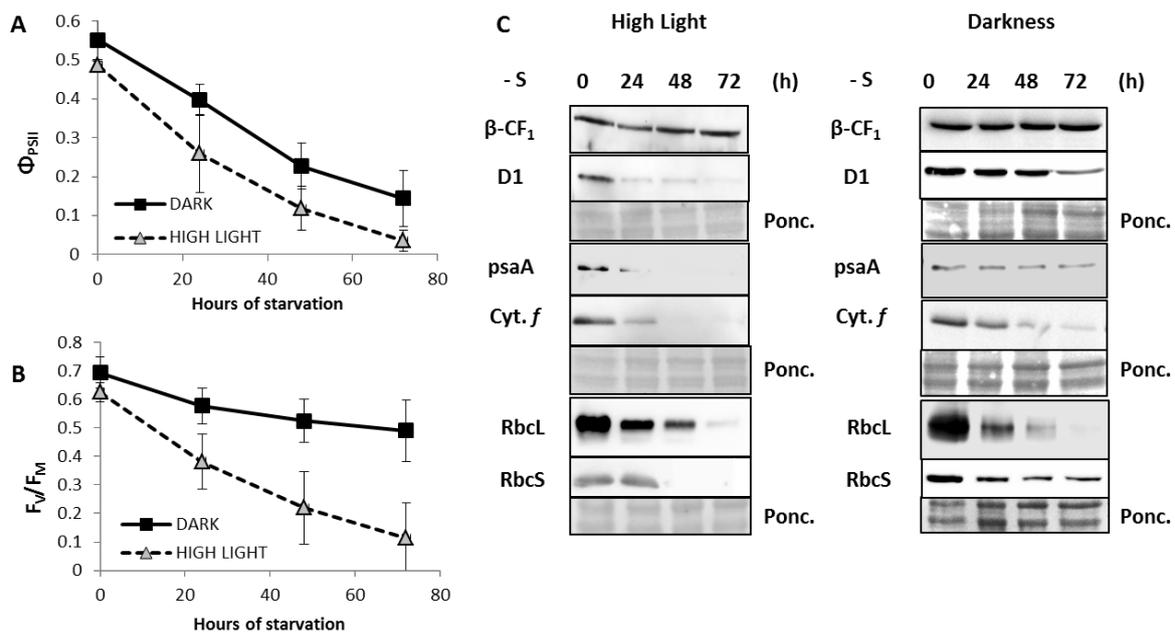
**(C)** Whole protein extracts of cells harvested at indicated time points after onset of sulfur starvation, probed with the indicated antibodies. To detect all proteins the samples were loaded at least three times on independent gels. Left panel shows protein levels of cells starved in heterotrophic conditions, right panel shows Min protein levels of cells starved in photo- autotrophic conditions.

### The degradation of cytochrome $b_6f$ and RuBisCO is light independent.

Cells were starved in sulfur in heterotrophic medium (TAP-S), either in darkness or at  $120\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , an intensity often used to trigger sulfur starvation-induced hydrogen production. Samples were analyzed as above for fluorescence kinetics and by immunoblots (Fig. 2). In darkness, WT-S24 exhibited responses similar to those observed under low light (Fig. 1):  $\Phi_{PSII}$  decreased dramatically without important changes of  $F_V/F_M$ . Protein immunoblotting confirmed the specific degradation of cyt.  $b_6f$  and RuBisCO (Fig. 2C).

We note that the behavior of WT-S24 differs from that of another wild type, WT-T222, used in a previous report. WT-T222 underwent PSII inactivation upon S-starvation in complete darkness (Malnoe et al, 2014). Indeed, we observed allelic variations between *Chlamydomonas* strains that display wild type photosynthesis in S-replete conditions: a cross between WT-S24 and WT-T222 illustrates the genetic determination of the “sulfur-sensitivity of PSII in the dark” (Supplemental Fig. S1)

By contrast, when cells were starved at  $120 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ , we still observed an inhibition of photosynthesis, but it was now mainly due to PSII inactivation, as indicated by the decrease in  $F_V/F_M$  values (Fig 2B). Analysis of protein extracts revealed that besides *cyt. b<sub>6</sub>f* and RuBisCO, PSI and PSII proteins were degraded at  $120 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  (Fig. 2C). Thus, upon sulfur starvation, high light induces the degradation of several photosynthetic proteins, as reported in many previous studies, (Wykoff *et al.* 1998; Antal *et al.* 2003), while under low light or darkness, a light-independent mechanism targets *cyt. b<sub>6</sub>f* and RuBisCO and leads to thylakoid remodeling in heterotrophic conditions.



**Fig. 2: effects of sulfur deprivation in darkness and under high light.**

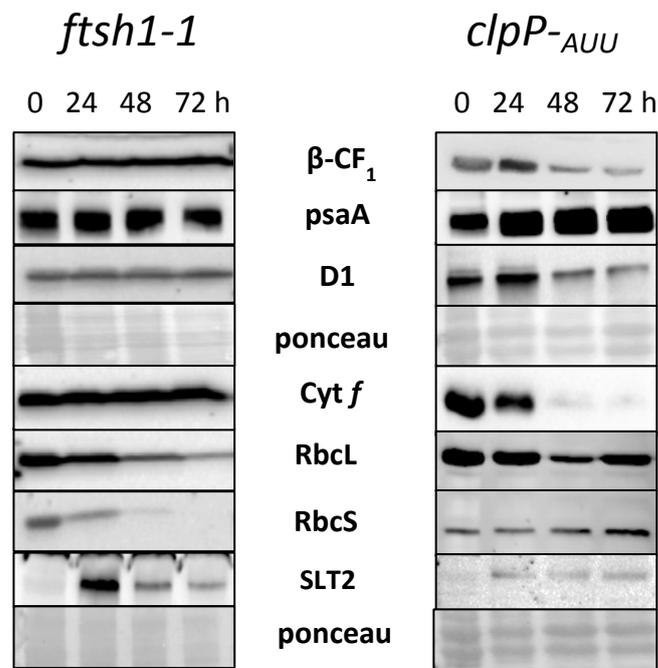
Evolution of  $\Phi_{\text{PSII}}$  (A) and  $F_V/F_M$  (B) during sulfur starvation of strain of WT-S24. Black/squares line: complete darkness; triangles/dashed line: high light  $120 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ . Data represent the mean of 3 experiments with SD. All starvations were performed in Heterotrophic medium (TAP-S).

(C) Whole protein extracts from high light (left panel) and darkness (right panel) samples, harvested at the indicated time points and probed with the indicated antibodies.

## **Specific degradation of cytochrome *b<sub>6</sub>f* and Rubisco are controlled by chloroplast proteases.**

To identify the mechanism of this selective protein loss, we investigated the role of the two major proteases localized in the chloroplast and involved in abiotic stress responses, ClpP (responsible for degradation of stromal proteins), and FtsH-1 (involved in the degradation of thylakoid membrane proteins) (Georgakopoulos *et al.* 2002; Sokolenko *et al.* 2002). We first used the mutant, *ftsh1-1*, which accumulates normal levels of an inactive FtsH protease (Malnoe *et al.*). When the *ftsh1-1* mutant is starved of sulfur in heterotrophic conditions under low light, cyt. *b<sub>6</sub>f* is preserved, but RuBisCO still disappears, even though the cells are struggling to find S, as demonstrated by the induction of the SLT2 transporter (Fig. 3A). The preservation of cyt. *b<sub>6</sub>f* is consistent with the very limited decrease in  $\Phi_{\text{PSII}}$  in the *Ftsh1-1* mutant (Suppl. Fig. S2). We then looked at the behavior upon sulfur starvation of the *clpP-AUU* mutant that displays a four-fold reduced amount of the Clp protease (Majeran *et al.*). Cyt. *b<sub>6</sub>f* was still degraded albeit at a lower rate but RuBisCO remained unaltered in this mutant. We note that PSII and ATP synthase were targeted to degradation at a later stage of starvation which is indicative of an enhanced susceptibility of the cells to the absence of sulfur when the activity of Clp is hampered.

Altogether these experiments show that, in the absence of sulfur, degradation of stromal RuBisCO is under the control of the stromal Clp protease whereas degradation of the integral cyt. *b<sub>6</sub>f* complex is under the control of the transmembrane FtsH protease.



**Fig. 3: Sulfur starvation in mutants of the main chloroplast proteases, in TAP-S and low light**

Whole protein extracts were harvested at the indicated time points and probed with the indicated antibodies.

### Sustained production of nitric oxide occurs under sulfur starvation

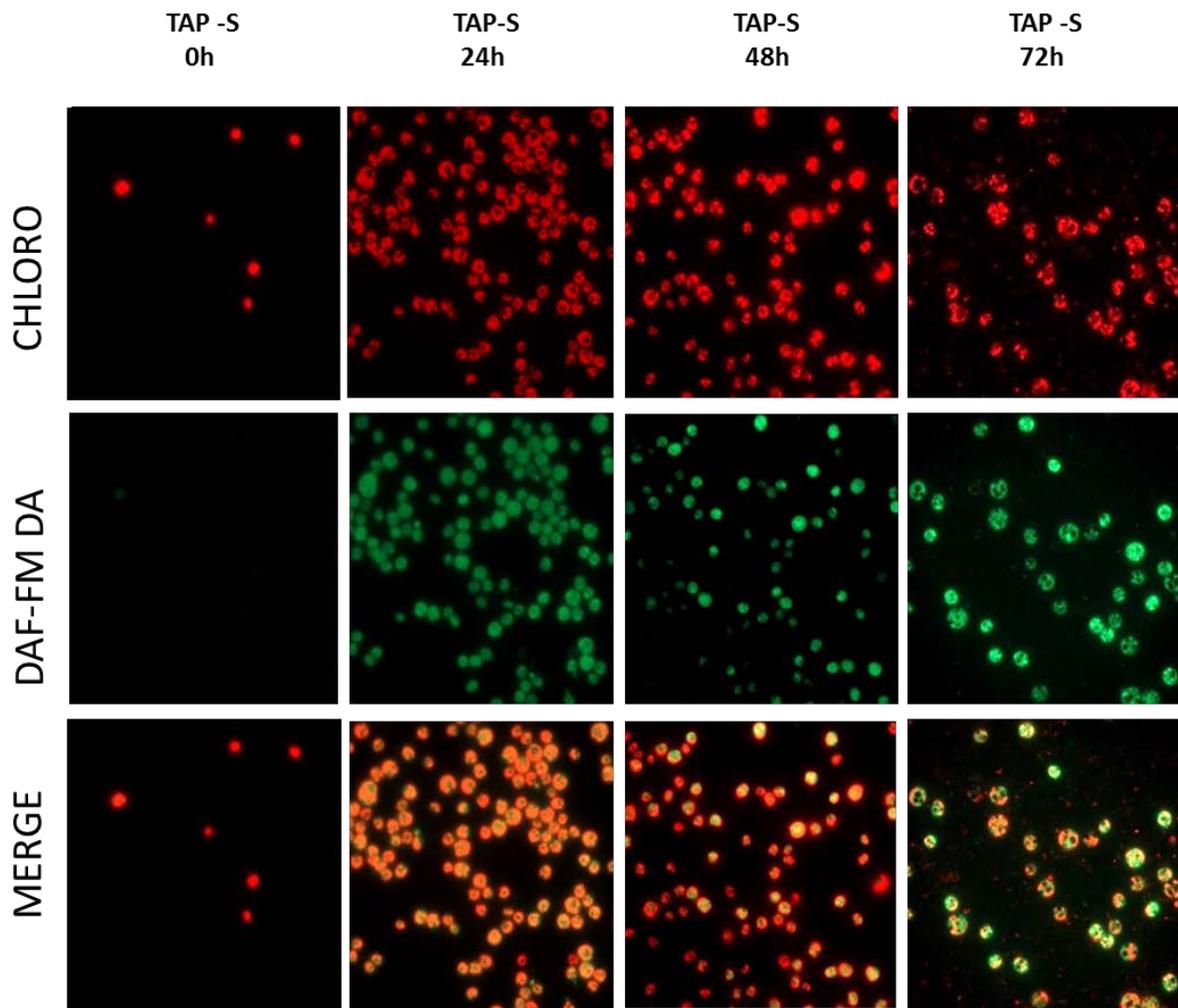
We have previously shown that nitric oxide (NO) is produced when *C. reinhardtii* is starved in nitrogen (Wei *et al.* 2014). NO is produced in a series of abiotic stresses during which it is suspected to act as a signaling molecule which may modulate enzymatic activities, protein localization and proteolytic susceptibility (REF). We sought to determine whether NO also is produced under sulfur limitation using a fluorescence microscopy approach. To detect endogenous NO production *in situ*, we monitored fluorescence levels after incubation with the NO-specific fluorescent probe 4-amino-5-methylamino-2',7'-difluoro-fluorescein diacetate (DAF-FM DA). This permeant molecule is naturally non-fluorescent. When it enters the cell it is esterified, becomes non-permeant, and remains trapped inside the cell. In the presence of NO (or of its oxidation products  $N_2O_3$  and  $NO^+$ ), it is converted into the highly fluorescent triazol derivative.

To observe NO production, WT-S24 cells were starved of sulfur and, at indicated time points, aliquots were harvested, incubated for 1 hour in the presence of with DAF-FM DA (5 $\mu$ M) before recording fluorescence levels. Fig. 4 shows chlorophyll auto-fluorescence in red and the DAF-FM DA signal in green. In cells kept in TAP medium green fluorescence was not observed, but only the red signal coming

from the chloroplasts (Suppl. Fig. S3 left panels). When cells were incubated with DEANONOate (a strong NO-donor), the signal of DAF-FM DA increased strongly, especially in the cytosolic compartment (Suppl. Fig. S1 middle panels). When cells were transferred in TAP-S medium, a faint-green signal appeared after 12-15 hours (data not shown), and became prominent after 24 hours of starvation (Fig. 4A). It then remained at a high level for the whole duration of the experiment, with a fluorescence emission originating from both the cytosol and plastid compartments. When chlorophyll fluorescence parameters were measured in parallel, we observed that NO production started together with the steep drop in  $\Phi_{PSII}$  (Fig 4C). Thus sulfur starvation led to a sustained NO production over four days, once cyt. *b<sub>6</sub>f* and RuBisCO were degraded, at variance with nitrogen starvation, where we observed a burst of NO slightly before protein degradation started.

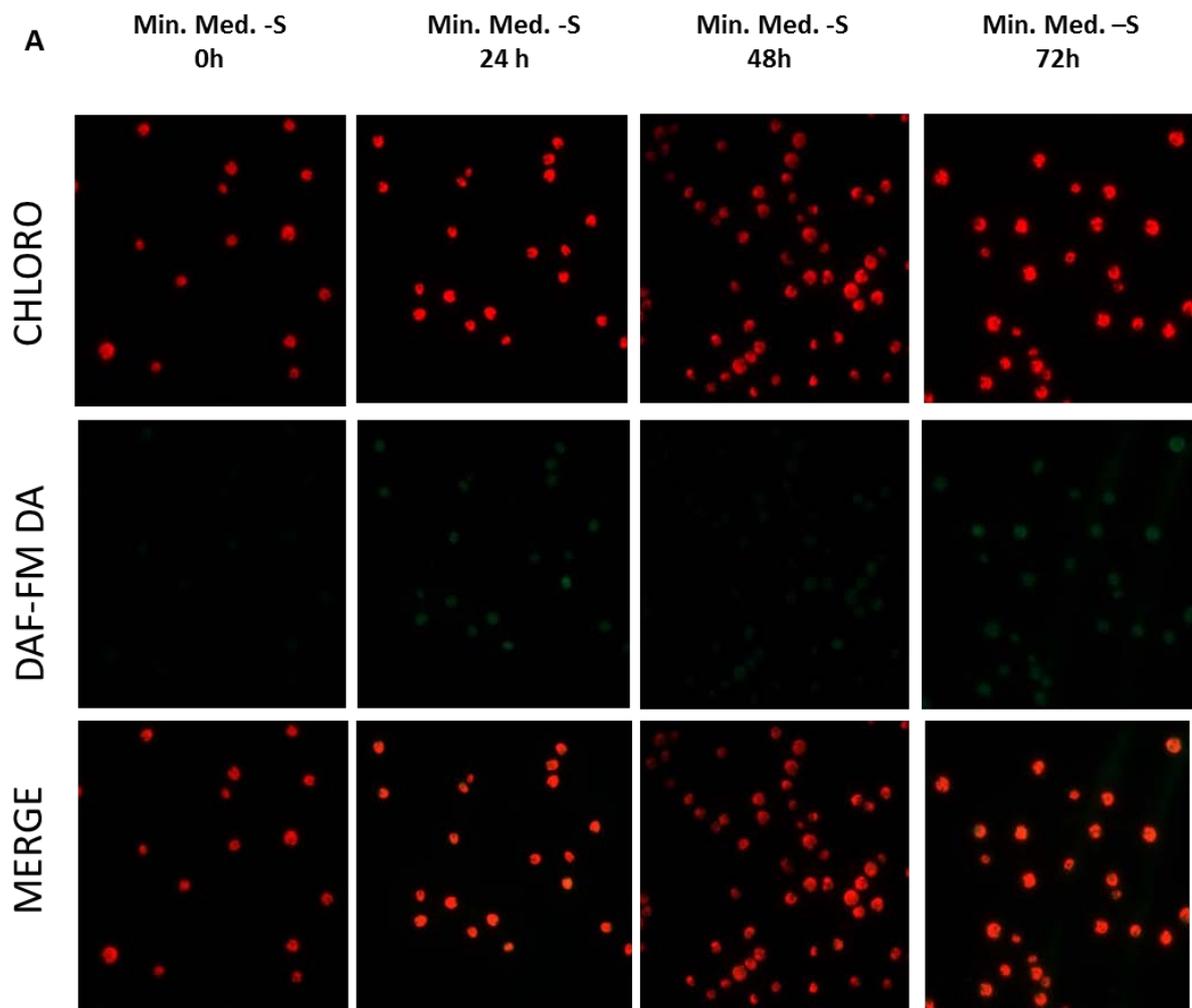
Since the response to sulfur starvation depends on growth conditions, we repeated the same NO measurements in phototrophic conditions (MM-S, Fig 5A) that lead to a limited decrease only in the quantum yield of fluorescence (Fig 5B). In these conditions we only observed a faint green fluorescence emission, indicating that no significant production of NO occurred. Thus, the absence of cyt. *b<sub>6</sub>f* and RuBisCO degradation correlates with the absence of NO production.

These observations constitute a first demonstration that NO production occurs upon sulfur limitation in *C. reinhardtii*, provided cells are in heterotrophic conditions. In order to get insights into the link between NO production and the remodeling of the photosynthetic apparatus, we designed a series of experiments using diverse NO donors and scavengers.



**Fig. 4: Nitric oxide production during sulfur starvation in heterotrophic growth conditions.**

Visualization of NO production *in vivo*, using the DAF-FM DA probe. Cells were starved in low light ( $15\mu\text{E m}^{-2} \text{s}^{-1}$ ), harvested at indicated time points and observed with a fluorescence microscope after washing step in sulfur free medium and 10-fold concentration.



**Fig. 5: Nitric Oxide production during sulfur starvation in photo-autotrophic growth conditions.**

Visualization of NO production *in vivo*, using the DAF-FM DA probe. Cells were starved in low light ( $15\mu\text{E m}^{-2} \text{s}^{-1}$ ), harvested at indicated time points and observed with a fluorescence microscope after washing step in sulfur free medium and 10-fold concentration

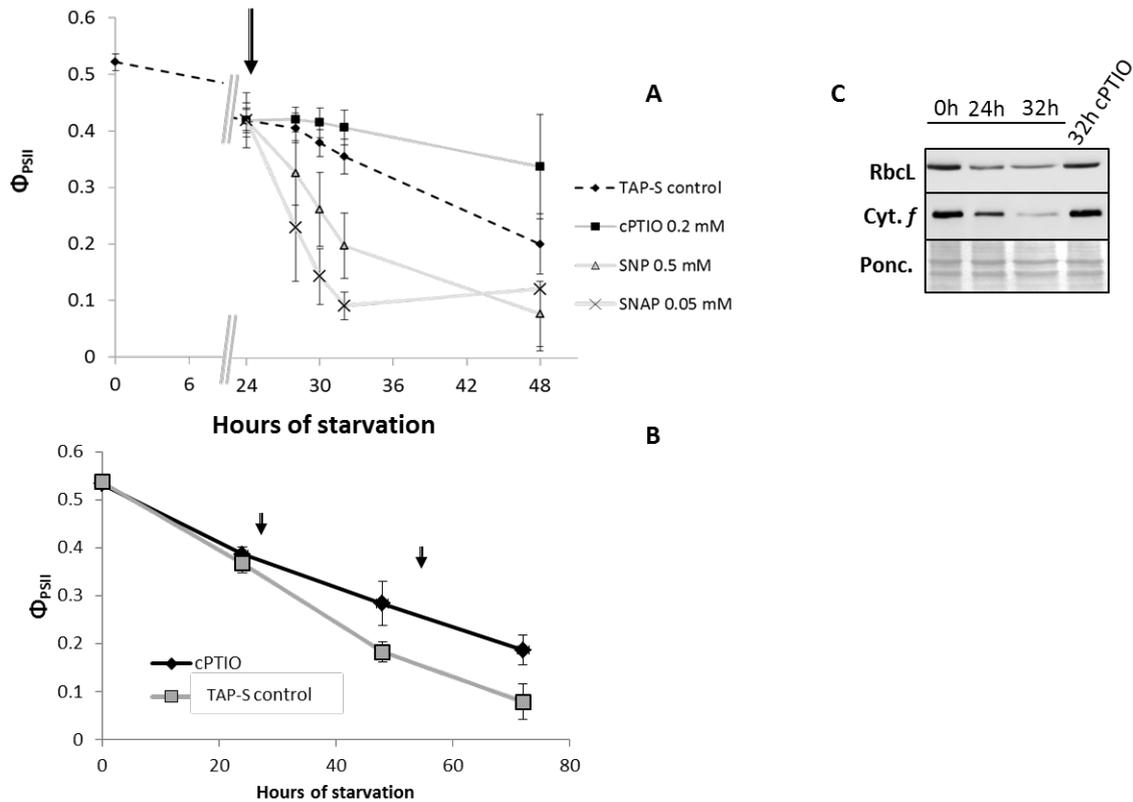
## Testing the involvement of nitric oxide as a triggering signal.

To investigate a possible direct role of NO production in the remodeling of the photosynthetic apparatus, we starved WT-S24 cells in TAP-S for 24 h and added the NO-donors sodium nitroprusside (SNP) and S-Nitroso-N-acetyl-DL-penicillamine (SNAP) in the presence or absence of the NO-scavenger Carboxy-PTIO potassium salt (cPTIO). After drug addition, we monitored the photosynthetic efficiency of each sample for 10 hours (one measure every 2 h), comparing it with the untreated control.

NO donors and scavengers were added 24 h after the onset of starvation because at this point photosynthetic parameters start to change significantly. The results, shown in Figure 6A, demonstrate that the amount of NO affects photosynthetic efficiency. Indeed, addition of SNP and SNAP both caused a more pronounced and faster decrease in quantum yield, with no major effect on  $F_V/F_M$  (Suppl. Fig. S4A). This stimulated inhibition of photosynthesis by SNP and SNAP was fully counteracted by cPTIO when added concomitantly (Suppl. Fig S4C-D). Thus the action of SNP and SNAP is likely due to NO release rather than to some unspecific effects of these drugs. Addition of cPTIO alone largely prevented photosynthesis inactivation (Fig. 6A, squares). The effect of cPTIO was also observed when it was added every 24 hours for the entire duration of the starvation experiment (Fig. 6B and Suppl. Fig. S4D), confirming that removing endogenous NO has a positive effect on photosynthetic efficiency, probably by blocking the signal triggering protein degradation.

We then analyzed the content in cyt. B6f and RuBisCO at the onset of starvation (0 h), before (24 h) and after (32 h) incubation with NO-donors and scavengers. We monitored the content in RuBisCO and Cyt *f* by immunoblotting and observed that their degradation was prevented in the presence of cPTIO (Fig.6C). Protein levels were even higher after 8 hours of cPTIO treatment than at the time of addition. These observations argue for a direct involvement of NO in the selective degradation of cyt. *b<sub>6</sub>f* and RuBisCO under sulfur starvation. Unfortunately, we were not able to obtain reproducible results for protein accumulation in the samples treated with SNP and SNAP. No significant differences in protein accumulation were observed. Taken together, these data identify NO as a signaling molecule for the remodeling of the photosynthetic apparatus under sulfur starvation. But at this point,

we are still lacking clues regarding the endogenous source of NO. To address this question, we analyzed S-starvation in the presence of inhibitors of NO synthesis.



**Fig. 6: Nitric oxide accumulation can effect the kinetics of photosynthesis inhibition and cytochrome  $b_6-f$  degradation.**

**(A)** Photosynthetic activity measured after addition (indicated by arrows) of NO-donors (SNAP 0.05 mM and SNP 0.5 mM) and NO-scavengers (cPTIO 0.2 mM); dashed line corresponds to the control (cells in TAP-S without any drug addition). Data show the mean of three independent experiments  $\pm$  SD.

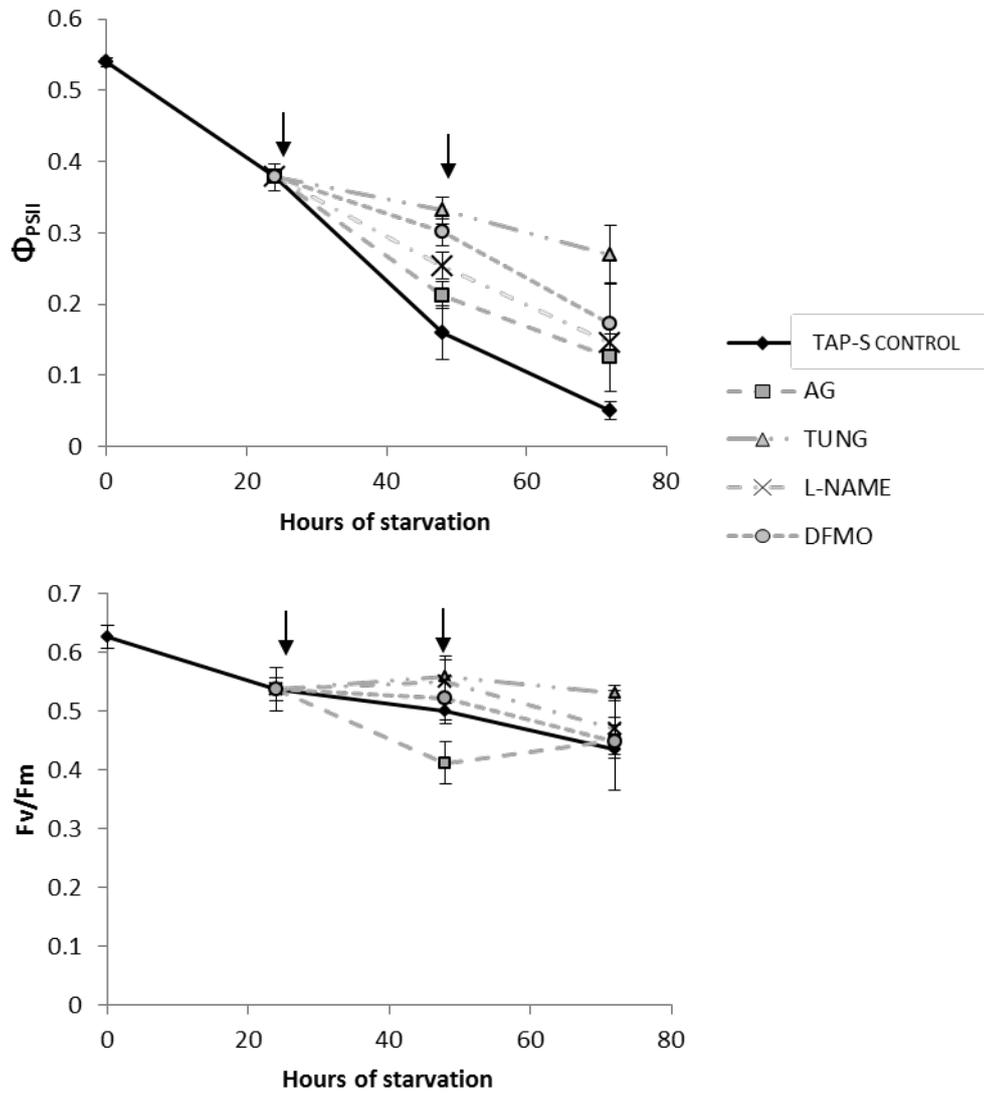
**(B)** Effects of cPTIO on quantum yield during sulfur starvation in TAP-S medium under low light. Arrows indicate drug addition (3 hours before measurements). Data show the mean of three independent experiments  $\pm$  SD.

**(C)** Accumulation of cytochrome  $f$  and Rubisco before and after cPTIO addition. Experiments performed in heterotrophic medium and at low light  $15\mu\text{E m}^{-2} \text{s}^{-1}$

## The source of NO

We investigated the source of NO during sulfur starvation of WT-S24 (*nit1-137 nit2-124 mt2*) a strain which lacks nitrate reductase (NR). We used specific inhibitors of the NR-independent NO-producing pathways. We chose L-N<sup>G</sup>-Nitroarginine methyl ester (L-NAME) and Amino guanidine (AG) to block the NOS-like dependent pathway; DL- $\alpha$ -Difluoromethylornithine (DFMO) to block the polyamine-dependent pathway and tungstate to block the synthesis of Molybdenum Cofactor (MoCo), a

cofactor required for the activity of 5 enzymes (nitrate reductase; sulfite reductase; xanthine oxido/reductase, aldehyde dehydrogenase and the Amidoxine Reducing Component, ARC, recently renamed NO-forming Nitrite Reductase or NOFNIR) all of which have been described as putative Nitrite-dependent producers of NO (Tewari *et al.* 2009; Wang *et al.* 2010; Maia *et al.* 2011; Wei *et al.* 2014; Chamizo-Ampudia *et al.* 2016). 24 h after the transfer of WT-S24 cells in sulfur-free medium (TAP-S), we added the various inhibitors every 24 h, to reach a 2 mM final concentration at the end of the experiment. The results clearly demonstrate that all pathways are involved in the production of NO upon sulfur starvation (figure 7). Indeed L-NAME, AG, DFMO and Tungstate all showed a positive effect on photosynthetic efficiency although with different kinetic effects. Interestingly, tungstate was the most effective in preserving photosynthesis, suggesting a prominent role for the Nitrite-dependent pathways, even in the absence of the NR enzyme. Since no significant effects were observed for cultures kept in TAP in the presence of these inhibitors (Suppl. Fig. S5), the preservation of photosynthesis in sulfur deficient conditions is unlikely to be due to secondary effects of these NO-inhibitors.



**Fig. 7: Effects of different NO-synthesis inhibitors on photosynthetic efficiency.**

Photosynthetic activity measured after addition (indicated by arrows) of L-NAME (2mM); DFMO (2 mM); Tungstate (2 mM) and Amino guanidine (2mM). Dashed line corresponds to the control (cells in TAP-S without any drug addition). Data show the mean of three independent experiments  $\pm$  SD. Experiments performed in heterotrophic medium and at low light  $15\mu\text{E m}^{-2} \text{s}^{-1}$

## DISCUSSION

When starved in sulfur *C. reinhardtii* slowly undergoes photosynthesis inactivation, which is completed over more than four days. This functional inhibition is achieved through the specific degradation of RuBisCO and cyt. *b<sub>6</sub>f*, borne respectively by the stromal and thylakoid proteases Clp and FtsH. However, our data are in contrast with previous studies on sulfur starvation, most of which have described a photosynthetic inhibition due to PSII inactivation/degradation (Wykoff *et al.* 1998; Grossman 2000; Zhang *et al.* 2002; Forestier *et al.* 2003; Zhang *et al.* 2004). These studies focused on H<sub>2</sub> production which required higher light intensities during starvation (80-300  $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) than those we used to observe the selective degradation of cyt. *b<sub>6</sub>f* and RuBisCO. Indeed, we had shown previously (Malnoe *et al.* 2014) and confirmed here that increasing the light intensity at which S-starvation is performed triggers a photoinhibitory process which drives PSII inactivation and degradation. This is a straightforward consequence of the lower metabolic demand for carbon fixation upon growth arrest, which leads to NADPH accumulation, over reduction of the photosynthetic electron transfer chain and ROS production which will damage PSI and PSII.

The light-independent degradation of RuBisCO when *C.reinhardtii* is starved in sulfur can be viewed as an attempt to remobilize sulfur. Indeed RuBisCO, the most abundant protein on Earth that amounts to about 30% of the soluble proteins in *C. reinhardtii* (Sugimoto *et al.* 2007; Michelet *et al.* 2013), is a major reservoir of sulfur. Being composed of 8 large and 8 small subunits that together contain 256 sulfur-containing residues, either cysteines or methionines, its degradation would allow the cells to cope with a transient decrease in external sulfur sources. This is less true for cyt. *b<sub>6</sub>f*, which, despite containing 27 methionines, 13 cysteines, and a [2Fe-2S] prosthetic group per monomer (Stroebel *et al.* 2003), is not a major source of sulfur compared to other photosynthesis proteins.

The rationale for this dual targeted protein degradation –RuBisCO and cyt. *b<sub>6</sub>f*– in sulfur deprived conditions may be better understood when compared with the very similar situation produced by nitrogen deprivation (Wei *et al.*, 2014). In both conditions, the central metabolism slows down drastically, leading to growth arrest and down regulation of gene expression. Thus, the energy demand for DNA replication, translation and protein synthesis dramatically decreases. At the same

time, catabolic pathways are activated in order to remobilize as much sulfur or nitrogen as possible. Up-regulation of catabolism leads to over accumulation of reduced carbon, mainly as starch and TAGs lipids (REF). Since carbon metabolism is intimately linked to nitrogen and sulfur metabolism, with which it shares a mutual genetic regulation (Kopriva *et al.* 2004), the cells try to avoid such imbalance between these macronutrients. The strategy to do so, in heterotrophic conditions, would be to operate a shift toward a respiratory metabolism at the expense of photosynthesis: RuBisCO degradation prevents carbon assimilation and thus over accumulation of additional carbon skeletons whereas cyt. *b<sub>6</sub>f* degradation blocks altogether the cyclic and linear electron flows, thus collapsing photosynthetic ATP and NADPH production. Mitochondrial respiration will provide enough energy to sustain the low cell metabolism, and consume at the same time the carbon skeletons in excess. It is of note that chlororespiration also is stimulated upon nitrogen deprivation (Wei *et al.*, 2014) and sulfur deprivation. Thus the chloroplast becomes a catabolic organelle to balance C, N and S levels in coordination with mitochondria. These responses to N and S deprivation are activated in heterotrophic conditions but not in phototrophic conditions. This contrast calls for a starvation response through a hierarchy of signals, the first one being the availability of reduced carbon in the medium. Its presence allows a sustained respiratory metabolism which should trigger the switch toward photosynthesis inactivation when the signal “sulfur or nitrogen deprivation” is perceived. This is consistent with the absence of photosynthesis inactivation when *C. reinhardtii* is starved in nitrogen sources in absence of mitochondrial respiration (Bulte *et al.* 1990; Wei *et al.* 2014). It is thus a situation similar to the absence of an external reduced carbon source (photoautotrophic conditions), which do not lead to degradation of RuBisCO and cyt. *b<sub>6</sub>f*, and preserve photosynthesis until the internal S-and N-resources are completely exhausted.

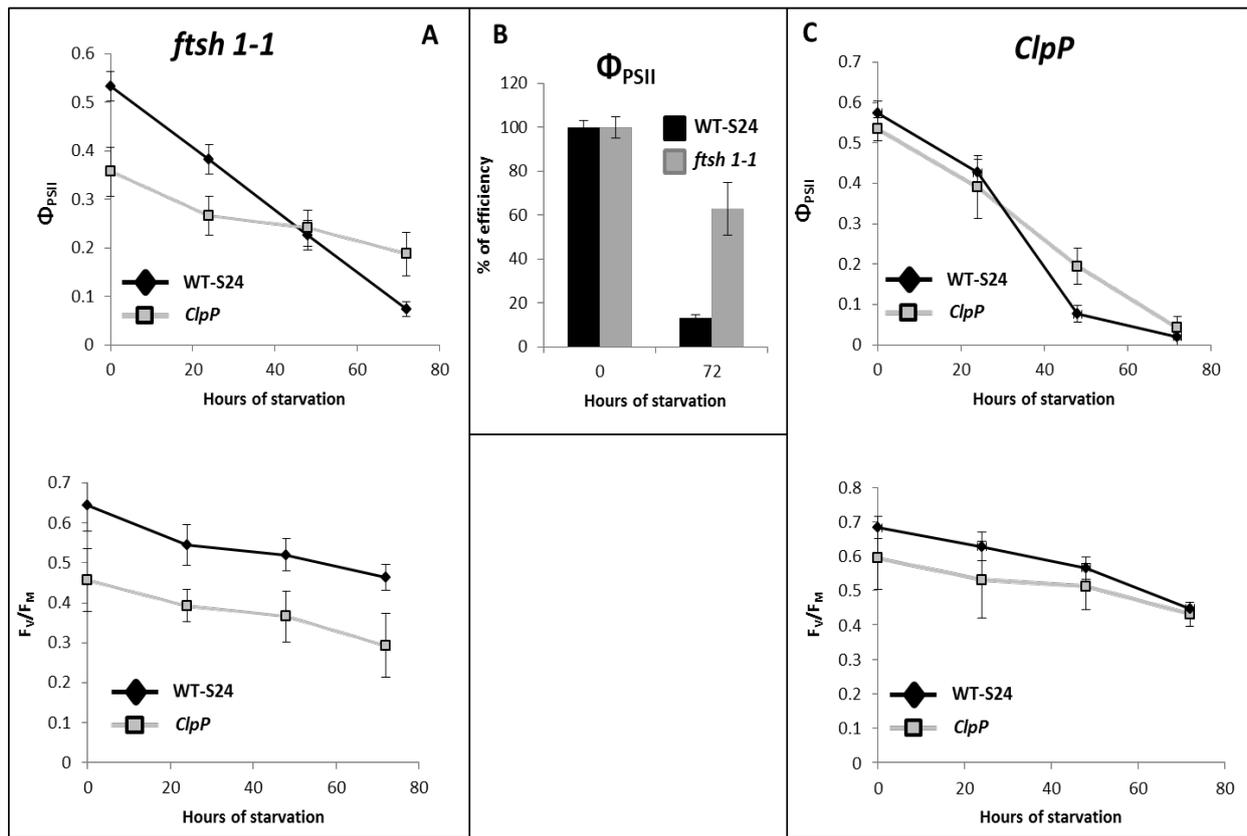
A key element in photosynthesis inactivation upon N or S deprivation is the production of NO, which is instrumental in the degradation of cyt *b<sub>6</sub>f* and rubisco (Wei *et al.* 2014 and this study). The strong NO-production observed just before protein degradation, and the use of NO-scavengers and producers in our experiments, corroborate its direct involvement in the degradation process. To do so, NO could either induce redox post-translational modifications such as nitrosylations or activate signaling pathways through the interaction with NO-responsive guanylate cyclase enzymes among others.

In *Chlamydomonas* two major NO synthesis pathways have been described. The major one is the Nitrite-dependent pathway, involving either nitrate reductase (NR) and the ARC enzyme (Chamizo-Ampudia *et al.* 2016), or an NR-independent process still to be characterized in molecular detail (Hemschemeier *et al.* 2013; Wei *et al.* 2014). The other pathway is the arginine dependent pathway, occurring either from the degradation of polyamines or through an as yet unidentified NOS-like enzyme (Barroso *et al.* 1999; Corpas *et al.* 2004; Tun *et al.* 2006; Yamasaki *et al.* 2006). Here, using various inhibitors of NO synthesis we provided evidence that the NR-independent Nitrite-dependent pathway plays a major role in the case of sulfur deprivation.

Glutathione is probably a key molecule at the crossroad between carbon, nitrogen and sulfur, playing a major role in stress responses and NO metabolism and signaling. Glutathione is the main redox buffer of the cell and, being a cysteine-containing tripeptide, it requires carbon skeletons as well as nitrogen and sulfur to be produced. In red algae under S-starvation, cysteine levels drop dramatically while glutathione accumulation shows a growth condition dependent behavior, with a 4-fold increase in heterotrophy and a decrease in photoautotrophy (Carfagna *et al.* 2015; Carfagna *et al.* 2016). We can thus imagine a similar scenario in *C. reinhardtii*, where starved cells sustain, at least in part, augmented GSH production providing carbon skeleton and N/S through sparing mechanisms. The maintenance of glutathione homeostasis may be required to protect proteins, nucleic acids and lipids against ROS-induced over-oxidation and nitrosative stress, events which are enhanced under sulfur deprivation, where the Benson Calvin cycle is blocked and the photosynthetic electron transfer chain becomes over-reduced. Moreover GSH interacts with NO to form the GSNO molecule, the main NO reservoir of the cell and the main transnitrosylating molecule. In fact, NO has been described to fulfill a signaling function either in couple with hemoglobins, after the direct interaction with heme groups (Ouellet *et al.* 2002; Perazzolli *et al.* 2004; Perazzolli *et al.* 2006; Smaghe *et al.* 2008; Hemschemeier *et al.* 2013), or as a nitrosylating agent coupled with GSH or with other proteins considered as alternative nitrosylases (Stamler *et al.* 2010; Zaffagnini *et al.* 2013; Zaffagnini *et al.* 2016). GSNO thus contributes a main nitrosylating mechanism but it also contributes to protein-mediated trans-nitrosylation. Notably, proteins containing a nitrosylated cysteine may be able to transfer their NO moiety to other proteins. Theoretically, this mechanism may lead to the nitrosylation

of proteins that are not themselves target of direct nitrosylation, thereby amplifying the signal (Kornberg *et al.* 2010; Stamler *et al.* 2010; Nakamura *et al.* 2013). The signaling pathway could be a multi-step cascade, with an increase in specificity at each step, starting from the highly reactive and less specific NO to end up with highly specific nitrosylases. This view is supported by the fact that nitric oxide, besides its involvement in physiological responses (Beligni *et al.* 2000; Neill *et al.* 2002; Pagnussat *et al.* 2002; He *et al.* 2004; Prado *et al.* 2004; Mishina *et al.* 2007; Tada *et al.* 2008; Lindermayr *et al.* 2010; Gibbs *et al.* 2014), has also been implicated in many stress responses with increased oxidative load in photosynthetic organisms (Delledonne *et al.* 1998; Garcia-Mata *et al.* 2001; Graziano *et al.* 2002; Feechan *et al.* 2005; Baudouin *et al.* 2006; Zhao *et al.* 2007; Lee *et al.* 2008; Besson-Bard *et al.* 2009; Hemschemeier *et al.* 2013) among which, nitrogen starvation (Wei *et al.* 2014). One should also keep in mind that GSH is also the main denitrosylating agent for most of nitrosylated proteins. Therefore the increased GSH accumulation could be a response to restore the appropriate GSH/GSNO ratio, which is crucial to determine the endogenous nitrosylation levels in all conditions (Zaffagnini *et al.* 2013).

In such intricate scenario with several details still lacking, an interesting perspective to work on is the analysis of endogenous protein nitrosylation levels. This information could be achieved using a proteomic approach coupled to biochemical labeling of nitrosothiols. Obtaining the nitrosylome pattern of S-starved cells, as well as the precise sites of nitrosylation, would provide valuable information on the underlying molecular mechanism. The identification of the target cysteines would allow production of mutants which could be exploited in functional studies to fully elucidate the interconnection between metabolic pathways, NO signaling, nitrosylation, and the selective degradation of cyt. *b<sub>6</sub>f* and RuBisCO.



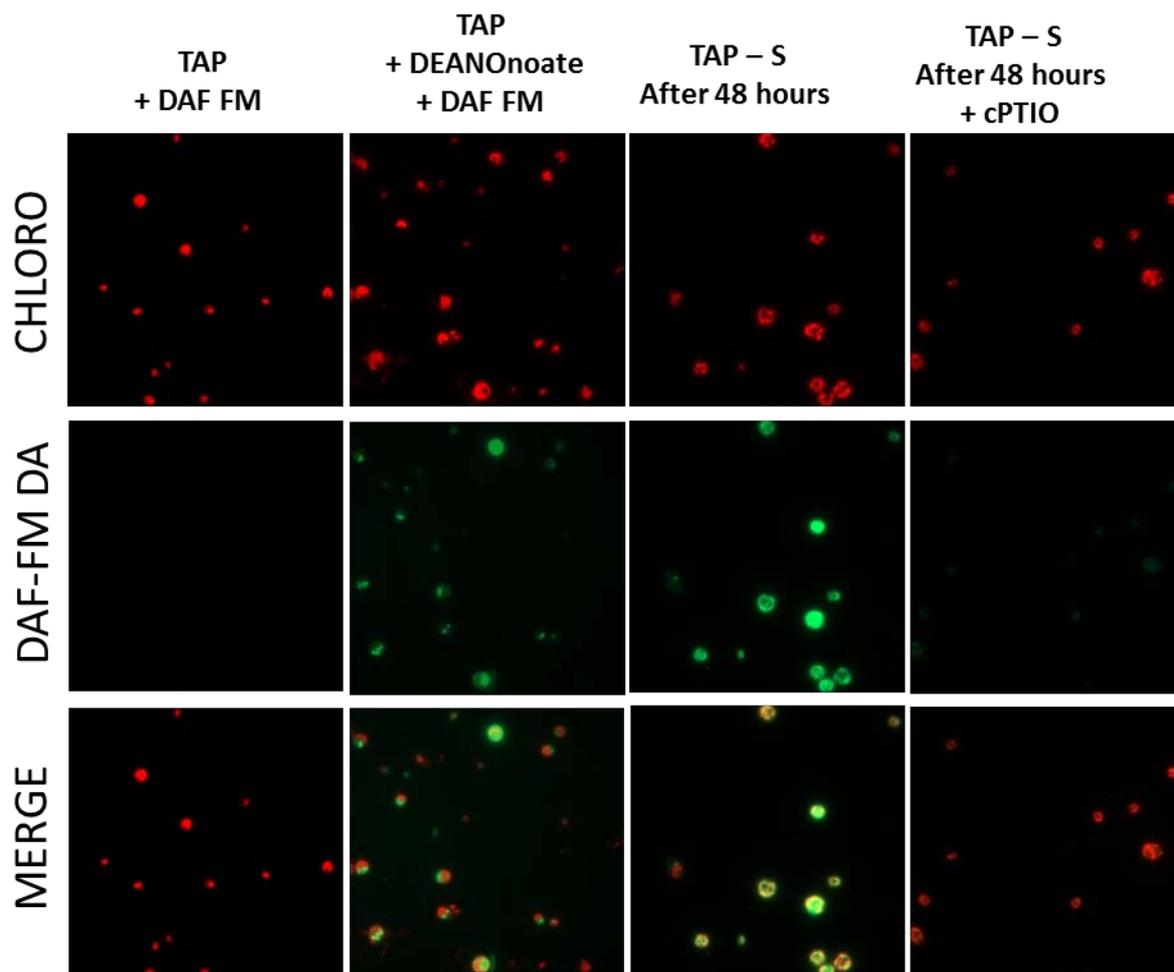
**Fig. S2: Sulfur starvation on the mutants *ftsh 1-1* and *ClpP*, defective for protease function.**

(A)  $\Phi_{PSII}$  and  $F_v/F_m$  of *ftsh 1-1* under starvation in TAP-S and  $15\mu\text{E m}^{-2} \text{s}^{-1}$ .

(B) Residual photosynthetic efficiency (as % of the initial efficiency) after 72h of starvation between *wts24* and *ftsh1-1*.

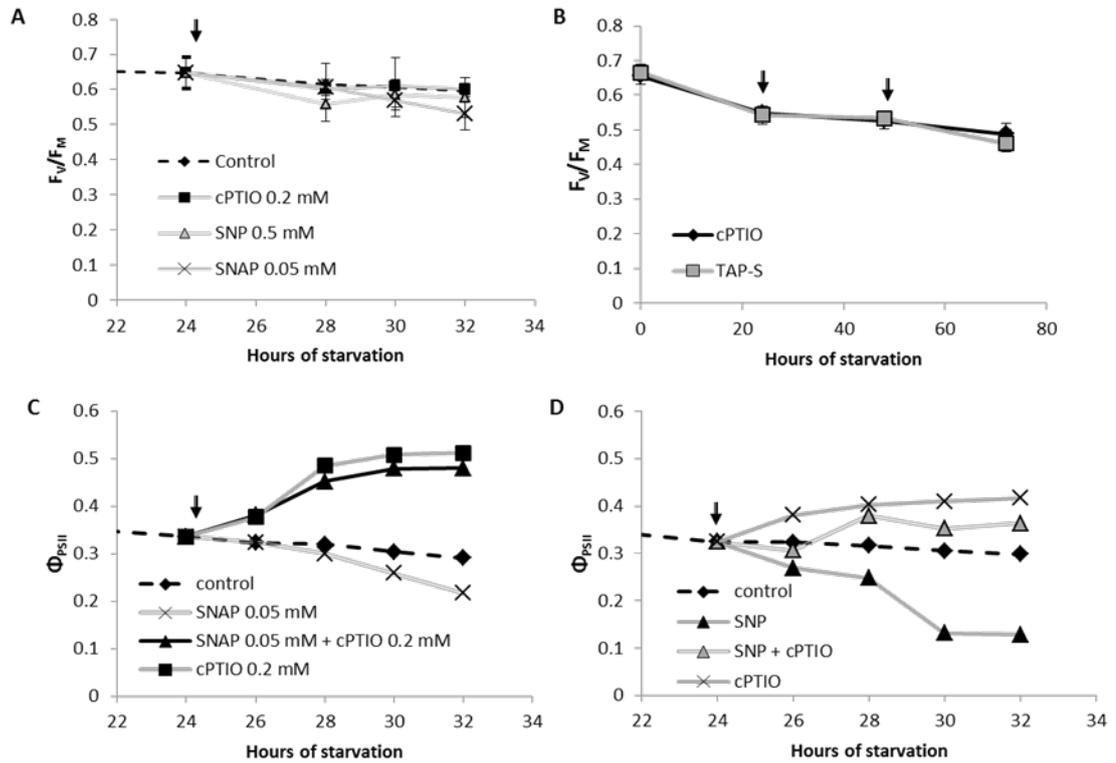
(C)  $\Phi_{PSII}$  and  $F_v/F_m$  of *ClpP* under starvation in TAP-S and  $15\mu\text{E m}^{-2} \text{s}^{-1}$ .

Data represent the mean of 5 independent experiments with SD



**Fig. S3: DAF-FM DA fluorescence can be seen only in presence of nitric oxide or under sulfur starvation.**

Visualization of NO production *in vivo*, using the DAF-FM DA probe. From left to right panels: not starved cells; not starved cells incubated with DEANOate; Starved cells after 48 h; Starved cells after 1 h of cPTIO incubation, to remove endogenous nitric oxide



**Fig. S4: Effects of NO donors and scavengers on  $F_v/F_m$ .**

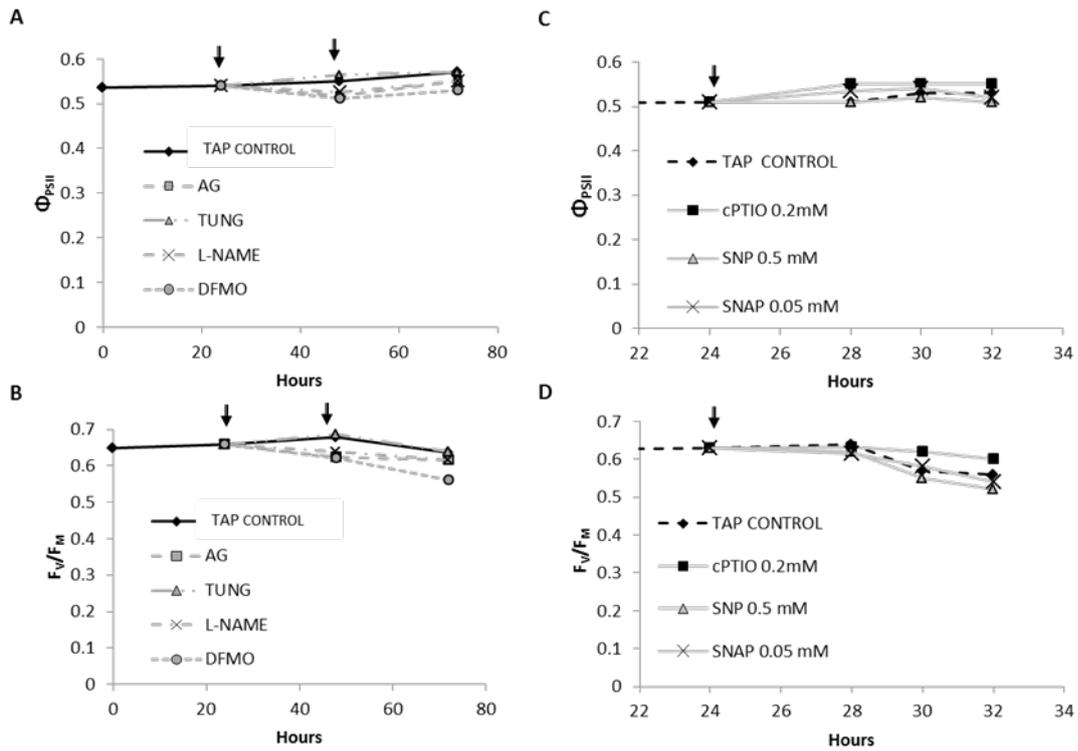
**(A)** PSII efficiency measured after addition of SNAP 0.05 mM, SNP 0.5 mM and cPTIO 0.2 mM; dashed line corresponds to the control (cells depleted in sulfur without any drug addition). Data show the mean of three independent experiments  $\pm$  SD.

**(B)** Effects on  $F_v/F_m$ . cPTIO was added every 24 hours over the course of sulfur starvation experiment. Data show the mean of three independent experiments  $\pm$  SD.

**(C)** Effects of SNAP and cPTIO, added together, on photosynthetic efficiency.

**(D)** Effects of SNP and cPTIO, added together, on photosynthetic efficiency.

Black arrows indicate drug addition.



**Fig. S5: Effects of different NO-synthesis inhibitors on photosynthetic efficiency in sulfur replete conditions.**

(A) (B) NO synthesis inhibitors effects on  $\Phi_{PSII}$  and  $F_v/F_m$  of WT-S24 cells kept in TAP at 15  $\mu$ E.

(C) (D) No-scavenger/donors effects on  $\Phi_{PSII}$  and  $F_v/F_m$  of WT-S24 cells kept in TAP at 15  $\mu$ E.

Arrows indicate drugs addition.

## IV.2 A light switch based on protein nitrosylation fine tunes photosynthetic light harvesting in *Chlamydomonas reinhardtii*

*Chlamydomonas reinhardtii* contains a protein, called NAB1 (nucleic acid binding protein 1), crucial in the response to light fluctuations (Berger *et al.* 2014). This protein has been identified in a screening for state transition mutants. The *nab1*<sup>-</sup> strain is an insertional mutant with characteristic phenotypes, among which we can cite higher sensitivity to high light; higher chlorophyll content with a decreased Chl a/b ratio, and basically a constitutive bigger LHCII antenna size (Fig.14). (Mussgnug *et al.* 2005)

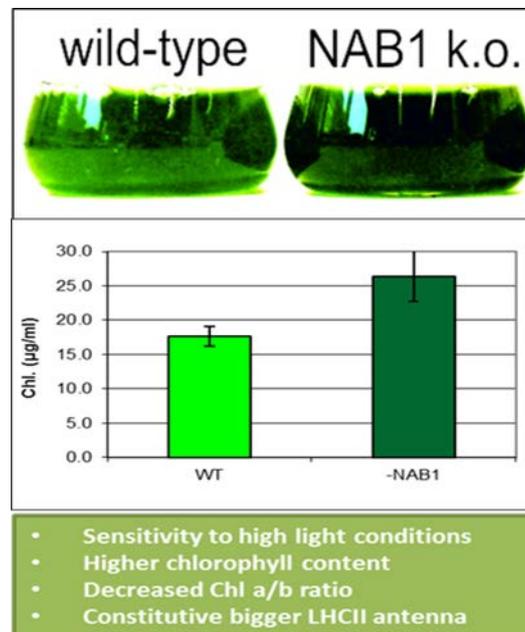


Figure 14: Phenotypic features of the *nab1* k.o. mutant strain. (adapted from Mussgnug *et al.* 2005)

NAB1 is a cytosolic 25 kDa translational repressor targeting mRNAs of the major light-harvesting complexes (LHCBMs 4/6). Once activated, it prevents target mRNA translation by sequestering the RNA into ribonucleoprotein complexes. NAB1 activity can be regulated at different levels. For example, growth conditions such as illumination and CO<sub>2</sub> availability can modify its expression level (Berger *et al.* 2014). Moreover, it can be post-translationally regulated through arginine methylation and/or cysteine RPTMs and, in this case, its RNA-binding affinity and specificity are decreased, blocking reversibly the repression activity (Wobbe *et al.* 2009).

The actual nature of the redox modification(s) affecting NAB1 activity is not well understood. It has been shown that oxidants such as oxidized glutathione (GSSG) or diamide, strongly decrease RNA-binding *in vitro*. However, oxidation does not lead to disulfide bond formation (as for TRX targets).

Indeed NAB1 possesses only two cysteines: Cys181 and Cys226 but the phenotype associated with Cys226 mutation is much more severe than that observed when mutating Cys181, suggesting a mechanism independent on disulfide bond formation. Beside these indications, the molecular mechanism and the nature of the redox modifications are still unknown, and my work aimed at shedding light on these aspects.

In the Article 3, entitled “A light switch based on protein nitrosylation fine tunes photosynthetic light harvesting in *Chlamydomonas*”, we describe in detail the redox-regulation of NAB1, presenting a new mechanism of antenna size modulation so far unknown. This mechanism involves nitric oxide and nitrosylation in the translational control of LHCBM mRNAs. The article has been published in April 2016 in the journal *Plant physiology*. The work has been performed in collaboration with the team of Pr. Olaf Kruse from the University of Bielefeld (Germany). My contribution was focused on the characterization of the nitrosylation/denitrosylation mechanisms.

## V Conclusions and perspectives

### V.1 The role of Nitric Oxide in the remodeling of the photosynthetic apparatus under sulfur starvation in *Chlamydomonas reinhardtii*

The response to sulfur deprivation in *Chlamydomonas* depends on growth conditions rather than on light intensity. Here we have focused our attention on the mechanism responsible for photosynthesis inactivation. Photosynthesis is shut down only in heterotrophy through selective degradation of cytochrome  $b_6f$  and Rubisco. One of the major plastidial proteases, FtsH1, seems to be involved in this event, as a mutant defective for the protein does not show degradation of cytochrome  $b_6f$  under starvation. Currently we are performing experiments with a second mutant defective for the soluble plastidial protease, ClpP, in order to observe if the protein controls Rubisco degradation in such conditions. However, many evidence point to a direct role of NO in coordination/regulation of this well-defined protein degradation. For instance, NO-donors and NO-scavengers, as well as NO-synthesis inhibitors, can all have a sensible impact on the photosynthetic efficiency of sulfur starved cells. NO-donors accelerate photosynthetic inactivation while scavengers and inhibitors slow down the process.

As previously discussed, NO has been described to be involved in response to hypoxia, heat stress, UV stress, salt stress and nitrogen starvation among other abiotic stresses, playing an active role as signaling molecule or second messenger (Domingos *et al.* 2015; Parankusam *et al.* 2017).

Produced from carbon skeletons, nitrogen and sulfur provided by scavenging processes, glutathione plays a key role in NO signaling. Indeed, while GSH reacts with NO to form GSNO, it is also the main denitrosylating molecule for most of the nitrosylated proteins. Therefore the GSH/GSNO ratio is crucial to define endogenous nitrosylation levels (Zaffagnini *et al.* 2013). However we can also imagine that the signal is propagated through proteins which are initially nitrosylated via GSNO and thereby become activated as trans-nitrosylases (Stamler *et al.* 2010), modifying other downstream targets, in a complex cascade. Although GSNO-dependent nitrosylation is considered as the main mechanism, recently different groups have demonstrated the existence of alternative nitrosylases, nitrosylated proteins that are able to transfer their NO moiety to other targets, with a high degree of specificity. Theoretically, this

mechanism may lead to the nitrosylation of proteins that are not themselves target of direct nitrosylation by NO or related molecules like GSNO, thereby amplifying the signal. At the moment, there are few examples of trans-nitrosylases in the literature: SNO–hemoglobin, SNO–glyceraldehyde-3-phosphate dehydrogenase, SNO–caspase 3 and SNO–Thioredoxin1 (Kornberg *et al.* 2010; Stamler *et al.* 2010; Nakamura *et al.* 2013; Zaffagnini *et al.* 2016). To date, nothing is known in plant systems. Independently from the mechanism of nitrosylation that remains speculative, NO signaling in this context could be propagated also through other signaling pathways (*e.g.* guanylate cyclase and truncated hemoglobins). Nevertheless, the outcome of the signal seems to be modulation of the metabolism which leads to the conversion of the chloroplast in a catabolic organelle and to the enhancement of mitochondrial respiration to provide energy for the cell.

Many questions remain open. The first, and maybe the most important, is about the nitric oxide source. According to our data, NO is accumulated in the chloroplast after one day of sulfur starvation. Moreover, the use of NO-synthesis inhibitors shows how both major pathways (Arginine dependent and NO<sub>2</sub> dependent) are activated under sulfur starvation in heterotrophy. However, the use of tungstate demonstrates that the scenario is complex and may involve alternative pathways. The strain used in this study is *nit1<sup>-</sup>/nit2<sup>-</sup>*, lacking all the enzymes responsible for nitrogen assimilation/reduction, among which the NR enzyme. Thus, the strain is virtually unable to accumulate NO<sub>2</sub> and to produce NO by NO<sub>2</sub> reduction via NR. However, the accumulation of NO<sub>2</sub> under sulfur deprivation can be achieved in consequence of catabolic processes, and could be sufficient to induce NO production in a NR-independent fashion. Indeed, the Molybdenum Cofactor (MoCo), the target of tungstate inhibition, is a prosthetic group present in other enzymes such as sulfite reductase; xanthine oxido/reductase; aldehyde dehydrogenase which have been associated to NO production in normoxic conditions (Tewari *et al.* 2009; Wang *et al.* 2010; Maia *et al.* 2011) (Corpas *et al.* 2001). Thus an interesting perspective for the future will be to study in more details how NO production is regulated during sulfur starvation and whether the NO<sub>2</sub>-dependent pathway contributes to NO production, as already suggested for N-starvation. In addition, it could be interesting to understand in which proportion the different synthetic pathways are involved.

Another particularly interesting perspective would be to disentangle the link between NO production and protein degradation. It is universally accepted that NO acts as a

second messenger in plant and animals, but an always increasing amount of studies show that the main effect on protein is induced by post translational modifications on cysteine (nitrosylation) and tyrosines (nitration) (Zaffagnini *et al.* 2016). Among these PTMs, nitrosylation is the only one that has been described and characterized as reversible and therefore suited for signaling functions. In the case of *Chlamydomonas*, 492 proteins have been identified as nitrosylation targets (Morisse *et al.* 2014). Among these targets we can find the major subunits of cytochrome *b<sub>6</sub>f*, both Rubisco subunits and the two proteases FtsH and ClpP. Thus it is possible to imagine that NO modifies these proteins, inducing conformational changes affecting protein stability or modulating their specificity/activity, and thereby triggers targeted degradation. The best approach to obtain a precise proof of the role of NO during S-starvation is the analysis of nitrosylation profiles of starved cell, to get a list of all targets with their respective cysteines. In order to obtain this information we envisage to couple the biochemical labeling of nitrosothiols (e.g. using the biotin switch technique) with a MS/MS quantitative approach. Unfortunately, I have performed biotin switch assays on starved cells several times with no significant or reproducible results. We believe these issues are related to the intrinsic difficulty of the biotin switch techniques *in vivo*. For instance nitrosothiols are highly unstable and should be labeled as fast as possible, avoiding direct light and working at 4°C. Moreover the technique has many drawbacks. A major one relies in the identification of false positives due to incomplete blocking or denitrosylation during sample handling. Moreover, some thiol blocking agents may react with other species such as sulfenic acids and in addition the specificity of the reduction step is difficult to establish unambiguously (Reisz *et al.* 2013; Couvertier *et al.* 2014). We envisage for the future to use direct methods for the identification of nitrosylated proteins. Promising approaches based on direct capture of S-nitrosocysteine residues have been proposed but need further confirmation of their potential for quantitative proteomic studies (Doulias *et al.* 2010; Faccenda *et al.* 2010; Wang *et al.* 2011). Overtaking the difficulties of these approaches and identifying the target cysteines will allow functional studies. For example, strains containing variants of cytochrome *b<sub>6</sub>f* or Rubisco where cysteine have been mutated could allow to determine whether and how blocking nitrosylation of key residues alters the response to sulfur starvation and the remodeling of the photosynthetic apparatus. Such studies could be initiated rapidly for Rubisco since cysteines undergoing nitrosylation *in vivo* after GSNO

treatment have been already identified in a previous study performed in the lab (Morisse *et al.* 2014).

To obtain further insights into the molecular details of signal propagation, we could also analyze further the mechanism of nitrosylation. For example, it would be useful to clarify the role of glutathione, the principal partner of NO in the cell, with which it forms GSNO, considered as major reservoir and major nitrosylating agent. An idea would be to measure glutathione concentration and redox state in the different growth conditions, as done for *Galdieria phlegrea* by Carfagna and colleagues (2016). In parallel, it would be interesting to evaluate the effect of GSH synthesis inhibitors on nitrosylation levels in starved cells. This would be helpful to gain information about the nitrosylation mechanism, to determine whether it requires GSNO or is directly carried out by NO and its radical species, as in the reaction with hemes. Unfortunately, my experiments with buthionine sulphoximine (BSO), a classical inhibitor of glutathione synthesis, did not yield reliable results but need to be repeated. We could also take advantage of the CRISPR technique (Shin *et al.* 2016; Kao *et al.* 2017) to produce GSNOR1 and GSNOR2 deficient strains or use strong promoters to generate overexpressing strains, in order to observe what happens when the endogenous nitrosylation levels are perturbed. GSNOR is the only enzyme capable of metabolize GSNO. It catalyzes the NADH-dependent transformation of GSNO into GSSG and ammonia. Since GSNOR can regulate GSNO cellular homeostasis, it indirectly controls the levels of protein nitrosothiols. Its importance has been demonstrated in mouse and in Arabidopsis, where knocking out GSNOR results in a high level of nitrosylated proteins, indicating that GSNO is in equilibrium with a pool of SNO-proteins (Liu *et al.* 2001; Liu *et al.* 2004; Feechan *et al.* 2005; Chaki *et al.* 2011). Conversely, GSNOR overexpression was found to decrease protein nitrosylation levels (Lin *et al.* 2012). We hypothesize that the signal should be perturbed in this type of strains and may have an impact on protein degradation and photosynthesis inactivation.

## V.2 A light switch based on protein s-nitrosylation fine-tunes photosynthetic light harvesting in *Chlamydomonas reinhardtii*

We have identified a novel type of redox regulation, involving again nitric oxide and nitrosylation. This regulatory mechanism fine-tunes the light harvesting capability of *Chlamydomonas* in response to fluctuating light conditions.

NO modifies through nitrosylation a specific protein, NAB1, at the level of cysteine 226. This event decreases the affinity of the protein toward LHCBM6/4 transcripts and, hence, its translation repressor activity. Interestingly, this nitrosylation seems to be constitutive, an unusual event, since for most proteins it normally occurs after a redox perturbation. As described previously (Benhar *et al.* 2008; Benhar 2015; Zaffagnini *et al.* 2016), constitutively nitrosylated proteins are rare (less than 10% of total targets) and preferentially denitrosylated via an alternate system: the thioredoxin system (constituted by TRX-NTR-NADPH). The vast majority of nitrosylated targets are conversely denitrosylated by GSH, which is considered as the main denitrosylating system in all organisms (Zaffagnini *et al.* 2016). In this work, we have obtained solid data which demonstrate how denitrosylation is performed by the cytosolic TRXh1, corroborating the hypothesis of constitutive nitrosylation, and making NAB1 one of the few examples of constitutive nitrosylated protein.

In a scenario where it is universally accepted that antennas tend to be bigger under low light and smaller under high light (Croce *et al.* 2014; Rochaix 2014), we propose a model in which NAB1 is nitrosylated under low light, being inactive and allowing translation of LHCBMs. In this situation the antenna gets bigger and ensures more efficient light harvesting. Conversely, when the light intensity gets higher, NAB1 is denitrosylated via TRXh1, which in turn is kept reduced by NTR using the reducing power of NADPH. Denitrosylated NAB1 becomes thus active and sequesters LHCBM mRNAs in ribonucleoprotein complexes, leading to a decrease of LHCII size. This efficient denitrosylation of NAB1 by the cytosolic TRX system could be considered as a fast strategy, acting in parallel with gene expression regulation, to adjust the activity state to the light conditions, in relation with the intracellular redox state.

However, our data did not provide insights into the mechanisms enabling the redox cross talk between chloroplast and cytosol. Some questions remain open for future studies.

For instance, how alterations of the cytosolic NADPH/NADP<sup>+</sup> ratio might be connected to photosynthetic activity in the chloroplast? We know many hypotheses regarding the retrograde signaling which may link the light capture capacity to the central metabolism of the cell and to the gene expression machinery (Pfannschmidt 2003; Pfannschmidt 2010). Nevertheless, among the putative players suggested, we believe that the malate-oxaloacetate shuttle could be a reasonable candidate for inter-compartment crosstalk (Scheibe 2004). Under elevated light conditions, light absorption capability overwhelms the carbon fixation rate and NADPH accumulates in the chloroplast. The excess of NADPH can be used to convert oxaloacetate into malate that can shuttle between chloroplast and cytosol and, after the export, is converted back into oxaloacetate with formation of NADH. In addition to the malate shuttle, triose phosphate metabolites produced by the CBC could also contribute to the inter-compartment crosstalk. Indeed, the cytosol contains a non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase (GAPN) that oxidizes G3P directly to 3-phosphoglycerate and NADPH (Rius *et al.* 2006; Zaffagnini *et al.* 2013). These shuttles could alter the redox state of the cytosol by increasing both the NADH/NAD<sup>+</sup> and NADPH/NADP<sup>+</sup> ratio, which would result in increased availability of reduced TRXh1 for the denitrosylation of NAB1. For these reasons we envisage future experiments to better understand the inter-compartment crosstalk as a message to modify NAB1 activity in response to light conditions.

Another question arising from our data is where does the NO comes from? and how can its production be regulated? We can hypothesize the existence of a pathway that ensures a basal (or minimal) accumulation of nitric oxide responsible for constitutive level of nitrosylation. However, data in this sense are still missing and a better understanding of NO synthesis pathways will probably be required to understand their contribution to the level of endogenous nitrosylation. Moreover, it is possible that alternative mechanisms of NO production could be responsible either for basal production of NO, or for a temporally well-defined and spatially regulated NO burst, activated in response to perturbation of the redox state. Nevertheless, it is realistic to speculate about the existence of alternative nitrosylases in *Chlamydomonas*, which would be responsible for a precise and constitutive basal nitrosylation. Such nitrosylases (*e.g.* TRXs or cytosolic GAPDH) are well known in other organisms and their role in the nitrosylation of particular targets has been established, as discussed in the previous section (IV.1).

In the future, it would be interesting to clarify the light-dependency of NAB1 nitrosylation. Namely, it could be interesting to better understand if there is a light-intensity threshold, above which NAB1 gets activated by TRXh1, or if the process is more gradual and follows strictly the light intensity fluctuations. A strategy to obtain these data would be to test NAB1 nitrosylation levels *in vivo* at different light intensities but will require a proteomic method more robust and reliable than the BST.

## VI General outlook

Living organisms in general, and photoautotrophs in particular, have evolved complex signaling networks to sense the environment and activate responses that lead to acclimation. In the case of photosynthesis, this complex network and its operation acquire particular importance since they are involved in the regulation of biomass and oxygen production, providing the basis of life on Earth. Despite our improving knowledge of signaling molecules, post-translational modifications, gene circuits and signaling cascades, our understanding of their interaction is still incomplete and further progresses are required to fully elucidate how the responses are integrated at the cellular level.

The “*omic*” era provides new tools to analyze these interactions, but further improvements are required to understand the links between environment, genome, proteome and metabolome modifications. For instance the use of global analytical approaches combined with innovative mathematical computation and bioinformatics could provide information to reconstitute the entire signaling network and the interconnections between each pathway, thereby understanding globally the mechanisms put in place by the cell to respond to a given stimulus. For the specific case of redox signaling in photoautotrophs, and at the level of photosynthesis regulation particularly, despite the great steps forward achieved in the last decade, we still don’t know how it is tuned and how it can be modulated in relation to other well characterized signals. Thus in the future it will be important to establish how, when, and through which players redox signaling is integrated in the stress response, understanding unequivocally its role.

On a larger scale, such an integrated view of photosynthesis regulation in response to abiotic stresses will provide tools to deeply understand the interaction between photoautotrophs and the environment, but also to understand the effect of human activity on ecosystems. This knowledge will be crucial to produce innovation in agricultural techniques, reducing their impact on ecosystems while improving qualitatively and quantitatively their production. Innovation is required because we will need to provide food to growing populations, in a planet where arable land area is decreasing, consumed by human development and desert extension, where water accumulation becomes critical, and where pollution levels are frightening.

Finally, a global view of regulation at the cellular level could provide fundamental knowledge exploitable for future biotechnological applications. Indeed “green technology” will be crucial to respond to other future challenges, as the conversion of conventional industry into a “green industry”, where, in order to reduce CO<sub>2</sub> emissions and pollution, we “grow” the products we need through synthetic biology processes instead of producing them with obsolete and energy consuming technologies, exploiting non-renewable resources, with all the environmental consequences we all know. This is also true for renewable energy production or biofuel production, technologies that will not be definitive solutions to the “sustainable development”, but will surely be helpful in the transition towards a postindustrial society, with sensitive lower footprint.

## VII Bibliography

- Adam, Z. and O. Ostersetzer (2001). "Degradation of unassembled and damaged thylakoid proteins." Biochem Soc Trans **29**(Pt 4): 427-430.
- Adam, Z., A. Rudella, et al. (2006). "Recent advances in the study of Clp, FtsH and other proteases located in chloroplasts." Curr Opin Plant Biol **9**(3): 234-240.
- Albertsson, P. (2001). "A quantitative model of the domain structure of the photosynthetic membrane." Trends Plant Sci **6**(8): 349-358.
- Albertsson, P. A. (1995). "The structure and function of the chloroplast photosynthetic membrane - a model for the domain organization." Photosynth Res **46**(1-2): 141-149.
- Allahverdiyeva, Y., H. Mustila, et al. (2013). "Flavodiiron proteins Flv1 and Flv3 enable cyanobacterial growth and photosynthesis under fluctuating light." Proc Natl Acad Sci U S A **110**(10): 4111-4116.
- Allahverdiyeva, Y., M. Suorsa, et al. (2015). "Photoprotection of photosystems in fluctuating light intensities." J Exp Bot **66**(9): 2427-2436.
- Allen, J. F. (1992). "Protein phosphorylation in regulation of photosynthesis." Biochim Biophys Acta **1098**(3): 275-335.
- Allen, J. F. (2004). "Cytochrome b6f: structure for signalling and vectorial metabolism." Trends Plant Sci **9**(3): 130-137.
- Alric, J. and X. Johnson (2017). "Alternative electron transport pathways in photosynthesis: a confluence of regulation." Curr Opin Plant Biol **37**: 78-86.
- Amtmann, A. and P. Armengaud (2009). "Effects of N, P, K and S on metabolism: new knowledge gained from multi-level analysis." Curr Opin Plant Biol **12**(3): 275-283.
- Andersson, I. and A. Backlund (2008). "Structure and function of Rubisco." Plant Physiol Biochem **46**(3): 275-291.
- Antal, T. K., T. E. Krendeleva, et al. (2003). "The dependence of algal H<sub>2</sub> production on Photosystem II and O<sub>2</sub> consumption activities in sulfur-deprived *Chlamydomonas reinhardtii* cells." Biochim Biophys Acta **1607**(2-3): 153-160.
- Antao, C. M. and F. X. Malcata (2005). "Plant serine proteases: biochemical, physiological and molecular features." Plant Physiol Biochem **43**(7): 637-650.
- Aoki, M., N. Sato, et al. (2004). "Differing involvement of sulfoquinovosyl diacylglycerol in photosystem II in two species of unicellular cyanobacteria." Eur J Biochem **271**(4): 685-693.
- Arnon, D. I. (1959). "Conversion of light into chemical energy in photosynthesis." Nature **184**: 10-21.
- Aro, E. M. (2016). "From first generation biofuels to advanced solar biofuels." Ambio **45** Suppl 1: S24-31.
- Aro, E. M., M. Suorsa, et al. (2005). "Dynamics of photosystem II: a proteomic approach to thylakoid protein complexes." J Exp Bot **56**(411): 347-356.
- Aro, E. M., I. Virgin, et al. (1993). "Photoinhibition of Photosystem II. Inactivation, protein damage and turnover." Biochim Biophys Acta **1143**(2): 113-134.
- Arsova, B., U. Hoja, et al. (2010). "Plastidial thioredoxin z interacts with two fructokinase-like proteins in a thiol-dependent manner: evidence for an essential role in chloroplast development in *Arabidopsis* and *Nicotiana benthamiana*." Plant Cell **22**(5): 1498-1515.
- Asada, K. (1999). "THE WATER-WATER CYCLE IN CHLOROPLASTS: Scavenging of Active Oxygens and Dissipation of Excess Photons." Annu Rev Plant Physiol Plant Mol Biol **50**: 601-639.
- Asada, K. (2006). "Production and scavenging of reactive oxygen species in chloroplasts and their functions." Plant Physiol **141**(2): 391-396.
- Baalman, E., R. Scheibe, et al. (1996). "Functional studies of chloroplast glyceraldehyde-3-phosphate dehydrogenase subunits A and B expressed in *Escherichia coli*: formation of highly active A4 and B4 homotetramers and evidence that aggregation of the B4 complex is mediated by the B subunit carboxy terminus." Plant Mol Biol **32**(3): 505-513.

- Badger, M. R., S. von Caemmerer, et al. (2000). "Electron flow to oxygen in higher plants and algae: rates and control of direct photoreduction (Mehler reaction) and rubisco oxygenase." Philos Trans R Soc Lond B Biol Sci **355**(1402): 1433-1446.
- Bailey, S., A. Melis, et al. (2008). "Alternative photosynthetic electron flow to oxygen in marine *Synechococcus*." Biochim Biophys Acta **1777**(3): 269-276.
- Ballicora, M. A., J. B. Frueauf, et al. (2000). "Activation of the potato tuber ADP-glucose pyrophosphorylase by thioredoxin." J Biol Chem **275**(2): 1315-1320.
- Ballottari, M., L. Dall'Osto, et al. (2007). "Contrasting behavior of higher plant photosystem I and II antenna systems during acclimation." J Biol Chem **282**(12): 8947-8958.
- Bariola, P. A., C. J. Howard, et al. (1994). "The *Arabidopsis* ribonuclease gene RNS1 is tightly controlled in response to phosphate limitation." Plant J **6**(5): 673-685.
- Barroso, J. B., F. J. Corpas, et al. (1999). "Localization of nitric-oxide synthase in plant peroxisomes." J Biol Chem **274**(51): 36729-36733.
- Basler, G., A. Kuken, et al. (2016). "Photorespiratory Bypasses Lead to Increased Growth in *Arabidopsis thaliana*: Are Predictions Consistent with Experimental Evidence?" Front Bioeng Biotechnol **4**: 31.
- Bassham, J. A. (1971). "The control of photosynthetic carbon metabolism." Science **172**(3983): 526-534.
- Bassham, J. A., A. A. Benson, et al. (1950). "The path of carbon in photosynthesis." J Biol Chem **185**(2): 781-787.
- Baudouin, E., L. Pieuchot, et al. (2006). "Nitric oxide is formed in *Medicago truncatula*-*Sinorhizobium meliloti* functional nodules." Mol Plant Microbe Interact **19**(9): 970-975.
- Baurle, I. (2016). "Plant Heat Adaptation: priming in response to heat stress." F1000Res **5**.
- Beligni, M. V. and L. Lamattina (2000). "Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants." Planta **210**(2): 215-221.
- Bellafore, S., F. Barneche, et al. (2005). "State transitions and light adaptation require chloroplast thylakoid protein kinase STN7." Nature **433**(7028): 892-895.
- Bellafore, S., P. Ferris, et al. (2002). "Loss of *Albino3* leads to the specific depletion of the light-harvesting system." Plant Cell **14**(9): 2303-2314.
- Benhar, M. (2015). "Nitric oxide and the thioredoxin system: a complex interplay in redox regulation." Biochim Biophys Acta **1850**(12): 2476-2484.
- Benhar, M., M. T. Forrester, et al. (2008). "Regulated protein denitrosylation by cytosolic and mitochondrial thioredoxins." Science **320**(5879): 1050-1054.
- Berger, H., O. Blifernez-Klassen, et al. (2014). "Integration of carbon assimilation modes with photosynthetic light capture in the green alga *Chlamydomonas reinhardtii*." Mol Plant **7**(10): 1545-1559.
- Berthold, T., E. D. von Gromoff, et al. (2012). "Exploring the electron transfer pathways in photosystem I by high-time-resolution electron paramagnetic resonance: observation of the B-side radical pair P700(+)/A1B(-) in whole cells of the deuterated green alga *Chlamydomonas reinhardtii* at cryogenic temperatures." J Am Chem Soc **134**(12): 5563-5576.
- Besson-Bard, A., A. Gravot, et al. (2009). "Nitric oxide contributes to cadmium toxicity in *Arabidopsis* by promoting cadmium accumulation in roots and by up-regulating genes related to iron uptake." Plant Physiol **149**(3): 1302-1315.
- Bohrer, A. S., V. Massot, et al. (2012). "New insights into the reduction systems of plastidial thioredoxins point out the unique properties of thioredoxin z from *Arabidopsis*." J Exp Bot **63**(18): 6315-6323.
- Bonaventura, C. and J. Myers (1969). "Fluorescence and oxygen evolution from *Chlorella pyrenoidosa*." Biochim Biophys Acta **189**(3): 366-383.
- Bottcher, B. and P. Graber (2000). "The structure of the H(+)-ATP synthase from chloroplasts and its subcomplexes as revealed by electron microscopy." Biochim Biophys Acta **1458**(2-3): 404-416.

- Brandes, H. K., F. W. Larimer, et al. (1996). "The molecular pathway for the regulation of phosphoribulokinase by thioredoxin f." *J Biol Chem* **271**(7): 3333-3335.
- Briantais, J. M., C. Vernet, et al. (1979). "A quantitative study of the slow decline of chlorophyll a fluorescence in isolated chloroplasts." *Biochim Biophys Acta* **548**(1): 128-138.
- Buchanan, B. B. (1991). "Regulation of CO<sub>2</sub> assimilation in oxygenic photosynthesis: the ferredoxin/thioredoxin system. Perspective on its discovery, present status, and future development." *Arch Biochem Biophys* **288**(1): 1-9.
- Buchanan, B. B., A. Holmgren, et al. (2012). "Fifty years in the thioredoxin field and a bountiful harvest." *Biochim Biophys Acta* **1820**(11): 1822-1829.
- Buchanan, B. B., P. P. Kalberer, et al. (1967). "Ferredoxin-activated fructose diphosphatase in isolated chloroplasts." *Biochem Biophys Res Commun* **29**(1): 74-79.
- Buchanan, B. B., P. Schürmann, et al. (1971). "Ferredoxin-activated fructose diphosphatase of spinach chloroplasts. Resolution of the system, properties of the alkaline fructose diphosphatase component, and physiological significance of the ferredoxin-linked activation." *J Biol Chem* **246**(19): 5952-5959.
- Bulte, L. and F. A. Wollman (1990). Mechanism and control of the inactivation of cytochrome *b6/f* complexes during gametogenesis in *C. reinhardtii*. *Current Research in Photosynthesis*. M. Baltscheffsky. **3**: 715-718.
- Bulte, L. and F. A. Wollman (1992). "Evidence for a selective destabilization of an integral membrane protein, the cytochrome *b6/f* complex, during gametogenesis in *Chlamydomonas reinhardtii*." *Eur J Biochem* **204**(1): 327-336.
- Burgess, S. J., B. Tamburic, et al. (2011). "Solar-driven hydrogen production in green algae." *Adv Appl Microbiol* **75**: 71-110.
- Cardol, P., B. Bailleul, et al. (2008). "An original adaptation of photosynthesis in the marine green alga *Ostreococcus*." *Proc Natl Acad Sci U S A* **105**(22): 7881-7886.
- Cardona, T., A. Sedoud, et al. (2012). "Charge separation in photosystem II: a comparative and evolutionary overview." *Biochim Biophys Acta* **1817**(1): 26-43.
- Carfagna, S., C. Bottone, et al. (2016). "Impact of Sulfur Starvation in Autotrophic and Heterotrophic Cultures of the Extremophilic Microalga *Galdieria phlegrea* (Cyanidiophyceae)." *Plant Cell Physiol* **57**(9): 1890-1898.
- Carfagna, S., G. Salbitani, et al. (2015). "Cross-Effects of Nitrogen and Sulphur Starvation in *Chlorella sorokiniana* 211/8K." *Natural Resources* **6**: 221-229.
- Cavalier-Smith, T. (1975). "Electron and light microscopy of gametogenesis and gamete fusion in *Chlamydomonas reinhardtii*." *Protoplasma* **86**(1-3): 1-18.
- Chaki, M., R. Valderrama, et al. (2011). "Mechanical wounding induces a nitrosative stress by down-regulation of GSNO reductase and an increase in S-nitrosothiols in sunflower (*Helianthus annuus*) seedlings." *J Exp Bot* **62**(6): 1803-1813.
- Chamizo-Ampudia, A., E. Sanz-Luque, et al. (2016). "A dual system formed by the ARC and NR molybdoenzymes mediates nitrite-dependent NO production in *Chlamydomonas*." *Plant Cell Environ* **39**(10): 2097-2107.
- Checchetto, V., E. Teardo, et al. (2013). "Regulation of photosynthesis by ion channels in cyanobacteria and higher plants." *Biophys Chem* **182**: 51-57.
- Chiadmi, M., A. Navaza, et al. (1999). "Redox signalling in the chloroplast: structure of oxidized pea fructose-1,6-bisphosphate phosphatase." *EMBO J* **18**(23): 6809-6815.
- Chibani, K., L. Tarrago, et al. (2011). "Biochemical properties of poplar thioredoxin z." *FEBS Lett* **585**(7): 1077-1081.
- Chitnis, P. R. (2001). "PHOTOSYSTEM I: Function and Physiology." *Annu Rev Plant Physiol Plant Mol Biol* **52**: 593-626.
- Claeys, H. and D. Inze (2013). "The agony of choice: how plants balance growth and survival under water-limiting conditions." *Plant Physiol* **162**(4): 1768-1779.
- Corpas, F. J., J. B. Barroso, et al. (2004). "Cellular and subcellular localization of endogenous nitric oxide in young and senescent pea plants." *Plant Physiol* **136**(1): 2722-2733.

- Corpas, F. J., J. B. Barroso, et al. (2001). "Peroxisomes as a source of reactive oxygen species and nitric oxide signal molecules in plant cells." *Trends Plant Sci* **6**(4): 145-150.
- Courteille, A., S. Vesa, et al. (2013). "Thioredoxin m4 controls photosynthetic alternative electron pathways in Arabidopsis." *Plant Physiol* **161**(1): 508-520.
- Couvertier, S. M., Y. Zhou, et al. (2014). "Chemical-proteomic strategies to investigate cysteine posttranslational modifications." *Biochim Biophys Acta* **1844**(12): 2315-2330.
- Cramer, W. A. and H. Zhang (2006). "Consequences of the structure of the cytochrome b6 complex for its charge transfer pathways." *Biochim Biophys Acta* **1757**(5-6): 339-345.
- Croce, R. and H. van Amerongen (2014). "Natural strategies for photosynthetic light harvesting." *Nat Chem Biol* **10**(7): 492-501.
- Cueto, M., O. Hernandez-Perera, et al. (1996). "Presence of nitric oxide synthase activity in roots and nodules of *Lupinus albus*." *FEBS Lett* **398**(2-3): 159-164.
- Dai, S., R. Friemann, et al. (2007). "Structural snapshots along the reaction pathway of ferredoxin-thioredoxin reductase." *Nature* **448**(7149): 92-96.
- DalCorso, G., P. Pesaresi, et al. (2008). "A complex containing PGRL1 and PGR5 is involved in the switch between linear and cyclic electron flow in Arabidopsis." *Cell* **132**(2): 273-285.
- Daniell, H., C. S. Lin, et al. (2016). "Chloroplast genomes: diversity, evolution, and applications in genetic engineering." *Genome Biol* **17**(1): 134.
- de Hostos, E. L., R. K. Togasaki, et al. (1988). "Purification and biosynthesis of a derepressible periplasmic arylsulfatase from *Chlamydomonas reinhardtii*." *J Cell Biol* **106**(1): 29-37.
- de Montaigu, A., E. Sanz-Luque, et al. (2010). "A soluble guanylate cyclase mediates negative signaling by ammonium on expression of nitrate reductase in *Chlamydomonas*." *Plant Cell* **22**(5): 1532-1548.
- Delledonne, M., Y. Xia, et al. (1998). "Nitric oxide functions as a signal in plant disease resistance." *Nature* **394**(6693): 585-588.
- Dellero, Y., M. Jossier, et al. (2016). "Decreased glycolate oxidase activity leads to altered carbon allocation and leaf senescence after a transfer from high CO<sub>2</sub> to ambient air in *Arabidopsis thaliana*." *J Exp Bot* **67**(10): 3149-3163.
- Dent, R. M., M. Han, et al. (2001). "Functional genomics of plant photosynthesis in the fast lane using *Chlamydomonas reinhardtii*." *Trends Plant Sci* **6**(8): 364-371.
- Depege, N., S. Bellafiore, et al. (2003). "Role of chloroplast protein kinase Stt7 in LHCII phosphorylation and state transition in *Chlamydomonas*." *Science* **299**(5612): 1572-1575.
- Desplats, C., F. Mus, et al. (2009). "Characterization of Nda2, a plastoquinone-reducing type II NAD(P)H dehydrogenase in *Chlamydomonas* chloroplasts." *J Biol Chem* **284**(7): 4148-4157.
- Domingos, P., A. M. Prado, et al. (2015). "Nitric oxide: a multitasked signaling gas in plants." *Mol Plant* **8**(4): 506-520.
- Doulias, P. T., J. L. Greene, et al. (2010). "Structural profiling of endogenous S-nitrosocysteine residues reveals unique features that accommodate diverse mechanisms for protein S-nitrosylation." *Proc Natl Acad Sci U S A* **107**(39): 16958-16963.
- Drapier, D., J. Girard-Bascou, et al. (1992). "Evidence for Nuclear Control of the Expression of the atpA and atpB Chloroplast Genes in *Chlamydomonas*." *Plant Cell* **4**(3): 283-295.
- Dunford, R. P., M. A. Catley, et al. (1998). "Purification of active chloroplast sedoheptulose-1,7-bisphosphatase expressed in *Escherichia coli*." *Protein Expr Purif* **14**(1): 139-145.
- Eberhard, S., G. Finazzi, et al. (2008). "The dynamics of photosynthesis." *Annu Rev Genet* **42**: 463-515.
- Erickson, E., S. Wakao, et al. (2015). "Light stress and photoprotection in *Chlamydomonas reinhardtii*." *Plant J* **82**(3): 449-465.
- Faccenda, A., C. A. Bonham, et al. (2010). "Gold nanoparticle enrichment method for identifying S-nitrosylation and S-glutathionylation sites in proteins." *J Am Chem Soc* **132**(33): 11392-11394.
- Feechan, A., E. Kwon, et al. (2005). "A central role for S-nitrosothiols in plant disease resistance." *Proc Natl Acad Sci U S A* **102**(22): 8054-8059.

- Feilke, K., Q. Yu, et al. (2014). "In vitro analysis of the plastid terminal oxidase in photosynthetic electron transport." *Biochim Biophys Acta* **1837**(10): 1684-1690.
- Fermani, S., F. Sparla, et al. (2007). "Molecular mechanism of thioredoxin regulation in photosynthetic A2B2-glyceraldehyde-3-phosphate dehydrogenase." *Proc Natl Acad Sci U S A* **104**(26): 11109-11114.
- Finazzi, G. and G. Forti (2004). "Metabolic Flexibility of the Green Alga *Chlamydomonas reinhardtii* as Revealed by the Link between State Transitions and Cyclic Electron Flow." *Photosynth Res* **82**(3): 327-338.
- Forestier, M., P. King, et al. (2003). "Expression of two [Fe]-hydrogenases in *Chlamydomonas reinhardtii* under anaerobic conditions." *Eur J Biochem* **270**(13): 2750-2758.
- Forti, G. and G. Caldiroli (2005). "State transitions in *Chlamydomonas reinhardtii*. The role of the Mehler reaction in state 2-to-state 1 transition." *Plant Physiol* **137**(2): 492-499.
- Foyer, C. H., A. J. Bloom, et al. (2009). "Photorespiratory metabolism: genes, mutants, energetics, and redox signaling." *Annu Rev Plant Biol* **60**: 455-484.
- Foyer, C. H., J. Neukermans, et al. (2012). "Photosynthetic control of electron transport and the regulation of gene expression." *J Exp Bot* **63**(4): 1637-1661.
- Foyer, C. H. and G. Noctor (2009). "Redox regulation in photosynthetic organisms: signaling, acclimation, and practical implications." *Antioxid Redox Signal* **11**(4): 861-905.
- Foyer, C. H. and G. Noctor (2011). "Ascorbate and glutathione: the heart of the redox hub." *Plant Physiol* **155**(1): 2-18.
- Foyer, C. H. and G. Noctor (2013). "Redox signaling in plants." *Antioxid Redox Signal* **18**(16): 2087-2090.
- Gallaher, S. D., S. T. Fitz-Gibbon, et al. (2015). "Chlamydomonas Genome Resource for Laboratory Strains Reveals a Mosaic of Sequence Variation, Identifies True Strain Histories, and Enables Strain-Specific Studies." *Plant Cell* **27**(9): 2335-2352.
- Garcia-Mata, C. and L. Lamattina (2001). "Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress." *Plant Physiol* **126**(3): 1196-1204.
- Geigenberger, P., A. Kolbe, et al. (2005). "Redox regulation of carbon storage and partitioning in response to light and sugars." *J Exp Bot* **56**(416): 1469-1479.
- Genty, B., J. Harbinson, et al. (1990). "The relationship between non-photochemical quenching of chlorophyll fluorescence and the rate of photosystem 2 photochemistry in leaves." *Photosynthesis Research* **25**(3): 249-257.
- Georgakopoulos, J. H., A. Sokolenko, et al. (2002). "Proteolytic activity against the light-harvesting complex and the D1/D2 core proteins of Photosystem II in close association to the light-harvesting complex II trimer." *Biochim Biophys Acta* **1556**(1): 53-64.
- Gerotto, C., A. Alboresi, et al. (2016). "Flavodiiron proteins act as safety valve for electrons in *Physcomitrella patens*." *Proc Natl Acad Sci U S A* **113**(43): 12322-12327.
- Ghirardi, M. L., L. Zhang, et al. (2000). "Microalgae: a green source of renewable H<sub>2</sub>." *Trends Biotechnol* **18**(12): 506-511.
- Gibbs, D. J., N. Md Isa, et al. (2014). "Nitric oxide sensing in plants is mediated by proteolytic control of group VII ERF transcription factors." *Mol Cell* **53**(3): 369-379.
- Gillet, J. N. (2015). "Ultrafast molecular dynamics of biofuel extraction for microalgae and bacteria milking: blocking membrane folding pathways to damaged lipid-bilayer conformations with nanomicelles." *J Biomol Struct Dyn* **33**(4): 690-705.
- Gohre, V., F. Ossenbuhl, et al. (2006). "One of two alb3 proteins is essential for the assembly of the photosystems and for cell survival in *Chlamydomonas*." *Plant Cell* **18**(6): 1454-1466.
- Golldack, D., C. Li, et al. (2014). "Tolerance to drought and salt stress in plants: Unraveling the signaling networks." *Front Plant Sci* **5**: 151.
- Graziano, M., M. V. Beligni, et al. (2002). "Nitric oxide improves internal iron availability in plants." *Plant Physiol* **130**(4): 1852-1859.
- Greiner, A., S. Kelterborn, et al. (2017). "Targeting of Photoreceptor Genes in *Chlamydomonas reinhardtii* via Zinc-Finger Nucleases and CRISPR/Cas9." *Plant Cell* **29**(10): 2498-2518.

- Grossman, A. (2000). "Acclimation of *Chlamydomonas reinhardtii* to its nutrient environment." Protist **151**(3): 201-224.
- Grossman, A. R. (2000). "Chlamydomonas reinhardtii and photosynthesis: genetics to genomics." Curr Opin Plant Biol **3**(2): 132-137.
- Grossman, A. R., C. Catalanotti, et al. (2011). "Multiple facets of anoxic metabolism and hydrogen production in the unicellular green alga *Chlamydomonas reinhardtii*." New Phytol **190**(2): 279-288.
- Grossman, A. R., M. Croft, et al. (2007). "Novel metabolism in *Chlamydomonas* through the lens of genomics." Curr Opin Plant Biol **10**(2): 190-198.
- Gutle, D. D., T. Roret, et al. (2016). "Chloroplast FBPase and SBPase are thioredoxin-linked enzymes with similar architecture but different evolutionary histories." Proc Natl Acad Sci U S A **113**(24): 6779-6784.
- Hansch, R. and R. R. Mendel (2009). "Physiological functions of mineral micronutrients (Cu, Zn, Mn, Fe, Ni, Mo, B, Cl)." Curr Opin Plant Biol **12**(3): 259-266.
- Harris, E. (1989). The Chlamydomonas sourcebook. San Diego, Academic Press.
- Harris, E. H. (1989). The Chlamydomonas source book: a comprehensive guide to biology and laboratory use. San Diego, Academic Press.
- He, Y., R. H. Tang, et al. (2004). "Nitric oxide represses the Arabidopsis floral transition." Science **305**(5692): 1968-1971.
- Hejazi, M. A. and R. H. Wijffels (2004). "Milking of microalgae." Trends Biotechnol **22**(4): 189-194.
- Hemschemeier, A., M. Duner, et al. (2013). "Hypoxic survival requires a 2-on-2 hemoglobin in a process involving nitric oxide." Proc Natl Acad Sci U S A **110**(26): 10854-10859.
- Hemschemeier, A., S. Fouchard, et al. (2008). "Hydrogen production by *Chlamydomonas reinhardtii*: an elaborate interplay of electron sources and sinks." Planta **227**(2): 397-407.
- Hill, R. and F. A. Y. Bendall (1960). "Function of the Two Cytochrome Components in Chloroplasts: A Working Hypothesis." Nature **186**(4719): 136-137.
- Holmgren, A. (1985). "Thioredoxin." Annu Rev Biochem **54**: 237-271.
- Horton, P., A. V. Ruban, et al. (1996). "Regulation of Light Harvesting in Green Plants." Annu Rev Plant Physiol Plant Mol Biol **47**: 655-684.
- Houille-Vernes, L., F. Rappaport, et al. (2011). "Plastid terminal oxidase 2 (PTOX2) is the major oxidase involved in chlororespiration in *Chlamydomonas*." Proc Natl Acad Sci U S A **108**(51): 20820-20825.
- Irihimovitch, V. and S. Yehudai-Resheff (2008). "Phosphate and sulfur limitation responses in the chloroplast of *Chlamydomonas reinhardtii*." FEMS Microbiol Lett **283**(1): 1-8.
- Jacquot, J. P., J. Lopez-Jaramillo, et al. (1995). "High-level expression of recombinant pea chloroplast fructose-1,6-bisphosphatase and mutagenesis of its regulatory site." Eur J Biochem **229**(3): 675-681.
- Jacquot, J. P., J. Lopez-Jaramillo, et al. (1997). "Cysteine-153 is required for redox regulation of pea chloroplast fructose-1,6-bisphosphatase." FEBS Lett **401**(2-3): 143-147.
- Jarvi, S., M. Suorsa, et al. (2015). "Photosystem II repair in plant chloroplasts--Regulation, assisting proteins and shared components with photosystem II biogenesis." Biochim Biophys Acta **1847**(9): 900-909.
- Jensen, P. E. and D. Leister (2014). "Chloroplast evolution, structure and functions." F1000Prime Rep **6**: 40.
- John Andrews, T. and S. M. Whitney (2003). "Manipulating ribulose bisphosphate carboxylase/oxygenase in the chloroplasts of higher plants." Arch Biochem Biophys **414**(2): 159-169.
- Johnson, G. N. (2005). "Cyclic electron transport in C3 plants: fact or artefact?" J Exp Bot **56**(411): 407-416.
- Joliot, P., D. Beal, et al. (2004). "Cyclic electron flow under saturating excitation of dark-adapted Arabidopsis leaves." Biochim Biophys Acta **1656**(2-3): 166-176.

- Joliot, P. and A. Joliot (2006). "Cyclic electron flow in C3 plants." Biochimica et Biophysica Acta (BBA) - Bioenergetics **1757**(5–6): 362-368.
- Kao, P. H. and I. S. Ng (2017). "CRISPRi mediated phosphoenolpyruvate carboxylase regulation to enhance the production of lipid in *Chlamydomonas reinhardtii*." Bioresour Technol.
- Kato, Y., X. Sun, et al. (2012). "Cooperative D1 degradation in the photosystem II repair mediated by chloroplastic proteases in *Arabidopsis*." Plant Physiol **159**(4): 1428-1439.
- Khan, M. S. (2007). "Engineering photorespiration in chloroplasts: a novel strategy for increasing biomass production." Trends Biotechnol **25**(10): 437-440.
- Kleine, T. and D. Leister (2016). "Retrograde signaling: Organelles go networking." Biochim Biophys Acta **1857**(8): 1313-1325.
- Klimyuk, V. I., F. Persello-Cartieaux, et al. (1999). "A chromodomain protein encoded by the *Arabidopsis* CAO gene is a plant-specific component of the chloroplast signal recognition particle pathway that is involved in LHCP targeting." Plant Cell **11**(1): 87-99.
- Koharyova, M. and M. Kolarova (2008). "Oxidative stress and thioredoxin system." Gen Physiol Biophys **27**(2): 71-84.
- Komenda, J., R. Sobotka, et al. (2012). "Assembling and maintaining the Photosystem II complex in chloroplasts and cyanobacteria." Curr Opin Plant Biol **15**(3): 245-251.
- Kopriva, S. and H. Rennenberg (2004). "Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism." J Exp Bot **55**(404): 1831-1842.
- Kornberg, M. D., N. Sen, et al. (2010). "GAPDH mediates nitrosylation of nuclear proteins." Nat Cell Biol **12**(11): 1094-1100.
- Koroban, N. V., A. V. Kudryavtseva, et al. (2016). "[The role of microRNA in abiotic stress response in plants]." Mol Biol (Mosk) **50**(3): 387-394.
- Kosourov, S., M. Seibert, et al. (2003). "Effects of extracellular pH on the metabolic pathways in sulfur-deprived, H<sub>2</sub>-producing *Chlamydomonas reinhardtii* cultures." Plant Cell Physiol **44**(2): 146-155.
- Kramer, D. M., T. J. Avenson, et al. (2004). "Dynamic flexibility in the light reactions of photosynthesis governed by both electron and proton transfer reactions." Trends Plant Sci **9**(7): 349-357.
- Krieger-Liszkay, A., P. B. Kos, et al. (2011). "Superoxide anion radicals generated by methylviologen in photosystem I damage photosystem II." Physiol Plant **142**(1): 17-25.
- Ku, M. S., R. K. Monson, et al. (1983). "Photosynthetic Characteristics of C(3)-C(4) Intermediate *Flaveria* Species : I. Leaf Anatomy, Photosynthetic Responses to O<sub>2</sub> and CO<sub>2</sub>, and Activities of Key Enzymes in the C(3) and C(4) Pathways." Plant Physiol **71**(4): 944-948.
- Kulheim, C., J. Agren, et al. (2002). "Rapid regulation of light harvesting and plant fitness in the field." Science **297**(5578): 91-93.
- Kuras, R. and F. A. Wollman (1994). "The assembly of cytochrome b6/f complexes: an approach using genetic transformation of the green alga *Chlamydomonas reinhardtii*." Embo J **13**(5): 1019-1027.
- Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(5259): 680-685.
- Laisk, A., H. Eichelmann, et al. (2007). "Rates and roles of cyclic and alternative electron flow in potato leaves." Plant Cell Physiol **48**(11): 1575-1588.
- Laloi, C. and M. Havaux (2015). "Key players of singlet oxygen-induced cell death in plants." Front Plant Sci **6**: 39.
- Lee, U., C. Wie, et al. (2008). "Modulation of nitrosative stress by S-nitrosoglutathione reductase is critical for thermotolerance and plant growth in *Arabidopsis*." Plant Cell **20**(3): 786-802.
- Lemaire, S. D., L. Michelet, et al. (2007). "Thioredoxins in chloroplasts." Curr Genet **51**(6): 343-365.
- Leustek, T. and K. Saito (1999). "Sulfate transport and assimilation in plants." Plant Physiol **120**(3): 637-644.
- Lichtenthaler, H. K. (1999). "The 1-Deoxy-D-Xylulose-5-Phosphate Pathway of Isoprenoid Biosynthesis in Plants." Annu Rev Plant Physiol Plant Mol Biol **50**: 47-65.

- Lin, A., Y. Wang, et al. (2012). "Nitric oxide and protein S-nitrosylation are integral to hydrogen peroxide-induced leaf cell death in rice." *Plant Physiol* **158**(1): 451-464.
- Lindahl, M., A. Mata-Cabana, et al. (2011). "The disulfide proteome and other reactive cysteine proteomes: analysis and functional significance." *Antioxid Redox Signal* **14**(12): 2581-2642.
- Lindermayr, C., S. Sell, et al. (2010). "Redox regulation of the NPR1-TGA1 system of *Arabidopsis thaliana* by nitric oxide." *Plant Cell* **22**(8): 2894-2907.
- Liu, L., A. Hausladen, et al. (2001). "A metabolic enzyme for S-nitrosothiol conserved from bacteria to humans." *Nature* **410**(6827): 490-494.
- Liu, L., Y. Yan, et al. (2004). "Essential roles of S-nitrosothiols in vascular homeostasis and endotoxic shock." *Cell* **116**(4): 617-628.
- Löffler, A., K. Glund, et al. (1993). "Amino acid sequence of an intracellular, phosphate-starvation-induced ribonuclease from cultured tomato (*Lycopersicon esculentum*) cells." *Eur J Biochem* **214**(3): 627-633.
- Maia, L. B. and J. J. Moura (2011). "Nitrite reduction by xanthine oxidase family enzymes: a new class of nitrite reductases." *J Biol Inorg Chem* **16**(3): 443-460.
- Majeran, W., F. A. Wollman, et al. (2000). "Evidence for a role of ClpP in the degradation of the chloroplast cytochrome b(6)f complex." *Plant Cell* **12**(1): 137-150.
- Malnoe, A., F. Wang, et al. (2014). "Thylakoid FtsH protease contributes to photosystem II and cytochrome b6f remodeling in *Chlamydomonas reinhardtii* under stress conditions." *Plant Cell* **26**(1): 373-390.
- Martin, N. C. and U. W. Goodenough (1975). "Gametic differentiation in *Chlamydomonas reinhardtii*. I. Production of gametes and their fine structure." *J Cell Biol* **67**(3): 587-605.
- Matsuda, Y. and B. Colman (1996). "A New Screening Method for Algal Photosynthetic Mutants (CO<sub>2</sub>-Insensitive Mutants of the Green Alga *Chlorella ellipsoidea*)." *Plant Physiol* **110**(4): 1283-1291.
- Matsuo, T. and M. Ishiura (2011). "*Chlamydomonas reinhardtii* as a new model system for studying the molecular basis of the circadian clock." *FEBS Lett* **585**(10): 1495-1502.
- Maul, J. E., J. W. Lilly, et al. (2002). "The *Chlamydomonas reinhardtii* plastid chromosome: islands of genes in a sea of repeats." *Plant Cell* **14**(11): 2659-2679.
- Maxwell, K. and G. N. Johnson (2000). "Chlorophyll fluorescence--a practical guide." *J Exp Bot* **51**(345): 659-668.
- Melis, A., L. Zhang, et al. (2000). "Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*." *Plant Physiol* **122**(1): 127-136.
- Mendez-Alvarez, S., U. Leisinger, et al. (1999). "Adaptive responses in *Chlamydomonas reinhardtii*." *Int Microbiol* **2**(1): 15-22.
- Merchant, S. S. and J. D. Helmann (2012). "Elemental economy: microbial strategies for optimizing growth in the face of nutrient limitation." *Adv Microb Physiol* **60**: 91-210.
- Merchant, S. S., S. E. Prochnik, et al. (2007). "The *Chlamydomonas* genome reveals the evolution of key animal and plant functions." *Science* **318**(5848): 245-250.
- Michelet, L., M. Zaffagnini, et al. (2013). "Redox regulation of the Calvin-Benson cycle: something old, something new." *Front Plant Sci* **4**: 470.
- Michelet, L., M. Zaffagnini, et al. (2008). "In vivo targets of S-thiolation in *Chlamydomonas reinhardtii*." *J Biol Chem* **283**(31): 21571-21578.
- Mikkelsen, R., K. E. Mutenda, et al. (2005). "Alpha-glucan, water dikinase (GWD): a plastidic enzyme with redox-regulated and coordinated catalytic activity and binding affinity." *Proc Natl Acad Sci U S A* **102**(5): 1785-1790.
- miles, D. (1980). [1] Mutants of higher plants: Maize. *Methods in Enzymology*. P. Anthony San, Academic Press. **Volume 69**: 3-23.
- Minagawa, J. and R. Tokutsu (2015). "Dynamic regulation of photosynthesis in *Chlamydomonas reinhardtii*." *Plant J* **82**(3): 413-428.

- Minoda, A., K. Sonoike, et al. (2003). "Decrease in the efficiency of the electron donation to tyrosine Z of photosystem II in an SQDG-deficient mutant of *Chlamydomonas*." *FEBS Lett* **553**(1-2): 109-112.
- Mishina, T. E., C. Lamb, et al. (2007). "Expression of a nitric oxide degrading enzyme induces a senescence programme in *Arabidopsis*." *Plant Cell Environ* **30**(1): 39-52.
- Mitchell, P. (1961). "Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism." *Nature* **191**: 144-148.
- Mitchell, P. (1966). "Chemiosmotic coupling in oxidative and photosynthetic phosphorylation." *Biol Rev Camb Philos Soc* **41**(3): 445-502.
- Mitra, M., H. Kirst, et al. (2012). "Modulation of the light-harvesting chlorophyll antenna size in *Chlamydomonas reinhardtii* by TLA1 gene over-expression and RNA interference." *Philos Trans R Soc Lond B Biol Sci* **367**(1608): 3430-3443.
- Mitra, M. and A. Melis (2010). "Genetic and biochemical analysis of the TLA1 gene in *Chlamydomonas reinhardtii*." *Planta* **231**(3): 729-740.
- Morisse, S., L. Michelet, et al. (2014). "Thioredoxin-dependent redox regulation of chloroplastic phosphoglycerate kinase from *Chlamydomonas reinhardtii*." *J Biol Chem* **289**(43): 30012-30024.
- Morisse, S., M. Zaffagnini, et al. (2014). "Insight into Protein S-nitrosylation in *Chlamydomonas reinhardtii*." *Antioxid Redox Signal* **21**(9): 1271-1284.
- Motohashi, K. and T. Hisabori (2006). "HCF164 receives reducing equivalents from stromal thioredoxin across the thylakoid membrane and mediates reduction of target proteins in the thylakoid lumen." *J Biol Chem* **281**(46): 35039-35047.
- Motohashi, K. and T. Hisabori (2010). "CcdA is a thylakoid membrane protein required for the transfer of reducing equivalents from stroma to thylakoid lumen in the higher plant chloroplast." *Antioxid Redox Signal* **13**(8): 1169-1176.
- Murata, N. (1969). "Control of excitation transfer in photosynthesis. I. Light-induced change of chlorophyll a fluorescence in *Porphyridium cruentum*." *Biochim Biophys Acta* **172**(2): 242-251.
- Mussgnug, J. H. (2015). "Genetic tools and techniques for *Chlamydomonas reinhardtii*." *Appl Microbiol Biotechnol* **99**(13): 5407-5418.
- Mussgnug, J. H., L. Wobbe, et al. (2005). "NAB1 is an RNA binding protein involved in the light-regulated differential expression of the light-harvesting antenna of *Chlamydomonas reinhardtii*." *Plant Cell* **17**(12): 3409-3421.
- Nakamura, T. and S. A. Lipton (2013). "Emerging role of protein-protein transnitrosylation in cell signaling pathways." *Antioxid Redox Signal* **18**(3): 239-249.
- Nandha, B., G. Finazzi, et al. (2007). "The role of PGR5 in the redox poisoning of photosynthetic electron transport." *Biochim Biophys Acta* **1767**(10): 1252-1259.
- Nawrocki, W. J., N. J. Tourasse, et al. (2015). "The plastid terminal oxidase: its elusive function points to multiple contributions to plastid physiology." *Annu Rev Plant Biol* **66**: 49-74.
- Nee, G., M. Zaffagnini, et al. (2009). "Redox regulation of chloroplastic glucose-6-phosphate dehydrogenase: a new role for f-type thioredoxin." *FEBS Lett* **583**(17): 2827-2832.
- Neill, S. J., R. Desikan, et al. (2002). "Hydrogen peroxide and nitric oxide as signalling molecules in plants." *J Exp Bot* **53**(372): 1237-1247.
- Ninnemann, H. and J. Maier (1996). "Indications for the occurrence of nitric oxide synthases in fungi and plants and the involvement in photoconidiation of *Neurospora crassa*." *Photochem Photobiol* **64**(2): 393-398.
- Nixon, P. J., F. Michoux, et al. (2010). "Recent advances in understanding the assembly and repair of photosystem II." *Ann Bot* **106**(1): 1-16.
- Obata, T. and A. R. Fernie (2012). "The use of metabolomics to dissect plant responses to abiotic stresses." *Cell Mol Life Sci* **69**(19): 3225-3243.
- Okegawa, Y. and K. Motohashi (2015). "Chloroplastic thioredoxin m functions as a major regulator of Calvin cycle enzymes during photosynthesis in vivo." *Plant J* **84**(5): 900-913.

- Ort, D. R. and N. R. Baker (2002). "A photoprotective role for O<sub>2</sub> as an alternative electron sink in photosynthesis?" *Curr Opin Plant Biol* **5**(3): 193-198.
- Ouellet, H., Y. Ouellet, et al. (2002). "Truncated hemoglobin HbN protects *Mycobacterium bovis* from nitric oxide." *Proc Natl Acad Sci U S A* **99**(9): 5902-5907.
- Pagnussat, G. C., M. Simontacchi, et al. (2002). "Nitric oxide is required for root organogenesis." *Plant Physiol* **129**(3): 954-956.
- Parankusam, S., S. S. Adimulam, et al. (2017). "Nitric Oxide (NO) in Plant Heat Stress Tolerance: Current Knowledge and Perspectives." *Front Plant Sci* **8**: 1582.
- Parry, M. A., A. J. Keys, et al. (2008). "Rubisco regulation: a role for inhibitors." *J Exp Bot* **59**(7): 1569-1580.
- Pearce, F. G. (2006). "Catalytic by-product formation and ligand binding by ribulose biphosphate carboxylases from different phylogenies." *Biochem J* **399**(3): 525-534.
- Pedersen, T. A., M. Kirk, et al. (1966). "Light-dark transients in levels of intermediate compounds during photosynthesis in air-adapted *Chlorella*." *Physiol Plant* **19**(1): 219-231.
- Peers, G., T. B. Truong, et al. (2009). "An ancient light-harvesting protein is critical for the regulation of algal photosynthesis." *Nature* **462**(7272): 518-521.
- Perazzolli, M., P. Dominici, et al. (2004). "Arabidopsis nonsymbiotic hemoglobin AHb1 modulates nitric oxide bioactivity." *Plant Cell* **16**(10): 2785-2794.
- Perazzolli, M., M. C. Romero-Puertas, et al. (2006). "Modulation of nitric oxide bioactivity by plant haemoglobins." *J Exp Bot* **57**(3): 479-488.
- Perez-Perez, M. E., A. Mauries, et al. (2017). "The Deep Thioresoxime in *Chlamydomonas reinhardtii*: New Insights into Redox Regulation." *Mol Plant* **10**(8): 1107-1125.
- Petersen, J., R. Teich, et al. (2006). "The GapA/B gene duplication marks the origin of Streptophyta (charophytes and land plants)." *Mol Biol Evol* **23**(6): 1109-1118.
- Petroutsos, D., R. Tokutsu, et al. (2016). "A blue-light photoreceptor mediates the feedback regulation of photosynthesis." *Nature* **537**(7621): 563-566.
- Pfannschmidt, T. (2003). "Chloroplast redox signals: how photosynthesis controls its own genes." *Trends Plant Sci* **8**(1): 33-41.
- Pfannschmidt, T. (2010). "Plastidial retrograde signalling--a true "plastid factor" or just metabolite signatures?" *Trends Plant Sci* **15**(8): 427-435.
- Pfundel, E. E. and R. A. Dilley (1993). "The pH Dependence of Violaxanthin Deepoxidation in Isolated Pea Chloroplasts." *Plant Physiol* **101**(1): 65-71.
- Picard-Bennoun, M. and P. Bennoun (1985). "Change in cytoplasmic ribosome properties during gametogenesis in the alga *Chlamydomonas reinhardtii*." *Curr Genet* **9**(3): 239-243.
- Pollock, S. V., W. Pootakham, et al. (2005). "Insights into the acclimation of *Chlamydomonas reinhardtii* to sulfur deprivation." *Photosynth Res* **86**(3): 475-489.
- Portis, A. R., Jr., C. Li, et al. (2008). "Regulation of Rubisco activase and its interaction with Rubisco." *J Exp Bot* **59**(7): 1597-1604.
- Prado, A. M., D. M. Porterfield, et al. (2004). "Nitric oxide is involved in growth regulation and re-orientation of pollen tubes." *Development* **131**(11): 2707-2714.
- Quisel, J. D., D. D. Wykoff, et al. (1996). "Biochemical characterization of the extracellular phosphatases produced by phosphorus-deprived *Chlamydomonas reinhardtii*." *Plant Physiology* **111**(3): 839-848.
- Raines, C. A. (2003). "The Calvin cycle revisited." *Photosynth Res* **75**(1): 1-10.
- Rasala, B. A., P. A. Lee, et al. (2012). "Robust expression and secretion of Xylanase1 in *Chlamydomonas reinhardtii* by fusion to a selection gene and processing with the FMDV 2A peptide." *PLoS ONE* **7**(8): e43349.
- Reisz, J. A., E. Bechtold, et al. (2013). "Thiol-blocking electrophiles interfere with labeling and detection of protein sulfenic acids." *Febs J* **280**(23): 6150-6161.
- Richter, M. L., H. S. Samra, et al. (2005). "Coupling proton movement to ATP synthesis in the chloroplast ATP synthase." *J Bioenerg Biomembr* **37**(6): 467-473.

- Rius, S. P., P. Casati, et al. (2006). "Characterization of an Arabidopsis thaliana mutant lacking a cytosolic non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase." Plant Mol Biol **61**(6): 945-957.
- Rochaix, J. D. (1995). "Chlamydomonas reinhardtii as the photosynthetic yeast." Annu Rev Genet **29**: 209-230.
- Rochaix, J. D. (2014). "Regulation and dynamics of the light-harvesting system." Annu Rev Plant Biol **65**: 287-309.
- Rochaix, J. D., S. P. Mayfield, et al. (1988). Plant Molecular Biology: A Practical Approach. IRL, Oxford.
- Rodolfi, L., G. Chini Zittelli, et al. (2009). "Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor." Biotechnol Bioeng **102**(1): 100-112.
- Rolland, N., A. Atteia, et al. (2009). "Chlamydomonas proteomics." Curr Opin Microbiol **12**(3): 285-291.
- Ruban, A. V., R. Berera, et al. (2007). "Identification of a mechanism of photoprotective energy dissipation in higher plants." Nature **450**(7169): 575-578.
- Sakihama, Y., S. Nakamura, et al. (2002). "Nitric oxide production mediated by nitrate reductase in the green alga Chlamydomonas reinhardtii: an alternative NO production pathway in photosynthetic organisms." Plant Cell Physiol **43**(3): 290-297.
- Salbitani, G., V. Vona, et al. (2015). "Sulfur Deprivation Results in Oxidative Perturbation in Chlorella sorokiniana (211/8k)." Plant Cell Physiol **56**(5): 897-905.
- Sato, A., R. Matsumura, et al. (2014). "Responsibility of regulatory gene expression and repressed protein synthesis for triacylglycerol accumulation on sulfur-starvation in Chlamydomonas reinhardtii." Front Plant Sci **5**: 444.
- Sato, N., K. Sonoike, et al. (1995). "Impaired photosystem II in a mutant of Chlamydomonas reinhardtii defective in sulfoquinovosyl diacylglycerol." Eur J Biochem **234**(1): 16-23.
- Scheibe, R. (1987). "NADP+-malate dehydrogenase in C3-plants: Regulation and role of a light-activated enzyme." Physiologia Plantarum **71**(3): 393-400.
- Scheibe, R. (2004). "Malate valves to balance cellular energy supply." Physiol Plant **120**(1): 21-26.
- Scheibe, R. and K. J. Dietz (2012). "Reduction-oxidation network for flexible adjustment of cellular metabolism in photoautotrophic cells." Plant Cell Environ **35**(2): 202-216.
- Schmollinger, S., T. Muhlhaus, et al. (2014). "Nitrogen-Sparing Mechanisms in Chlamydomonas Affect the Transcriptome, the Proteome, and Photosynthetic Metabolism." Plant Cell **26**(4): 1410-1435.
- Schreiner, Ø., T. Lien, et al. (1975). "The capacity for arylsulfatase synthesis in synchronous and synchronized cultures of Chlamydomonas reinhardtii." Biochimica et Biophysica Acta (BBA) - Enzymology **384**(1): 180-193.
- Schroda, M., D. Hemme, et al. (2015). "The Chlamydomonas heat stress response." Plant J **82**(3): 466-480.
- Schroter, Y., S. Steiner, et al. (2010). "Analysis of oligomeric protein complexes in the chloroplast sub-proteome of nucleic acid-binding proteins from mustard reveals potential redox regulators of plastid gene expression." Proteomics **10**(11): 2191-2204.
- Schuenemann, D., S. Gupta, et al. (1998). "A novel signal recognition particle targets light-harvesting proteins to the thylakoid membranes." Proc Natl Acad Sci U S A **95**(17): 10312-10316.
- Schürmann, P. and B. B. Buchanan (2008). "The ferredoxin/thioredoxin system of oxygenic photosynthesis." Antioxid Redox Signal **10**(7): 1235-1274.
- Sears, B. B., J. E. Boynton, et al. (1980). "The Effect of Gametogenesis Regimes on the Chloroplast Genetic System of CHLAMYDOMONAS REINHARDTII." Genetics **96**(1): 95-114.
- Sevilla, F., D. Camejo, et al. (2015). "The thioredoxin/peroxiredoxin/sulfiredoxin system: current overview on its redox function in plants and regulation by reactive oxygen and nitrogen species." J Exp Bot **66**(10): 2945-2955.
- Shin, S. E., J. M. Lim, et al. (2016). "CRISPR/Cas9-induced knockout and knock-in mutations in Chlamydomonas reinhardtii." Sci Rep **6**: 27810.

- Smagghe, B. J., J. T. Trent, 3rd, et al. (2008). "NO dioxygenase activity in hemoglobins is ubiquitous in vitro, but limited by reduction in vivo." *PLoS ONE* **3**(4): e2039.
- Sokolenko, A., E. Pojidaeva, et al. (2002). "The gene complement for proteolysis in the cyanobacterium *Synechocystis* sp. PCC 6803 and *Arabidopsis thaliana* chloroplasts." *Curr Genet* **41**(5): 291-310.
- Sparla, F., A. Costa, et al. (2006). "Redox regulation of a novel plastid-targeted beta-amylase of *Arabidopsis*." *Plant Physiol* **141**(3): 840-850.
- Sparla, F., P. Pupillo, et al. (2002). "The C-terminal extension of glyceraldehyde-3-phosphate dehydrogenase subunit B acts as an autoinhibitory domain regulated by thioredoxins and nicotinamide adenine dinucleotide." *J Biol Chem* **277**(47): 44946-44952.
- Stamler, J. S. and D. T. Hess (2010). "Nascent nitrosylases." *Nat Cell Biol* **12**(11): 1024-1026.
- Stroebel, D., Y. Choquet, et al. (2003). "An atypical haem in the cytochrome b(6)f complex." *Nature* **426**(6965): 413-418.
- Sugimoto, K., N. Sato, et al. (2007). "Utilization of a chloroplast membrane sulfolipid as a major internal sulfur source for protein synthesis in the early phase of sulfur starvation in *Chlamydomonas reinhardtii*." *FEBS Lett* **581**(23): 4519-4522.
- Sundberg, E., J. G. Slagter, et al. (1997). "ALBINO3, an *Arabidopsis* nuclear gene essential for chloroplast differentiation, encodes a chloroplast protein that shows homology to proteins present in bacterial membranes and yeast mitochondria." *Plant Cell* **9**(5): 717-730.
- Suvarna, Y., N. Maity, et al. (2016). "Emerging Trends in Retrograde Signaling." *Mol Neurobiol* **53**(4): 2572-2578.
- Tada, Y., S. H. Spoel, et al. (2008). "Plant immunity requires conformational changes [corrected] of NPR1 via S-nitrosylation and thioredoxins." *Science* **321**(5891): 952-956.
- Takahashi, H., S. Clowez, et al. (2013). "Cyclic electron flow is redox-controlled but independent of state transition." *Nat Commun* **4**: 1954.
- Tamoi, M., T. Ishikawa, et al. (1996). "Molecular characterization and resistance to hydrogen peroxide of two fructose-1,6-bisphosphatases from *Synechococcus* PCC 7942." *Arch Biochem Biophys* **334**(1): 27-36.
- Tamoi, M., M. Nagaoka, et al. (2006). "Contribution of fructose-1,6-bisphosphatase and sedoheptulose-1,7-bisphosphatase to the photosynthetic rate and carbon flow in the Calvin cycle in transgenic plants." *Plant Cell Physiol* **47**(3): 380-390.
- Tewari, R. K., P. Kumar, et al. (2009). "Nitric oxide retards xanthine oxidase-mediated superoxide anion generation in *Phalaenopsis* flower: an implication of NO in the senescence and oxidative stress regulation." *Plant Cell Rep* **28**(2): 267-279.
- Thieulin-Pardo, G., T. Remy, et al. (2015). "Phosphoribulokinase from *Chlamydomonas reinhardtii*: a Benson-Calvin cycle enzyme enslaved to its cysteine residues." *Mol Biosyst* **11**(4): 1134-1145.
- Tikhonov, A. N. (2014). "The cytochrome b6f complex at the crossroad of photosynthetic electron transport pathways." *Plant Physiol Biochem* **81**: 163-183.
- Tokutsu, R. and J. Minagawa (2013). "Energy-dissipative supercomplex of photosystem II associated with LHCSR3 in *Chlamydomonas reinhardtii*." *Proc Natl Acad Sci U S A* **110**(24): 10016-10021.
- Towbin, H., T. Staehelin, et al. (1979). "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications." *Proc Natl Acad Sci U S A* **76**(9): 4350-4354.
- Trost, P., S. Fermani, et al. (2006). "Thioredoxin-dependent regulation of photosynthetic glyceraldehyde-3-phosphate dehydrogenase: autonomous vs. CP12-dependent mechanisms." *Photosynth Res* **89**(2-3): 263-275.
- Tun, N. N., C. Santa-Catarina, et al. (2006). "Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings." *Plant Cell Physiol* **47**(3): 346-354.
- Turkina, M. V., J. Kargul, et al. (2006). "Environmentally modulated phosphoproteome of photosynthetic membranes in the green alga *Chlamydomonas reinhardtii*." *Mol Cell Proteomics* **5**(8): 1412-1425.

- Vallon, O., L. Bulte, et al. (1993). "Extensive accumulation of an extracellular L-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*." Eur J Biochem **215**(2): 351-360.
- Walters, R. G., A. V. Ruban, et al. (1994). "Higher plant light-harvesting complexes LHCIIa and LHCIIc are bound by dicyclohexylcarbodiimide during inhibition of energy dissipation." Eur J Biochem **226**(3): 1063-1069.
- Walters, R. G., F. Shephard, et al. (2003). "Identification of mutants of *Arabidopsis* defective in acclimation of photosynthesis to the light environment." Plant Physiol **131**(2): 472-481.
- Wang, B. L., X. Y. Tang, et al. (2010). "Nitric oxide is involved in phosphorus deficiency-induced cluster-root development and citrate exudation in white lupin." New Phytol **187**(4): 1112-1123.
- Wang, C., X. Chen, et al. (2017). "Artificial miRNA inhibition of phosphoenolpyruvate carboxylase increases fatty acid production in a green microalga *Chlamydomonas reinhardtii*." Biotechnol Biofuels **10**: 91.
- Wang, F., Y. Qi, et al. (2017). "The High Light Response and Redox Control of Thylakoid FtsH Protease in *Chlamydomonas reinhardtii*." Mol Plant **10**(1): 99-114.
- Wang, H. and M. Xian (2011). "Chemical methods to detect S-nitrosation." Curr Opin Chem Biol **15**(1): 32-37.
- Watanabe, M., H. M. Hubberten, et al. (2010). "General regulatory patterns of plant mineral nutrient depletion as revealed by serat quadruple mutants disturbed in cysteine synthesis." Mol Plant **3**(2): 438-466.
- Wei, L., B. Derrien, et al. (2014). "Nitric oxide-triggered remodeling of chloroplast bioenergetics and thylakoid proteins upon nitrogen starvation in *Chlamydomonas reinhardtii*." Plant Cell **26**(1): 353-372.
- Wei, L. L., B. Derrien, et al. (2014). "Nitric Oxide-Triggered Remodeling of Chloroplast Bioenergetics and Thylakoid Proteins upon Nitrogen Starvation in *Chlamydomonas reinhardtii*." Plant Cell **26**(1): 353-372.
- Wen, Z. Y. and F. Chen (2003). "Heterotrophic production of eicosapentaenoic acid by microalgae." Biotechnol Adv **21**(4): 273-294.
- Wendehenne, D., A. Pugin, et al. (2001). "Nitric oxide: comparative synthesis and signaling in animal and plant cells." Trends Plant Sci **6**(4): 177-183.
- Wenderoth, I., R. Scheibe, et al. (1997). "Identification of the cysteine residues involved in redox modification of plant plastidic glucose-6-phosphate dehydrogenase." J Biol Chem **272**(43): 26985-26990.
- Whitney, S. M., R. L. Houtz, et al. (2011). "Advancing our understanding and capacity to engineer nature's CO<sub>2</sub>-sequestering enzyme, Rubisco." Plant Physiol **155**(1): 27-35.
- Wingler, A., P. J. Lea, et al. (2000). "Photorespiration: metabolic pathways and their role in stress protection." Philos Trans R Soc Lond B Biol Sci **355**(1402): 1517-1529.
- Wobbe, L., O. Blifernéz, et al. (2009). "Cysteine modification of a specific repressor protein controls the translational status of nucleus-encoded LHCII mRNAs in *Chlamydomonas*." Proc Natl Acad Sci U S A **106**(32): 13290-13295.
- Wollman, F. A. (2001). "State transitions reveal the dynamics and flexibility of the photosynthetic apparatus." Embo J **20**(14): 3623-3630.
- Woloskiuk, R. A. and B. B. Buchanan (1978). "Activation of Chloroplast NADP-linked Glyceraldehyde-3-Phosphate Dehydrogenase by the Ferredoxin/Thioredoxin System." Plant Physiol **61**(4): 669-671.
- Wraight, C. A. and A. R. Crofts (1970). "Energy-dependent quenching of chlorophyll alpha fluorescence in isolated chloroplasts." Eur J Biochem **17**(2): 319-327.
- Wykoff, D. D., J. P. Davies, et al. (1998). "The regulation of photosynthetic electron transport during nutrient deprivation in *Chlamydomonas reinhardtii*." Plant Physiol **117**(1): 129-139.
- Xin, C. P., D. Tholen, et al. (2015). "The benefits of photorespiratory bypasses: how can they work?" Plant Physiol **167**(2): 574-585.

- Yamasaki, H. and M. F. Cohen (2006). "NO signal at the crossroads: polyamine-induced nitric oxide synthesis in plants?" *Trends Plant Sci* **11**(11): 522-524.
- Yang, D., D. Song, et al. (2015). "Lipidomic Analysis of *Chlamydomonas reinhardtii* under Nitrogen and Sulfur Deprivation." *PLoS ONE* **10**(9): e0137948.
- Yang, X. J. and P. M. Finnegan (2010). "Regulation of phosphate starvation responses in higher plants." *Ann Bot* **105**(4): 513-526.
- Yehudai-Resheff, S., S. L. Zimmer, et al. (2007). "Integration of chloroplast nucleic acid metabolism into the phosphate deprivation response in *Chlamydomonas reinhardtii*." *Plant Cell* **19**(3): 1023-1038.
- Zaffagnini, M., M. Bedhomme, et al. (2012). "The emerging roles of protein glutathionylation in chloroplasts." *Plant Sci* **185-186**: 86-96.
- Zaffagnini, M., M. Bedhomme, et al. (2012). "Redox regulation in photosynthetic organisms: focus on glutathionylation." *Antioxid Redox Signal* **16**(6): 567-586.
- Zaffagnini, M., M. De Mia, et al. (2016). "Protein S-nitrosylation in photosynthetic organisms: A comprehensive overview with future perspectives." *Biochim Biophys Acta* **1864**(8): 952-966.
- Zaffagnini, M., S. Fermani, et al. (2013). "Plant cytoplasmic GAPDH: redox post-translational modifications and moonlighting properties." *Front Plant Sci* **4**: 450.
- Zaffagnini, M., L. Michelet, et al. (2007). "The thioredoxin-independent isoform of chloroplastic glyceraldehyde-3-phosphate dehydrogenase is selectively regulated by glutathionylation." *FEBS J* **274**(1): 212-226.
- Zaffagnini, M., L. Michelet, et al. (2014). "High-resolution crystal structure and redox properties of chloroplastic triosephosphate isomerase from *Chlamydomonas reinhardtii*." *Mol Plant* **7**(1): 101-120.
- Zaffagnini, M., S. Morisse, et al. (2013). "Mechanisms of nitrosylation and denitrosylation of cytoplasmic glyceraldehyde-3-phosphate dehydrogenase from *Arabidopsis thaliana*." *J Biol Chem* **288**(31): 22777-22789.
- Zhang, L., T. Happe, et al. (2002). "Biochemical and morphological characterization of sulfur-deprived and H<sub>2</sub>-producing *Chlamydomonas reinhardtii* (green alga)." *Planta* **214**(4): 552-561.
- Zhang, L. P., S. K. Mehta, et al. (2008). "Copper-induced proline synthesis is associated with nitric oxide generation in *Chlamydomonas reinhardtii*." *Plant Cell Physiol* **49**(3): 411-419.
- Zhang, N. and A. R. Portis, Jr. (1999). "Mechanism of light regulation of Rubisco: a specific role for the larger Rubisco activase isoform involving reductive activation by thioredoxin-f." *Proc Natl Acad Sci U S A* **96**(16): 9438-9443.
- Zhang, N., P. Schurmann, et al. (2001). "Characterization of the regulatory function of the 46-kDa isoform of Rubisco activase from *Arabidopsis*." *Photosynth Res* **68**(1): 29-37.
- Zhang, P., M. Eisenhut, et al. (2012). "Operon flv4-flv2 provides cyanobacterial photosystem II with flexibility of electron transfer." *Plant Cell* **24**(5): 1952-1971.
- Zhang, Z., J. Shrager, et al. (2004). "Insights into the survival of *Chlamydomonas reinhardtii* during sulfur starvation based on microarray analysis of gene expression." *Eukaryot Cell* **3**(5): 1331-1348.
- Zhao, D. Y., Q. Y. Tian, et al. (2007). "Nitric oxide is involved in nitrate-induced inhibition of root elongation in *Zea mays*." *Ann Bot* **100**(3): 497-503.
- Zhu, L. D., Z. H. Li, et al. (2016). "Strategies for Lipid Production Improvement in Microalgae as a Biodiesel Feedstock." *Biomed Res Int* **2016**: 8792548.

**Titre :** titre (en français Rôle de l'oxyde nitrique dans le remodelage de l'appareil photosynthétique lors de stress abiotiques chez *Chlamydomonas reinhardtii*)

**Mots clés :** Acclimatation à la lumière, Carence en soufre, Nitrosylation, Oxyde nitrique, Photosynthèse, Régulation redox.

**Résumé :** La régulation de la photosynthèse est cruciale pour les organismes photoautotrophes et est habituellement opérée par la modulation de l'absorption de la lumière ou par la réorientation des électrons vers des puits alternatifs afin de redistribuer l'énergie entre plusieurs voies métaboliques. Parmi les différents mécanismes décrits, le remodelage de l'appareil photosynthétique est crucial dans des conditions de carences nutritives ou de fluctuations de la lumière. Il est bien connu que l'oxyde nitrique (NO) joue un rôle de signalisation dans de nombreuses réponses au stress abiotique, agissant comme second messenger et/ou modifiant les protéines cibles par des modifications post-traductionnelles redox. Sa participation a été récemment décrite au cours de la carence en azote chez *Chlamydomonas reinhardtii*. Ce travail se concentre sur le remodelage de l'appareil photosynthétique lors de la carence en soufre et lors des fluctuations de lumineuses chez *Chlamydomonas reinhardtii*, avec un intérêt particulier pour la voie de signalisation impliquée dans ces réponses. Tout d'abord, nous avons caractérisé la carence en soufre en conditions d'hétérotrophie ou de photoautotrophie. En faible lumière ou à l'obscurité, l'inactivation photosynthétique est obtenue grâce à la dégradation spécifique de la Rubisco et du cytochrome b6f et ne se produit qu'en présence de carbone réduit dans le milieu. Nous avons également montré une forte production de NO après le début de la carence, avec des sondes fluorescentes sensibles au NO visualisées par microscopie confocale. Nous fournissons des preuves pharmacologiques que la production de NO intracellulaire régit cette voie de dégradation.

Par ailleurs, nous fournissons des preuves claires de l'existence d'un circuit régulateur qui contrôle la traduction cytosolique du LHCII en réponse à des changements de quantité de lumière. Ce circuit nécessite la protéine de liaison à l'ARN cytosolique NAB1 pour réprimer la traduction de certains ARNm de LHCII. La nitrosylation spécifique de la Cys-226 diminue l'activité de NAB1 et a été démontrée *in vitro* et *in vivo*. La forme moins active et nitrosylée de NAB1 se trouve dans les cellules acclimatées à un apport de lumière limité, ce qui permet l'accumulation de protéines des antennes et la capture efficace de la lumière. En revanche, une intensité lumineuse plus élevée provoque la dénitrosylation de NAB1, activant ainsi la répression de la synthèse des protéines LHCII et diminuant ainsi la pression de la lumière au niveau du PSII. La dénitrosylation de NAB1 est efficacement réalisée par le système thiorédoxine cytosolique *in vitro*. À notre connaissance, NAB1 est le premier exemple de dénitrosylation induite par un stimulus dans le contexte de l'acclimatation photosynthétique. Dans l'ensemble, nos données suggèrent un rôle pivot pour la signalisation NO dans le contrôle des réponses au stress environnemental

**Title :** Titre en anglais) The Role of Nitric Oxide in the remodeling of the photosynthetic apparatus under abiotic stress in *Chlamydomonas reinhardtii*

**Keywords :** Nitric oxide; Sulfur starvation; Photosynthesis; Redox regulation; Nitrosylation; light acclimation.

**Abstract :** The regulation of photosynthesis is crucial for photoautotrophic organisms and is usually operated by the modulation of light absorption or by redirection of electrons towards alternative sinks, in order to redistribute energy among several metabolic pathways. Between different mechanisms described, the remodeling of the photosynthetic apparatus is crucial under conditions of nutrient starvation or light fluctuations. It is well known that nitric oxide (NO) plays a signaling role in many abiotic stress responses, acting as a second messenger and/or modifying target proteins through redox post translational modifications. Its involvement has been recently described during nitrogen starvation in *Chlamydomonas reinhardtii*. This work focuses on the remodeling of the photosynthetic apparatus upon sulfur starvation and light fluctuations in *Chlamydomonas reinhardtii*, with particular interest for the signaling pathway involved in the responses. First we characterized sulfur starvation under heterotrophy and photo-autotrophy. Photosynthetic inactivation under low light and darkness is achieved through specific degradation of Rubisco and cytochrome *b<sub>6</sub>f* and occurs only in the presence of reduced carbon in the medium. We have also shown a strong NO production after the onset of starvation, with NO-sensitive fluorescence probes visualized by confocal microscopy.

We provide pharmacological evidence that intracellular NO production governs this degradation pathway using NO scavengers, NO synthesis inhibitors and NO donors. Furthermore, we provide clear evidence for a regulatory circuit that controls cytosolic LHCII translation in response to light quantity changes. This circuit requires the cytosolic RNA-binding protein NAB1 to repress translation of certain LHCII mRNAs. Specific nitrosylation of Cys-226 decreases NAB1 activity and could be demonstrated *in vitro* and *in vivo*. The less active, nitrosylated form of NAB1 is found in cells acclimated to limiting light supply, which permits accumulation of light harvesting proteins and efficient light capture. In contrast, elevated light supply causes NAB1 denitrosylation, thereby activating the repression of light-harvesting protein synthesis and decreasing the light pressure at the level of PSII. Denitrosylation of NAB1 is efficiently performed by the cytosolic thioredoxin system *in vitro*. To our knowledge, NAB1 is the first example of stimulus-induced denitrosylation in the context of photosynthetic acclimation. Taken together, our data suggest a pivotal role for NO-signaling in the control of environmental stress responses