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# Light stress and photoprotection in green algae, mosses and diatoms

Giulio Rocco Stella

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# Light Stress and Photoprotection in Green algae, Mosses and Diatoms

Giulio R. Stella

Ph.D. thesis submitted to

*University of Verona*

and

*University Pierre et Marie Curie*

September 13<sup>th</sup>, 2016

## Abstract

The molecular bases of responses to light excess in photosynthetic organisms having different evolutionary histories and belonging to different lineages are still not completely characterized. Therefore I explored the functions of photoprotective antennae in green algae, mosses and diatoms, together with the role of the two xanthophyll cycles present in diatoms.

I studied the Light Harvesting Complex Stress-Related (LHCSR/LHCX) proteins in different organisms. In the green alga *Chlamydomonas reinhardtii*, LHCSR3 is a protein important for photoprotection. I used site-specific mutagenesis *in vivo* and *in vitro* and identified three residues of LHCSR3, exposed to the thylakoidal lumen, that can sense pH changes. These residues are essential for Non-Photochemical Quenching (NPQ) induction *in vivo* and are responsible for LHCSR3 activation in excessive light conditions (chapter 2).

In the moss *Physcomitrella patens* I studied the *in vitro* spectroscopic and quenching characteristics of different pigment-binding mutants of the protein LHCSR1, to understand how energy is transfer and thermally dissipate in this protein. I found that at least eight chlorophylls are bound to LHCSR1 and I identified the pigments with the lowest energy levels that could be the traps for energy transfer (chlorophylls A2 and A5, chapter 3).

LHCSRs in diatoms are named LHCXs, and in the model organism *Phaeodactylum tricorutum* I found that multiple abiotic stress signals converge to differently regulate the four isoforms of LHCXs, modulating proteins contents and providing a way to fine-tune light harvesting and photoprotection (chapter 4).

The other main driver of photoprotection in diatoms is the xanthophyll cycle. Two cycles are present in diatoms, the violaxanthin-zeaxanthin (also present in plants) and the diadinoxanthin-diatoxanthin one (specific to diatoms). In addition to that, *P. tricorutum* have expanded the protein families involved in this photoprotective process. I found that the the knock-down of VDR and VDL2 (putative violaxanthin de-epoxidase enzymes) leads to the accumulation of violaxanthin and zeaxanthin, and that this accumulation have a negative effect in the development of NPQ. This suggests that zeaxanthin does not participate in the enhancing of NPQ in diatoms and may even interferes with the action of diatoxanthin (chapter 5).

Thanks to these studies done on different organisms, we gained a deeper knowledge on the shared characteristics and on the peculiar features about photoprotection in green algae, mosses and diatoms.





Université Pierre et Marie Curie

Ecole doctorale 515 Complexité du vivant  
Laboratoire de Biologie Computationnelle et Quantitative

et

Università degli Studi di Verona

# Light Stress and Photoprotection in Green Algae, Mosses and Diatoms

Par Giulio Rocco Stella

Thèse de doctorat de Biologie

Dirigée par Dr. Angela Falciatore, Prof. Roberto Bassi et Dr. Fayza Daboussi

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## Light Stress and Photoprotection in Green Algae, Mosses and Diatoms

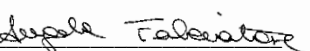
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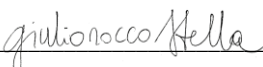
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Light Stress and Photoprotection in Green Algae, Mosses and Diatoms

Giulio Rocco Stella

Tesi di Dottorato

Parigi, 13 settembre 2016

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# Nomenclature

ATP	Adenosine triphosphate
ATPase	Chloroplastic ATP synthase
Ax	Antheraxanthin
CD	Circular Dichroism
Chl	Chlorophyll
CL	Control transgenic line
Cyt-b6f	Cytochrome-b6f
DD	Diadinoxanthin-diatoxanthin pool
Ddx	Diadinoxanthin
DES	De-epoxidation state
DGDG	Digalactosyldiacylglycerol
Dtx	Diatoxanthin
FCP	Fucoxanthin Chl a/c binding protein
Fx	Fucoxanthin
LHC	Light Harvesting Complex
LHCSR	Light Harvesting Complex Stress-Related protein
MGDG	Monogalactosyldiacylglycerol
NADPH	Nicotinammide adenina dinucleotide fosfato
NPQ	Non-photochemical quenching of chlorophyll fluorescence
Nx	Neoxanthin
PBR	Photobioreactors
PC	Phosphatidylcholine
PC	Plastocyanin

PSI	Photosystem I
PSII	Photosystem II
qP	Photochemical quenching
So	Electronic energetic ground state
SQDG	Sulfoquinovosyldiacylglycerol
TALENs™	Transcription activator-like endonucleases
VAZ	Violaxanthin-antheraxanthin-zeaxanthin pool
VDE	Violaxanthin de-epoxidase
VDL	Violaxanthin de-epoxidase like
VDR	Violaxanthin de-epoxidase related
Vx	Violaxanthin
XC	Xanthophyll Cycle
ZEP	Zeaxanthin epoxidase
Zx	Zeaxanthin

## Ph.D. context

Much of life on Earth depends on photosynthesis, a process in which solar light energy is used to produce oxygen and organic compounds from atmospheric CO<sub>2</sub> and water. Photosynthetic organisms with different evolutionary histories have developed different strategies to adapt their photosynthetic apparatus to changing light conditions and to optimize photosynthetic yield in different environments. In stressful light conditions, when the amount of photons absorbed by the chloroplast largely exceed the capacity for light utilization in photosynthesis, these organisms show very efficient photoprotective mechanisms, including Non-Photochemical Quenching (NPQ), a short-term photoprotective response allowing dissipation of the excess excitation energy as heat. The molecular bases of responses to light excess in photosynthetic organisms belonging to different evolutionary lineages are still not completely characterized. Therefore, the main aim of my Ph.D. was to address this important topic by exploring photoprotection mechanisms in three different model systems, representatives of green algae (*Chlamydomonas reinhardtii*), mosses (*Physcomitrella patens*) and diatoms (*Phaeodactylum tricornerutum*).

I started my Ph.D. in 2013 in Verona (Italy), in the laboratory of Prof. Roberto Bassi, working on the characterization of the *in vitro* properties of the LHCSR proteins from *C. reinhardtii* and *P. patens*. Because of my strong interest to work in the applied research field and to perform a research experience abroad, after nine months I grabbed the chance to join a European Marie Curie Initial Training Network (ITN) named AccliPhot, devoted to study the acclimation of photosynthesis. Here I initially worked in Paris with one of the partners of the AccliPhot consortium, the private company Collectis S.A., under the supervision of Dr. Fayza Daboussi (and co-supervised by Dr. Angela Falciatore, from UPMC, and Prof. Roberto Bassi, from Verona).

In 2010, Collectis set up a research team to focus on diatoms, with the aim of improving the genetic resources for diatom gene functional studies. In particular novel targeted nucleases approaches for diatom genomic engineering would have been optimized and used to gather information about the regulators of diatom photoprotective mechanisms. Therefore, in the following months of my Ph.D., I developed engineered site-specific endonucleases, notably Transcription Activator-Like Effector Nucleases (TALENs<sup>TM</sup>), to knock-out genes involved in photoprotection in diatoms.

Unfortunately, Collectis decided to stop the project on diatoms and microalgae in mid 2014, after just six months I had started. I thus joined the lab of Dr. Angela Falciatore, at the University of Paris VI (UPMC), and we decided to continue the project using an already established reverse genetic approaches (RNAi and over-expression) to study genes involved in photoprotection<sup>1</sup>.

My “final” Ph.D. is co-tutored between the University of Verona and the University of Paris VI (UPMC), under the co-supervision of Prof. Bassi, Dr. Falciatore and Dr. Daboussi.

In spite of all these adversities and adjustments, my Ph.D. project was still devoted, as at the very beginning, to the study of photosynthesis and its regulation. In particular, I focused on the characterization of photoprotective antennae in green algae, mosses and diatoms, and on the functions of the two xanthophyll cycles present in diatoms, that are still largely unknown. This research activity engaged strong collaborations between two research units: the team of Dr. Angela Falciatore (UPMC, Paris, FR), expert of genetics and genomics applied to the study of light regulation in diatoms, and the team of Prof. Roberto Bassi (Univ. of Verona, IT), expert in biochemistry of membrane proteins, physiology and biophysics of photosynthetic organisms.

In line with the aim of better understand photoprotection in green algae, mosses and diatoms,

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<sup>1</sup>After an agreement between UPMC and Collectis, the TALENs<sup>TM</sup> I developed are now available, but this project is now re-starting, thus results regarding this initial part of my Ph.D. are not present in the thesis.

I studied Light Harvesting Complex Stress-Related (LHCSR) proteins in all these organisms (with the results presented in chapters 2, 3 and 4) and the role of the two xanthophyll cycles in diatoms (chapter 5). More detailed information about the main goals of my work and on the organization of this thesis are presented in the paragraph “Aim of the thesis”, just after the general introduction in chapter 1, while conclusions and future perspectives can be found in chapter 6.

## Résumé de la thèse (Français)

Une grande partie de la vie sur la Terre dépend de la photosynthèse, un processus au cours duquel l'énergie lumineuse solaire est utilisée pour produire de l'oxygène et des composés organiques à partir du CO<sub>2</sub> atmosphérique. Les organismes photosynthétiques, vivant dans des environnements variables, ont développé différentes stratégies pour adapter leur appareil photosynthétique au changement de conditions lumineuses afin d'optimiser leur rendement photosynthétique. Dans des conditions de lumière stressantes, lorsque la quantité de photons absorbés par le chloroplaste dépasse largement la capacité d'utilisation de la lumière par la photosynthèse, ces organismes montrent des mécanismes photoprotectifs très efficaces, dont le Quenching Non-Photochimique (NPQ), une réponse photoprotective à court terme permettant la dissipation de l'énergie d'excitation en excès sous forme de chaleur.

Les bases moléculaires des réponses aux excès de lumière dans les différents organismes photosynthétiques ayant des histoires évolutives et appartenant à différentes lignées ne sont toujours pas complètement caractérisées. Le but de mon projet de thèse était de fournir de nouvelles informations sur la photosynthèse et sa régulation dynamique dans des conditions de luminosité changeantes, en mettant l'accent sur la caractérisation des antennes photoprotectives dans les algues vertes, les mousses et les diatomées. De plus, je voulais aussi explorer la fonction de deux cycles de xanthophylles dans les diatomées, qui sont encore largement inconnus.

Le projet de Ph.D., co-encadré entre l'Université de Vérone et de l'Université de Paris VI (UPMC), est développé dans le cadre d'une Marie Curie Initial Training Network (ITN) nommé AccliPhot et consacrée à l'étude de l'acclimatation de la photosynthèse à des changements de lumière et à d'autres changements de conditions environnementales chez les plantes et les algues. Elle implique une collaboration interdisciplinaire en utilisant des approches expérimentales et théoriques complémentaires (<http://www.accliphot.eu/>). L'activité de recherche engage des collaborations fortes entre les deux unités de recherche : l'équipe du Dr. Angela Falciatore (directeur de thèse, UPMC, Paris, FR), expert de la génétique et de la génomique appliquée à l'étude de la régulation de la lumière chez les diatomées, et l'équipe du Prof. Roberto Bassi (co-superviseur, Univ. de Vérone, IT), expert en biochimie des protéines membranaires, en physiologie et biophysique des organismes photosynthétiques.

Au début du projet, une société privée, Collectis S.A., était impliquée en tant que troisième partenaire, sous la coordination du Dr. Fayza Daboussi. En 2010, Collectis a mis en place un groupe de recherche sur les diatomées, dans le but d'améliorer les ressources génétiques pour des études fonctionnelles. En particulier, de nouvelles approches, en ingénierie ciblée du génome chez les diatomées, devaient être optimisées et utilisées pour recueillir des informations sur la régulation des mécanismes de photoprotection chez ces organismes. Par conséquent, dans les premiers mois de ma thèse, j'ai développé une endonucléase à site spécifique (Transcription Activator-Like Effector Nucleases (TALENs<sup>TM</sup>)), pour déléter des gènes codant des protéines potentiellement impliquées dans la photoprotection des diatomées. Malheureusement, Collectis a décidé d'arrêter le projet sur les microalgues et les diatomées en 2014, et mon sujet initial a été modifié pour utiliser des approches plus traditionnelles afin d'étudier ces gènes, telles que la stratégie anti-sens et la surexpression. Après un accord entre l'UPMC et Collectis, le TALENs<sup>TM</sup> que j'ai développé est maintenant disponible, mais n'a pas pu être utilisé dans le cadre de mon doctorat.

Pour mieux comprendre la photoprotection dans les algues vertes, les mousses et les diatomées, j'ai étudié les protéines Light Harvesting Complex Stress-Related (LHCSR) dans tous ces organismes. Dans les algues vertes, en particulier chez *Chlamydomonas reinhardtii*, LHCSR3 est une protéine importante pour le NPQ. L'activation du NPQ nécessite un pH bas dans le lumen des thylakoïdes, qui est induit dans des conditions d'excès de lumière et qui est détecté par les



protéines LHCSR grâce à des résidus acides exposés au lumen. Dans le chapitre 2 j'ai muté par une approche de mutagenèse spécifique différents résidus afin d'identifier les résidus de LHCSR3 responsables de la détection du pH. Les résidus protonables exposés au lumen, aspartate et glutamate, ont été mutés en asparagine et glutamine, respectivement. La surexpression de ces protéines mutées dans une souche de *C. reinhardtii*, dépourvue de toutes les isoformes de LHCSR a permis d'identifier les résidus Asp<sup>117</sup>, Glu<sup>221</sup>, et Glu<sup>224</sup> comme essentiels pour l'induction du NPQ dépendant de LHCSR3. L'analyse des protéines recombinantes portant les mêmes mutations, repliées *in vitro* avec des pigments, a permis de montrer que la capacité de répondre à un pH faible par réduction de la durée de vie de fluorescence présente dans la protéine de type sauvage, a été perdue chez la protéine mutée. Cela indique que les acides aminés identifiés sont en effet important pour l'activation du NPQ par LHCSR3.

A la différence du rôle solitaire des LHCSRs dans les algues vertes, le NPQ dans le modèle de mousse *Physcomitrella patens*, est sous le contrôle de deux protéines : PSBS, présente également dans les plantes supérieures, et des LHCSRs. PSBS ne lie pas les pigments et son action dans la photoprotection est probablement due à son interaction avec d'autres antennes du photosystème II. Au contraire, la protéine LHCSR1 lie des pigments et a été montré comme le principal moteur du NPQ chez *P. patens*, notamment après la liaison de la xanthophylle zéaxanthine, suggérant que cette protéine peut être directement impliquée dans le processus de quenching. Cependant, pour comprendre les caractéristiques précises de transfert d'énergie et de quenching de LHCSR1, l'organisation exacte et la distance entre les chromophores est nécessaire. Les structures cristallines des protéines-antennes n'ont pas la résolution suffisante pour obtenir toutes ces informations, cependant le rôle des différents chromophores a été étudié dans le passé à l'aide de mutant de fixation d'antennes, ne possédant pas les résidus spécifiques qui coordonnent chaque pigment. Dans le chapitre 3, je montre les résultats *in vitro* de spectroscopie ainsi que les caractéristiques de quenching de différents mutants de liaison de pigment de la protéine LHCSR1 de *P. patens*. Nous avons muté chaque résidu liant la chlorophylle et analysé les protéines reconstituées à la fois par absorption et par fluorescence. En particulier, nous avons concentré notre attention sur les chlorophylles A2 et A5, qui sont censées être impliquées dans les mécanismes de quenching induits par l'interaction avec les caroténoïdes dans les sites L1 et L2.

Les protéines LHCSR sont également présentes dans les diatomées, où ils sont nommés LHCXs. Les diatomées sont des organismes phytoplanctoniques qui poussent avec succès dans l'océan, où les conditions de lumière sont très variables. Des études sur les mécanismes moléculaires de l'acclimatation à la lumière dans la diatomée marine *Phaeodactylum tricornerutum* montrent que les enzymes de de-époxydation des caroténoïdes et LHCX1 sont les deux facteurs principaux qui contribuent à dissiper l'énergie de la lumière en excès au travers du NPQ. Dans le chapitre 4, j'ai étudié le rôle des protéines de la famille LHCX dans les diatomées en réponse au stress. L'analyse des données génomiques disponibles montre que la présence de plusieurs gènes LHCX est une caractéristique conservée des espèces de diatomées vivant dans des niches écologiques différentes. En outre, une analyse des niveaux de transcriptions, de l'accumulation des protéines et de l'activité photosynthétique indique que les LHCXs sont régulées en fonction de l'intensité de lumière, de la disponibilité en nutriments et qu'elles modulent la capacité NPQ chez *P. tricornerutum*. Nous avons pu conclure que des signaux multiples de stress abiotiques convergent pour réguler la teneur en LHCX des cellules, fournissant un moyen d'affiner la collecte de la lumière et la photoprotection. En outre, nos données indiquent que l'expansion de la famille des gènes LHCX reflète une diversification fonctionnelle dont pourraient bénéficier des cellules répondant à des environnements marins très variables.

L'autre acteur principal de la photoprotection dans les diatomées est l'accumulation de la xanthophylle de-époxyde la diatoxanthine. Le cycle de xanthophylles, la conversion de la violaxanthine (Vx) en zéaxanthine (Zx) dans des conditions de forte luminosité, est l'un des acteur

principal du NPQ dans les plantes. Les diatomées possèdent également un deuxième cycle de xanthophylle permettant la conversion de la diadinoxanthine (Ddx) en diatoxanthine (Dtx) dans des conditions de forte luminosité. La concentration de Dtx est strictement corrélée au niveau NPQ, et le rôle réel de la Zx dans la photoprotection dans les diatomées a été mis en doute. Pour comprendre la régulation et la fonction de ces deux cycles de xanthophylle, dans le chapitre 5 j'ai dérégulé chacun des gènes putatifs codant des protéines potentiellement impliquées dans ce processus chez *Phaeodactylum tricornerutum*. J'ai concentré mon attention sur deux protéines, VDR et VDL2, qui ont été prédites comme des dé-époxydase permettant la conversion de la Vx en Zx et de la Ddx en Dtx. J'ai trouvé que les mutants knock-down pour ces deux protéines accumulent plus de xanthophylles du pool Vx-Zx et moins du pool Ddx-Dtx que la souche sauvage, indiquant un rôle de VDR et VDL2 dans la voie de biosynthèse des xanthophylles. La plus grande quantité de Vx et Zx eu un effet négatif dans le développement du NPQ dans les mutants knock-down, montrant que la Zx ne participe pas au renforcement du NPQ des diatomées.

Grâce à ces études effectuées sur les différents organismes, nous avons acquis une connaissance plus approfondie sur les caractéristiques communes et sur les caractéristiques particulières de la photoprotection chez les algues vertes, les mousses et les diatomées. L'ensemble de ces résultats est discuté dans le chapitre 6.

## Riassunto della tesi (Italiano)

Gran parte della vita sulla Terra dipende dalla fotosintesi, un processo in cui l'energia della luce solare è utilizzata per produrre ossigeno e composti organici a partire dalla CO<sub>2</sub> atmosferica e dall'acqua. Gli organismi fotosintetici che vivono in ambienti diversi hanno sviluppato diverse strategie per adattare il loro apparato fotosintetico alle diverse condizioni di luce e per ottimizzare la resa fotosintetica. In condizioni di luce stressante, quando la quantità di fotoni assorbiti dal cloroplasto supera la capacità di utilizzo della luce nella fotosintesi, questi organismi mostrano meccanismi di fotoprotezione molto efficienti, compresa lo smorzamento non-fotochimico (NPQ), una risposta fotoprotettiva a breve termine che permette la dissipazione dell'energia di eccitazione in eccesso sotto forma di calore. Le basi molecolari delle risposte all'eccesso di luce in organismi fotosintetici con differenti storie evolutive e appartenenti a diverse linee non sono ancora completamente caratterizzati.

Pertanto, l'obiettivo del mio progetto di dottorato è stato quello di fornire nuove informazioni sulla fotosintesi e la sua regolazione dinamica in condizioni di luce mutevoli, concentrandomi sulla caratterizzazione delle antenne fotoprotettiva in alghe verdi, muschi e diatomee. Inoltre, ho voluto esplorare la funzione dei due cicli delle xantofille presenti nelle diatomee, che sono ancora in parte sconosciuti. Il progetto del dottorato di ricerca, in co-tutela tra l'Università di Verona e l'Università di Parigi VI (UPMC), è stato sviluppato nell'ambito di un Marie Curie Initial Training Network (ITN) chiamato AccliPhot, dedicato a studiare l'acclimatazione della fotosintesi ai cambiamenti di luce e altre condizioni ambientali nelle piante e nelle alghe. Si tratta di una collaborazione interdisciplinare, utilizzando approcci sperimentali e teorici complementari (<http://www.accliphot.eu/>). L'attività di ricerca ha visto forti collaborazioni tra due unità di ricerca: il team del Dr. Angela Falciatore (supervisore Ph.D., UPMC, Paris, FR), esperto di genetica e genomica applicata allo studio della regolazione mediata dalla luce nelle diatomee, ed il laboratorio del Prof. Roberto Bassi (co-supervisore, Univ. di Verona, IT), esperto in biochimica delle proteine di membrana, fisiologia e biofisica degli organismi fotosintetici.

All'inizio del progetto, una società privata, Collectis S.A., era coinvolta come terzo partner, con il coordinamento del Dr. Fayza Daboussi. Nel 2010, Collectis aveva istituito un gruppo di ricerca sulle diatomee, con l'obiettivo di migliorare le risorse genetiche per studi funzionali. In particolare nuovi approcci mirati per l'ingegneria genomica nelle diatomee dovevano essere ottimizzati ed utilizzati per raccogliere informazioni sui regolatori dei meccanismi di fotoprotezione. Perciò, nei primi mesi del mio dottorato, ho sviluppato endonucleasi ingegnerizzate sito-specifiche, in particolare Transcription Activator-Like Effector Nucleases (TALENs<sup>TM</sup>), per eliminare geni coinvolti nella fotoprotezione nelle diatomee. Purtroppo, Collectis ha deciso di fermare il progetto sulle diatomee e microalghe nel 2014, quindi il mio progetto iniziale è stato modificato per utilizzare approcci più tradizionali per studiare i geni coinvolti nella fotoprotezione, come gene knock-down e sovra-espressione. Dopo un accordo tra UPMC e Collectis, le TALENs<sup>TM</sup> che ho sviluppato sono ora disponibili, ma questo progetto è appena ripartito, quindi i risultati riguardanti questa parte iniziale del mio dottorato non sono presenti nella tesi.

In linea con l'obbiettivo di comprendere meglio la fotoprotezione nelle alghe verdi, muschi e diatomee, ho studiato le proteine Light Harvesting Complex Stress-Related (LHCSR) in tutti questi organismi. Nelle alghe verdi, in particolare in *Chlamydomonas reinhardtii*, LHCSR3 è una proteina importante per l'NPQ. L'attivazione dell'NPQ richiede un basso pH nel lumen dei tilacoidi, che è indotto in condizioni di luce in eccesso ed è rilevato dai residui acidi esposti al lumen. Nel capitolo 2 ho usato mutagenesi sito-specifica *in vivo* ed *in vitro* per l'identificazione dei residui di LHCSR3 che sono responsabili per il rilevamento del pH del lumen. I residui protonabili esposti al lumen, aspartati e glutammati, sono stati mutati in asparagine e glutammine, rispettivamente. Con l'espressione in un mutante privo di tutte le isoforme LHCSR, i residui Asp<sup>117</sup>,

Glu<sup>221</sup>, and Glu<sup>224</sup> hanno dimostrato di essere essenziali per l'induzione di NPQ dipendente da LHCSR3 in *C. reinhardtii*. L'analisi di proteine ricombinanti con le stesse mutazioni, ripiegate *in vitro* con pigmenti, hanno mostrato che la capacità di rispondere al basso pH, diminuendo il tempo di vita della fluorescenza, presente nella proteina wild-type, era perduto. Ciò indica che gli amminoacidi individuati sono effettivamente importanti per l'NPQ dato da LHCSR3.

Diversamente dal ruolo solitario delle proteine LHCSR nelle alghe verdi, l'NPQ nello muschio modello *Physcomitrella patens* è sotto il controllo di due proteine: PSBS, presente anche nelle piante superiori, e LHCSR1. PSBS non lega pigmenti e la sua azione nella fotoprotezione è probabilmente mediata dall'interazione con altre antenne del fotosistema II. Al contrario, la proteina LHCSR1 lega pigmenti ed ha dimostrato di essere il motore principale dell'NPQ in *P. patens*, in particolare dopo il legame della xantofilla zeaxantina, indicando che questa proteina potrebbe essere direttamente coinvolta nel processo di smorzamento. Tuttavia, per comprendere le caratteristiche precise di trasferimento di energia e smorzamento di LHCSR1, sarebbe necessario conoscere l'organizzazione precisa e la distanza tra tutti i cromofori. Poiché strutture cristalline di proteine antenna non hanno la risoluzione sufficiente per ottenere tutte queste informazioni, il ruolo dei diversi cromofori è stato studiato in passato con l'aiuto di antenne mutate, prive di specifici residui che coordinano ciascun pigmento. Nel capitolo 3, sono riportati i risultati dello studio *in vitro* delle caratteristiche spettroscopiche e di smorzamento dei diversi mutanti privi dei pigmenti della proteina LHCSR1 di *P. patens*. Abbiamo mutagenizzato ogni residuo legante le clorofille identificato ed analizzato le proteine ricostituite, sia per l'assorbimento che per la fluorescenza. In particolare, abbiamo focalizzato la nostra attenzione sulle clorofille A2 e A5, che dovrebbero essere coinvolte nel meccanismo di dissipazione mediato dall'interazione con i carotenoidi nei siti L1 e L2.

Le proteine LHCSR sono presenti anche nelle diatomee, dove sono denominate LHCXs. Le diatomee sono organismi del fitoplancton che si sviluppano con successo nell'oceano, dove le condizioni di luce sono molto variabili. Gli studi dei meccanismi molecolari di acclimatazione alla luce nella diatomea marina *Phaeodactylum tricorutum* mostrano che la de-epossidazione dei carotenoidi e la proteina LHCX1 contribuiscono a dissipare l'energia della luce in eccesso attraverso l'NPQ. Nel capitolo 4 ho indagato il ruolo della famiglia LHCX nelle risposte allo stress nelle diatomee. L'analisi dei dati genomici disponibili mostra che la presenza di geni multipli per LHCX è una caratteristica conservata nelle diatomee che vivono in diverse nicchie ecologiche. Inoltre, l'analisi dei livelli di trascrizioni delle quattro LHCX di *P. tricorutum* in relazione con l'espressione delle proteine e l'attività fotosintetica indica che le LHCX sono differenzialmente regolate dalle diverse intensità di luce e dalla concentrazione di nutrienti, modulando la capacità di NPQ. Concludiamo che più segnali di stress abiotici convergono per regolare il contenuto di LHCX nelle cellule, fornendo un modo per regolare la raccolta di luce e fotoprotezione. Inoltre, i nostri dati indicano che l'espansione della famiglia genica LHCX riflette la diversificazione funzionale dei suoi componenti, che potrebbero beneficiare le cellule che vivono in ambienti oceanici altamente variabili.

L'altro attore principale della fotoprotezione nelle diatomee è l'accumulo della xantofilla diatoxantina. Il ciclo delle xantofille, con la conversione di violaxantina (Vx) in zeaxantina (Zx) in condizioni di luce elevata, è uno dei principali meccanismi di NPQ nelle piante. Le diatomee possiedono anche un secondo ciclo delle xantofille, convertendo diadinoxanthin (Ddx) in diatoxanthin (Dtx) in condizioni di luce alta. La concentrazione di Dtx è strettamente correlata al livello di NPQ, ed il ruolo effettivo della Zx nella fotoprotezione nelle diatomee è stato messo in discussione. Per comprendere la regolazione e la funzione di questi due cicli delle xantofille, nel capitolo 5 ho deregolato ogni gene putativamente coinvolto in questo processo in *Phaeodactylum tricorutum*. Ho concentrato la mia attenzione su due proteine, VDR e VDL2, che sono state predette per essere coinvolte nella de-epossidazione di Vx a Zx e di Ddx a Dtx. Ho trovato

che i mutanti knock-down per queste due proteine accumulano più xantofille del gruppo Vx-Zx e meno del gruppo Ddx-Dtx rispetto al wild-type, indicando un ruolo di VDR e VDL2 nella via biosintetica delle xantofille. La maggiore quantità di Vx e Zx ha un effetto negativo nello sviluppo dell'NPQ nei mutanti, dimostrando che la Zx non partecipa all'aumento dell'NPQ nelle diatomee.

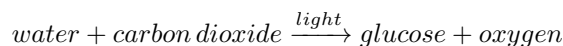
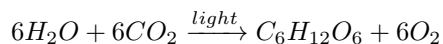
Grazie a questi studi condotti su organismi differenti, abbiamo acquisito una conoscenza più approfondita sulle caratteristiche condivise e su quelle peculiari sulla fotoprotezione nelle alghe verdi, muschi e diatomee, come discusso nel il capitolo 6.

# Chapter 1

## Introduction

In this chapter, I present some general concepts about photosynthesis and photoprotection, focusing first on what we know about plants and green algae. More information on diatoms are reported in the last part of the chapter, where I describe the characteristics of the organisms I worked with.

### 1.1 Oxygenic photosynthesis



Most of life on Earth is sustained by oxygenic photosynthesis, arguably one of the most important biochemical processes that allowed complex life to evolve on our planet. Photosynthetic organisms use water and carbon dioxide to produce glucose and oxygen; light is the energy source that drives the process, while water is used as an electron donor to reduce carbon dioxide into organic carbon. Oxygen, to our luck, is formed as a side product.

Historically, oxygenic photosynthesis has been divided into two main parts: the light phase and the dark phase. The former, as the name suggests, requires light and oxidizes water to produce a reduced molecule (NADPH), an high energy compound (ATP) and molecular oxygen. The dark phase uses NADPH and ATP to produce organic carbon from  $CO_2$  and does not require light (Benson (1950)).

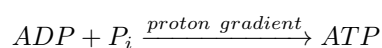
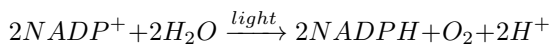
In eukaryotes, the two phases of photosynthesis take place in the chloroplast (figure 1.1), an intracellular organelle derived

from an endosymbiotic event that happened approximately one and a half billion years ago, between an ancient eukaryotic heterotroph and a prokaryotic phototroph. Due to their origin, chloroplasts of plants and green algae are surrounded by two membranes: the outer one, more permeable, and the inner one, more selective, both originated from the gram-negative prokaryotic phototroph (Keeling (2013); CAVALIER-SMITH (1982)). Chloroplasts of organisms that underwent a secondary endosymbiotic event, like brown algae and diatoms, are instead surrounded by four membranes; we will talk about their “special” features in detail in section §1.5.4, so hold on on that.

In all eukaryotic phototrophs, an internal membranes system, called thylakoidal membranes, divides the bulk of the chloroplast in two main parts: the lumen, inside the thylakoidal membranes, and the stroma, outside of the thylakoids (figure 1.1). The reactions

of the light phase of photosynthesis happen in the thylakoids membranes and lumen, while dark reactions take place in the stroma. Thylakoidal membranes in plants and green algae can be usually divided in two other regions: stacked layers of membranes, called grana, and interconnecting regions, the stroma lamellae. These two membrane domains host different proteins of the photosynthetic apparatus, and their abundance can be modified according to light conditions to finely tune the light reactions (Eberhard et al. (2008); Croce and van Amerongen (2014)).

The light phase is the first part of photosynthesis, in which light is absorbed and used to drive an “unfavorable” chemical reaction *i.e.* the oxidation of water. The light phase is a series of incredibly well coordinated and interrelated reactions, involving four membrane protein complexes, all located in thylakoids: photosystems I and II (PSI and PSII), cytochrome- $b_6f$  (Cyt- $b_6f$ ) and ATP synthase (ATPase) (Eberhard et al. (2008); Xiong and Bauer (2002)). These complexes allow the transport of electrons from water to  $NADP^+$  and the formation of a proton gradient between the two sides of the membrane; both these events are needed for the production of ATP and NADPH (figure 1.2).



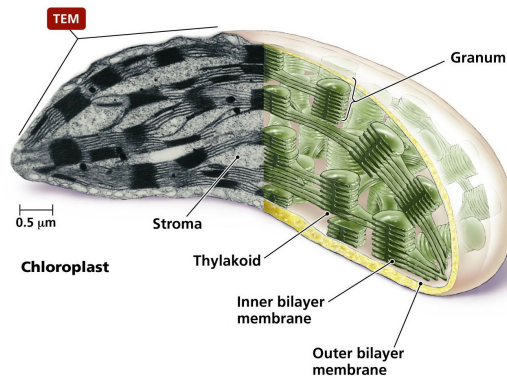
Let’s now follow the journey of an electron through the photosynthetic transport chain, starting from light absorption up to  $NADP^+$  reduction. Light phase begins in PSII with the absorption of a photon: the energy of each quantum of light absorbed is transferred to a special pair of chlorophylls, located in the core of PSII, called P680. This special pair initiates the first photochemical reaction of the process, a charge separation. The electron separated from the special pair is immediately transferred

to a pheophytin, then to two plastoquinones:  $Q_A$ , strongly bound to the PSII, and  $Q_B$ , more labile and that can be liberate in the lipid phase of the thylakoidal membrane. In the meantime, the electronic gap of the special couple is filled by an electron deriving from the oxidation of water. The capture of four photons allows the complete oxidation of water to molecular oxygen and the reduction of two plastoquinones  $Q_B$ , which are released into the thylakoidal membranes as plastoquinols. Water oxidation also releases four protons in the thylakoidal lumen, while the reduction of the two plastoquinones requires the acquisition of four protons from the stroma. The net result is the transfer of four protons from the stroma to the lumen through the absorption of four photons.

Once released into the membranes, plastoquinols diffuse from PSII to Cyt- $b_6f$ , where they are oxidized and each releases the two electron derived from the special pair, liberating at the same time two protons in the lumen. The two electrons then follow different paths: through various heme groups and iron-sulfur clusters, one of them will reduce plastocyanin (PC), a lumenal-soluble copper-containing protein. The other electron will instead be taken by a new plastoquinone that, after having acquired a second electron and taken two protons from the stroma, will be re-released in the lipid bilayer in the form of plastoquinol. This (re)cycle allows the pumping of other protons from the stroma to the lumen, thus increasing the energy yield of the process (Shikanai et al. (2002); Eberhard et al. (2008)), a wonderful example of how evolution was able to create incredibly well tuned and coordinated processes<sup>1</sup>.

Meanwhile, the PSI, just like PSII, captures a photon, transferring its energy to a special pair of chlorophylls (P700) to perform another charge separation. The electron released is transferred to a chlorophyll *a* ( $A_0$ ) and to a plastoquinone, it passes through three iron-sulfur clusters and arrives to ferredoxin, an iron-protein loosely associated to the membrane that can accept two electrons. The reduced PC from Cyt- $b_6f$  meanwhile move to

<sup>1</sup>Don’t take it to literally. Evolution does not create teleologically (*i.e.* with an intent), it’s just random events and selective pressure. ;-)



**Figure 1.1 – Chloroplast structure.** On the left, a Transmission Electron Micrography (TEM) of a plant chloroplast. On the right, a schematic representation view of the different structures of the organelle. Image from Pearson Education, Inc., publishing as Benjamin Cummings.

ward PSI, donating its electron to restore the special pair P700. The electrons transport ends with ferredoxin giving its electrons to reduce  $\text{NADP}^+$  to NADPH, a reaction catalyzed by the enzyme ferredoxin-NADP<sup>+</sup> reductase (Buchanan (1991)).

The journey of our electron is ended but photosynthesis is not: the pumping of protons ( $\text{H}^+$ ) from the stroma to the lumen created a pH gradient ( $\Delta\text{pH}$ ) and an electric field gradient ( $\Delta\Psi$ ) between the two sides of the membrane. As all uneven distribution of matter, the proton gradient can be use as an energy source, in this specific case to drive the production of

ATP by the ATPase enzyme (Mitchell (1961)).

The difference of redox potential of the various chemicals involved in the light phase of photosynthesis is summarized by the Z-scheme (Holland et al. (2002), figure 1.3), which shows how light energy is used to drive the unfavorable oxidation of water up to the reduction of  $\text{NADP}^+$ .

The products of the light phase (*i.e.* ATP and NADPH) are then used to sustain the metabolic needs of the cell, for example to fix inorganic carbon through the dark phase (Benson (1950)) or to synthesize organic compounds like aminoacids and lipids.

## 1.2 Photosynthetic pigments

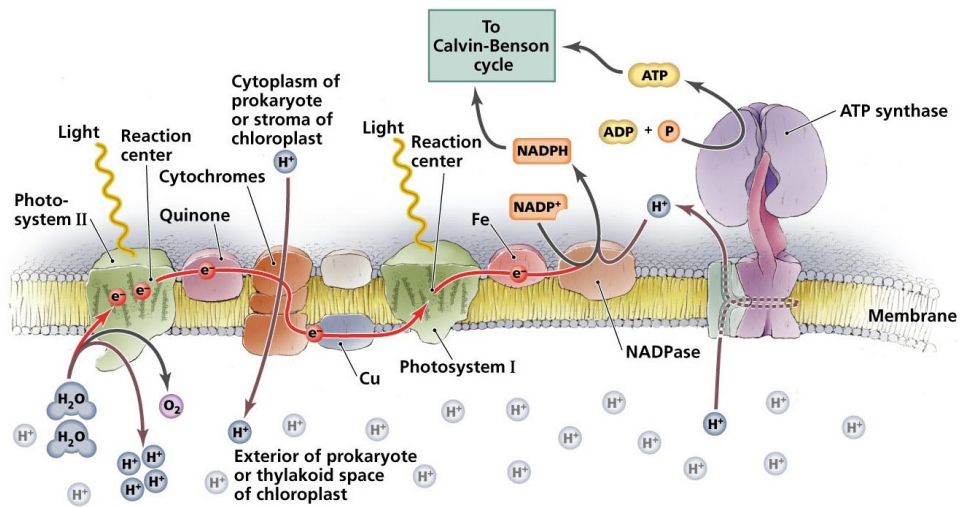
The two photosystems (PSI and PSII) are composed of pigment-binding proteins that can absorb light and use its energy to fuel metabolisms. I will now describe the properties of pigments to later discuss the characteristics and functions of the whole pigment-protein complexes.

Light absorption by the photosynthetic apparatus is possible thanks to the existence of special molecules that can interact with visible light, due to their extensive conjugated double bonds systems (van Amerongen et al. (2000)).

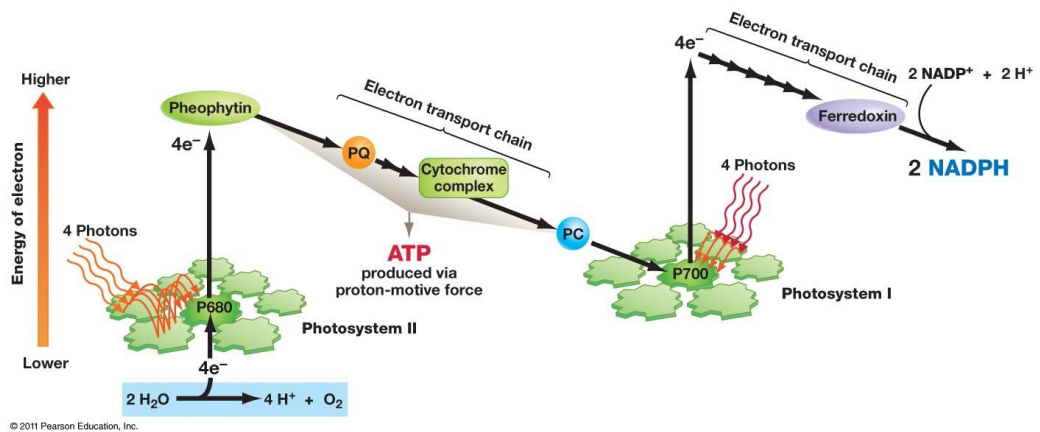
These molecules are called photosynthetic pigments and are divided in two main classes: chlorophylls and carotenoids. Photosynthetic pigments have external electrons that can absorb photons and “jump” to an electronic excited state. Since electronic states are discrete, these external electrons can only absorb photons with the right amount of energy to “jump” to an excited state<sup>2</sup>. The energetic levels of electronic states are mostly determined by the molecular structure of pigments (in particular by the conjugated double bonds systems and

<sup>2</sup>The energy of photons is determined by their frequency (or their wavelength), as described by Planck law:  $E = h\nu = (hc)/\lambda$  (where  $h$  is Planck constant,  $\nu$  is photon frequency,  $c$  is the speed of light and  $\lambda$  is photon wavelength).





**Figure 1.2 – Light phase reactions.** Schematic representation of the photosynthetic apparatus and of the reactions involved in the light phase of photosynthesis. Stroma is on the upper side of the membrane, lumen in the lower one. The two photosystems are indicated in green, cytochrome- $b_6f$  in brown and ATP synthase in purple. Electrons extracted from water are transported through the electron transport chain (red arrows) to reduce  $NADP^+$ , while the proton gradient is used by the ATP synthase to generate ATP. ATP and NADPH are then used to sustain in the dark phase (Calvin-Benson cycle) to fix  $CO_2$  into organic carbon. Image © Pearson Education, Inc., publishing as Benjamin Cummings.



**Figure 1.3 – Z-Scheme of Electron Transport Chain in Photosynthesis.** Z-Scheme of the electron transport chain. Vertical axis indicates electron potential. Green polygons represent chlorophylls. Abbreviations refer to: P680: special pair of chlorophylls in PSII. PQ: plastoquinone. PC: plastocyanin. P700: special pair of chlorophylls in PSI. Image © 2011 Pearson Education, Inc.

residue groups), thus changing the colors that are absorbed by these molecules (as described in section 1.2.1 and section 1.2.2). Once the electron reaches an excited state (called  $S_1$  or  $S_2$ ), it tends to relax to its ground state ( $S_0$ ), as I explain in section 1.2.3. Let's now see the two main classes of pigments in more details, starting with chlorophylls.

### 1.2.1 Chlorophylls

Chlorophylls (Chl) are photosynthetic pigments that can absorb mainly blue and red light (figure 1.4 E). The blue absorption band is caused by the  $S_0$ - $S_2$  electronic transition (called Soret transition), while the red absorption band correspond to the  $S_0$ - $S_1$  (or  $Q_y$ ) transition.  $S_2$  excited state (populated after the absorption of a blue photon) relaxes to  $S_1$  so quickly that no photochemical reaction can happens in such a short amount of time (see section 1.2.3 for an explanation of excited state relaxation). Electrons in the  $S_1$  excited state (populated after the absorption of a red photon or by the relaxation from  $S_2$ ) take more time before returning to the ground state  $S_0$ , and can be thus used in photosynthetic reactions (like charge separation in P700 or P680). This means that a blue photon, even if it's more energetic, actually provokes the same photochemical response as a red one in the photosynthetic apparatus, because of the fast relaxation of  $S_2$  to  $S_1$  (van Amerongen et al. (2000); Croce and van Amerongen (2014)). Thus photosynthetic rate is mostly influenced by the number of absorbed photons (measured as  $\mu\text{mol of photons}/(\text{m}^2 * \text{s})$ ) rather than by the absorbed energy (which is different between red and blue photons and is usually measured in  $\text{Watt}/\text{m}^2$ ).

All Chls share a common tetrapyrrole structure (the porphyrin ring) and are synthesized from glutamic acid (Meinecke et al. (2010)). Chl *a* (figure 1.4 A) is present in almost all photosynthetic organisms, while the other forms are specific of different groups: plant and green algae, for example, synthesize Chl *b* (figure 1.4 B), while diatoms and brown algae use Chls *c*, which lacks the hydrophobic phytol tail (figure 1.4 C-D, Ballottari et al. (2012); Alberte

et al. (1981)).

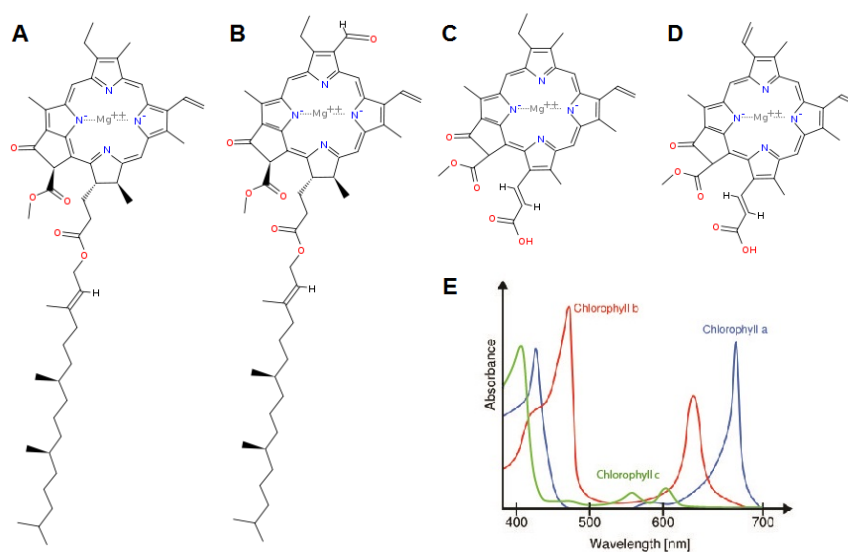
The different chemical groups bound to the porphyrin ring allow the tuning of the absorption properties of the molecule (figure 1.4 E) by changing the energy gaps between the electronic ground state and the excited states (Croce and van Amerongen (2014)). Absorption properties can also be changed by the external environment, in particular by the polarity of the solvent: in this way, Chls bound to photosynthetic proteins can absorb different wavelengths *in vivo*, depending on the protein environment in which they are burred (Bassi et al. (1999)). Proteins can bind Chls by the interaction of a nucleophilic aminoacid with the magnesium in the center of the porphyrin ring (Jordan et al. (2001); Liu et al. (2004)), and the presence of pigments is often indispensable for the correct folding and stability of photosynthetic proteins (Paulsen et al. (1993) and chapter 3).

### 1.2.2 Carotenoids

The second class of photosynthetic pigments is composed by carotenoids: these are isoprenoid compounds with a  $C_{40}$  backbone, derived from the biosynthetic pathway of terpenes in the chloroplast (Eisenreich et al. (2001), figure 1.6). Carotenoids consist of a long chain of carbon atoms with conjugated double bonds and with two end rings of six carbon atoms (figure 1.5). They can distinguished into two different categories: carotenes, composed of only carbon and hydrogen (*e.g.*  $\beta$ -carotene), and xanthophylls, which contains also oxygen (*e.g.* lutein or zeaxanthin).

In nature, more than 600 different carotenoids have been discovered and they are responsible for many of the orange/brown/yellowish colors that we see (for example in carrots, yolk and tomatoes) (Estebana et al. (2015); Kuczynska et al. (2015)). Their economic importance is quite relevant, since they are used as antioxidants and vitamins precursors, in a market worth millions of dollars every year (Mikami and Hosokawa (2013); Peng et al. (2011)).

Despite this huge variety, the carotenoids



**Figure 1.4 – Chlorophylls structure and absorption spectra.** Molecular structures of chlorophylls *a* (A), *b* (B), *c1* (C) and *c2* (D). E: absorption spectra in organic solvent of the different chlorophyll forms: Chl *a*, blue line; Chl *b*, red line; Chl *c*, green line. Images modified from <https://en.wikipedia.org/wiki/Chlorophyll>.

present in plants and green algae are mainly six:  $\beta$ -carotene, neoxanthin, violaxanthin, lutein, zeaxanthin and antheraxanthin.  $\beta$ -carotene is mainly present in photosystem reaction centers, while the others are related to peripheral antenna complexes (Yamamoto and Bassi (1996)). Violaxanthin and zeaxanthin, together with antheraxanthin, play an important role in photoprotection since they are involved in the xanthophyll cycle (see section 1.4.3).

Diatoms and brown algae lack lutein, but accumulate three other carotenoids: fucoxanthin, mainly used for light harvesting, diadinoxanthin and diatoxanthin, also involved in the photoprotective mechanism of the xanthophyll cycle (Kuczynska et al. (2015)).

The spectroscopic properties of carotenoids (as already seen for chlorophylls) derive from the presence of double conjugates bonds (which form a delocalized  $\pi$ -orbital), from the chemical groups present in the end rings and from the polarity of the environment (Andersson et al. (1991)). The absorption in the visible range is between 350 and 550 nm (transition  $S_0$ - $S_2$ ) while the transition  $S_0$ - $S_1$  is not allowed (Croce and van Amerongen (2014)); for this reason carotenoids do not absorb in the red region of

the spectrum (figure 1.5).

Carotenoids biosynthesis starts with the condensation of isopentenyl diphosphate and dimethylallyl-diphosphate (figure 1.6). A series of condensation form geranyl geranyl diphosphate (GGPP, a  $C_{20}$  molecule that is also the precursor of the phytol hydrophobic tail of chlorophyll *a* and *b*), which then reacts with another GGPP to give phytoene. The desaturation of phytoene form  $\zeta$ -carotene first and then lycopene. In green algae and plants, lycopene can be transformed to either  $\beta$ -carotene or to  $\alpha$ -carotene (figure 1.6), while diatoms can only produce  $\beta$ -carotene and lack  $\alpha$ -carotene and lutein (Dambek et al. (2012); Grossman et al. (2004); Bertrand (2010)). The hydroxylation of  $\beta$ -carotene results in the formation of the first xanthophyll, zeaxanthin, which is then transformed to antheraxanthin, violaxanthin and finally neoxanthin. This is the last step in xanthophyll biosynthesis in plants and green algae, while in diatoms the process goes on to form diatoxanthin, diadinoxanthin and fucoxanthin, as we will see in chapter 5.

All these carotenoids have various roles in photosynthetic organisms: together with light harvesting and energy transfer (Gradi-

narum et al. (2000)), they are also important for membrane regulation (Ruban and Johnson (2010); Havaux and Niyogi (1999)), photoprotection (Havaux and Niyogi (1999)), antioxidant regulation (Dall'Osto et al. (2007)) and chloroplast-to-nucleus signaling (Alboresi et al. (2011)). Binding of carotenoids to proteins is mediated via hydrophobic interactions and is also implicated in protein folding and structure stability (Plumley and Schmidt (1987); Paulsen et al. (1993)).

### 1.2.3 Excited state relaxation

We already said that an electron that interacts with a photon with the right energy goes from the ground state ( $S_0$ ) to an excited state (indicated as  $S_1, S_2, \dots, S_n$ ). In this state the electron is not stable and will quickly relax to its ground state. There are various ways for the electron to relax, and the prevalence of one mechanism over the other depends on its decay kinetic constant: the faster the decay, the more electrons will relax using that mechanism. The most important relaxation mechanisms are (figure 1.7, Croce and van Amerongen (2014); van Amerongen et al. (2000)):

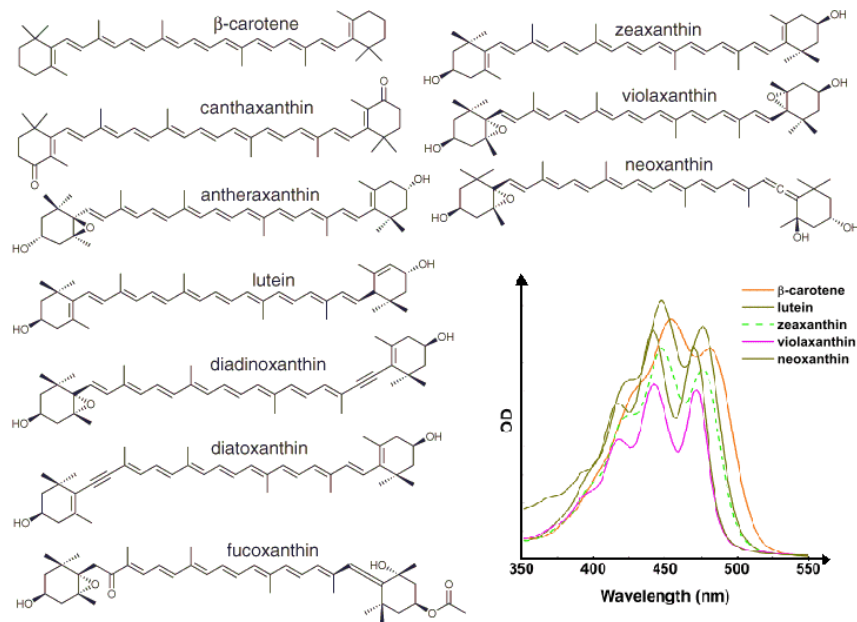
- Vibrational relaxation: is the mechanism that relaxes an electron that reaches an excited vibrational state, from which it will move very rapidly ( $10^{-12}$  s) to the fundamental vibrational energy level, giving its energy to other molecules (*e.g.* through collisions with the solvent);
- Internal conversion: is a non-radiative horizontal transition (without changes in the energy of the electron), consisting in the transition between vibrational levels of the state  $S_n$  to those of the  $S_{n-1}$  level. This process also happens very quickly ( $10^{-12}$  s);
- Fluorescence: is the emission of a photon with an energy corresponding to the difference between the energy levels  $S_1$  and  $S_0$ . It happens at a lower speed compared to the previous mechanisms ( $10^{-7}$ -

$10^{-9}$  s). The emitted photon usually has longer wavelength (and therefore less energy) than that of the one absorbed to pass from  $S_0$  to  $S_1$ , due to the energy loss of the vibrational levels (Stokes shift);

- Intersystem crossing: is a horizontal transition from a singlet excited state to a triplet excited state ( $T_1$ ) by the relatively slow inversion of the electron spin ( $10^{-8}$  s). The excited triplet state molecule can then react with other molecules in the same state, such as molecular oxygen; in this case it will generate singlet oxygen ( $^1O_2$ ), a very reactive species that can damage the photosynthetic apparatus (as described in section §1.4);
- Phosphorescence: once arrived in the excited triplet ( $T_1$ ) the electron, in order to return to the state  $S_0$ , can undergo a new spin reversal and emit a photon. Such emission is called phosphorescence and occurs with times ranging from  $10^{-4}$  s to a few tens of seconds.

In addition to these intramolecular mechanisms, energy can also be dissipated by processes involving other nearby molecules:

- Exciton transfer: is the de-excitation of a donor molecule with the parallel excitation of an acceptor molecule through Coulomb coupling of their dipole moments. Such transfer can take place only if the donor and the acceptor are close by (1-10 nm), with the right orientation of their dipole moments and if there is an overlap of the emission spectrum of the donor with the absorption spectrum of the acceptor;
- Photochemistry: in the case of photosynthetic pigments, energy is used for charge separation in the reaction center. This mechanism requires the presence of an electron acceptor molecule and is what happens in the special pairs P680 and P700 (section §1.1).



**Figure 1.5 – Carotenoids structure and absorption spectra.** Molecular structures and absorption spectra of different carotenoids. Image modified from <http://photobiology.info/Solovchenko.html> and Guaratini et al. (2009).

### 1.3 Photosystems and Antenna proteins

In both photosystems we can distinguish two components: a core complex, where the charge separation occurs, and a peripheral system of antennae, deputed to the collection of light and to the transfer of energy to the reaction center (figure 1.8).

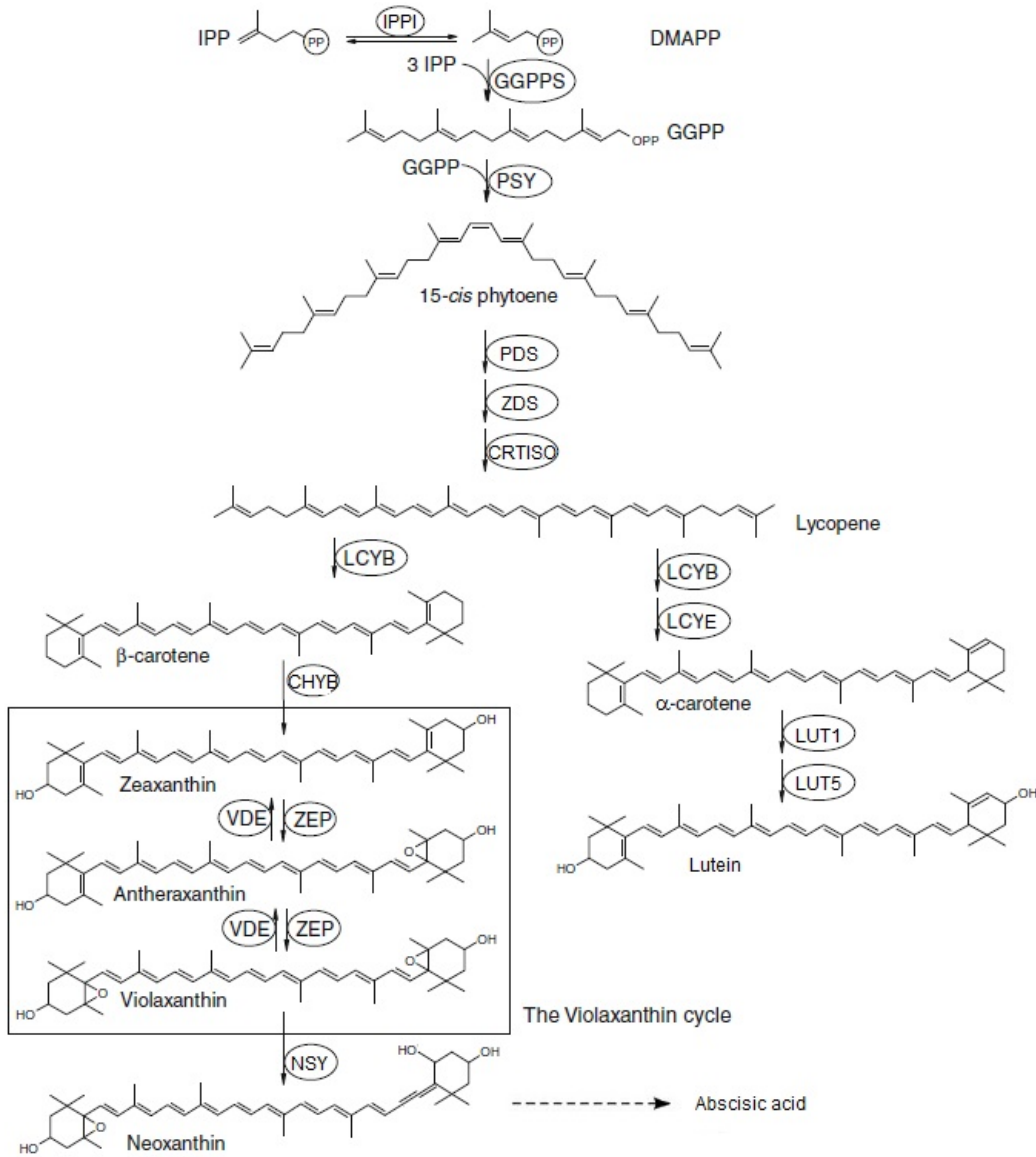
The genes encoding the core proteins are mostly part of the plastid genome: they are very conserved in all photosynthetic organisms and have the prefixes *Psa* and *Psb* in their names for the PSI and PSII components, respectively.

The antenna proteins are instead encoded in the nuclear genome and belong to the LHC gene family (Light Harvesting Complex) (Dall’Osto et al. (2015)). These genes show a greater sequence divergence than those of the core, likely the result of the adaptations of the various organisms to different environments (Hoffman et al. (2011); Xiong and Bauer (2002); Wobbe et al. (2015)).

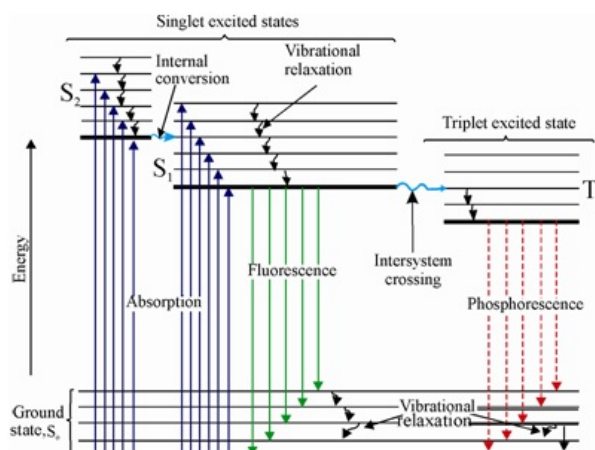
Photosystem II (PSII) is a membrane-integral multiprotein complex, localized mainly

in the grana; in PSII starts the linear electrons transport chain of the light phase of photosynthesis, with the oxidation of water to molecular oxygen. The PSII core complex is organized as a dimer and consists of the proteins D1 and D2 (*psbA* and *psbD* genes), the Oxygen Evolving Complex (deputy to water photolysis) and the internal antennae CP43 and CP47 (Chlorophyll binding Proteins of 43 and 47 kDa) (figure 1.8 A). The reaction center contains the special pair of chlorophylls (P680) and all the necessary co-factors for the electrons transport (pheophytin,  $Q_A$  and  $Q_B$ ) (Ballottari et al. (2012)).

PSII external antennae of plants and green algae are chlorophyll a/b binding proteins, encoded by nuclear genes and then imported into the chloroplast thanks to the presence of a special sequence at the N-terminal, called transit peptide. LHC antennae all have a similar structural organization, featuring three trans-membrane  $\alpha$ -helices and conserved Chl binding residues (Xiong and Bauer (2002)). PSII an-



**Figure 1.6 – Carotenoids biosynthesis in green algae and plants.** Biosynthetic pathway of carotenoids in green algae and plants. Abbreviations correspond to: DMAPP, dimethylallyl-diphosphate; IPP, isopentenyl diphosphate; GGPP, geranyl geranyl diphosphate. The abbreviations used for the enzymes are: IPPI (also called IDI), IPP-DMAPP isomerase; GGPPS, GGPP synthase; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS,  $\zeta$ -carotene desaturase; CRTISO, carotenoid isomerase; LCYB, lycopene- $\beta$ -cyclase; LCYE, lycopene- $\epsilon$ -cyclase; CHYB,  $\beta$ -carotene hydroxylase; LUT, Lutein Deficient 1 and 5; ZEP, zeaxanthin epoxidase; VDE, violaxanthin de-epoxidase; NSY, neoxanthin synthase. Image modified from Coesel (2007) and Bertrand (2010).



**Figure 1.7 – Jablonski diagram.** Jablonski diagram indicating various relaxation path from a electronic excited states. Electronic ground state  $S_0$  (and its vibrational levels) are represented by the lower black horizontal lines, and electron excitation after the absorption of a photon is indicated by blue arrows. Electrons reaching the  $S_2$  excited state quickly relax to the  $S_1$  state via vibrational relaxation (black arrows) and internal conversion (light blue arrows). Relaxation from the  $S_1$  state can either happen via fluorescence emission (green arrows) or internal conversion (not represented). Intersystem crossing from  $S_1$  can lead to a triplet excited state ( $T_1$ ), with relax through the emission of phosphorescence (red dashed arrows). Image from <https://www.quora.com/What-is-the-Jablonski-diagram>

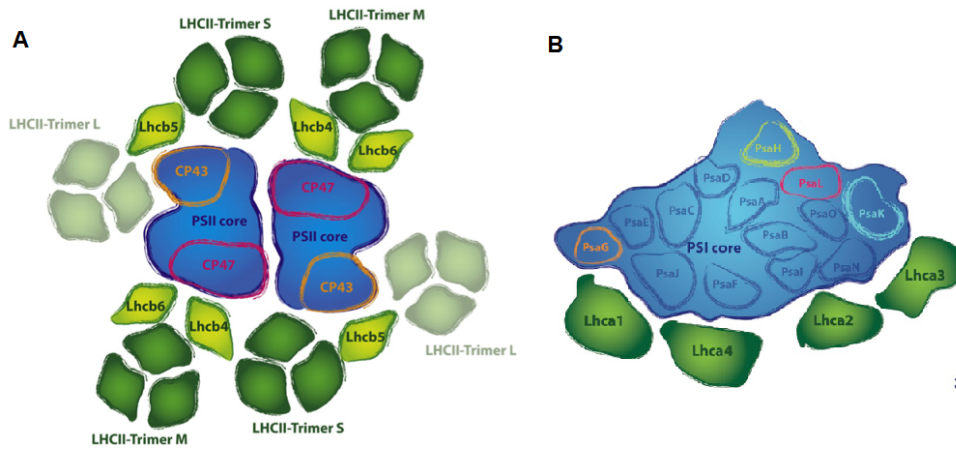
tennae can be divided into trimeric major antennae (LHCII, Dall’Osto et al. (2015)), and monomeric minor antennae (LHCb 4-5-6 Bassi et al. (1993), figure 1.8 A).

Both major and minor antenna proteins increase the amount of absorbed photons, but they can also provide photoprotection and regulate the amount of energy that is transfer to the reaction centers (Dall’Osto et al. (2015); Niyogi (1999); Bassi et al. (1993)). The amount of LHCII attached to each reaction center can be regulated in dependence on environmental conditions, as plants grown in high light show a lower content of LHCII compared to plants grown in low light conditions (Bailey et al. (2001); Ballottari et al. (2007)). LHCII trimers can be separated from PSII with different detergent methods (from here comes the subdivision of LHCII into strongly, medium and loosely bound to the core center, figure 1.8 A, Ballottari et al. (2012)), and their three-dimensional structure shows the typical features of LHC proteins (Liu et al. (2004)), with three trans-membrane  $\alpha$ -helices (A, B and C) (figure 1.9) and each LHCII subunit binding 14 Chls and 4 carotenoids in plants (Liu et al. (2004)).

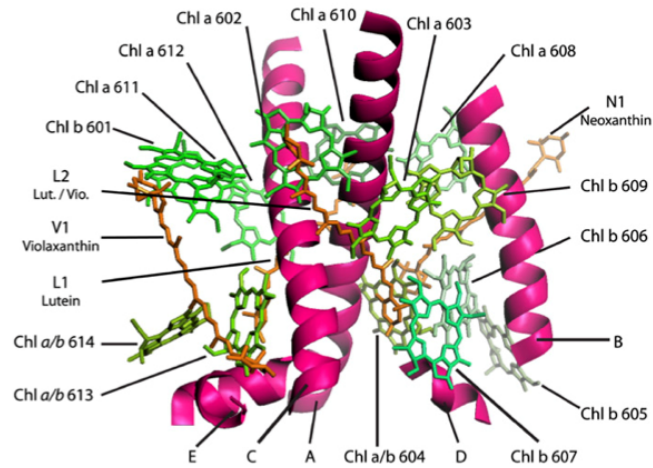
Photosystem I (PSI) is highly conserved in

plants, algae and cyanobacteria: the core consists of multiple subunits (Scheller et al. (2001), figure 1.8 B), where the special Chls pair P700 is located in PsaA and PsaB proteins. In plants and algae, PSI also binds LHC proteins, that compose its external antenna system (Bassi et al. (1992); Ikeda et al. (2008), figure 1.8 B), and these proteins show the same structural organization as the ones from PSII, with three trans-membrane  $\alpha$ -helices. In higher plants, only four antennae are bound to each PSI core, and their number does not change with light conditions (Ballottari et al. (2007)), with the exception of the migration of LHCII from PSII to PSI during state transitions (section §1.4). In green algae, instead, antenna size of PSI is much bigger, and up to nine PSI-specific LHCs are bound to it (Bassi et al. (1992)) and can be modulated in response to different light conditions (Le Quiniou et al. (2015)).

Diatoms photosystems differ from those of the green lineage, in particular regarding the external antennae system and the organization of the thylakoidal membranes. For a specific discussion and description of diatoms antenna proteins see section §1.5.4.



**Figure 1.8 – Organization of photosystem II and photosystem I.** Structural organization of photosystem II (panel A) and photosystems I (panel B) super complexes. Core complexes are indicated in blue, antenna proteins in green. Trimers of LHCII are distinguished in strongly bound to the complex (S), medium (M) and weakly bound (L). Images modified from Ballottari et al. (2012) and Girardon (2013).



**Figure 1.9 – Structure of a monomer LHCII antenna.** X-ray crystal structure of a LHCII monomer from *Spinacia oleracea*. The various chlorophylls (Chl) and the four binding sites of carotenoids are indicated (L1, L2, and N1 V1). Image from Liu et al. (2004).



## 1.4 Photoprotective mechanisms

Light is (of course) essential for photosynthesis. Nevertheless, the rate of photosynthesis does not linearly increase with light intensity, reaching a saturating point when other reactions became limiting. In high light, or under other stressful conditions, the absorption of photons exceeds the capacity of the downstream reactions to consume the products of the light phase and reaction centers get saturated (Barber and Andersson (1992); Wobbe et al. (2015)). As a consequence, there is an increase in the concentration of chlorophylls in the excited singlet ( $^1\text{Chl}^*$ ) and an excessive acidification of thylakoid lumen (Niyogi (1999)). The increase of  $^1\text{Chl}^*$  causes an increase in the probability of intersystem crossing (see section 1.2.3), with the generation of chlorophylls in the triplet excited state ( $^3\text{Chl}^*$ ) that can react with oxygen, thus giving singlet oxygen ( $^1\text{O}_2$ ), a highly reactive and toxic species (Niyogi (1999)). The life time of  $^1\text{Chl}^*$  in PSII is much higher than that in PSI (since in the case of PSI, charge separation and recombination of the special pair P700 is faster than in PSII, Slavov et al. (2008); Jeong et al. (2002)) thus making PSII the main production site of  $^1\text{O}_2$ , which can damage the structure of the photosynthetic apparatus and lead to photoinhibition (Niyogi (1999); Eberhard et al. (2008)).

Singlet oxygen, formed by the interaction with  $^3\text{Chl}^*$ , can produce various other harmful chemical species (figure 1.10), known as Reactive Oxygen Species (ROS). Via monovalent reduction, singlet oxygen can give superoxide oxygen ( $\text{O}_2^{\bullet-}$ ), which can then be reduced to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radical ( $\text{OH}^\bullet$ ). The latter species in particular causes serious damage to the photosynthetic apparatus and to cellular structures, for example oxidizing lipids in a chain reaction. Thylakoidal membranes are particularly abundant in unsaturated fatty acids, so lipid oxidation is a real threat, and ROS production must be kept strictly under control to avoid damages (Aro et al. (1993); Knox and Dodge (1985)).

To avoid the formation of  $^3\text{Chl}^*$  or to pro-

tect themselves from ROS damages, photosynthetic organisms have developed different photoprotective strategies, which are the main topic of this thesis and that I will briefly introduce in the next sections.

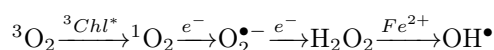
### 1.4.1 Short-term and long-term responses to excessive light

Responses to light stress can in part be distinguished based on their velocity of action or activation (Eberhard et al. (2008)). Rapid responses do not require changes in gene expression, as their components are already in place.

There are light stresses that occur slowly (*e.g.* the variation of sun light during the day) or quickly (*e.g.* an area of underbrush suddenly exposed to direct sunlight because of the upper canopy moved by the wind or, in case of a marine organism, the water turbulence), and the duration of the stress can be variable as well.

To deal with these adverse conditions, plants and algae have mechanisms that can act on different time scales:

- Photochemical quenching (qP): it is a fast mechanism (on a millisecond time scale) that consists in the relaxation of excited Chls through the normal photosynthetic process, so by energy transfer and charge separation (see figure 1.2);
- ROS scavenging: various molecules, such as ascorbate, glutathione,  $\alpha$ -tocopherol and some xanthophylls, act as traps for ROS by reacting with them and avoiding damages to cellular components like membrane lipids or the photosynthetic apparatus;
- Non-Photochemical Quenching (NPQ): it's a mechanism that can be activated in few seconds and that dissipates  $^1\text{Chl}^*$  in form of heat. This quenching is measurable as the progressive reduction of chlorophyll fluorescence in response to illumination (figure 1.11). Short and intense pulses of light saturate all photochemical reactions, in order to eliminate



**Figure 1.10 – Reactive Oxygen Species formation.** Scheme of reactions that lead to the various reactive oxygen species from molecular oxygen. Chemical species represented are: molecular triplet oxygen ( ${}^3\text{O}_2$ ), singlet oxygen ( ${}^1\text{O}_2$ ), superoxide oxygen ( $\text{O}_2^{\bullet-}$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radical ( $\text{OH}^\bullet$ ).

the photochemical quenching component (Schreiber et al. (1986)) and NPQ is calculated as  $(F_m - F'_m)/F'_m$ . NPQ can be further subdivided into different components (namely qE, qZ and qI) as I will described in section 1.4.2;

- State transitions: in plants and green algae, under conditions that favor the excitation of one of the two PSs, the movement of LHCII antenna proteins between PSII and PSI allows to re-balance the excitation energy in the photosynthetic apparatus in few minutes (Eberhard et al. (2008); Wobbe et al. (2015));
- Non-assimilatory electron sinks: metabolic reactions that consume NADPH and ATP, other than the Calvin Cycle, can alleviate energy overload on the photosynthetic apparatus. These non-assimilatory electron sinks may include, for example, photorespiration (Wallsgrave et al. (1987)) or water-to-water cycle (Asada (1999)) and their regulation can be quite fast;
- Chloroplast movement (qM): chloroplast relocation and self-shading in plant cells can reduce the amount of absorbed energy in case of excessive light in few tens of minutes (Cazzaniga et al. (2013); Dall'Osto et al. (2014));
- Light avoidance: in case of motile microalgae, the whole organism can responds to excessive light conditions by swimming far away from the light source, while plants can change the orientation of their leaves to reduce light absorption, thus diminishing the energy overload (Bennett and Golestanian (2015)). These mechanisms can require tens of minutes to hours to be effective;

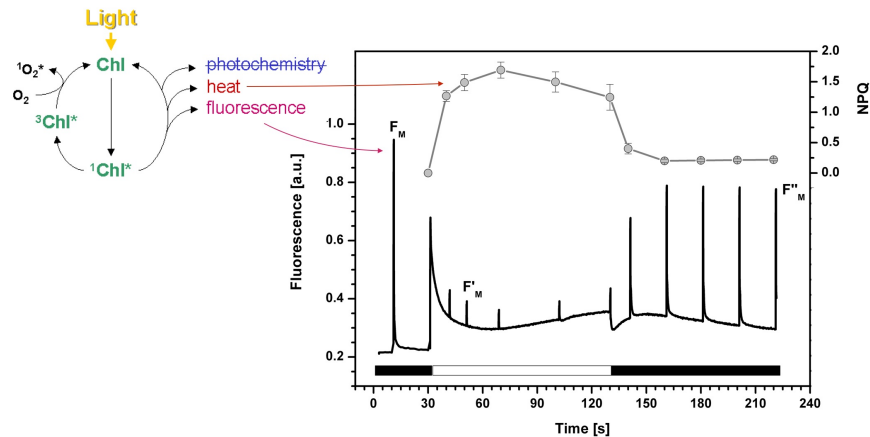
Slower mechanisms, which require important changes in gene expression, fall into the category of photoacclimation responses:

- Pigment synthesis: *de novo* synthesis of photoprotective pigments (such as those involved in ROS scavenging and in the xanthophyll cycle), which happens in some hours, increases the capacity of the photosynthetic organisms to resist to high light stresses (Lavaud (2002); Gilmore and Ball (2000));
- Re-balance of light and dark reactions: after several hours or days of sustained high light conditions, the antenna size of photosystems is generally reduced and components of electrons transport chain and  $\text{CO}_2$  fixation are increased, in order to re-balance the amount of absorbed light with the capacity of energy utilization (Bonente et al. (2012); Pfannschmidt et al. (1999); Wobbe et al. (2015); Ballottari et al. (2007));
- Modification of plant morphology: reduction of leaves surface and increase in their thickness, smaller chloroplasts with fewer grana stacks and lower chlorophyll content are all common characteristics of plants responses to prolonged (weeks) high light conditions (Yano (2001)).

#### 1.4.2 PSBS, LHCSR and LHCX proteins

As photoprotection via Non-Photochemical Quenching (NPQ) is a relevant part of this thesis, I will discuss this process a bit more in detail.

We can distinguish three main components of NPQ: the first one is called qE and is activated by the acidification of thylakoid lumen. A second component is qZ, which is due to the bind-



**Figure 1.11 – Chlorophyll de-excitation processes, chlorophyll fluorescence trace and corresponding NPQ.** On the left is represented a scheme of chlorophyll (Chl) excitation, due to light absorption, and de-excitation, due to photochemistry, heat dissipation or fluorescence emission. Chl triplet state conversion ( $^3\text{Chl}^*$ ) and reaction with molecular oxygen is also depicted. On the right, an example of fluorescence kinetic (black line, left vertical axes) in an alga subjected to actinic light that saturate photosynthesis (light box), followed by dark exposure (black box). NPQ is calculated as  $(F_m - F'_m)/F'_m$  and is represented as a grey line in the upper part of the graph (right vertical axes). Images modified from [www.photosynthesis.ch/fluorescence.html](http://www.photosynthesis.ch/fluorescence.html) and Kana et al. (2012).

ing of zeaxanthin to LHC proteins, while the last component is qI, associated with photoinhibition and other long-lasting reduction in PSII fluorescence. These three NPQ components can be distinguished by their relaxation kinetics during the dark exposure in an NPQ measurement (figure 1.11, Eberhard et al. (2008); Dall'Osto et al. (2014)).

The qI component is the slowest one, with a relaxation time of hours, and it's actually composed of more than one mechanisms, like the damage, inactivation and repairment of the core proteins of PSII (Eberhard et al. (2008); Krause (1988)).

A medium-lasting component (10-30 minutes relaxation time) was initially thought to be related to state transitions (the movement of LHCII antennae between PSII and PSI) and therefore called qT. Results from the *Arabidopsis thaliana str7* mutant, which is blocked in state transitions, showed no change in the qT component (Bellafiore et al. (2005)), thus challenging the interpretation that this NPQ component is due to the movement of LHCII antennae. qT would be rather due to the accumulation of zeaxanthin (Miloslavina et al. (2008);

Dall'Osto et al. (2005)), and should be called qZ (Nilkens et al. (2010)).

The qE component is the fastest one and it's activated by excessive acidification of thylakoid lumen, which in the model plant *Arabidopsis thaliana* is detected via the reversible protonation of the protein PSBS. PSBS is a four-transmembrane  $\alpha$ -helixes protein, with two glutamic acid residues facing the lumen: these residues can be protonated in low pH conditions (Li et al. (2000b); Ruban et al. (2007); Li et al. (2004)), leading to the activation of NPQ via the interaction of PSBS with other LHC antennae (Gerotto et al. (2015)).

In green algae, *PSBS* gene is present but the detection of proteins has been a long-lasting dilemma, since apparently the gene was transcribed but no protein could be found (Peers et al. (2009); Allmer et al. (2006); Bonente et al. (2008)). Only very recently this mystery arrived to a turning point, when PSBS was firstly detected transiently in cells exposed to high light and low  $\text{CO}_2$  (Correa-Galvis et al. (2016)). More stable expression of PSBS was found after UV-B treatment, where an involvement of this protein in qE development has also

been demonstrated (Guillaume Allorent and Michel Goldschmidt-Clermont, personal communication).

In “normal” high light conditions, when PSBS is not present in green algae, the function of pH-sensor and NPQ activator is performed by other proteins, called LHCSR (Light Harvesting Complex Stress-Related proteins, Peers et al. (2009)).

Mosses (and in particular *Physcomitrella patens*) are a special case for what concern photoprotection and LHCSR proteins: they are somewhat in the middle between vascular plants and green algae, since PSBS and LHCSR proteins are present together and they are both active in photoprotection (Alboresi et al. (2010)). The regulation and involvement in NPQ of LHCSR proteins in *Chlamydomonas reinhardtii* and in *Physcomitrella patens* are further described in section §1.5.2 and in section 1.5.3, respectively.

Lastly, also diatoms (see section §1.5.4) possess LHCSR proteins, here called LHCX, which are still active in photoprotection (Bailleul et al. (2010)). In the model diatom *Phaeodactylum tricoratum*, four different isoforms of LHCX are present, and an involvement in photoprotection and photosynthesis regulation as been demonstrated for the isoform LHCX1 (Bailleul et al. (2010)). Knock-down mutants for LHCX1 show in fact lower NPQ capacity and lower growth, highlighting the essential role of this protein in photosynthesis regulation. Different amounts of LHCX1 protein were also detected in various ecotypes of *P. tricoratum*, nicely correlating with the maximal NPQ capacity of each strain (Bailleul et al. (2010)). More recently, the involvement of LHCX proteins in NPQ has been demonstrated also in centric diatoms, where they possibly control aggregation and reorganization of antenna proteins in high light conditions (Ghazaryan et al. (2016)).

### 1.4.3 The Xanthophyll Cycle

PSBS and LHCSR/LHCX are not the only proteins involved in the qE ( $\Delta$ pH-dependent) component of NPQ. The second main actor is the

xanthophyll cycle (XC), a series of reactions that convert violaxanthin (Vx) into zeaxanthin (Zx) in excessive light conditions (Havaux and Niyogi (1999)). These reactions are catalyzed by a luminal soluble enzyme, violaxanthin de-epoxidase (VDE, Niyogi et al. (1997); Yamamoto and Kamite (1972)), which requires ascorbate as a co-substrate and that is activated by the reduction of pH in the lumen. In acid conditions, VDE dimerizes and binds to thylakoidal membranes (in particular to monogalactosyldiacilglycerol lipid domains) to convert Vx into Zx (Hager and Holocher (1994); Arnoux et al. (2009); Goss and Jakob (2010)). Dimerization is thought to allow VDE to perform the simultaneous de-epoxidation of both Vx rings, directly producing Zx (Arnoux et al. (2009)).

VDE belongs to the lipocalin protein family, having a central lipocalin conserved domain for the binding of the substrate, flanked by an N-terminal cysteine-rich domain and a C-terminal glutamic acid-rich region (Coesel et al. (2008)). The back reaction (from Zx to Vx) is instead catalyzed by a stromatic enzyme (possibly associated to the thylakoid membrane), zeaxanthin epoxidase (ZEP, Jahns et al. (2009)). ZEP has a neutral optimum pH and utilizes oxygen, FAD and NADPH as co-substrates (Buch et al. (1995)). In plants and green algae, ZEP is always active, but it’s much slower than VDE, ensuring effective accumulation of Zx upon excessive light and a subsequent slow reconversion to Vx in low light conditions (Siefermann and Yamamoto (1975)).

Zx has a fundamental role in photoprotection, performing ROS scavenging (Dall’Osto et al. (2010)),  $^3\text{Chl}^*$  quenching (Dall’Osto et al. (2012)) and NPQ regulation (Niyogi et al. (1998, 2001)). Different quenching mechanisms have been hypothesized, which propose either the direct involvement of Zx (and or lutein) to form a Zx radical cation in the minor antennae (Ahn et al. (2008)) or the transfer of energy from chlorophylls to the  $S_1$  state of a carotenoid (Ruban et al. (2007)). Indirect roles of Zx in quenching have been proposed as well, like the action of this xanthophyll as an allosteric regulator of antenna proteins to enhance Chl dimer

interaction (Muller et al. (2010)).

The violaxanthin-zeaxanthin cycle is present in viridiplantae (plants and green algae) and chromista (brown algae and diatoms), even though some studies point to its presence also in rhodophyta (red algae, Estebana et al. (2015); Schubert et al. (2006)). In diatoms, together with the violaxanthin-zeaxanthin cycle, a second XC is present, involving two pigments that are absent in the green lineage: diadinoxanthin and diatoxanthin (Lohr and Wilhelm (1999)).

The XC in diatoms have some peculiar characteristics when compared to the one present in plants. VDE is active not only in acidic environments but already at neutral pH (Jakob

et al. (2001)), and has a higher  $K_M$  for the co-factor ascorbate (Grouneva et al. (2006)). ZEP protein is much faster in catalyzing the epoxidation reaction, but it is completely inhibited by low lumenal pH (*i.e.* in high light conditions, Goss et al. (2006)). Diatoxanthin is also able to sustain NPQ even in the absence of a proton gradient (Goss et al. (2006)), while NPQ in plants immediately collapses when an uncoupler is added, even if Zx is still present.

Together with these different characteristics, diatoms also show an expansion of the genes which encode the enzymes putatively involved in the epoxidation and de-epoxidation reactions, as described in section §1.5.5.

## 1.5 My three working organisms: green algae, mosses and diatoms

Now that the main characteristics of photosynthesis and photoprotection have been described, I will present some information regarding the organisms I worked with, namely green algae, diatoms and mosses, and the differences that distinguish them one from the other. These organisms all have some common characteristics regarding their photosynthetic apparatus, but major differences exist as well, due to their different evolutionary histories. Comparative analysis can give us extensive information regarding the most important and conserved characteristics of dissipative mechanisms. I will start by describing green algae and mosses, which both have chloroplasts derived from primary endosymbiosis. As diatoms have some more peculiar features, mainly due to the different origin of their chloroplast, I will describe them in a separate section (section §1.5.4).

### 1.5.1 The green lineage

Photosynthetic organisms are hugely differentiated, from cyanobacteria to plants, but oxygenic photosynthesis probably evolved just once in ancient prokaryotic cyanobacteria and spread among eukaryotes through various endosymbiotic events (Howe et al. (2008)). The

primary endosymbiotic event formed three lineages, all belonging to the plant kingdom (figure 1.12 and figure 1.15): chlorophyta (green algae), rhodophyta (red algae) and glaucophyta (Keeling (2013)). In the organisms belonging to these groups, the plastid is surrounded by two membranes that correspond to the inner and outer membranes of the ancient gram-negative cyanobacterium (CAVALIER-SMITH (1982)). Land plants (embryophytes) lately evolved from green algae and the two are part of the same clade of Viridiplantae (Becker (2007); Baldauf (2008)) sharing most of the photosynthetic apparatus architecture that has been described previously.

Chloroplasts of Viridiplantae are generally organized similarly, with thylakoidal membranes that usually form grana stacks and stroma lamellae, a segregation between PSI and PSII in these membrane structures, the presence of chlorophyll *b* in antenna proteins (Ballottari et al. (2012); Koziol et al. (2007)) and the production of starch as carbohydrate storing molecule (Busi et al. (2014)). Also mosses (clade Viridiplantae, phylum bryophytes) show these characteristics (Koziol et al. (2007); Alboresi et al. (2008)). They are considered, from a functional and evolutionary point of view, to

be in between green algae and vascular plants. Because of that, the use of mosses in studies regarding photosynthesis, photoprotection and the adaptation in the transition from aquatic to terrestrial environments is steadily growing (Quatrano et al. (2007); Schaefer et al. (1991); Alboresi et al. (2010); Gerotto et al. (2011); Schaefer (2001); Alboresi et al. (2008)). An interesting characteristic of mosses is, for example, the concurrent presence of the two antenna proteins involved in the onset of NPQ, PSBS and LHCSR (Alboresi et al. (2010)), both active in photoprotection.

In the next two sessions, I will introduce the two “green organisms” addressed in this thesis: the green alga *Chlamydomonas reinhardtii* and the moss *Physcomitrella patens*.

### 1.5.2 *Chlamydomonas reinhardtii*

*Chlamydomonas reinhardtii* is the model organism of the chlorophyta phylum (green algae). It is a flagellated, unicellular alga of about 10  $\mu\text{m}$  that lives in fresh water (Proschold (2005)). It was chosen as a model organism for the many advantages it offers: ease of cultivation (it is able to grow both in autotrophy and in heterotrophy, using acetate as a complementary carbon source), rapid growth and short mitotic life cycle, haploidy, the ability to generate various mutant strains and to control the sexual cycle (Grossman (2000); Nickelsen (2005); Dent et al. (2005); Rochaix (1995)).

The nuclear, mitochondrial and plastidial genomes of *C. reinhardtii* have been completely sequenced (Merchant et al. (2007)) and there are collections of EST, cDNA and mutant strains. The sexual cycle can be induced in the laboratory by stress conditions (such as nitrogen deprivation), thus generating gametes. When two gametes of opposite mating type (+ and -) come together, they give a diploid zygote, devoid of flagella and surrounded by a thick wall to withstand the stress conditions. As soon as the environmental conditions return favorable, the zygote begins the meiosis, generating four haploid cells (Proschold (2005); Grossman (2000)). The nuclear genome of 120

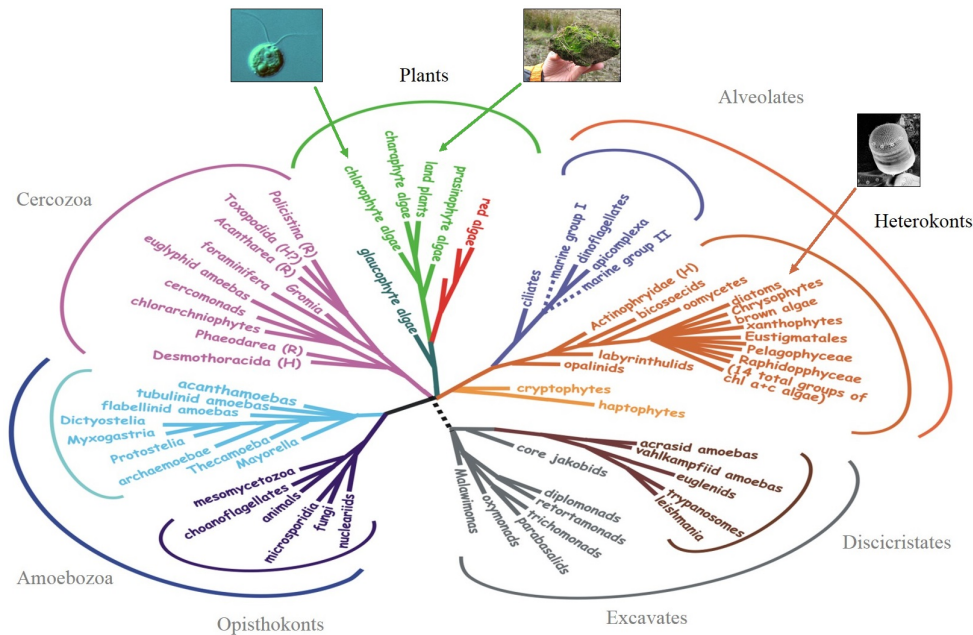
Mbp is haploid in the vegetative stage, with a high GC content (62%) (Merchant et al. (2007)).

*C. reinhardtii* possesses two apical flagella for mobility, a single chloroplast, which occupies 50% of the cell volume, and a special organelle, called pyrenoid, containing high concentration of RuBisCo (Ribulose-1,5-bisphosphate carboxylase oxygenase) for carbon fixation and the accumulation of starch (figure 1.13).

Compared to land plants, thylakoidal membranes are poorly stacked, PSI antenna system is bigger, with 9 LHCA instead of just 4 as in plants (Wobbe et al. (2015)), and the number of subunits is modulated in response to external conditions (Le Quiniou et al. (2015)). *C. reinhardtii* also lack the minor antenna LHCB6 (CP24), which is instead present in plants and mosses (Büchel (2015)).

As already mentioned in section 1.4.2, *C. reinhardtii* have the *PSBS* gene, but the protein is not present in normal or in high light conditions (Peers et al. (2009); Allmer et al. (2006); Bonente et al. (2008)). In *C. reinhardtii*, three *LHCSR* (Light Harvesting Complex Stress-Related) genes are present, called *LHCSR1*, *LHCSR3.1* and *3.2*, with the last two encoding the same aminoacid sequence (*LHCSR3* protein, Merchant et al. (2007); Bonente et al. (2011)). *LHCSR1* is constitutively accumulated, but can induce only little qE, while *LHCSR3* is induced by excessive light conditions or low  $\text{CO}_2$  (Peers et al. (2009); Thuy Binh Truong (2011); Correa-Galvis et al. (2016)) and is responsible for most of the qE activity in *C. reinhardtii*. *LHCSR3* in particular has been studied in the last years because of its possible involvement not only in qE activation (with the perception of low lumenal pH due to the excessive light conditions) but also in direct quenching (Thuy Binh Truong (2011); Peers et al. (2009); Bonente et al. (2011)). This protein, differently from PSBS, can in fact bind pigments and may act as a quenching antenna (Bonente et al. (2011); Liguori et al. (2013); Tokutsu and Minagawa (2013); Liguori et al. (2016)).

During my Ph.D., I studied the pH-sensing



**Figure 1.12 – Phylogenetic tree of the eight major eukaryotic groups.** Phylogenetic tree of eukaryotes representing the eight major groups: green algae and mosses belong to the plant kingdom (Plantae), while diatoms are part of Heterokonts. Image modified from Baldauf et al. (2004).

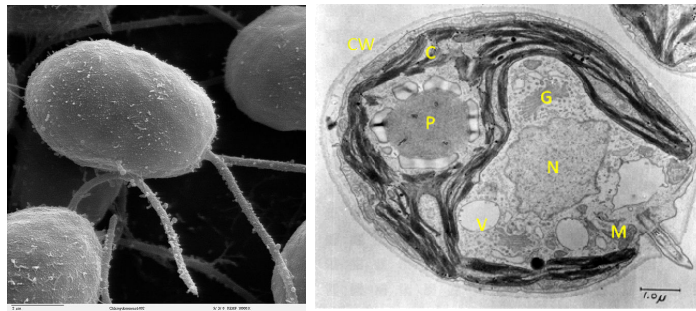
and quenching properties of LHCSR3 protein from *C. reinhardtii*, trying to understand how the protein is activated and if it can really sense luminal pH and switch on NPQ in response to this signal; the results I obtained are presented in chapter 2.

### 1.5.3 *Physcomitrella patens*

*Physcomitrella patens* is the model organism of the bryophyta phylum (mosses). It is a moss of a few centimeters size (figure 1.14) that can be found in temperate climates. Since it lacks roots and a xylem system, it can't control its internal state of hydration, so it inhabits only very humid environments, such as the edges of pools of water. As all mosses, it lacks a vascular system and does not produce lignin, two factors that limit the size of the plant due to poor water distribution between the various parts of the organism and no mechanical support from a "rigid" molecule.

As already said, mosses are gaining momentum in plant biology to study water-to-

land transition, since they present intermediate characteristics between algae and land vascular plants (Alboresi et al. (2010); Rensing et al. (2008); Alboresi et al. (2008)). Bryophyta shared a common ancestor with vascular plants about 200-400 millions years ago (Sanderson et al. (2004)), and it has been shown that *P. patens* have at least 66% of homologous genes with the model plant *Arabidopsis thaliana* (Nishiyama et al. (2003)). *P. patens* was chosen as a model organism for the many advantages it offers: ease of cultivation, haploidy in most of its life cycle, easy vegetative propagation and the possibility to generate mutants with homologous recombination (Quatrano et al. (2007); Schaefer et al. (1991); Cove (2005); Cove et al. (2009); Frank et al. (2005); Schaefer and Zryd (1997); Kamisugi et al. (2006)). The nuclear, mitochondrial and plastidial genomes of *P. patens* have been completely sequenced (Terasawa et al. (2007); Rensing et al. (2008); Sugiyama et al. (2003)) and there are collections of EST and transcriptomic data available (Cove (2005); Xiao et al. (2011)).



**Figure 1.13** – *Chlamydomonas reinhardtii* electron micrographs. Left: *Chlamydomonas reinhardtii* electron micrograph with a scanner electron microscopy. Right: electron micrograph with a transmission electron microscopy showing ultrastructural details of the cell. Abbreviations indicate: CW, cell wall; C, chloroplast; M, mitochondria; N, nucleus; V, vacuole; P, pyrenoid; G, Golgi apparatus. Images from <http://remf.dartmouth.edu/imagesindex.html> and [http://am.celllibrary.org/ascb\\_il/render\\_image/37252/0/0/](http://am.celllibrary.org/ascb_il/render_image/37252/0/0/).

Some interesting information have emerged from the study of antenna proteins in *P. patens*, showing that some of the genes are shared between all the green lineage (e.g. *LHCA1-2-3* and *LHCB 4-5*) while others seems to be specific of plants (e.g. *LHCB3* and *6*) (Alboresi et al. (2008)). An apparently unique antenna protein, found only in mosses, is instead *LHCB9*: this is the first PSII-associated protein that has a red-shifted absorption spectrum, a characteristic that could optimize light absorption under the canopy (Alboresi et al. (2011)).

As already discussed in section 1.4.2, mosses are an interesting case of study when it comes to photoprotection: sitting in between vascular plants and green algae, they posses both the proteins involved in NPQ trigger mentioned so far, PSBS and LHCSR, both of which are active in photoprotection (Alboresi et al. (2010); Gerotto et al. (2012)). This makes mosses unique organisms to study NPQ process and its evolution upon land colonization. In *P. patens*, two LHCSR proteins are present (LHCSR1 and LHCSR2), which have a 91% sequence similarity. LHCSR1 is the most abundant and important in photoprotection (Alboresi et al. (2010)) and it is strongly induced by high light treatment, while LHCSR2 does not respond to high light but rather to cold stress (Gerotto et al. (2011)).

PSBS probably does not bind pigments (Domini et al. (2002); Bonente et al. (2008)), al-

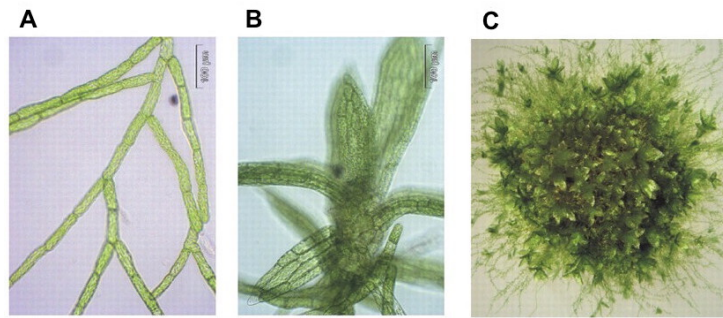
though it has been reported to be able to bind xanthophylls *in vitro* (Aspinall-O’Dea et al. (2002)), and thus the real quenching site in plant is in the LHCs (Ahn et al. (2008); Johnson et al. (2011)). Differently from PSBS, LHCSR proteins do bind pigments (Bonente et al. (2011); Pinnola et al. (2013)), and is therefore possible that these proteins are not only the pH sensors but also active quenchers (chapter 2). Differently from what has been shown in *C. reinhardtii*, LHCSR quenching activity in *P. patens* is strongly increased by the presence of zeaxanthin (Pinnola et al. (2013)), synthesized from violaxanthin under excessive light conditions via the xanthophyll cycle (Demmig et al. (1987)), but the exact energy transfer or quenching role of each chromophore is nowadays unknown.

To address this issue, I studied the *in vitro* pigment-binding characteristics and quenching activity of LHCSR1 protein from *Physcomitrella patens*, and the results of this work are presented and discussed in chapter 3.

### 1.5.4 Diatoms

Diatoms are marine microalgae spread in all the oceans, and they occupy a huge variety of ecological niches, from the poles to tropical seas; they are the most diverse group of phytoplankton and, although they represent less than 1% of the global biomass of photosynthetic organisms, they account for about 20% of the pri-





**Figure 1.14 – *Physcomitrella patens* micrographs.** Micrographs of protonema (A) and developing gametophores (B) of *P. patens* in liquid cultures, bar represent 100  $\mu\text{m}$ . (C) Cultures of *P. patens* after eight week of growth, bar represent 1 mm. Image modified from von Schwartzberg et al. (2007).

mary production on Earth. Diatoms are also important for the biogeochemical cycles of various elements in the oceans, such as nitrate, iron and silicon (Armbrust (2009); Falkowski (1998); Granum et al. (2005); Roberts et al. (2007)).

Perhaps the more striking (and beautiful) characteristic that distinguish diatoms from other algae is their silica cell wall, called frustule: it is composed of two halves, fitting into each other as a Petri dish (Falcatore and Bowler (2002)). The pattern of this wall is genetically encoded and allows to distinguish the different species from a morphological point of view (figure 1.16 A-D). Various authors have proposed different functions for the silicified cell wall of diatoms, such as increased cell density to induce sinking (to avoid strong light or to find nutrients, possible only for bigger diatoms, Waite et al. (1997); Purcell (1977)), restricted access of protoplast to parasites and resistance to grazers (Raven and Waite (2004)). For the presence of this special cell wall, diatoms are particularly involved in the biogeochemical cycle of silicon, which is incorporated into their frustule several times before sinking on the sea floor (Tréguer et al. (1995); Raven and Waite (2004)).

The presence of the frustule influence also the vegetative reproduction of diatoms: two daughter cells derived from a mitotic division receive one of the two halves each, and these became, in both cases, the new bigger halves of their new frustule. This means that one cell

will reconstruct the frustule has the mother cell, while the other will build a smaller theca. This process repeats over and over, until the daughter cell reaches a threshold size, below which the sexual cycle is induced and the cell restores its initial size (Chepurnov et al. (2004)).

Diatoms separated from other groups of secondary endosymbionts around 250 millions years ago (Sorhannus (2007)) and become ones of the most important primary producers about 100 millions years ago (Falkowski and Knoll (2007)), splitting in two distinct divisions: centric and pennates diatoms (Armbrust (2009)). These two groups are differentiated by the symmetry of their cell wall, radial for centric diatoms and bilateral for pennates; they also have physiological differences, notably their “swimming habits” (centric diatoms are usually planktonic, while pennate tends to be benthic organisms) and in the usage and storage of iron (Falcatore and Bowler (2002)). Centric open-ocean living diatoms, for example, lowered their iron requirement by modifying their photosynthetic apparatus, replacing iron electron-transport proteins with copper-containing ones (Strzepek and Harrison (2004); Peers and Price (2006)). Pennates diatoms, on the other side, produce ferritin and can storage iron to sustain photosynthetic processes even when iron in the environment decrease (Marchetti et al. (2009)).

Let’s now dive into a diatom cell, focusing in particular on the organelle where photosynthesis happens.

Unlike green algae and plants, diatoms

have a chloroplast surrounded by four membranes, which is the legacy of the secondary endosymbiotic event from which their plastid derives (Howe et al. (2008); CAVALIER-SMITH (1982)). In this endosymbiotic event, which occurred approximately 500 millions years ago, an ancient heterotroph engulfed an eukaryotic red algae (Keeling (2009); Moustafa et al. (2009); Armbrust (2009)); the endosymbiont became the plastid of stramenopiles, the group that includes diatoms and brown algae (figure 1.15). Other endosymbiotic events probably took place over time, notably with green algae, giving to diatoms a complex and diversified set of genes with different origins that probably contribute to their ecological success (Frommolt et al. (2008); Bowler et al. (2008); Oudot-Le Secq et al. (2007); Armbrust et al. (2004); Prihoda et al. (2012); Moustafa et al. (2009)).

Analysis of nitrogen metabolism provided evidences of the diversity of diatoms genomes: these microalgae possess a complete urea cycle, similar to the one found in metazoans, which is supposed to facilitate the recovery after a prolonged nitrogen limitation (Allen et al. (2011)), together with some nitrogen storing mechanisms (Rogato et al. (2015)). The urea cycle is absent in any other phototroph, and its presence in diatoms is indicative of the complex mix-and-match that characterizes their genomes, with the legacy of the multiple endosymbiotic events that happened during diatoms evolution (Allen et al. (2011); Frommolt et al. (2008); Armbrust et al. (2004); Bowler et al. (2008); Moustafa et al. (2009)).

Due to their different evolutionary origin, chloroplasts in diatoms have some peculiar characteristics that distinguish them from the green lineage. Therefore, a direct extrapolation on the structure and physiology of their chloroplast from Viridiplantae is not always applicable. For example, the presence of four membranes surrounding the plastid modified the import system of nuclear-encoded plastid-target proteins, so that these proteins have to carry

a bipartite targeting sequence (composed of a signal peptide and a transit peptide) to enter the chloroplast<sup>3</sup> (Apt et al. (2002); Agrawal and Striepen (2010); Kroth and Strotmann (1999)).

Also the inside structure of chloroplasts and the photosynthetic apparatus architecture in diatoms are different than those in plants: thylakoidal membranes are not divided into grana stack and stroma lamellae, but are all loosely stacked in layers of three membranes that go along all the length of the plastid (figure 1.16 E, Berkaloff et al. (1990); Bedoshvili et al. (2009); Flori et al. (2016)). Due to the lack of grana and stroma lamellae, the segregation of PSI and PSII in thylakoidal membranes has been a long question for diatoms. A recent study shows that the two photosystems are located in two different regions of the thylakoids, with PSI mainly present in the outer membranes layers, while PSII concentrates in the inner ones (Serena Flori and Giovanni Finazzi, personal communication).

Another important difference between diatoms and the green lineage is related to...their color: green algae and plants are, as we all know, green. This is due to their pigment content, in particular the abundance of Chl over carotenoids. Diatoms are instead brown, and their color is due to the presence of high levels of the carotenoid fucoxanthin (which is absent in plants): they also accumulate Chl *c* instead of Chl *b* (figure 1.4), lack the lycopene  $\epsilon$ -cyclases (LCYE) and are thus not able to produce  $\alpha$ -carotene and lutein (Dambek et al. (2012); Grossman et al. (2004); Bertrand (2010)). Diatoms can instead produce three carotenoids that are absent in chlorophyta: the already mentioned fucoxanthin, a light harvesting pigment, diadinoxanthin and diatoxanthin, involved in the second xanthophyll cycle present in diatoms (together with the violaxanthin-zeaxanthin cycle, see section 1.4.3).

Differences between diatoms and the green lineage regards also the regulation of photosynthesis, like the control of the ratio of the products of the light phase: linear electrons

<sup>3</sup>This is also due to the fact that secondary endosymbiotic-derived plastids are topologically located in the endomembrane system and not in the cytoplasm, as primary plastids (Keeling (2013)), so that a bipartite targeting sequence is needed.

transport produce an imbalanced amount of NADPH and ATP with respect to what are the needs of the Calvin-Benson cycle, making too much NADPH (or too little ATP). Viridiplantae usually regulates ATP/NADPH ratio with cyclic electron flow around PSI and/or with the water-to-water cycles (Allen (1975)). In diatoms, instead, it as been shown that cyclic electron flow is very low and that re-equilibration of ATP/NADPH is achieved thanks to the exchange of ATP and NADPH between chloroplast and mitochondrion, which are in physical contact between each other (Bailleul et al. (2015)).

Antenna proteins of PSI and PSII differ from their plants and green algae counterparts, binding Chl *c* instead of Chl *b*, together with high amounts of fucoxanthin (hence their name, Fucoxanthin Chl *a/c* binding Protein, FCP, Lepetit et al. (2007)). The antenna size in low light of high light do not differ significantly and no state transition have be detected so far (Lepetit et al. (2012)). FCP complexes are organized as trimers or oligomers (Szabó et al. (2008); Guglielmi et al. (2005); Nagao et al. (2013)) and currently we still miss a strong evidence of PSI or PSII specific antenna proteins (Büchel (2015)). Three different FCP antenna proteins families are present in diatoms: LHCF, the major light harvesting proteins and the most similar to plant antennae, LHCR, related to red algae and mostly attached to PS I, and LHCX, functional homologous to LHCSR proteins and usually induced by excessive light conditions (Eppard et al. (2000); GREEN (2007); Koziol et al. (2007); Büchel (2015); Taddei et al. (2016)).

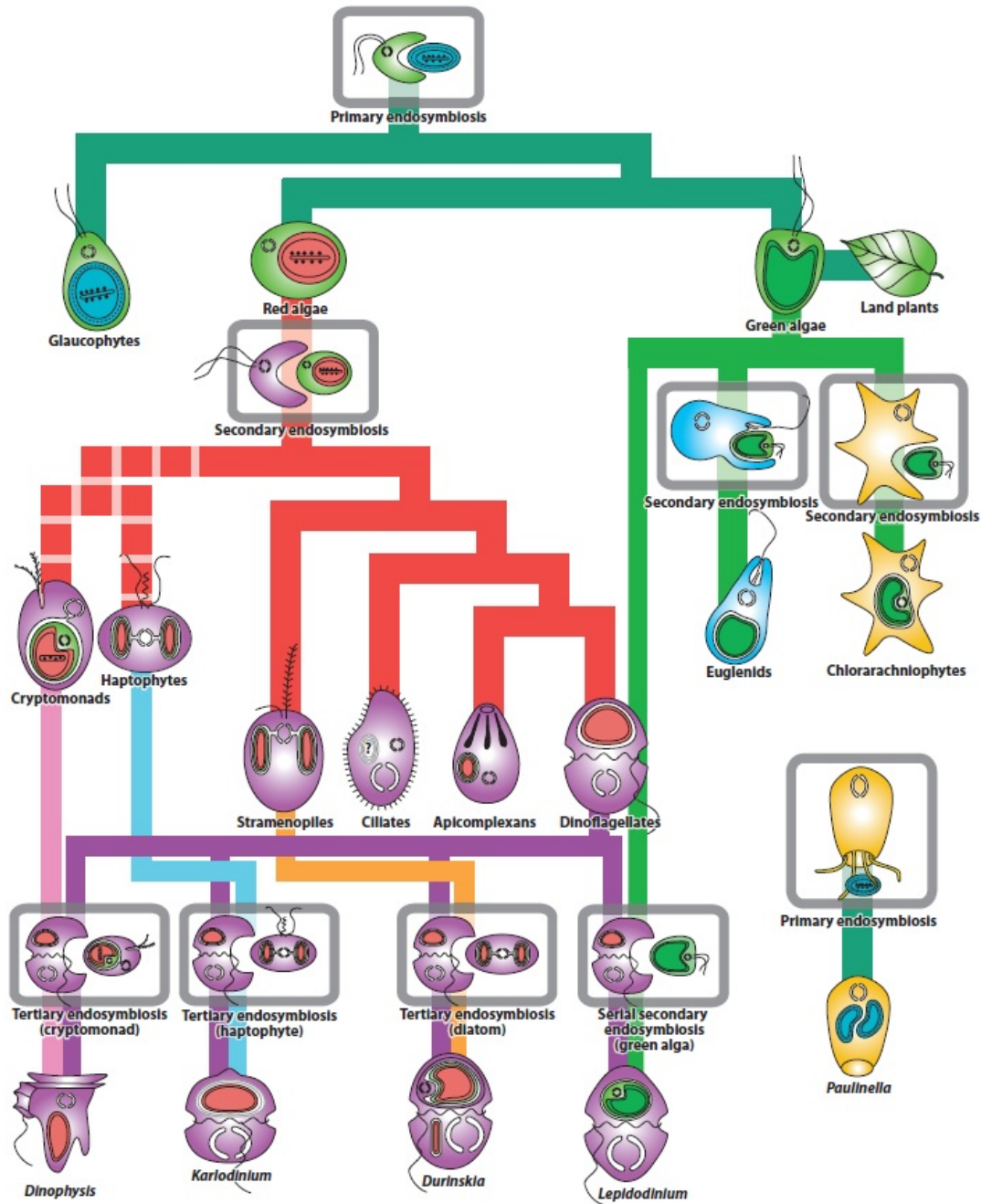
As already mentioned, together with the violaxanthin cycle, diatoms posses a second xanthophyll cycle: in response to high light, diadinoxanthin is de-epoxidized to diatoxanthin (Lohr and Wilhelm (1999)), and the opposite reaction happens in low light. The de-epoxidation is catalyzed by the lumenal enzyme violaxanthin de-epoxidase (VDE, Lavaud et al. (2012)), which in diatoms has a different regulation than in plants: the protein is in fact active already at neutral pH, while plant VDE is only active at acidic pH (Jakob et al. (2001)),

and has an higher  $K_M$  for the co-factor ascorbate (Grouneva et al. (2006)). Also the enzyme that catalyze the opposite reaction, the zeaxanthin epoxidase (ZEP), has different characteristics in diatoms: in particular, its activity is completely inhibited by the thylakoidal proton gradient and its catalytic rate constant is almost 20 times higher that that of plants ZEP (Goss et al. (2006); Mewes and Richter (2002)).

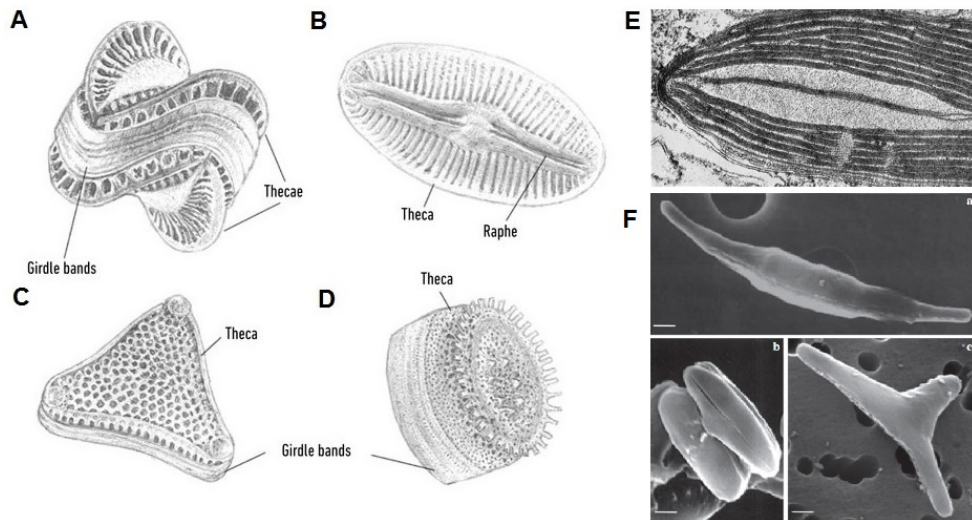
Non-Photochemical Quenching (NPQ) in diatoms is positively correlated with diatoxanthin accumulation, even if the  $\Delta pH$  is abolished (Goss et al. (2006)), indicating the fundamental importance of this xanthophyll in photoprotection. Thylakoidal membranes are particularly enriched in anionic lipids (like sulfoquinovosyldiacylglycerol, SQDG, and phosphatidylcholine, PC) but neutral lipids (monogalactosyldiacylglycerol, MGDG, and digalactosyldiacylglycerol, DGDG) form shield-like domains around FCP proteins. In these domains, high concentration of diadinoxanthin can be more easily solubilized and be available for fast de-epoxidation by VDE (Lepetit et al. (2012)).

An interesting aspect to notice is that diatoms, in nature, are dominant in turbulent water environments, where the amount and quality of light is very unpredictable (Smetacek (1999); Armbrust (2009); MacIntyre et al. (2000); Margalef (1977)). In stressful light conditions, when the amount of photons absorbed by the chloroplast largely exceed the capacity for light utilization in photosynthesis, these microalgae show very efficient photoprotective mechanisms, including higher NPQ compared to most land plants (Depauw et al. (2012); Lavaud (2002)). This characteristic is of great interest for the comprehension of diatoms high photosynthetic efficiency in different light conditions. The high NPQ also imply a great adaptability to different environmental conditions which, however, are not yet understood at molecular level. One of the aims of my thesis was to better understand the regulation of NPQ in diatoms, as I'll discuss later.

Let's now see the main characteristics of the model species I used to study diatoms photosynthesis, *P. tricornutum*.



**Figure 1.15 – Major endosymbiotic events in plastid evolution.** At the top, the primary endosymbiotic event between an ancient heterotroph and a cyanobacterium, which originates plastids and the three main groups of photosynthetic eukaryotes (glaucophytes, red and green algae). Plants and green algae all derive from this first symbiosis (right branch). Secondary endosymbiotic events occurred between an heterotroph and a red alga (probably multiple times), creating a large variety of groups, including stramenopiles (to which diatoms belong). Image from Keeling (2013).



**Figure 1.16 – Diatoms structure.** Diatoms external structures of (A) centric diatom, *Campylodiscus* sp.; (B) raphid pennate diatom, *Diploneis* sp.; (C) multipolar centric diatom, *Triceratum* sp.; (D) centric diatom, *Cyclotella* sp.. (E) Transmission electron micrograph of the chloroplast in the diatom *Melosira varians*, where the three layers of thylakoidal membranes spanning from one side of the plastid to the other are visible. (F) Scanning electron micrograph of the three morphotypes of *P. tricornutum*, scale bar 1  $\mu\text{m}$ . Images from <http://www.microbiologysociety.org>, Richard Crawford in J. Dodge's *Fine Structure of Algal Cells* (1975), Academic Press and De Martino et al. (2007).

### 1.5.5 *Phaeodactylum tricornutum*

*P. tricornutum* is a pennate diatom and a model organism for molecular studies. Its nuclear, plastidial and mitochondrial genomes have been sequenced (Bowler et al. (2008); Oudot-Le Secq et al. (2007)), and whole genome transcriptomes and proteomes are available in various conditions (Allen et al. (2008); Nymark et al. (2009); Grouneva et al. (2011); Nymark et al. (2013); Jungandreas et al. (2014); Valle et al. (2014)).

In natural conditions, *P. tricornutum* is a coastal species that mainly occurs in unstable environments such as estuaries and rock pools; various accessions (or ecotypes) have been found and characterized, from the English Channel and Finland coasts to Micronesia islands, presenting different physiological adaptation (De Martino et al. (2007)). It can exist in different morphotypes (oval, fusiform and tri-radiate, figure 1.16 F) and can grow in the absence of silicon in the media, thus producing no cell wall (Borowitzka and Volcani (1978)). This factor allows easier manipulation and genetic

transformation, which helped for the establishment of *P. tricornutum* as the model species for pennate diatoms, despite its relatively low ecological importance (De Martino et al. (2007)).

*P. tricornutum* photosynthetic antenna system is composed of membrane antenna proteins called FCP, organized as trimers and hexamers (Lepetit et al. (2007)). Each trimer is mainly composed of LHCF proteins, in particular LHCF5, LHCF10/2 and LHCF4 (Büchel (2015)). Each subunit is smaller than LHCFII proteins, ranging between 17 and 19 kDa, and so far no PSII supercomplex has been ever isolated. In the genome of *P. tricornutum*, 17 genes for LHCF, 14 for LHCFII and 4 for LHCFX have been found (Bowler et al. (2008)) and all are expressed at the transcript level (Nymark et al. (2009)). Each FCP subunit approximately binds eight Chl *a*, two Chl *c* and eight fucoxanthin molecules (Lepetit et al. (2010); Premvardhan et al. (2009, 2010)).

Regarding its photoprotective characteristics, *P. tricornutum* is able to reach high levels of NPQ, particularly in light conditions that induce an accumulation of diadinoxanthin

thin (Lavaud (2002)). In its genome there are seven genes putatively involved in the two xanthophyll cycles (XCs, section 1.4.3), three for the epoxidase reactions (zeaxanthin epoxidases *ZEP1*, *2* and *3*), to convert zeaxanthin to violaxanthin or diatoxanthin to diadinoxanthin, and four for the opposite reactions (violaxanthin de-epoxidase *VDE*, VDE-like *VDL1* and *2*, and the VDE-related *VDR*) (Coesel et al. (2008); Eilers et al. (2016)). The expansion of these gene families is in contrast with what happens in plants, where only one *VDE*, one *VDR* and one *ZEP* are present. Nowadays the precise role of these seven genes in the XCs is not known, thus I decided to investigate their activity in photoprotection, modulating the expression of these proteins by generating knock down and over expressing lines in *P. tricornutum*. The results regarding this research are presented in chapter 5.

A feature that looks to be conserved between diatoms and green algae is the presence of LHCSR proteins (called LHCX proteins in diatoms): LHCXs possess the putative pigment-binding residues (Bailleul et al. (2010); Zhu and Green (2010) and chapter 3) and, oppositely to what has been proposed by Bailleul et al. (2010), also the pH-sensing aminoacids to detect lumen acidification identified in green algae and mosses (chapter 4). It has been also shown that different ecotypes of *P. tricornutum* have different NPQ maximal level, and that this variability could be mainly due to a different level of accumulation of the photoprotective antennae LHCXs (Bailleul et al. (2010)). Nevertheless, LHCXs exact roles are still unclear. Diatoms, in fact, expanded this protein family and the different members seem to have a different regulation.

I addressed this topic during my Ph.D., in collaboration with another student of the lab, Lucilla Taddei: our results are reported in chapter 4, where we correlated the expression of LHCX proteins with photosynthetic and photoprotective activity in response to different environmental conditions.

## 1.5.6 Genetic manipulation of *P. tricornutum*

As *P. tricornutum* is the organism with which I performed functional characterization *in vivo*, I will introduce some of the molecular tools that I used during my Ph.D. to manipulate gene expression in this alga.

Thanks to the set up of different methods of transformation, as particle bombardment (Falcone et al. (1999); Apt et al. (1996)), conjugation (Karas et al. (2015)) and electroporation (Miyahara et al. (2013); Niu et al. (2012)), and to the availability of a range of antibiotic for mutant selection (Zaslavskaja et al. (2000)), it is possible to manipulate gene expression in *P. tricornutum*.

Gene over-expression is usually achieved by using the strong promoter of the gene encoding the fucoxanthin Chlorophyll a/c-binding Protein. This approach has become routine thanks to the generation of diatom specific Gateway<sup>®</sup> Destination vectors (Siaut et al. (2007)). Gateway<sup>®</sup> technology allows fast and efficient transfer of DNA fragments between plasmids, using the recombinant machinery of phage  $\lambda$ , instead of the more classical restriction and ligation reactions.

Different Destination vectors for gene over-expression are available for the diatom community, including constructs that permit to easily tag the protein of interest with enhanced-YFP, enhanced-CFP or HA-tag (Siaut et al. (2007)).

A second approach, which has been extensively used in this thesis, is gene silencing via RNA interference (RNAi, De Riso et al. (2009)). Gene silencing in diatoms mainly relies on two strategies: the first uses antisense DNA fragments complementary to the gene of interest, and the second one uses inverted repeat sequences. Both these methods produce double-strand RNA (dsRNA), which can inhibit the expression of the target gene by triggering post-transcriptional cleavage of its mRNA (Hamilton (1999)). Short RNAs can also lead to transcriptional gene silencing via methylation or chromatin remodeling in the DNA sequence of the target gene (Chan (2008)).

The expression of antisense fragments or

inverted-repeat complementary sequences separated by a spacer (which leads to the formation of double-strand RNA) have been proven to be similarly effective in silencing of endogenous and exogenous genes in diatoms (De Riso et al. (2009)). The precise mechanism of gene silencing is still unknown, as in *P. tricornutum*, the putative components of the RNAi machinery show a lower conservation compared to the more canonical ones present in plants and other eukaryotes (De Riso et al. (2009)).

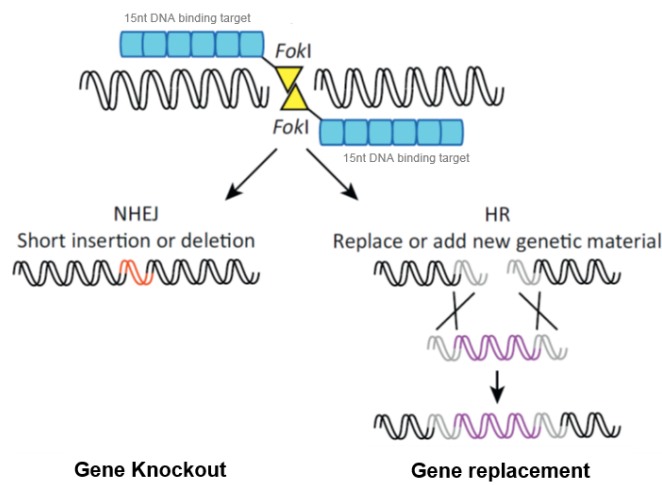
More recently, gene knock-out techniques, using site-specific endonucleases, have been demonstrated to work efficiently in diatoms, using both TALENs™ (Daboussi et al. (2014)) and CRISPR/Cas9 (Nymark et al. (2016)).

TALENs™ (transcription activator-like endonucleases) are engineered site-specific endonucleases, whose DNA-binding domain (TALE) derives from proteins produced by the plant pathogens of the genus *Xanthomonas* (Boch et al. (2009); Moscou and Bogdanove (2009)). This DNA-binding domain is composed of multiple repetitions (14-24 repeats) of 34-long aminoacid sequences. The specificity of the DNA-binding domain results from two polymorphic amino acids, the so-called repeat variable diresidues (RVDs), located at positions 12 and 13 of a repeated unit and responsible for the specific recognition of a unique nucleotide (Boch et al. (2009); Moscou and Bogdanove (2009)). TALE RVDs can be assembled in a particular number and order to target specifically any genomic locus, providing an adaptive tool for genome engineering. The original TALEN™ architecture consisted of a custom

TALE DNA-binding domain linked to the N-terminal end of the non-specific FokI nuclease domain (figure 1.17), and because FokI needs to dimerize to catalyze a double-strand break in the DNA, TALENs™ work by pairs (Li et al. (2011)).

Once the TALENs™ perform a double-strand DNA break, the repair mechanisms of the cell will repair the damage by two endogenous mechanisms: homologous recombination (HR) or non homologous end joining (NHEJ) (Voytas (2013)). The former uses a homologous piece of DNA to repair the double-strand break, while the latter just sticks back together the two DNA broken ends. HR can be used to introduce exogenous DNA sequences in a specific locus (for gene knock-in or gene replacement), while NHEJ can, in some cases, introduce errors in the repaired DNA (mainly insertions or deletions), thus leading to a possible frame-shift and to the inactivation of the targeted gene (figure 1.17).

These strategies have been successfully applied in diatoms (Daboussi et al. (2014); Weyman et al. (2014); Fortunato et al. (2016)) and, when I was working at the private company Collectis, I produced several TALENs™ to knock-out genes involved in photosynthesis and photoprotection (as describe in chapter 6). I am currently selecting and screening these knock-out mutants, so to provide further understanding of diatoms photosynthesis and to establish a more of a routine use of these techniques in our laboratory.



**Figure 1.17 – TALENs™ mechanisms of action.** TALENs™ are composed of a DNA-binding domain (light blue) and a FokI endonuclease domain (yellow triangle). FokI can introduce a double strand DNA break only when it dimerize, hence when the two TALENs™ arms are bound to the target DNA sequence at the right distance. Once a double DNA strand break is introduced, there are two possible pathways for its repair: homologous recombination (HR) and non homologous end joining (NHEJ). HR can be used to introduce or replace a gene by adding a DNA fragment with homologous flanking regions, while NHEJ can lead to short insertions or deletions that can cause a frameshift and thus the inactivation of the target sequence. Image modified from Doyle et al. (2013).





# Aims of the thesis

Photosynthetic organisms with different evolutionary histories have developed different strategies to adapt their photosynthetic apparatus to changing light conditions, and to optimize photosynthetic yield under different environmental conditions. Photoprotective mechanisms that are active in excessive light conditions can also differ, but the extent and significance of this diversification are still not completely characterized at a molecular and functional level.

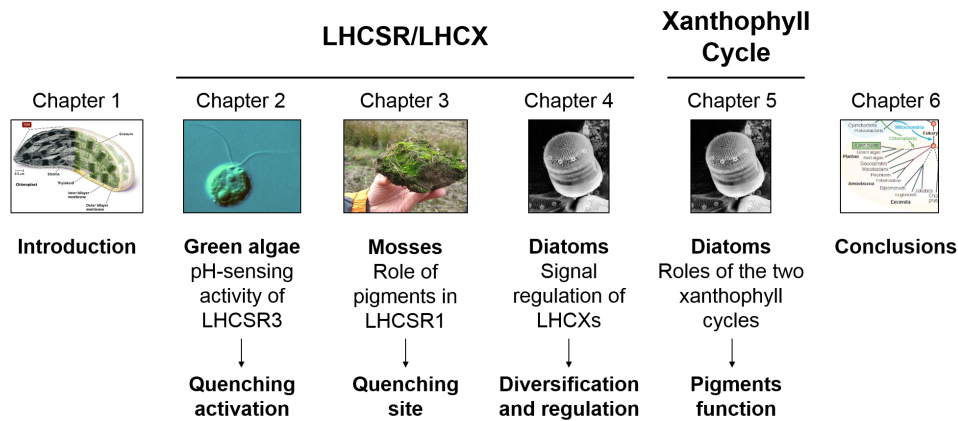
Therefore, the aim of my Ph.D. project was to provide novel information about photosynthesis and its dynamic regulation under changing light conditions, by focusing on the characterization of photoprotective antennae in green algae, mosses and diatoms. Moreover, I also explored the functions of the two diatom xanthophyll cycles, that are still largely unknown.

Green algae, diatoms and mosses have some common characteristics regarding their photosynthetic apparatus, but major differences as well. In particular all these organisms possess multiple photoprotective antennae of the LHCSR/LHCX family and different pigments of the xanthophyll cycle (s). Thus a comparative analysis can provide novel information regarding how photoprotective mechanisms were either conserved or diverged during evolution. This is of major interest for basic biology, since it can allow to comprehend the precise function and mechanisms of action of energy dissipation processes.

To better understand photoprotection in green algae, mosses and diatoms, I studied Light Harvesting Complex Stress-Related (LHCSR/LHCX) proteins in all these organisms. These proteins are supposed to combine both the PSBS pH-sensing function and the quenching activity of antennae proteins. I started my Ph.D. studying green algae, in particular in *Chlamydomonas reinhardtii*, in the laboratory of Prof. Roberto Bassi in Verona, where I used site-specific mutagenesis for the identification of the residues in LHCSR3 that are responsible for sensing luminal pH and activation of thermal dissipation. A mutant version of LHCSR3 lacking three protonatable aminoacids was found to be unable to activate NPQ *in vivo* and to be insensitive to pH changes *in vitro*. Results regarding this part of my work are presented in chapter 2 (published as Ballottari et al. (2016) in the Journal of Biochemistry).

After green algae, I continued my research on LHCSR proteins in mosses: in chapter 3, I report the results of the *in vitro* investigation of the spectroscopic and quenching characteristics of different pigment-binding mutants of the protein LHCSR1 from the moss *Physcomitrella patens*. Since the protein LHCSR1, contrary to PSBS, binds pigments and has been shown to be the main driver of NPQ in *P. patens*, in particular upon the binding of the xanthophyll zeaxanthin, it has been hypothesized that this protein may be directly involved in the quenching process. I analyzed reconstituted proteins mutated in each of the chlorophyll-binding residue identified, both with absorption and fluorescence analyses. I found that LHCSR1 binds at least eight chlorophylls and three carotenoids and that chlorophylls A2 and A5 are the lowest energy pigments in the protein.

LHCSR proteins are also present in diatoms, the third organism that I studied, where they are named LHCXs. Diatoms grow successfully in oceans where light conditions are highly variable and present an expansion of the LHCX protein family, but their exact role is not understood.



**Figure 1.18** – Scheme of the topics treated in the chapters of the thesis. LHCSR/LHCX proteins have been studied in different aspects in the various organisms (the pH-sensing and activation of the protein in green algae, the role of the different pigments bound to it in mosses, and the regulation by environmental stresses of the various isoforms in diatoms), while the roles of the two xanthophyll cycles has been addressed in diatoms.

In the laboratory of Dr. Angela Falciatore, in Paris, I investigated the role of the LHCXs in diatom stress responses (chapter 4), showing a relationship between the different expression of *LHCX* genes and photosynthetic activity under various light intensities and nutrient starvation conditions. Data indicate that the expansion of the LHCX gene family reflects a functional diversification of its members, which could benefit cells responding to highly variable ocean environments (chapter published as Taddei, Stella et al. (2016) in the Journal of Experimental Botany).

In chapter 5 (presented as paper in preparation), I report my results regarding the other main driver of photoprotection in diatoms, the xanthophyll cycles. Differently from the green lineage, diatoms possess both the violaxanthin and the diadinoxanthin cycles, and the amount of diatoxanthin is strictly correlated to NPQ level. The actual role of zeaxanthin in photoprotection in diatoms has thus been questioned, and to understand the regulation and function of these two xanthophyll cycles, I knocked-down each of the putative gene involved in this process in the model system *Phaeodactylum tricornerutum*. Characterization of knock-down mutants for VDR and VDL2, performed in Paris and in Verona, showed that these transgenic lines had a lower NPQ and accumulated more pigments of the violaxanthin cycle and less of the diadinoxanthin cycle. None of these lines was impaired in the de-epoxidation of violaxanthin or diadinoxanthin, indicating that VDR and VDL2 are involved in the biosynthesis of pigments, rather than in the xanthophyll cycles themselves. Accumulation of violaxanthin and zeaxanthin had a negative effect on NPQ, likely because of the interference of these pigments with diatoxanthin activity.

Thanks to these studies done on different organisms, I gained a deeper knowledge on the shared characteristics and on the peculiar features about photoprotection in green algae, mosses and diatoms. Conclusions and perspectives of this work are presented in chapter 6.

## Chapter 2

# Identification of pH-sensing sites in the Light Harvesting Complex Stress-Related 3 protein essential for triggering non-photochemical quenching in *Chlamydomonas reinhardtii*

### Summary of the work

Since the year 2000, photoprotection in plants was known to rely on the protein PSBS (Li et al. (2000)), which was later found to be a pH-sensor for the thylakoid lumen and to be activated in excessive light conditions (Li et al. (2004)). PSBS seemed not to be expressed in green algae (Bonente et al. (2008)), and in 2009, Peers et al. discovered that light harvesting complex stress-related proteins (LHCSR3) can regulate photoprotection in *Chlamydomonas reinhardtii* in a PSBS-independent manner. Further studies, conducted in the laboratory of Prof. Roberto Bassi, showed that LHCSR3 from *C. reinhardtii* is a pigment-binding protein and that it is able to quench fluorescence *in vitro* in response to pH changes (Bonente et al. 2011).

At the beginning of my Ph.D. in Verona (Italy), we were thus wondering if LHCSR proteins had a pH-sensing mechanism similar to the one of PSBS, were two glutamate get protonated and activate NPQ when pH in the thylakoid lumen drops. In the group of Prof. Bassi, we decided to address this question analyzing LHCSR3 protein from *C. reinhardtii*, both *in vivo* and *in vitro*, in collaboration with the laboratory of Krishna K. Niyogi in Berkeley (CA, USA). I built the 3D model of LHCSR3 and analyzed the sequences alignment to identify possible protonatable residues, by checking their predicted biochemical features as well as their conservation in different organisms. The *in vivo* mutagenesis, conducted in Berkeley, of the residues I identified, pointed to an essential role of residues Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup> in NPQ development. We thus mutated the same aminoacids and I refolded LHCSR3 WT and mutant proteins *in vitro* to study their characteristics in response to pH changes. I measured the spectroscopic features of the two

LHCSR3 and found that also *in vitro* the mutated protein had a lower response to acid pH in its fluorescence emission properties and a lower capacity to bind DCCD (a chemical that binds to protonatable residues) with respect to WT. Finally, fluorescence lifetime measurements were conducted in Berkeley with the proteins I produced, confirming the important role of residues Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup> in pH response in LHCSR3.

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I was responsible of LHCSR3 structure modeling, sequence analysis, *in vitro* re-folding of wild-type and mutant proteins, as well as their biochemical and spectroscopic characterization (with the exception of lifetime fluorescence measurements).

# Identification of pH-sensing Sites in the Light Harvesting Complex Stress-related 3 Protein Essential for Triggering Non-photochemical Quenching in *Chlamydomonas reinhardtii*\*

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Light harvesting complex stress-related 3 (LHCSR3) is the protein essential for photoprotective excess energy dissipation (non-photochemical quenching, NPQ) in the model green alga *Chlamydomonas reinhardtii*. Activation of NPQ requires low pH in the thylakoid lumen, which is induced in excess light conditions and sensed by lumen-exposed acidic residues. In this work we have used site-specific mutagenesis *in vivo* and *in vitro* for identification of the residues in LHCSR3 that are responsible for sensing lumen pH. Lumen-exposed protonatable residues, aspartate and glutamate, were mutated to asparagine and glutamine, respectively. By expression in a mutant lacking all LHCSR isoforms, residues Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup> were shown to be essential for LHCSR3-dependent NPQ induction in *C. reinhardtii*. Analysis of recombinant proteins carrying the same mutations refolded *in vitro* with pigments showed that the capacity of responding to low pH by decreasing the fluorescence lifetime, present in the wild-type protein, was lost. Consistent with a role in pH sensing, the mutations led to a substantial reduction in binding the NPQ inhibitor dicyclohexylcarbodiimide.

Photosynthetic organisms convert sunlight absorbed by chlorophyll into chemical energy by reducing CO<sub>2</sub> into sugars with electrons extracted from water, yielding O<sub>2</sub>. However, molecular oxygen can react with chlorophyll triplets (<sup>3</sup>Chl\*)<sup>6</sup> to yield singlet oxygen, one of several types of reactive oxygen species, which damage biological molecules (1). Because <sup>3</sup>Chl\* originates from <sup>1</sup>Chl\*, prevention of photooxidative stress can be obtained by quenching <sup>3</sup>Chl\*, scavenging reactive oxygen species, or by quenching <sup>1</sup>Chl\*. In addition, the photon absorption rate can be regulated by chloroplast relocation within the cell or changes in leaf orientation (1–3).

Of particular importance are the non-photochemical quenching (NPQ) mechanisms that quench <sup>1</sup>Chl\* and dissipate excess excitation energy as heat when light absorption exceeds the capacity of photochemical reactions. NPQ includes several components, the major and fastest of which is energy-dependent quenching (qE), which is sensitive to uncouplers (4, 5). qE is a feedback process triggered by thylakoid lumen acidification (4, 6–10). Saturation of downstream reactions leads to depletion of ADP and P<sub>i</sub>, the substrates of ATPase, which prevents the efflux of protons generated by photosynthetic electron transport from the thylakoid lumen to the stroma, leading to lumen acidification.

Genetic analysis of qE activation led to the identification of PSBS and LHCSR (11–15) as gene products required for qE in the model plant *Arabidopsis thaliana* and the green alga *Chlamydomonas reinhardtii*, respectively (12, 13, 16–18). In *C. reinhardtii*, two LHCSR isoforms, LHCSR1 and LHCSR3, are active in qE. LHCSR3 strongly accumulates in excess light, whereas LHCSR1 is constitutively present even at low light levels (13, 19). Also, LHCSR-like proteins with qE activity have been identified in diatoms (20–23). A special case is found in mosses, where both PSBS and LHCSR proteins are present and involved

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<sup>6</sup> The abbreviations used are: <sup>3</sup>Chl\*, chlorophyll triplets; NPQ, non-photochemical quenching;  $\alpha$ -DM, *n*-dodecyl- $\alpha$ -D-maltopyranoside; Car, carotenoid; Chl, chlorophyll; qE, energy-dependent quenching; LHCSR, light harvesting complex stress-related; DCCD, dicyclohexylcarbodiimide; PSBS, photosystem II subunit S.

in qE induction (15, 24, 25). Although the fundamental mechanisms of quenching activity by PSBS and LHCSR are the subjects of intense investigation (26), they must be different because LHCSR is a chlorophyll- and xanthophyll-binding protein where quenching of  $^1\text{Chl}^*$  can be catalyzed as shown by its short fluorescence lifetime (18). In contrast, pigment-binding sites are not conserved in PSBS, suggesting that the quenching activity is elicited within interacting proteins (18, 25, 27). As for the capacity for sensing the lumenal pH, PSBS and LHCSR share the property of binding dicyclohexylcarbodiimide (DCCD), a protein-modifying agent that covalently binds to acidic residues involved in reversible protonation events (28). Indeed, we have previously shown that two glutamate residues in PSBS are responsible for both the DCCD binding *in vitro* and the NPQ activity *in vivo* (17). Sequence analysis of LHCSR proteins showed multiple conserved acidic residues exposed to the lumen as potential sites of protonation. Recombinant LHCSR3 from *C. reinhardtii* has been shown to be pH responsive and to undergo a switch to a dissipative state in acidic solution (18, 27). Mutation analysis has located eight putative pH-sensing residues in the C terminus of LHCSR3 (27), whereas PSII super-complexes containing LHCSR3 with a stoichiometry LHCSR3:PSII of 0.28 were reported to undergo a decrease in fluorescence lifetime when exposed to pH 5 (29).

Here, we have performed a detailed investigation of the pH-sensing activity in LHCSR3 from *C. reinhardtii*, including identification of lumen-exposed protonatable residues that have been mutated to non-protonatable ones. The effect of these mutations has been analyzed both by fluorescence lifetime analysis of the proteins refolded *in vitro* and by measuring NPQ activity *in vivo* upon expression in a mutant lacking both LHCSR3 and LHCSR1. This comprehensive procedure led to the identification of three residues that are crucial for pH-dependent quenching *in vivo* and *in vitro* and are also responsible, to a large extent, for the binding of the qE inhibitor DCCD.

## Experimental Procedures

**LHCSR3 Structure Modeling**—LHCSR3 protein structures were obtained using homology modeling techniques with the on-line servers I-TASSER (30, 31) version 1.1. The model with the best C-score (confidence score) was selected for further analysis.

**Site-directed Mutagenesis of Acidic Residues**—The LHCSR3.1 genomic clone plasmid LHCSR3/GwypBC1 from previous complementation experiments (13) was used for site-directed mutagenesis of each acidic residue reported in the text. The QuikChange® Site-directed Mutagenesis Kit was used according to the manufacturer's instructions.

**Transformation and Isolation of Site-directed Mutants**—The plasmid LHCSR3/GwypBC1 containing each or multiple mutations was transformed into either *npq4* (13) or *npq4 lhcsr1* (32) and transformants were selected for paromomycin resistance. At least 300 colonies were picked for each line and patched onto HS minimal medium to grow in high light (400  $\mu\text{mol}$  of photons  $\text{m}^{-2} \text{s}^{-1}$ ). NPQ was measured by chlorophyll fluorescence video imaging (Imaging-PAM, Walz). Selected colonies, as judged by their NPQ value relative to the parent strain, were further cultured in liquid HS to measure via a pulse-amplitude-

modulated fluorometer (FMS2, Hansatech) and for immunoblots, as previously described (13).

**Recombinant Protein Overexpression, Purification, and In Vitro Refolding**—LHCSR3 coding sequence was cloned in pET28 expression vector and expressed in *Escherichia coli* as previously described (18). Purified apoprotein was refolded *in vitro* in the presence of pigments as reported in Refs. 18 and 33.

**Pigment Analysis**—Pigments bound by recombinant LHCSR3 were measured as described in Ref. 34.

**SDS-PAGE, Coomassie Staining, DCCD Binding, and Western Blot**—SDS-PAGE was performed as reported in Ref. 18. SDS-PAGE gel was then stained with Coomassie-R as described in Ref. 35. DCCD binding properties of recombinant LHCSR3 proteins were estimated by incubating the refolded protein with [ $^{14}\text{C}$ ]DCCD and subsequent autoradiography evaluation of the binding as previously described (18, 28, 36). Western blot analysis was performed as described in Ref. 13.

**Fluorescence Lifetime Measurements**—Fluorescence decay kinetics were measured on recombinant LHCSR3 protein using a time-correlated Single Photon Counting apparatus similar to the one described in Ref. 10. 150-fs pulses centered at 820 nm are generated by a Ti:Sapphire oscillator (Coherent Mira 900F) at 76 MHz repetition rate. The pulses are frequency-doubled in a 1-mm thick BBO crystal and their repetition rate is reduced by a factor of 8 with a pulse picker (Spectra Physics model 3980). The resulting pulses are centered at 410 nm, with 12-nm bandwidth full width half-maximum, and with an energy at sample of  $\sim 10$  pJ/pulse. The fluorescence emitted by the sample passes through a polarizer set at magic angle, followed by either a monochromator (Horiba Jobin-Ivon H-20) or a long-pass filter. The detection system is composed of a MCP/PMT detector (Hamamatsu R3809U), electrically cooled to  $-30$  °C. The detector is connected to a PC computer with a DCC-100 detector control card (Becker-Hickl). The full width half-maximum of the instrument response function is measured to be 45–55 ps. The samples are held in 1- or 2-mm thick quartz cuvettes (Starna Cells), and kept at  $\sim 12$  °C during the measurements with a home-built nitrogen cooling system.

**Steady-state Absorption, Fluorescence, and Circular Dichroism Measurements**—Room temperature absorption spectra were recorded using an SLM-Aminco DK2000 spectrophotometer, in 10 mM HEPES, pH 7.5, 0.2 M sucrose, and 0.03% *n*-dodecyl- $\alpha$ -D-maltopyranoside. The wavelength sampling step was 0.4 nm. Fluorescence emission spectra were measured using a Jobin-Yvon Fluoromax-3 device. Circular dichroism (CD) spectra were measured at 10 °C on a Jasco 600 spectropolarimeter using a R7400U-20 photomultiplier tube: samples were in the same solution described for the absorption with an OD of 1 at the maximum in the Q<sub>y</sub> transition. The measurements were performed in a 1-cm cuvette. Denaturation temperature measurements were performed by following the decay of the CD signal at 682 nm when increasing the temperature from 20 to 80 °C with a time slope of 1 °C/min and a resolution of 0.2 °C. The thermal stability of the samples was determined by finding the  $T_{1/2}$  of the signal decay.

**Dynamic Light Scattering Measurements**—The size of aggregates induced by detergent dilution was determined by dy-

## LHCSR3 pH Sensing Sites for Non-photochemical Quenching

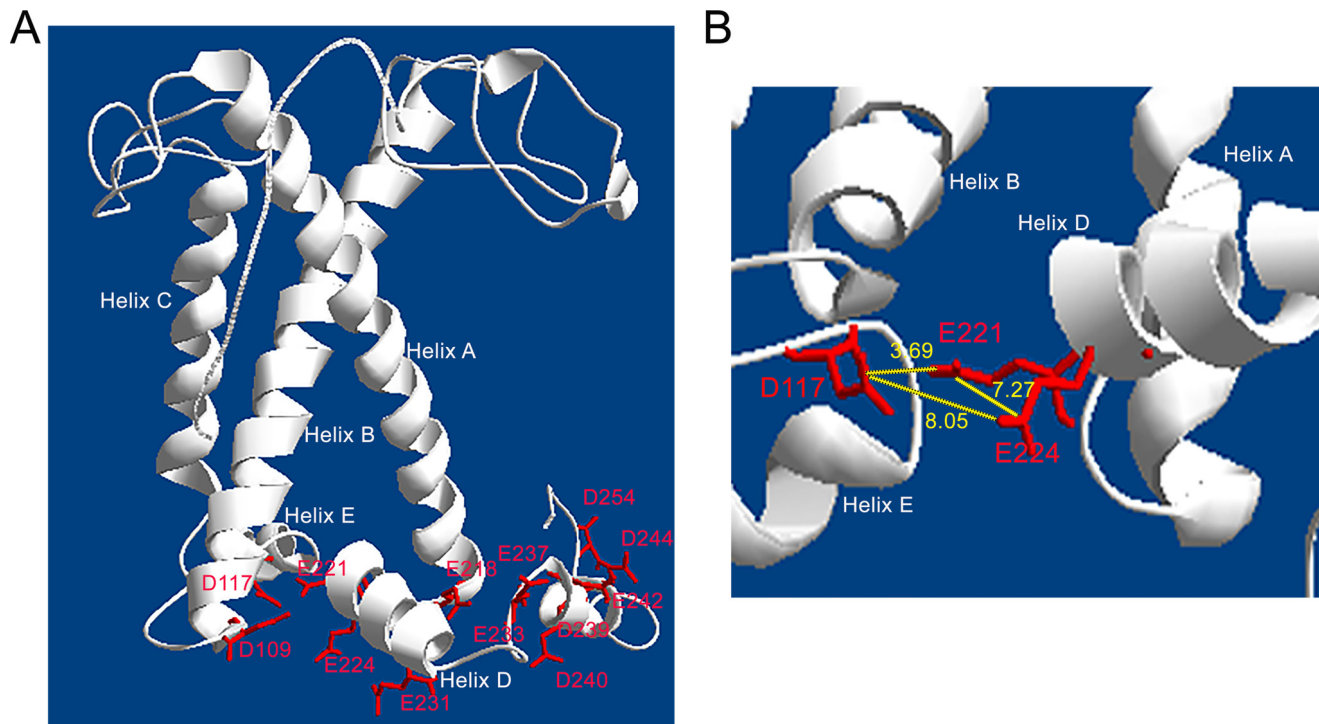


FIGURE 1. **Three-dimensional model of LHCSR3.** Panel A, LHCSR3 structure modeled on LHCII and CP29 crystallographic structures. Putative protonatable sites are indicated. Panel B, zoom view on Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup> residues; the distance between the different residues is indicated in yellow (Å).

**TABLE 1**

### Evaluation of the accessibility to the solvent of LHCSR3 lumen-exposed residues

Solvent accessibility was predicted using I-TASSER software which scores each protein residue with a 0–9 figure with high score indicating higher probability for solvent exposure. Lumen-exposed regions are reported together with the solvent-accessibility score for each residue shown below. Amino acid position, within the LHCSR3 sequence is indicated above.

	100	120	220	240	255
<b>Sequence</b>	...GFVVGEQLQDFPLFFNWDGRV.....VEQTEIFEHLALRFEKEAILELDDIERDLGLPVTPLPDNLKSL				
<b>Prediction</b>	...010003313322222333232.....1464300310242044221220341454232330313641475				

dynamic light scattering using ZETASIZER NANO S instrumentation as described in Refs. 37 and 38.

## Results

**Structural Model of LHCSR3**—A model of LHCSR3 (Fig. 1) was created using as a template the three-dimensional structure of other LHC proteins, LHCII, CP29, and LHCI (39–41), with the aim of identifying protonatable residues exposed to the thylakoid lumen. The analysis of the protein model is consistent with LHCSR3 conserving the three trans-membrane  $\alpha$ -helices (helix A, B, and C) and two amphipathic helices (helix D and E) revealed from crystallographic analysis of CP29 and LHCII; a short additional helix was predicted at the C-terminal domain, significantly more extended than in LHCII or CP29. LHCSR3 bears several acidic amino acid residues (*i.e.* aspartate and glutamate) predicted to face the thylakoid lumen. In particular, the C-terminal domain contains eight acidic residues: Glu<sup>231</sup>, Glu<sup>233</sup>, Glu<sup>237</sup>, Asp<sup>239</sup>, Asp<sup>240</sup>, Glu<sup>242</sup>, Asp<sup>244</sup>, and Asp<sup>254</sup>, as described in a previous report (27). Additional residues exposed to the lumen include Glu<sup>221</sup>, Glu<sup>224</sup> at the luminal end of Helix D, the residues Asp<sup>109</sup> and Asp<sup>117</sup> in the loop between Helix B and Helix E, and residue Glu<sup>218</sup>, located in the loop connecting

Helix A and Helix D. To evaluate the accessibility of these residues to the solvent, the LHCSR3 protein sequence was analyzed by I-TASSER software (30, 31), which ranks the probability of a residue to be solvent-exposed within a range of 1 to 9, with a higher number indicating a higher probability. As reported in Table 1, the values obtained for most of the selected glutamates and aspartates were in the range of 3–4 with the exception of Glu<sup>233</sup> and Glu<sup>237</sup> (scoring 2) and Asp<sup>254</sup> (scoring 6).

**Identification of the LHCSR3 Protonatable Sites Involved in Luminal pH Sensing**—The evaluation of the conservation of specific residues in sequences from different species can facilitate the identification of residues that are crucial for protein function. When the LHCSR3 protein sequence from *C. reinhardtii* was compared with homologous sequences from other organisms, either microalgae or mosses (Table 2), several residues appeared to be highly conserved (Fig. 2A). Most of the chlorophyll-binding sites previously identified in LHCSR3 from *C. reinhardtii* can also be found in LHCSR proteins from other species, namely the residues binding chlorophyll at the A1, A4, and A5 sites, according to the nomenclature previously used for LHCII chlorophyll binding sites (18, 42). However,



**TABLE 2**

**LHCSR-like protein sequences used for the determination of conserved residues**

Protein sequences were selected by BLAST search using LHCSR3 mature protein sequence as query. Each sequence was selected for having a score >150 and *e*-value <6  $e^{-41}$ .

Protein sequence	Organism
XP_001696064.1	<i>C. reinhardtii</i> LHCSR3
XP_001696125.1	<i>C. reinhardtii</i> LHCSR1
XP_002948670.1	<i>V. carteri</i> f. <i>nagariensis</i>
ADP89594.1	<i>Chlamydomonas</i> sp. ICE-L LHCSR2
Q03965.1	<i>C. moewusii</i>
XP_001768071.1	<i>P. patens</i> LHCSR2
ABD58893.1	<i>Acutodesmus obliquus</i>
XP_005647960.1	<i>Coccomyxa subellipsoidea</i> C-169
ADY38581.1	<i>U. linza</i>
ADU04518.1	<i>U. prolifera</i>
XP_005848576.1	<i>C. variabilis</i>
ABD37894.1	<i>M. viride</i>
XP_002178699.1	<i>Phaeodactylum tricornutum</i>
XP_002295258.1	<i>Thalassiosira pseudonana</i>
CAA04403.1	<i>Cyclotella cryptica</i>
AHH80644.1	<i>Durinskia baltica</i>
DAA05890.1	<i>Bigeloviella natans</i>
CCO66741.1	<i>Bathycoccus prasinos</i>
EGB07306.1	<i>A. anophagefferens</i>
EJK65083.1	<i>Thalassiosira oceanica</i>
ABV22207.1	<i>Karlodinium veneficum</i>
CBJ27803.1	<i>Ectocarpus siliculosus</i>
ABA55525.1	<i>Isochrysis galbana</i>
XP_003079276.1	<i>O. tauri</i>
AAAY27550.1	<i>O. tauri</i>
XP_001417976.1	<i>O. lucimarinus</i>

some variability can be found for the A2, B5, and A3 sites. Finally, a residue at the position of binding site B6 (glutamate) was found only in a few sequences. As for the lumen-exposed residues from *C. reinhardtii*, their conservation is far from complete among the different sequences. In particular, as reported in Fig. 2B, the C-terminal domain, which was reported to be the knob of a dimmer switch to control the transition to a dissipative state (27), is present only in *Chlamydomonas* species, *Volvox carteri*, and *Aureococcus anophagefferens*, whereas the number of acidic residues within this domain is variable in the different organisms (Fig. 2B). LHCSR-like sequences from *Ostreococcus tauri*, *Ostreococcus lucimarinus*, and *Chlorella variabilis* show a single protonatable glutamate residue in the C terminus. Moreover, this domain was significantly shorter in the remaining sequences. In contrast, the residues in Helix E, Glu<sup>221</sup> or Glu<sup>224</sup> in LHCSR3 from *C. reinhardtii*, were conserved in 18 of 26 sequences analyzed. As for residue Glu<sup>218</sup>, this is only found in *C. reinhardtii*, *Chlamydomonas moewusii*, and *V. carteri*, whereas in *Chlamydomonas* sp. ICE a conservative replacement to aspartate was identified. Nevertheless, a glutamate was found very close to this position and shifted toward the N terminus in all the other accessions, suggesting it might have a conserved functional role. The analysis of the conservation of residues Asp<sup>109</sup> and Asp<sup>117</sup> showed that Asp<sup>109</sup> is present in 9 accessions, whereas Asp<sup>117</sup> is present in 17 accessions as aspartate, or replaced by glutamate in sequences from *Ulva linza* and *Ulva prolifera*. It is worth noting, however, that all the accessions that do not bear Asp<sup>117</sup>, do have one or more aspartate residues within 1–3 positions, the only exception being the sequence from *Mesostigma viride* lacking aspartates or glutamates in that protein domain. On the basis of these results, Asp<sup>109</sup>, Asp<sup>117</sup>, Glu<sup>218</sup>, Glu<sup>221</sup>, Glu<sup>224</sup>, Glu<sup>231</sup>, and

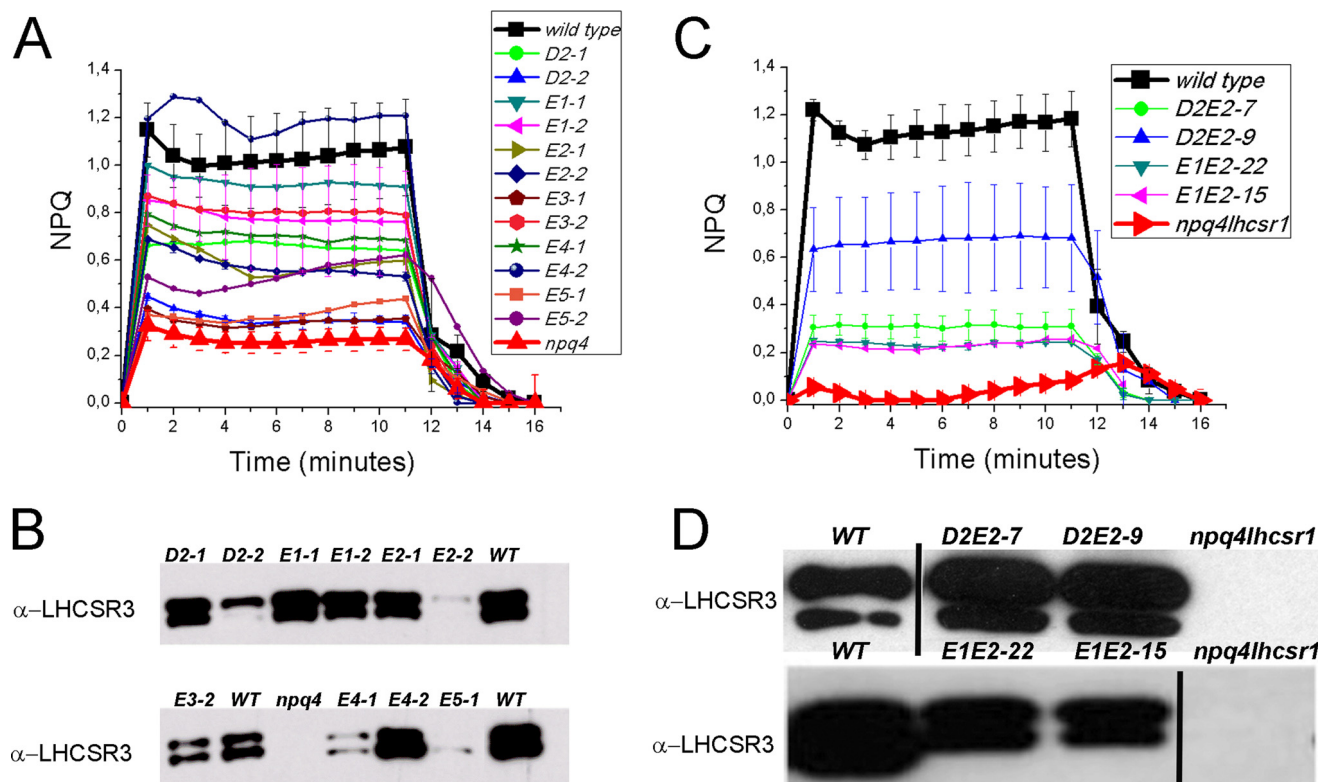
Glu<sup>233</sup> were selected for further investigation and renamed, respectively, D1, D2, E3, E1, E2, E4, and E5 for simplicity.

**In Vivo Mutation Analysis**—To test the importance of these protonatable residues for LHCSR3 function, each of the selected aspartate or glutamate residues was mutagenized *in vitro* to asparagine or glutamine, respectively, and the resulting mutant LHCSR3 genomic DNA sequence was used to transform the *npq4* mutant strain lacking LHCSR3 expression (13). After selection of transformants based on paromomycin resistance, these were screened to determine the effect of the amino acid replacement on the NPQ activity. Fig. 3A shows that when each individual acidic residue was mutated, the NPQ amplitude was reduced, but a significant level of quenching was still present. Indeed, the transformant lines exhibited an NPQ level proportional to the level of LHCSR3 protein accumulation as assessed by Western blotting (Fig. 3B), suggesting redundancy of the proton-sensing residues in LHCSR3. Strains transformed with LHCSR3 variants mutated at residue D1 did not show any accumulation of LHCSR3, suggesting a major role of this residue in stabilizing protein folding. Therefore, combinations of multiple mutations within the same protein were generated and tested. When D2, E1, and E2 residues were mutated together, the triple mutant had an NPQ amplitude similar to that of the *npq4* strain. Indeed, of more than 300 colonies of the triple mutant D2E1E2 in the *npq4* background, none had higher NPQ than *npq4*, despite the accumulation of the mutant LHCSR3 protein at wild-type level.

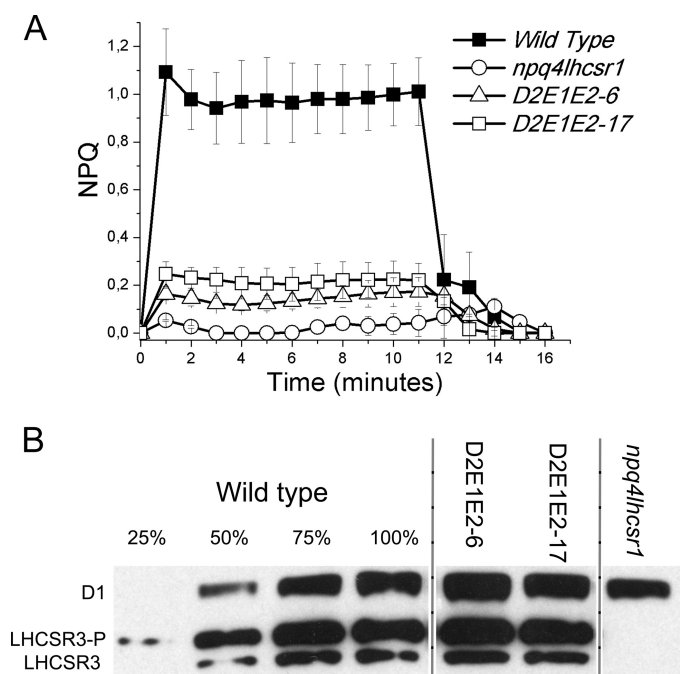
To improve the signal to background ratio in the NPQ assays, subsequent transformations were done with the *npq4 lhcsr1* double mutant (32). This system would allow for better resolution of the effect that the mutated LHCSR3 protein has on NPQ, independent of the LHCSR1 isoform that remains in the *npq4* mutant. Combinations of the double mutations, D2E2 and E1E2, were made and transformed into *npq4 lhcsr1* (Fig. 3, C and D). These double mutations impaired but did not completely eliminate the qE function of LHCSR3, because the expressed LHCSR3 protein still conferred some NPQ in the *npq4 lhcsr1* background (Fig. 3, C and D). Lines of the E1E2 mutant accumulating LHCSR3 at 50–60% with respect to the wild-type had ~20% of wild-type qE (Fig. 3C). Two lines from the D2E2 transformations with more than wild-type LHCSR3, had only ~30 and ~50% of the qE found in wild-type, respectively (Fig. 3C). The D2E1E2 triple mutant version of LHCSR3 was then transformed into the *npq4 lhcsr1* genotype. As shown in Fig. 4, two independent lines expressed the D2E1E2 mutant protein at a level close to the wild-type LHCSR3 protein level, but they exhibited the lowest qE activity observed (~25% of the wild-type level). The residual qE induction observed in the triple D2E1E2 mutant could be related to the activity of one or more of the other protonatable sites that are still present in the mutant. Unfortunately, the addition of further mutations in the LHCSR3 gene resulted in a loss of protein accumulation, suggesting a strong destabilization of the protein or some impairment in protein import into the thylakoid membranes.

**In Vitro Reconstitution of LHCSR3 Recombinant Protein Mutated on Protonatable Sites**—The function of the three identified protonatable sites in LHCSR3 was next investigated *in vitro* using recombinant proteins. In particular, the LHCSR3





**FIGURE 3. NPQ measurements and immunoblot analysis of LHCSR3 protein levels in *npq4* lines expressing site-specific mutant versions of LHCSR3 affecting protonatable sites.** Panel A, NPQ measurements on WT, *npq4* mutant, and transgenic lines with LHCSR3 proteins carrying a single mutation on putative protonatable sites D2, E1–5. Panel B, immunoblot analysis of LHCSR3 accumulation on genotypes analyzed in panel A; immunoblot analysis of the D1 subunit of PSII is shown as a control for loading. Panel C, NPQ measurements on WT, *npq4lhcsr1* mutant, and transgenic lines with LHCSR3 proteins with double mutations on putative protonatable sites D2E2 and E1E2. Panel D, immunoblot analysis of LHCSR3 accumulation on genotypes analyzed in panel C. In all cases three independent biological replicates were analyzed. The experiments were reproduced two times.



**FIGURE 4. NPQ measurements and immunoblot analysis of LHCSR3 protein levels in *npq4lhcsr1* lines expressing site-specific mutant versions of LHCSR3 affecting protonatable sites.** NPQ measurements on WT, *npq4lhcsr1* mutant, and transgenic lines with LHCSR3 proteins mutated on D2, E1, and E2 protonatable sites (panel A). Panel B, immunoblot analysis of LHCSR3 accumulation; immunoblot analysis of the D1 subunit of PSII is shown as a control for loading. In all cases three independent biological replicates were analyzed. The experiments were reproduced three times.

ences, neither in the Soret nor in the Qy spectral regions, suggesting that the mutations introduced no changes in the pigment organization in the complex. Similarly, circular dichroism spectra of the two samples were virtually identical (Fig. 5B). To assess if the mutations introduced could induce some level of protein destabilization, the thermal stability of recombinant WT and D2E1E2 mutant proteins was measured by following the change of the amplitude of the CD signal at 681 nm when slowly increasing the temperature of the samples. The melting temperatures ( $T_m$ ), calculated by fitting to a sigmoidal function, are reported in Table 4:  $T_m$  was similar for WT and the D2E1E2 mutant, 41.8 and 40.2 °C, respectively, suggesting that substitution of the three acidic residues in the D2E1E2 mutant did not alter the stability of the pigment-protein complex.

**[ $^{14}$ C]DCCD Binding of Recombinant LHCSR3 Proteins—**DCCD is an inhibitor of qE in *Chlamydomonas* (18). Its binding to acidic residues indicates reversible protonation events. An enhanced DCCD binding with respect to other LHC proteins has been reported for LHCSR3 (18), in agreement with its pH-sensing function. To assess the proton-binding activity of the Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup> residues, DCCD binding was measured in WT and D2E1E2 mutant proteins. *In vitro* refolded proteins were incubated with [ $^{14}$ C]DCCD, and the amount of  $^{14}$ C bound by LHCSR3 was determined by autoradiography. The level of  $^{14}$ C bound by LHCSR3 WT and D2E1E2 was then normalized to the protein amount loaded into the SDS-PAGE

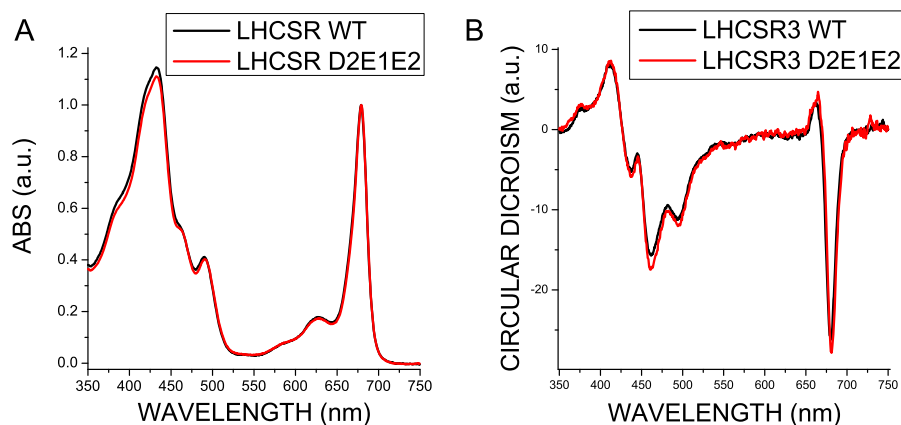
## LHCSR3 pH Sensing Sites for Non-photochemical Quenching

**TABLE 3**

**Pigment analysis of recombinant LHCSR3 proteins**

Pigment analysis was performed by HPLC and fitting of absorption spectrum of pigment acetone extracts with chlorophylls and carotenoids spectral forms as described in Ref. 34. The experiments were performed two times, with three independent biological replicates each time.

	Chl total	Chl <i>a</i>	Chl <i>b</i>	Viola	Lute	Neo	$\beta$ -car	Chl/Car	Chl <i>a/b</i>	Car total
WT	7	6.29	0.71	0.41	1.56	0.06	0.00	3.38	8.86	2.07
S.D.	/	0.10	0.05	0.03	0.07	0.01	0.00	0.31	0.64	0.19
D2E1E2	7	6.51	0.49	0.28	1.63	0.00	0.00	3.61	13.29	1.94
S.D.	/	0.09	0.07	0.03	0.05	0.01	0.00	0.22	1.91	0.11



**FIGURE 5. Absorption and circular dichroism spectra of LHCSR recombinant proteins.** Absorption spectra (panel A) and circular dichroism (panel B) in the visible region of LHCSR3 WT and D2E1E2 mutant refolded *in vitro* in the presence of chlorophylls and carotenoids. The experiments were reproduced three times, each time with two independent biological replicates.

**TABLE 4**

**Thermal stability of LHCSR WT and D2E1E2 recombinant proteins refolded *in vitro***

Thermal stability ( $T_m$ ) was evaluated following the decay of the CD signal at 682 nm when increasing the temperature from 20 to 80 °C. The thermal stability of the samples was determined by finding the  $T_{1/2}$  of the signal decay. The experiments were performed two times, with two independent biological replicates each time.

	$T_m$	S.D.
	°C	
WT	41.8	2.3
D2E1E2	40.2	0.9

gel quantified by Coomassie staining (Fig. 6). As reported in Fig. 6, both WT and D2E1E2 mutant bound [ $^{14}$ C]DCCD, but binding to the D2E1E2 mutant was decreased by 40% with respect to WT. This result supports the hypothesis of multiple protonatable sites in LHCSR3, of which Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup> account for at least 40% of the DCCD-binding activity of this protein.

**Fluorescence Lifetimes of Recombinant LHCSR3 Proteins—**Fluorescence lifetime measurements on recombinant LHC proteins allow investigation of their excitation energy conserving *versus* quenching properties (44). To investigate *in vitro* the pH-dependent regulation of LHCSR3 quenching activity, fluorescence decay kinetics of WT LHCSR3 and the D2E1E2 mutant were measured using a single photon counting device at neutral pH (7.5) and at low pH (5) in detergent solution of 0.03% *n*-dodecyl- $\alpha$ -D-maltopyranoside ( $\alpha$ -DM). As reported in Fig. 7A, the fluorescence decay kinetics of WT and the D2E1E2 mutant can be satisfactorily fitted with three exponentials with associated time constants of 4 ns, 1.9 ns, and ~200 ps. The relative amplitudes were 38–44, 48–52, and 7.4–9.7%, respectively, with an average lifetime

of 2.6–2.7 ns (Table 5). Decays were similar at both pH 7.5 and 5, suggesting no pH-dependent response of quenching reactions.

This result is in agreement with a previous report on LHCSR3 fluorescence lifetime in detergent (27), suggesting that interaction of detergent micelles with the protein prevents the switch to a dissipative conformation. pH sensitivity of the LHCSR fluorescence lifetime can be better detected at a low detergent/protein ratio leading to moderate aggregation, which reproduces protein-protein interactions occurring in the protein-crowded thylakoid membrane (45). Fig. 7B shows the fluorescence lifetimes of recombinant WT LHCSR3 and the D2E1E2 mutant as measured upon incubation in a detergent concentration of 0.003%  $\alpha$ -DM. These measuring conditions induced a faster decay of emitted fluorescence at either pH 5 or 7.5 for both LHCSR3 WT and D2E1E2 mutant. However, whereas at pH 7.5 the two proteins showed the same decay profile, at pH 5 LHCSR3 WT fluorescence decay was much faster than at pH 7.5, whereas the fluorescence decay of LHCSR3 D2E1E2 was the same as at pH 7.5. Decays of LHCSR3 WT at pH 7.5, and D2E1E2 at pH 7.5 and 5 were fitted to three exponentials with time constants of 2.5 ns, 0.9 ns, and 140 ps with amplitudes of 40, 31, and 29%, respectively, with an average lifetime of ~1.4 ns (Table 5). LHCSR3 WT decay traces at pH 5 were similarly fitted to three exponentials, but in this case the major amplitude was associated to the fastest component (140 ps) with amplitude of 42%, whereas those with 0.8 and 2.5 ns showed amplitudes of 33 and 25%, respectively (Table 5). Because aggregation is well known to influence the lifetime of LHC proteins (46), the aggregation size of WT and D2E1E2 proteins at 0.03 and 0.003% DM was measured by dynamic light scattering as previously reported (38), yielding the average aggregate size (113.3  $\pm$

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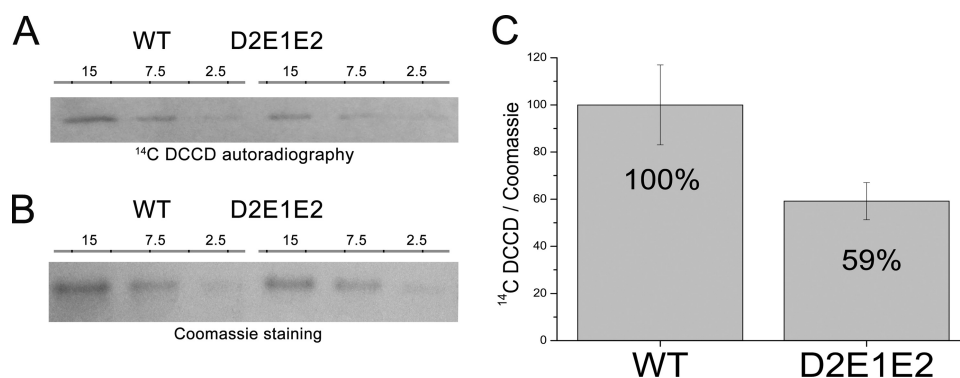


FIGURE 6.  $^{14}\text{C}$ DCCD binding in LHCSR3 recombinant WT and D2E1E2 mutant. *Panel A*, autoradiography of recombinant LHCSR3 WT and the D2E1E2 mutant treated with  $^{14}\text{C}$ DCCD; microliters of sample ( $0.2 \mu\text{g}/\mu\text{l}$  of chlorophylls) loaded on SDS-PAGE are reported (15, 7.5, and  $2.5 \mu\text{l}$ ). *Panel B*, Coomassie staining of SDS-PAGE used for autoradiography. *Panel C*, ratio of the level of  $^{14}\text{C}$  observed by autoradiography signals and protein quantity obtained by densitometric analysis of Coomassie-stained gels. The experiments were performed two times; each time two independent biological replicates were analyzed with three technical replicates with different loading volume as indicated.

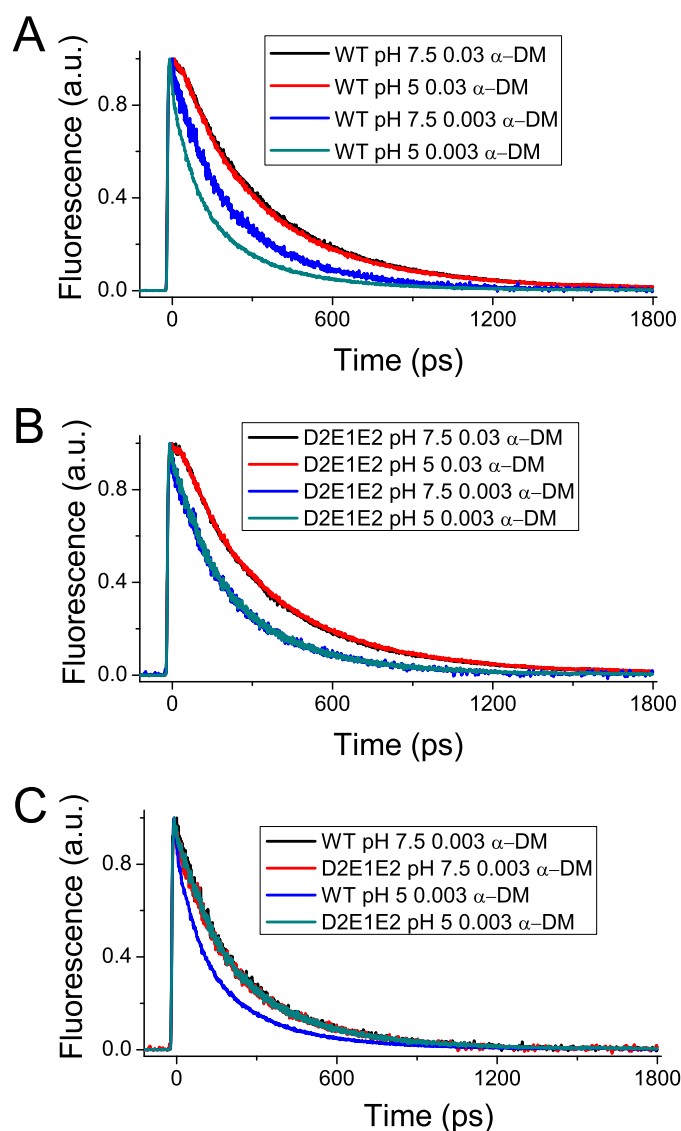


FIGURE 7. **Fluorescence decay kinetics.** Fluorescence decay kinetics of recombinant LHCSR3 WT (*panel A*) and D2E1E2 mutant (*panel B*) at pH 7.5 or 5.0 in the presence of high (0.03%) or low (0.003%) detergent ( $\alpha$ -DM) concentrations. The experiment was performed two times, each time with two independent biological replicates.

TABLE 5

### Fluorescence lifetimes of LHCSR3 WT and D2E1E2 mutant

The decay traces reported at Fig. 7 were fitting using three exponential functions. The amplitude (A) and time constants ( $\tau$ ) for each exponential are reported in the table. The average lifetimes for each sample are calculated as  $\sum A_i \tau_i$ .

	A1	$\tau_1$	A2	$\tau_2$	A3	$\tau_3$	$\tau_{\text{avg}}$
WT, pH 7.5, 0.03% $\alpha$ -DM	44%	4.04	48%	1.88	8%	0.21	2.69
WT, pH 5, 0.03% $\alpha$ -DM	40%	4.22	51%	1.91	10%	0.19	2.71
D2E1E2, pH 7.5, 0.03% $\alpha$ -DM	36%	4.37	54%	1.93	10%	0.18	2.63
D2E1E2, pH 5, 0.03% $\alpha$ -DM	39%	4.20	52%	1.83	9%	0.23	2.61
WT, pH 7.5, 0.003% $\alpha$ -DM	40%	2.61	31%	1.00	29%	0.14	1.39
D2E1E2, pH 7.5, 0.003% $\alpha$ -DM	41%	2.65	30%	0.98	29%	0.14	1.42
WT, pH 5, 0.003% $\alpha$ -DM	25%	2.50	33%	0.84	42%	0.14	0.97
D2E1E2, pH 5, 0.003% $\alpha$ -DM	41%	2.63	30%	0.99	29%	0.14	1.41

8.6 in the case of WT and  $126.9 \pm 18.2$ ). LHCSR3 WT and D2E1E2 in low detergent condition at pH 5 formed aggregates with similar size with a radius of 100 nm, implying that the difference in fluorescence quenching observed between WT and D2E1E2 is likely due to the different dissipative conformations that can be reached by the two proteins.

**Fluorescence Emission Spectra at 77 K**—Activation of quenching mechanisms has been previously associated *in vivo* with induction of far-red fluorescence emission forms at 77 K (38, 47, 48). In particular, aggregation-dependent quenching in LHC proteins at low pH was shown to lead to far-red emission in both trimeric and monomeric isoforms, and this feature has been correlated with the extent of excitation energy quenching (38). The fluorescence emission spectra at 77 K of LHCSR3 WT and D2E1E2 recombinant proteins were measured to investigate the correlation of far-red emission forms with the activation of quenching mechanisms. The measurements were performed at high (0.03%) or low (0.003%) detergent concentrations and at pH 7.5 or 5.0. As reported in Fig. 8, fluorescence emission spectra were almost identical at high detergent conditions for both WT and D2E1E2 samples at pH 7.5 or 5 with a peak at 682 nm. At low detergent, instead, a clear shift of the emission peak to 685 or 687 nm was observed for D2E1E2 and WT, respectively. The most evident change in the spectra, however, was observed at low detergent concentrations and pH 5, where both WT and D2E1E2 dramatically increased their far-red emission forms, with the formation of a defined peak at 735

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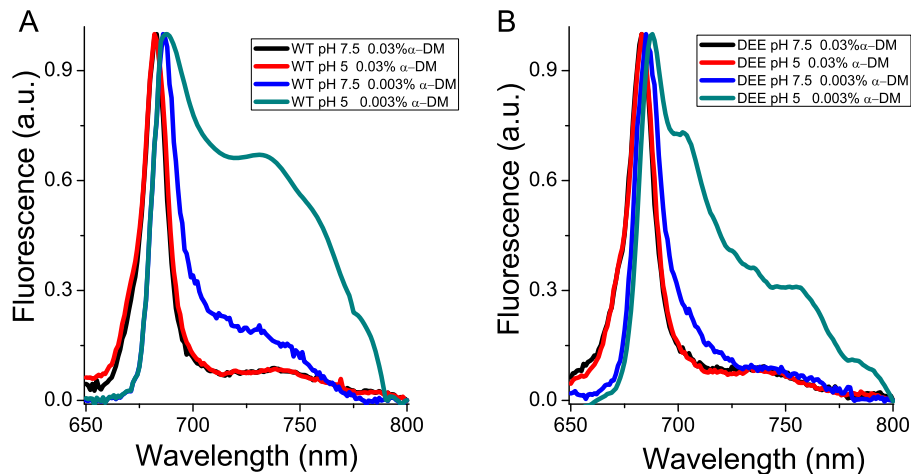


FIGURE 8. **77 K fluorescence emission spectra.** Fluorescence emission spectra of recombinant LHCSR3 WT (panel A) and the D2E1E2 mutant (panel B) at 77 K measured at pH 5 or 7.5 in the presence of 0.03%  $\alpha$ -DM or 0.003%  $\alpha$ -DM. The experiments were reproduced three times, each time with two independent biological replicates.

nm that was far more intense in WT *versus* D2E1E2. These results support the presence of a positive relationship between protonation of specific residues, the appearance of far-red emission forms in the spectra, and the activation of quenching mechanisms in LHCSR3.

### Discussion

All oxygenic photosynthetic organisms are endowed with mechanisms for thermal dissipation of excess absorbed light energy. The triggering of these mechanisms can either be controlled directly by light as for the Orange Carotenoid-binding protein of cyanobacteria (49, 50) or by low luminal pH caused by excess light as in the case of PSBS in plants and LHCSR in unicellular algae (10, 13, 18, 26, 51, 52). LHCSR3 is of particular interest because the quenching and the pH-sensing activities are merged in the same protein subunit (18, 27, 53) making this protein a relatively simple system for the molecular analysis of NPQ. The case of plants is more complex because the pH is sensed by PSBS, whereas quenching occurs in an interacting pigment-binding partner (17, 26). LHCSR3 has been reported to undergo functional changes depending on pH (18, 27, 53). In this work, the function of LHCSR3 as a sensor of lumen pH has been investigated *in vivo* by site-specific mutagenesis of putative protonatable residues. The LHCSR3 structure was modeled on the basis of LHCII and CP29 structures (39, 40) (Fig. 1) allowing for identification of 13 potentially protonatable aspartate and glutamate residues located within lumen-exposed domains at the C terminus, at Helices D and E, and at the loops between Helices A and D and between Helices B and E. Among these residues, Asp<sup>117</sup>, Glu<sup>218</sup>, Glu<sup>221</sup>, Glu<sup>224</sup>, Glu<sup>231</sup>, and Glu<sup>233</sup> were selected based on their high conservation among LHCSR-like sequences and were targeted for site-specific mutagenesis and functional analysis *in vivo*. The complementation of *npq4* and *npq4 lhcsr1* mutants with sequences carrying mutations affecting these protonatable residues identified Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup> as the key residues for the pH sensitivity of LHCSR3 *in vivo*. Any single mutation of an acidic amino acid residue failed to yield significant effects on qE. When double mutants affecting two different putative protona-

table sites were obtained, a substantial decrease in qE relative to the LHCSR3 protein level could be observed, suggesting a cooperative behavior (Fig. 4). The triple D2E1E2 mutant expressed in *npq4 lhcsr1* showed an even greater impairment of function, suggesting that Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup> are key residues for pH sensing in LHCSR3 from *C. reinhardtii*.

These results are consistent with the significant decrease observed in DCCD binding to LHCSR3 recombinant proteins mutated on Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup>, *i.e.* a reduction by 41% (Fig. 6). The fact that DCCD can still be bound by the D2E1E2 mutant is not surprising, because structure modeling revealed the presence of 10 additional acidic residues, including 8 glutamate and aspartate residues at the C terminus that are likely to bind DCCD in the D2E1E2 mutant. It is interesting to note that the mutation of 23% of the putative protonatable residues, as in the case of D2E1E2 mutant, led to a 41% reduction in DCCD-binding activity of LHCSR3, suggesting that these residues have a special cooperative role in transducing the luminal pH signal, as shown by a 72% of reduction in qE *in vivo* (Fig. 4). The presence of additional glutamate and aspartate residues at the C terminus in LHCSR3 is a peculiar feature of *Chlamydomonas* spp. LHCSR proteins (Fig. 2), whereas other LHCSR-like proteins have a shorter C terminus extension (Fig. 2B). Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup> are more conserved in the different LHCSR-like sequences analyzed. Nevertheless, it is worth noting that a substantial level of variability is present in the position and number of acidic residues, which might reflect the need for complementarity with interacting proteins putatively involved as partners in qE activity (54, 55). Alternatively, the density of lumen-exposed acidic residues might be related to the response sensitivity for triggering qE, which is particularly strong in *Chlamydomonas*, for which light saturation of photosynthesis occurs at lower irradiances (56), whereas NPQ amplitude is fully reached already at 200  $\mu\text{mol m}^{-2} \text{s}^{-1}$  illumination (57).

It is interesting to compare the distribution of acidic residues in *Chlamydomonas* to that of *Physcomitrella patens* LHCSR1, which only harbors 4 of the 13 putative protonatable residues identified in LHCSR3. *P. patens* LHCSR1 has been shown to

require zeaxanthin for a significant level of activity (25), whereas *Chlamydomonas* NPQ is not dependent on zeaxanthin accumulation (18). Zeaxanthin binding has been shown to confer cooperativity to NPQ in higher plants (58–60). It is tempting to propose that zeaxanthin might replace the effect of the 9 additional lumen-exposed acidic residues in promoting the switch of LHCSR from conservative to dissipative conformations. Indeed, we verified that faster fluorescence decay was triggered by acidic pH in WT LHCSR3 but not in the D2E1E2 mutant, implying that the protonation of Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup> has a special role in triggering quenching events within LHCSR3 *in vitro* (Fig. 7). A previous report has shown that mutation of 9 acidic residues at the C terminus, including Glu<sup>224</sup>, led to impaired pH sensitivity of LHCSR3 *in vitro* (27). Here we show that 72% of qE activity *in vivo* was dependent on the mutation of only three protonatable residues, consistent with loss of pH responsiveness *in vitro*. Residual NPQ activity in D2E1E2 is likely due to the presence of several other protonatable residues in the D2E1E2 mutant, partially inducing a small NPQ activation in the triple mutant. By the way it could not be excluded that the protonation of other LHCBM subunits, as LHCBM1 (18), would contribute to pH-dependent triggering of a low NPQ activity in D2E1E2.

As previously reported, LHCSR3 does not respond significantly to pH variation when the protein is dissolved in detergent such as  $\alpha$ -DM, whereas the pH sensitivity becomes evident when detergent is substituted by nano-polymers (27) or decreased to levels below the critical micelle concentration. This latter condition induces the formation of small particle arrays, mimicking protein-protein interactions in the thylakoid membrane (47), a condition likely to also occur in PSII-LHCSR supercomplexes (53). Recent results showed that high detergent conditions favor monodispersion of LHCS and shift their conformation far from the dissipative state toward a state poorly responding to pH variations (45). This is likely due to the induction of a relaxed protein conformation that decreases pigment-pigment interactions within the complexes with respect to the state present in the native membrane environment. Structural analysis suggested that conformational changes involved in quenching are subtle (61, 62) and involve small changes in Chl-Chl and xanthophyll-Chl interactions (11) thus making the relaxed structure unfavorable to trigger quenching. The main effect induced at pH 5 on fluorescence decay kinetics of WT LHCSR3 was an increased amplitude of the 140-ps ( $\tau_3$ ) component, which favorably compares with the 65- and 305-ps components recently identified as induced *in vivo* upon qE activation in *C. reinhardtii* (10) and the 200-ps component identified when measuring fluorescence lifetimes of LHCSR3-binding PSII supercomplexes at pH 5 (53).

The mechanism by which LHCSR3 dissipates excitation energy quenching is still debated. High yield of a carotenoid radical cation has been previously reported (18), and formation of these radical species has been previously related to NPQ in plants (63, 64). Here, we present evidence that aggregation-dependent quenching is also active in LHCSR3, as in other LHC proteins (38, 45, 46, 59, 62, 65, 66). Interestingly, a strong red-shift in fluorescence emission was associated with the low pH effect in the LHCSR3 WT, but not in the D2E1E2 mutant (Fig.

5). The formation of these far-red emitting forms is dependent on pH and protonation of Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup>. The correlation between far-red emission and switch to a dissipative state has been previously reported for plant LHCII (47), possibly resulting from a strong coupling between chlorophylls (67). It is interesting to note that the only Chl-binding residues fully conserved through LHCSR-like sequences are those associated with sites A1, A4, and A5 (Fig. 2), as putative ligands for Chl 601, Chl 610, and Chl 609 (39). Site A1 has been previously reported to be crucial for protein stability in most LHC proteins (34, 43) acting as a bridge between Helices A and B. Chl-binding sites A4 and A5, instead, are located in proximity of the Car-binding site L2, with a special role in <sup>3</sup>Chl\* quenching (68). Together with the Chl in site B5, these Chls form a strongly coupled cluster (40, 69), which has been associated to Car radical cation formation (11). Interactions between Chls and between Chls and xanthophylls are likely involved in LHCSR3 quenching activity. The structural model of LHCSR3 in Fig. 1 shows that the three acidic residues Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup> are relatively close to each other, with an estimated distance of 3.69, 7.27, and 8.05 Å, respectively (Asp<sup>117</sup>-Glu<sup>221</sup>, Glu<sup>221</sup>-Glu<sup>224</sup>, and Asp<sup>117</sup>-Glu<sup>224</sup> in Fig. 1B). The proximity of these residues, shown to be crucial and cooperative in transducing pH sensing, suggests that upon their protonation the overall structure of LHCSR3 might undergo adjustments that reduce the distance/relative orientation between the helices as a result of reduced electrostatic repulsion in the lumen-exposed domain. In particular, a different distance between Helix D and Helix E might be induced by protonation of Asp<sup>117</sup>, Glu<sup>221</sup>, and Glu<sup>224</sup>. This could be transduced into changes in the relative orientation of Helices A and B, with a consequent reorganization of Chl-Chl and Chl-Car interactions. The correspondence between protein aggregation *in vitro* and NPQ activation *in vivo* has been previously investigated, showing a similarity between the conformational change induced *in vitro* by aggregation of LHC proteins and conformational changes observed *in vivo* upon NPQ induction (14). In addition, we cannot exclude protein aggregation *in vitro* forces LHCSR3 subunits to establish some peculiar protein-protein interactions required for LHCSR3 activity *in vivo*. LHCSR3 proteins can be found as dimers in thylakoid membranes (18, 70), suggesting that possibly some specific protein-protein interactions are needed for LHCSR3 activity. The finding of LHCSR3 proteins as dimers agrees with recent crystallization of PSBS at low pH in the dimeric state (71), suggesting a possible common strategy for protein activation by formation of homo- or heterodimers and rearrangements of PSII supercomplexes. However, it should be pointed out that whereas LHCSR3 is a chlorophyll- and carotenoid-binding protein, PSBS was reported to bind a single chlorophyll at most. These different pigment-binding properties suggest that whereas LHCSR3 can be a direct quencher of excitation energy located on its pigments, PSBS function is more likely restricted to pH sensing, whereas triggering quenching is activated within interacting LHC proteins. Finally, it is interesting to compare the effects of mutation on protonatable residues in LHCSR3 as compared with PSBS. Recently, the structure at low pH of PSBS from spinach was revealed (71), showing the DCCD binding site at residue Glu<sup>173</sup>. Previously it was shown indeed that in

## LHCSR3 pH Sensing Sites for Non-photochemical Quenching

*A. thaliana* mutation of two PSBS lumen-exposed glutamate residues, Glu<sup>122</sup> (corresponding to Glu<sup>173</sup> in PSBS from spinach) and Glu<sup>226</sup>, yielded complete loss of qE *in vivo* and DCCD binding *in vitro*. The effect of individual mutations was additive, with changes to non-protonatable residues at each residue leading to 50% loss in both functions (17). This is clearly not the case for LHCSR, because no effect was observed upon mutation at single residues and even the D2E1E2 mutant still retained 28% of qE and 59% of DCCD binding. Thus, it appears that pH-dependent triggering is far more cooperative in LHCSR3 than in PSBS, with a number of contributing protonation events depending on species that have been reported to differ in the relative contribution by  $\Delta\text{pH}$  and  $\Delta\psi$  to the transmembrane pH gradient (72, 73). Also, responsiveness of different species to light intensity and adaptation to specific environments (15, 57) might be tuned by the number and distribution of lumen-exposed protonatable residues in LHCSR. These results are complementary to those recently reported (27) showing that pH responsiveness, as determined by fluorescence lifetime *in vitro*, was lost by mutation of 9 acidic residues at the C terminus (including Glu<sup>224</sup>, also studied in the present work) to non-protonatable species. The observed cooperativity between acidic residues might well explain this result.

**Author Contributions**—K. K. N., R. B., and M. B. conceived the work, designed the experiments, and wrote the paper. K. K. N. coordinated the experiments about *Chlamydomonas* complementation (Figs. 3 and 4), whereas R. B. and G. F. coordinated the experiments *in vitro*. M. B. and G. R. S. performed all the experiments reported with the exception of *Chlamydomonas* complementation and mutant screening and characterization. T. B. T. and E. E. performed the work described in Figs. 3 and 4. G. R. F. and E. D. R. contributed to the results reported in Fig. 7. All authors analyzed the results, contributed to writing, and approved the final version of the manuscript.

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## Chapter 3

# *In vitro* characterization of chlorophyll-binding sites of LHCSR1 from *Physcomitrella patens*

### Summary of the work

As discussed in chapter 2, LHCSR proteins have conserved the residues binding pigments present in other light harvesting complexes. This characteristic distinguishes LHCSRs from PSBS, their functional homologues in plants, which does not bind pigments. This suggests that LHCSR proteins might have additional functions beside lumenal pH-sensing. In fact, chlorophylls and xanthophylls bound to LHCSRs could be involved in quenching reactions.

The moss *Physcomitrella patens* is of particular interest to the study of photoprotection, as this organism uses both LHCSRs and PSBS for NPQ (Alboresi et al. (2010)). In *P. patens*, LHCSR1 has been demonstrated to be the most important LHC protein involved in NPQ (Alboresi et al. (2010)), with its quenching-induced activity being strongly enhanced by the presence of zeaxanthin (Pinnola et al. (2013)). These results point to a possible involvement of LHCSR1 in hosting quenching reactions in *P. patens*, and prompted us to investigate the role of the chromophores bound to LHCSR1.

To understand the energy transfer and quenching characteristics in LHC proteins, we need to know the precise organization and distance between all chromophores, yet no crystal structures of LHCSR proteins have not been obtained. In order to gain information on the chromophore organization in LHCSR1, we undertook site-directed mutagenesis of the pigment-binding residues conserved in the protein sequence, to study the quenching activity of mutant proteins both *in vitro* and *in vivo*. During my working period in Verona, I contributed to the *in vitro* characterization of these mutant proteins, while the *in vivo* study is currently on going. I built a 3D model of LHCSR1 structure and found that most of the putative pigment-binding residues had a conserved position and orientation with respect to the crystal structures of LHCII and CP29. I then refolded the WT and all the chlorophyll-binding mutants of LHCSR1 *in vitro* and analyzed their spectroscopic characteristics. I identified chlorophylls A2 and A5 as the putative low energy pigments in LHCSR1, and I thus decided to focus my attention on these chromophores. I conducted the fluorescence measurements in aggregation, but the results regarding mutants A2 and A5 were unclear. Following my moving to Paris to undertake the work on diatoms presented in the next two chapters, work on LHCSR1 was continued by Federico Perozeni and Matteo Bal-

lottari. Fluorescence lifetime measurements on *in vitro* reconstituted mutant proteins, together with the *in vivo* complementation, will hopefully allow us to uncover the role of the different chromophores and to identify the site of quenching reactions within LHCSR1.

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I was responsible of LHCSR1 structure modeling, *in vitro* re-folding of wild-type and mutant proteins, as well as their biochemical and spectroscopic characterization

**Abstract** Excess energy dissipation as non-photochemical quenching (NPQ) in the model moss *Physcomitrella patens*, is under the control of two proteins: PSBS, presents in plants, and Light Harvesting Complex Stress-Related proteins (LHCSR), found in algae. PSBS does not bind pigments and its action in photoprotection is likely dependent on the interaction with other antennae of photosystem II. On the contrary, the protein LHCSR1 binds pigments and has been shown to be important for NPQ in *P. patens*, pointing to the hypothesis that this protein might be directly involved in the quenching process. However, to understand the precise energy transfer and quenching characteristics of LHCSR1, the exact organization and distance between all chromophores is needed. Since crystal structures of LHCSR members are not yet available, the role of individual chromophores has been studied previously by using mutant antennae, lacking specific residues that coordinate each pigment.

Here, we report the *in vitro* investigation of the spectroscopic and quenching characteristics of different pigment-binding mutants of the LHCSR1 protein from *P. patens*. We mutagenized each chlorophyll-binding residue identified and analyzed reconstituted proteins both with absorption and fluorescence analyses. In particular, we focused our attention on chlorophylls A2 and A5, which have been proposed to be involved in quenching mechanisms mediated by the interaction with carotenoids in L1 and L2 sites. We found that chlorophylls A2 and A5 are indeed the lowest energy pigments in LHCSR1, but their involvement in thermal dissipation is not clearly assessed yet.

### 3.1 Introduction

Oxygenic photosynthesis allows plants and algae to reduce carbon dioxide into sugars, using light as an energy source and water as an electron donor. The side product of this reaction is molecular oxygen, which can be harmful since it can react with chlorophyll triplets (<sup>3</sup>Chl\*) to yield singlet oxygen, a reactive oxygen species (ROS) that can damage membranes and photosynthetic proteins (Niyogi (1999)). Prevention of <sup>3</sup>Chl\* formation and scavenging of ROS is therefore an important protective mechanism for all photosynthetic organisms. In particular, upon land colonization, plants had to evolve new ways to adapt to the higher

oxygen and lower CO<sub>2</sub> concentration present in the atmosphere in comparison to the water environment (Rensing et al. (2008); Gerotto and Morosinotto (2013)).

Energy absorbed in excess can be thermally dissipated via non-photochemical quenching (NPQ), which is the fastest protective mechanism to quench singlet chlorophylls (<sup>1</sup>Chl\*) before they convert to <sup>3</sup>Chl\* and can react with oxygen (Niyogi (1999)). Several components contribute to NPQ (Eberhard et al. (2008)): the major and fastest of these components is qE, or energy-dependent quenching, which requires a  $\Delta$ pH gradient across the thylakoid

membrane (Rees et al. (1992); Ruban et al. (1992); de Bianchi et al. (2010)). The  $\Delta\text{pH}$  is generated by the increased rate of photosynthesis in excessive light conditions, that increases protons concentration in the lumen (Eberhard et al. (2008)).

In plants and algae, different proteins have been identified to be responsible for the activation of qE: in the model plant *Arabidopsis thaliana*, the protein PSBS can sense lumen acidification via the protonation of two glutamic acids (Li et al. (2000b, 2002, 2004)), while the actual quenching happens in the PSII antennae (Ahn et al. (2008); Johnson et al. (2011)). In the green alga *Chlamydomonas reinhardtii*, although the *PSBS* gene is present (Peers et al. (2009)), the protein is only transiently expressed in high light (Correa-Galvis et al. (2016)) or is present only in very specific conditions, as for example upon UV-B exposure (Guillaume Allorent and Michel Goldschmidt-Clermont, personal communication). Most of qE in green algae is therefore under the control of other proteins, called LHCSR (Light harvesting complex stress-related, Peers et al. (2009)), and similar proteins have also been identified in diatoms (Bailleul et al. (2010)).

PSBS probably does not bind pigments (Dominici et al. (2002); Bonente et al. (2008)), although it has been reported to bind xanthophylls *in vitro* (Aspinall-O'Dea et al. (2002)), and thus the real quenching site in plant is thought to be located in the interacting LHC antennae (Ahn et al. (2008); Johnson et al. (2011); Gerotto et al. (2015)). Differently from that, LHCSR proteins do bind pigments (Bonente et al. (2011); Pinnola et al. (2013) and chapter 2), and it is therefore possible that these proteins are not only the pH sensors but also active quenchers (chapter 2).

The moss *Physcomitrella patens* is of great interest for the study of photoprotective mechanisms, since both PSBS and LHCSR proteins are expressed and active in NPQ (Alboresi et al. (2010); Gerotto et al. (2012)). In this organism, two LHCSR proteins are present (LHCSR1 and LHCSR2), which have a 91% sequence similarity. LHCSR1 is the most abundant and important in photoprotection (Alboresi et al. (2010))

and is strongly induced by high light treatment, while LHCSR2 does not respond to light but rather to cold stress (Gerotto et al. (2011)).

PSBS and LHCSR independent involvement in photoprotection in *P. patens* has been demonstrated with genetic approaches, using knock-out mutants, showing that they work in an additive way (Gerotto et al. (2012); Alboresi et al. (2010)).

LHCSR folding depends on the presence of chlorophylls and carotenoids (Bonente et al. (2011)), and the quenching activity of LHCSR in *P. patens* is strongly increased by the presence of zeaxanthin (Pinnola et al. (2013)), synthesized from violaxanthin under excessive light conditions via the xanthophyll cycle (Demmig et al. (1987)).

Different quenching models for LHC proteins have been proposed in the last years: the first one, proposed by Ahn et al. (2008), involves the formation of a carotenoid radical cation in the monomeric antennae, which then undergoes charge recombination to dissipate the excitation energy. Ruban et al. (2007) proposed instead the Chl quenching occurs by the transfer of energy from Chl to the  $S_1$  forbidden excited state of carotenoids, which rapidly decays to the ground state via internal conversion. Finally, Muller et al. (2010) hypothesized a carotenoid-independent mechanisms, where a strongly coupled Chl dimer can perform charge separation, followed by a recombination that quench the excitation energy.

To understand the energy transfer and quenching in LHC proteins we need to know the precise organization and distance between all chromophores, also with the orientation of their transition dipole and energy levels (Bassi et al. (1999)). Crystal structures of antenna proteins so far obtained (Pan et al. (2011); Liu et al. (2004)) do not have the sufficient resolution to get all these information. For this reason, the role of the different chromophores has been studied in the past with the help of mutated antennae, lacking specific residues that coordinate the different pigments (Bassi et al. (1999); Formaggio et al. (2001); Ballottari et al. (2009)).

Here, we report the *in vitro* investigation

of the spectroscopic and quenching characteristics of different pigment-binding mutants of the LHCSR1 protein from *P. patens*. We mutagenized each chlorophyll-binding residue identified and analyzed reconstituted proteins both with absorption and fluorescence analyses. In particular, we focused our attention on chloro-

phylls A2 and A5, which were supposed to be involved in quenching mechanisms mediated by the interaction with carotenoids in L1 and L2 sites. We found that chlorophylls A2 and A5 are indeed the lowest energy pigments in LHCSR1, but that their involvement in thermal dissipation is still unclear.

## 3.2 Material and Methods

**LHCSR1 sequence analysis:** Protein alignments were performed with CLUSTAL OMEGA (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). Identification of putative chlorophyll-binding residues was done by manually comparing LHCSR1 sequence with the identified pigment-binding residues in LHCII and CP29 crystal structures (Liu et al. (2004); Pan et al. (2011)).

**LHCSR1 Structure Modeling:** LHCSR1 protein structures were obtained by using homology modeling techniques with the on-line servers I-TASSER (Zhang (2008); Roy et al. (2010)) version 1.1. The model with the best C-score (confidence score) was selected for further analysis. In the model of LHCSR1 with pigments, each chromophore was manually positioned, using the software Swiss-PdbViewer (Guex and Peitsch (1997)) and maintaining the same distance and orientation from their binding residues in the protein, with respect to the distance and orientation measured in CP29 crystal structure.

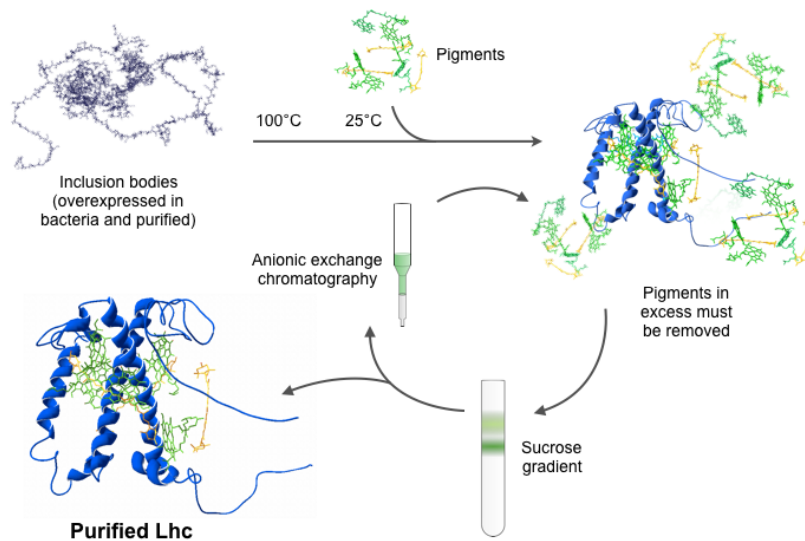
**Site-directed Mutagenesis of Acidic Residues:** The LHCSR1 coding sequence was cloned in a pET28 plasmid (see Girardon (2013)). Site-directed mutagenesis of each acidic residue reported in the text was done with the QuikChange Site-directed Mutagenesis Kit, used according to the manufacturer's instructions.

**Recombinant Protein Overexpression, Purification, and *in Vitro* Refolding:** LHCSR1 coding sequence was cloned in pET28

expression vector and expressed in *Escherichia coli* as previously described (Bonente et al. (2011)). Purified apoprotein was refolded *in vitro* in the presence of pigments as reported in Bonente et al. (2011); Giuffra et al. (1996) (figure 3.1). Zeaxanthin-containing proteins were re-folded using pigments extracted from spinach and de-epoxidated *in vitro*. Presence of zeaxanthin and de-epoxidation level (70%) was checked via HPLC.

**Pigment Analysis:** Pigments bound by recombinant LHCSR1 proteins were measured by HPLC as described in Ballottari et al. (2009).

**Steady-state Absorption, Fluorescence, and Circular Dichroism Measurements:** Room temperature absorption spectra were recorded using an SLM-Aminco DW2000 spectrophotometer, in 10 mM HEPES, pH 7.5, 0.2 M sucrose, and 0.03% n-dodecyl-D-maltopyranoside ( $\alpha$ -DM). The wavelength sampling step was 0.4 nm. Fluorescence emission spectra at room temperature and at 77K were measured using a Jobin-Yvon Fluoromax-3 device with proteins solubilized at pH 7.5 or 5, 0.2 M sucrose, and 0.03% or 0.003%  $\alpha$ -DM. Circular dichroism (CD) spectra were measured at 10 °C on a Jasco 600 spectropolarimeter using a R7400U-20 photomultiplier tube: samples were in the same solution described for the absorption, with an OD of 1 at the maximum in the  $Q_y$  transition. The measurements were performed in a 1-cm cuvette.



**Figure 3.1 – *In vitro* refolding procedure.** Scheme depicting the *in vitro* refolding procedure: the protein is expressed in bacterial cells, where it's accumulated as inclusion bodies. The protein is extracted and purified from bacteria, denatured and mixed with pigments. To induce the correct folding, cycles of freezing and thawing are used. Once the protein is folded, the pigments in excess are removed with a series of sucrose gradient ultra centrifugation and anion exchange chromatography steps.

### 3.3 Results

**Identification of the LHCSR1 pigment-binding sites:** Antenna proteins bind chlorophylls by coordinating the central magnesium in the porphyrin ring, usually using nucleophilic aminoacids (Jordan et al. (2001); Liu et al. (2004)). It has been shown previously that LHCSR3 (in *C. reinhardtii*) and LHCSR1 (in *P. patens*) have various pigment-binding residue in conserved positions (Bonente et al. (2011); Girardon (2013)). In LHCSR1, the chlorophyll-binding sites A1 (610 in the new nomenclature from Liu et al. (2004)), A2 (612), A3 (613), A4 (602), A5 (603) and B5 (609) are in fact present in conserved positions with respect to LHCII and CP29 (figure 3.2), the antenna proteins for which a crystal structure is available (Liu et al. (2004); Pan et al. (2011)). The sites B3 (614) and B6 (606) are instead slightly shifted in LHCSR1 with respect to LHCII and CP29 and it was not clear if they could still retain their pigment-binding activity.

In order to investigate the orientation and positions of these chlorophyll-binding residues, we created a homology-based model

of LHCSR1 (figure 3.3), using as a template the three-dimensional structure of other LHC proteins, LHCII, CP29, and LHCI (Liu et al. (2004); Pan et al. (2011); Amunts et al. (2010)). As previously determined for the protein LHCSR3, from *C. reinhardtii* (chapter 2), the protein model for LHCSR1 from *P. patens* has three trans-membrane  $\alpha$ -helices (helix A, B, and C) and two amphipathic helices (helix D and E), like those present in CP29 and LHCII.

From the superimposition of the structures (figure 3.3) it was possible to observe that, in LHCSR1, the six conserved chlorophyll-binding sites (A1, A2, A3, A4, A5 and B5) also had a position and an orientation of the amino acids very similar to the crystal structures of LHCII and CP29. The site B6 (E149, helix C), shown in green in figure 3.3, was instead oriented in different directions in the various models obtained with the on-line server I-TASSER. This binding site, in LHCII (Q131) and CP29 (E195) coordinates the chlorophyll in between helices C and B, while in three out of five models of LHCSR1, the conserved residue is oriented to-



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P.p. LHCSR1  ADKVS PDPEV VPP-----NVLEYAKGMPGVCAPF 29
S.o. LHCb1.1 MRKSAGKPKNVSSGSPWYGP-----DRVKYLGPFSGEAPSY 36
A.t. CP29     AAPKKS AKKTV TTRDPLWY PGAI SPDWLDGSLVGDYGFDPFGLGKPAEYLFQDIDSLDQN 60

P.p. LHCSR1  PNI FDPADLL-ARAASSPRPIKELN-----RWRESEITHGRVAMLASLGFIVQEQL 79
S.o. LHCb1.1 LTGEVFPGDYG-WDTAGLSADPETFS-----KNRELEVIHCRWAMLGALGCVFPELL 86
A.t. CP29     LAKNL AGDVIGTRTEAADAKSTPFQPYSEVFGIQRFRECELIHGRWAMLATLGALSVEWL 120
                                     A4 A5 A1

P.p. LHCSR1  QDYSLFYN-FDGQISGPAIYHFQQVEARGAV-----FWEP L LFAIALCEAYRVG- 123
S.o. LHCb1.1 ARNGVKFGEAVWFKAGSQIFSEGGLDYLGNSPLVHAQSILAIWACQVILMGAVEGYRVAG 146
A.t. CP29     TG-----VTWQDAGKVELVDG-SSYLGQP---LPFSISTLIWIEVLVIGYIEFQRNAE 169
                                     B6 B5

P.p. LHCSR1  LGWATPRSEDFNTLRDDYEPGNLGFDPGLGLPSDPAERKDMQTKELNNGRLAMIAIAAFVA 183
S.o. LHCb1.1 -----GPLGEVVDPLYPGG-SFDPLGLAE-DPEAFAELKVKKEIKNGRLAMFMSFGFFV 196
A.t. CP29     -----LDS---EKRLYPGKFFDPLGLAA-DPEKTAQLQLAEIKHARLAMVAFLGFAV 217
                                     A1 A2 A4

P.p. LHCSR1  QELVSGEEIFVHLFKRLGL----- 207
S.o. LHCb1.1 QAIVTGKGPLENLADHLADPVNNAWNFATNFVPGN 233
A.t. CP29     QAAATGKGPLENNWATHLS DPLHTTIIDTFSSS---- 250
                                     A3 B3

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**Figure 3.2 – Protein sequence alignment of LHCSR1 with LHCII and CP29.** LHCSR1 protein sequence aligned with LHCII (LHCb1.1 from spinach) and CP29 (from *Arabidopsis thaliana*). Conserved chlorophyll-binding sites are underlined and indicated in red.

wards the membrane. Again, it was difficult, given the variability in the structure predictions, to say whether or not this site is still binds a pigment in LHCSR1.

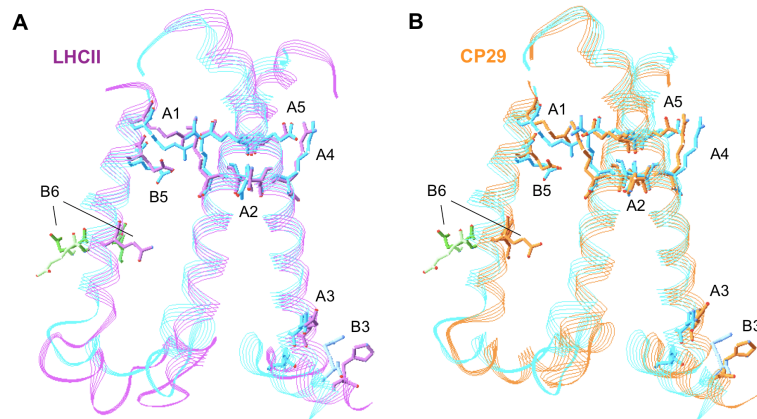
The B3 site present in LHCSR1 (H236) was closer to helix A and to the site A3, with respect to what happen in LHCII and CP29, but has the same orientation and may therefore still be implicated in pigment binding.

**Mutation and *in vitro* reconstitution of LHCSR1 recombinant proteins:** To investigate the conservation of the pigment-binding properties of the eight putative chlorophyll-binding sites in LHCSR1, we decided to mutate those residues and reconstitute the proteins *in vitro*. With site-directed mutagenesis, the LHCSR1 cDNA sequence was modified to substitute the putative chlorophyll-binding sites with residues that are not able to coordinate chlorophylls (Girardon (2013), table 3.1) and the proteins were expressed in *E. coli* and purified. Purified WT and the eight mutant apoproteins were then refolded *in vitro* in the presence of chlorophylls and carotenoids (Bonente et al. (2011); Liguori et al. (2013); Giuffra et al. (1996)).

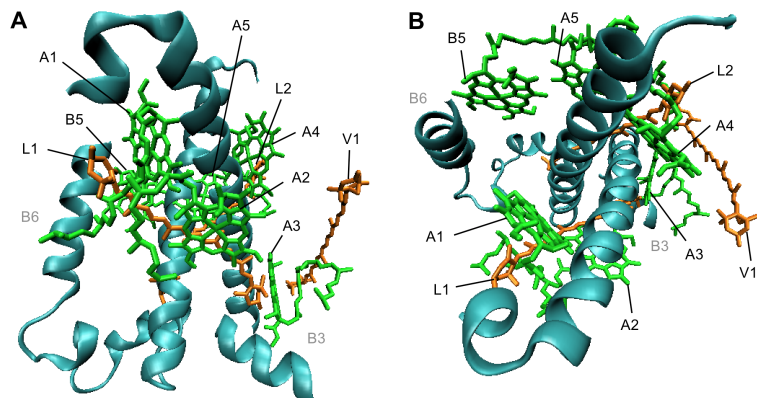
**Circular Dichroism spectra:** To verify the correct folding of the LHCSR1 WT and mutant proteins, we first measured Circular Dichroism (CD) spectra. The CD spectrum of a pigment-protein complex depends on the asymmetric-protein environment around the pigments and on the interaction between different chromophores. Positive and/or negative CD signals are therefore present only if the protein-pigment complex is folded.

LHCSR1 WT and mutant proteins all had optical activity, with the exception of mutants lacking sites A1 and A4 (figure 3.5 D). This indicates that these two mutations destabilize the protein, which cannot properly fold *in vitro*.

**Fluorescence emission spectra at room temperature:** The correct folding of the reconstituted proteins was also assessed by measuring the fluorescence emission spectra at room temperature. If all the pigments are bound to the protein and correctly oriented, the energy absorbed by carotenoids and chlorophyll *b* is efficiently transfer to chlorophyll *a*, which has a lower excited energy state, and fluorescence emission only comes from chlorophyll *a* (Chl *a*).



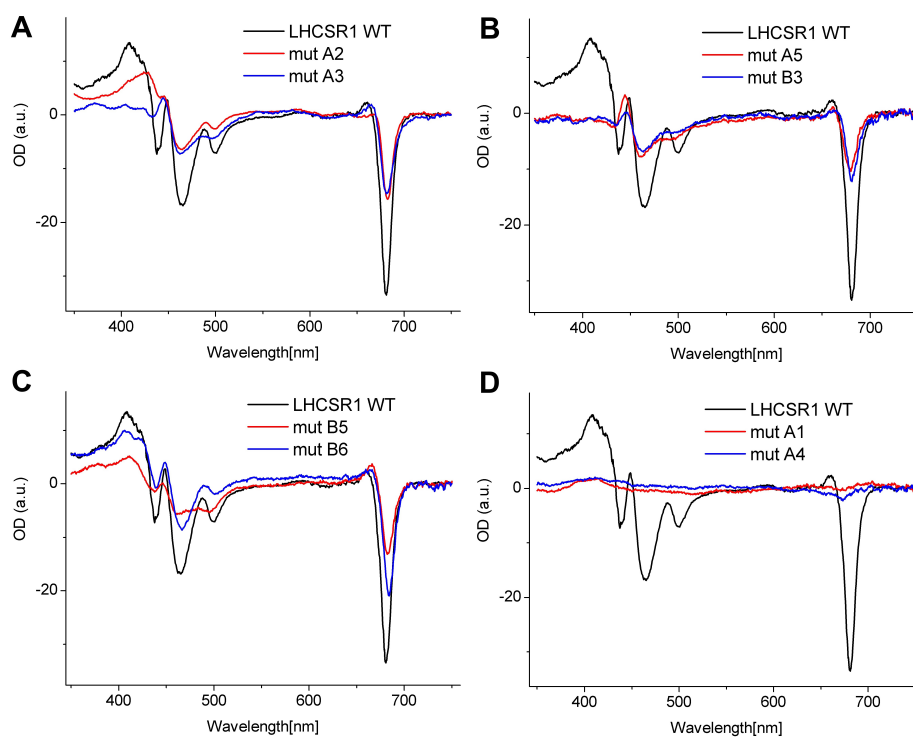
**Figure 3.3 – Three-dimensional model of LHCSR1 with LHCII and CP29.** LHCSR1 model superimposed on LHCII (A) and CP29 (B) crystallographic structures. Pigment-binding sites are indicated for LHCII (in purple) and CP29 (in orange); the conserved residues in LHCSR1 are indicated in blue. The different orientations of the B6 site, predicted in the various models obtained from I-TASSER, are represented in green.



**Figure 3.4 – Three-dimensional model of LHCSR1 with pigments.** LHCSR1 model with pigments ligands. Chlorophylls are depicted in green, carotenoids in orange. Panel A: front view. Panel B: top view. Pigments were positioned maintaining the same distance and orientation from their binding protein residue, with respect to the distance and orientation measured in CP29 crystal structure. Putative chlorophylls in site B3 and B6 were omitted.

Binding site	A1	A2	A3	A4	A5	B3	B5	B6
Mutations	E209V	N212F	Q226L	R213L	H99F	H237L	E159V	E149Q

**Table 3.1 – Mutations in pigment-binding residues in LHCSR1.** Single mutations introduced to the eight hypothetical chlorophyll-binding sites in LHCSR1 protein.



**Figure 3.5 – Circular dichroism spectra of LHCSR1 recombinant proteins.** Circular dichroism in the visible region of LHCSR1 WT and pigment-binding mutants refolded *in vitro*. All samples were diluted at the same 680 nm absorption. (A) WT and mutants A2 and A3. (B) WT and mutants A5 and B3. (C) WT and mutants B5 and B6. (D) WT and mutants A1 and A4. a.u. arbitrary units.

In the WT and in mutants A2, A3, A5, B3, B5 and B6, fluorescence emission spectra always resemble that of Chl *a*, even upon excitation of Chl *b* (475nm) and carotenoids (500nm), indicating an efficient energy transfer (figure 3.6). WT and B6 mutant emitted at 683 nm, A3, B3 and B5 mutant proteins all have a peak at 682.5 nm. A2 mutant had a mixim emission at 682 nm while A5 mutants have a shifted peak at 680 nm.

Mutants A1 and A4, once again, confirmed their incorrect folding, even though some energy transfer was still visible from the excitation of Chl *b*, with a peak at 660 nm and a shoulder at 676 nm, and from carotenoids, that transfer some energy to Chl *a* (figure 3.6 B and E).

**HPLC pigment analysis:** To verify the loss of pigments in the mutated proteins, we characterized the LHCSR1 holoproteins by HPLC. Due to their incorrect folding, mutants A1 and A4 were not analyzed.

We normalized the total pigment content to eight Chl per protein in the WT LHCSR1 (Pinnola et al. (2015)), obtaining that 7 of the pigment-binding sites were occupied by Chl *a*, one by Chl *b* and three by carotenoids (table 3.2). The carotenoids bound in reconstituted samples were mainly violaxanthin and lutein, as in Pinnola et al. (2015) and in agreement with previous reports on LHCSR3 from *C. reinhardtii* (Bonente et al. (2011); Liguori et al. (2013) and chapter 2). The presence of one Chl *b* per protein is instead in contrast with what was found by expressing LHCSR1 in tobacco, where substoichiometric amounts of this pigment were bound to the protein (Pinnola et al. (2015)).

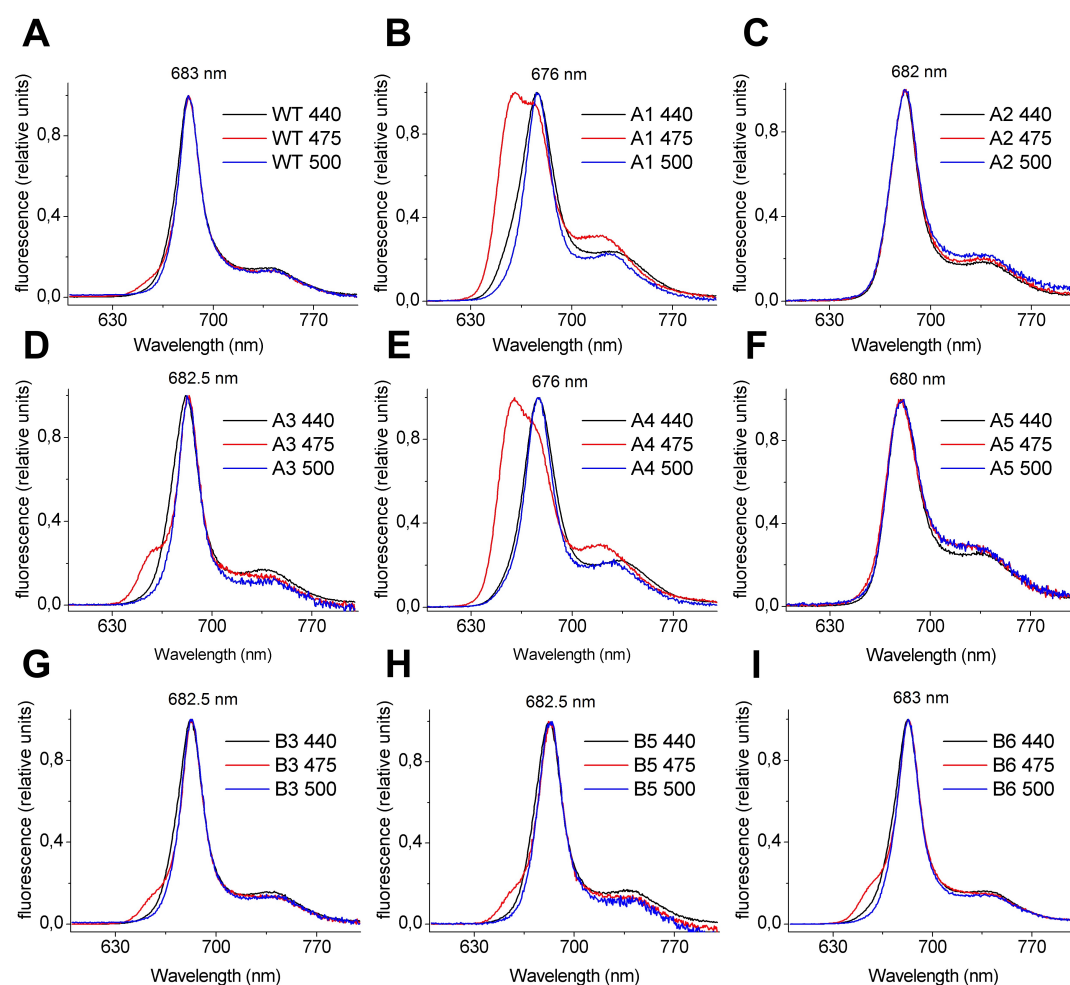
The pigment content of the mutant LHCSR1 proteins were normalized to seven Chl per protein, assuming that all the mutations led to the loss of the respective pigment and that no other chlorophyll was lost. In all cases, the Chl *a/b* ratios were lower compared to WT, indicating that the putative pigments lost were always a Chl *a*. In all mutants the Chl/Car ratio was increased, with the exception of the A5 and B6 mutants: upon normal-

ization to 7 Chls, all mutants retained at least two carotenoids, likely located at the inner sites L1 and L2 (figure 3.4). Only the A2 mutant had a stronger reduction of carotenoids content, in particular of lutein. The mutation A2 affects a chlorophyll-binding residue located close to the binding site of the carotenoid L1, which is occupied by lutein in all LHC proteins so far analyzed (Bassi et al. (1999); Formaggio et al. (2001); Ballottari et al. (2009)). This mutation probably destabilize the L1 site, so that one lutein is missing. Conversely, the mutation of chlorophyll A5, which is next the site L2, did not cause modifications in the carotenoid content. Most of the carotenoids lost in the other mutants are likely those present at the most peripheral carotenoid binding sites (V1-like or N1-like binding sites), which are more unstable compared to the inner carotenoid binding sites (L1 and L2).

From the proteins sequence alignment and from the three-dimensional model of LHCSR1, the presence of the chlorophyll-binding sites B3 and B6 was questionable. The HPLC data suggest indeed that these sites do coordinate a pigment, due to the reduction of Chl *a/b* ratio. The mutation of the B3 site also leads to the loss of one lutein, probably destabilizing the L1 site due to the lack of one chlorophyll.

The normalization of the B6 mutant to eight Chl, under the hypothesis that this site does not bind a pigment, would lead to an higher total carotenoid content (3.86 carotenoids per protein) with respect to the WT. An increase in the binding of carotenoids in the B6 mutant sounds unlikely, an indication that this protein lacks one Chl *a*.

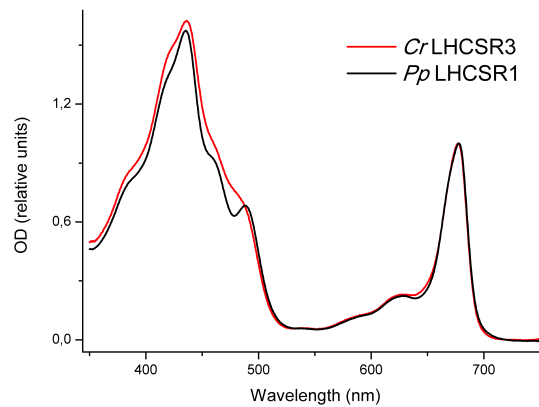
**Absorption spectra:** The absorption spectra in the visible region of WT LHCSR1 was identical to the one from the native protein isolated by Pinnola et al. (2013) and Pinnola et al. (2015), and it was also very similar to that of LHCSR3 from *C. reinhardtii* (figure 3.7). This suggests a similar pigment organization in the two complexes. The  $Q_y$  peak of LHCSR1 was 678 nm, confirming to be more red-shifted than the others PSII antenna proteins (Pinnola et al. (2013)).



**Figure 3.6 – Room temperature fluorescence spectra of recombinant LHCSR1 proteins.** Fluorescence spectra at room temperature of the WT and the pigment-binding mutants of LHCSR1 protein. The proteins were illuminated at 440, 475 and 500 nm to excite preferentially Chl *a*, Chl *b* and carotenoids respectively. Fluorescence emission was normalized to the maximum for each sample, wavelength of the peak is indicated for each graph. (A) LHCSR1 WT. (B) mutant A1. (C) mutant A2. (D) mutant A3. (E) mutant A4. (F) mutant A5. (G) mutant B3. (H) mutant B5. (I) mutant B6.

LHCSR1	Chl tot	Chl <i>a</i>	Chl <i>b</i>	Viola	Lute	Neo	Chl/Car	Chl <i>a/b</i>	Car tot	$\Delta$ Chl <i>a</i>
WT	8	7.02	0.98	0.31	2.76	0.02	2.59	7.18	3.09	
mut A2	7	6.05	0.95	0.09	1.24	0.04	5.09	6.33	1.38	-0.98
mut A3	7	6.12	0.88	0.08	1.81	0.05	3.60	6.95	1.94	-0.90
mut A5	7	6.04	0.96	0.31	2.45	0.33	2.26	6.27	3.10	-0.98
mut B3	7	5.90	1.10	0.18	1.73	0.22	3.28	5.35	2.13	-1.12
mut B5	7	6.03	0.97	0.17	1.87	0.15	3.21	6.22	2.18	-0.99
mut B6	7	5.89	1.11	0.39	2.81	0.17	2.07	5.32	3.38	-1.13

**Table 3.2 – Pigment analysis of recombinant LHCSR1 proteins.** Pigment analysis were performed by HPLC. Pigment composition of the WT protein was normalized to eight chlorophyll (Chl) per protein, while pigment-binding mutants were normalized to seven chlorophyll per protein.  $\Delta$  Chl *a* indicates the missing chlorophyll *a* in each mutant protein.



**Figure 3.7 – Absorption of LHCSR1 and LHCSR3 recombinant proteins.** Absorption spectra in the visible region of LHCSR1 (*P. patens*, in black) and LHCSR3 (*C. reinhardtii*, in red) WT proteins refolded *in vitro* in the presence of chlorophylls and carotenoids. Proteins were solubilized in 10 mM HEPES, pH 7.5, 0.2 M sucrose, and 0.03%  $\alpha$ -DM.

We measured the absorption spectra of all the correctly reconstituted mutant proteins, normalizing the  $Q_y$  peak to the pigment content detected via HPLC. From the differential spectra between LHCSR1 WT and the mutated proteins it is possible to obtain the absorption properties of the missing chlorophyll (figure 3.8).

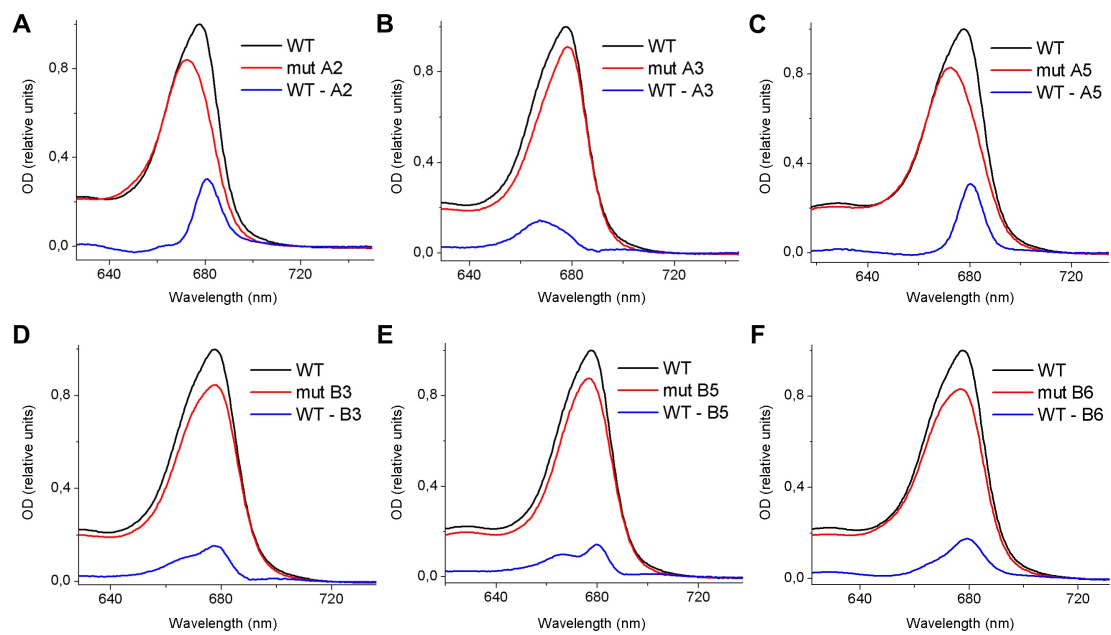
For the mutants A2 and A5, we obtained a similar peak of the missing chromophores at 682 nm and 681 nm, respectively, indicating a comparable protein environment surrounding these two pigments, which leads to a similar energy transition level. The differential spectra of B5 and B6 mutants show instead a peak at 680 nm and a shoulder (especially visible in B5) at 665 nm, probably due to the loss of the interaction of these pigments with another chlorophyll. Mutants A3 and B3 have a major peak at 667 and 677 nm respectively, with a shoulder at 677 nm for A3 and at 667 nm for B3. This may indicate an interaction between these two pigments in LHCSR1, as their major and minor peaks have the same wavelength.

**Fluorescence emission spectra in aggregation:** Two of the proposed quenching mechanism models are based on the interaction between chlorophylls and carotenoids (Ahn et al. (2008); Ruban et al. (2007)). Molecular dynamic simulations suggested a high flex-

ibility of chlorophylls in A2 and A5 sites in LHCII (with a switch from quenching to unquenching state, Liguori et al. (2015)). Moreover, chlorophylls bound to sites A2 and A5 are in the proximity of lutein ligands in L1 and L2 sites in CP29 and LHCII (Liu et al. (2004); Pan et al. (2011)) and the spectra of these two chromophores in LHCSR1 mutants (figure 3.7 A and C) are the most red-shifted ones (*i.e.* have the lowest energy). This suggests that A2 and A5 chlorophylls could be the final acceptors of excitation energy of LHCSR1, thus we decided to investigate deeper the mutants of LHCSR1 lacking these two pigments, to assess if their quenching activities were modified by the mutations.

We measured fluorescence emission spectra at pH 7 and at pH 5, since LHCSR1 is supposed to be active in photoprotection when the thylakoid lumen becomes acidic, just like LHCSR3 in *C. reinhardtii* does (see chapter 2).

As previously reported for LHCSR3, the change in conformation was impaired by the interaction of detergent micelles with the protein (chapter 2), and a decreased detergent concentration was effective in recovering pH sensitivity in LHCSR. Low detergent concentration leads in fact to a moderate aggregation of LHC proteins, reproducing protein-protein interactions occurring in the thylakoid membrane and reducing fluorescence lifetimes (Petrou et al.



**Figure 3.8 – Absorption spectra of recombinant LHCSR1 proteins.** Absorption spectra in the  $Q_y$  region of the *in vitro* refolded LHCSR1 proteins. Absorption spectra on the mutant proteins (in red) were normalized according to the pigment content detected with the HPLC analysis (with seven chlorophylls present out of eight), in comparison to the WT protein (in black), as described in Bassi et al. (1999). The differential spectrum between the WT and each mutant is represented in blue. (A) LHCSR1 WT and A2 mutant. (B) LHCSR1 WT and A3 mutant. (C) LHCSR1 WT and A5 mutant. (D) LHCSR1 WT and B3 mutant. (E) LHCSR1 WT and B5 mutant. (F) LHCSR1 WT and B6 mutant.

(2014); Horton et al. (1991) and chapter 2).

Upon aggregation, LHCSR1 WT protein show a small pH sensitivity, with a red-shifted emission at pH 5 (figure 3.9). The A2 mutant have a similar behavior, even if the emission maxims at both pH 7 and pH 5 were blue-shifted by 1 nm with respect to WT. The A5 mutant maintains its blue-shifted emission, already seen in non-aggregated state (figure 3.6), but strongly responds to pH drop by changing its peak emission to the same wavelength as the WT protein at pH 5 (figure 3.9 C).

Fluorescence emission was measured at low temperature, since *in vivo* measurements have highlighted an increased far-red emission at 77K when quenching is active (Ballottari et al. (2010); van Oort et al. (2007); Miloslavina et al. (2008)). We also reconstituted LHCSR1 WT protein in the presence of zeaxanthin, as *in vivo* this xanthophyll was reported to be crucial for LHCSR quenching activity in *P. patens* (Pinnola et al. (2013)).

WT LHCSR1 protein, reconstituted with a pigment mixture in the presence or absence of zeaxanthin, showed the same fluorescence emission at 77K, even at low pH, when deter-

gent concentration was high ( $\alpha$ -DM0.03%, figure 3.10 A and B). Mutants A2 and A5, reconstituted only without zeaxanthin, have a blue-shifted emission compared to WT protein, and they both seem not to be affected by low pH.

As high detergent concentration could impaired the switch to the quenching conformation, we also measured 77K fluorescence spectra with LHCSR1 in an aggregated state, lowering the detergent concentration below the critical micellar concentration. In this situation, a clear difference between pH 7 and pH 5 emerged, with a peak above 700 nm showing up in low pH conditions. This peak was more evident in the protein reconstituted with zeaxanthin (figure 3.10 B), even though here the spectrum was really noisy due to the strong quenching of fluorescence.

The spectrum of the A2 mutant protein in aggregated state at pH 5 also shows a peak above 700 nm, which seems to be higher than in the WT protein reconstituted without zeaxanthin (figure 3.10 A and C). Mutant A5, instead, show a lower intensity in the long-wavelength peak compared to WT.

### 3.4 Discussion

Photosynthetic organisms need mechanisms to dissipate the excess of absorbed light energy (section §1.4). In plants and green algae, the trigger of thermal dissipation is the acidification of thylakoid lumen, and it can be sensed by PSBS protein in plants and LHCSR protein in unicellular algae (Amarnath et al. (2012); Peers et al. (2009); Bonente et al. (2011, 2008,?); Niyogi and Truong (2013)). Mosses, from this point of view, are of particular interest, since both the above mentioned proteins are present in the same organism and are active in photo-protection (Alboresi et al. (2010)).

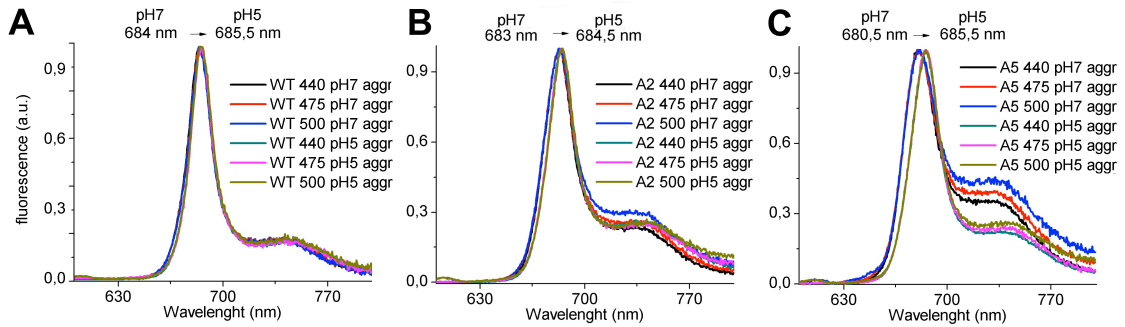
LHCSR proteins, contrary to PSBS (Dominici et al. (2002)), bind pigments. Because of that, LHCSRs could potentially be not only the pH-sensors but also host quenching activity

themselves (Bonente et al. (2011); Liguori et al. (2013); Tokutsu and Minagawa (2013)), while in plants these two functions are separated (Li et al. (2004); Bonente et al. (2008)). LHCSR3 in *Chlamydomonas reinhardtii* has been reported to undergo functional changes depending on pH (Bonente et al. (2011); Liguori et al. (2013); Tokutsu and Minagawa (2013) and chapter 2).

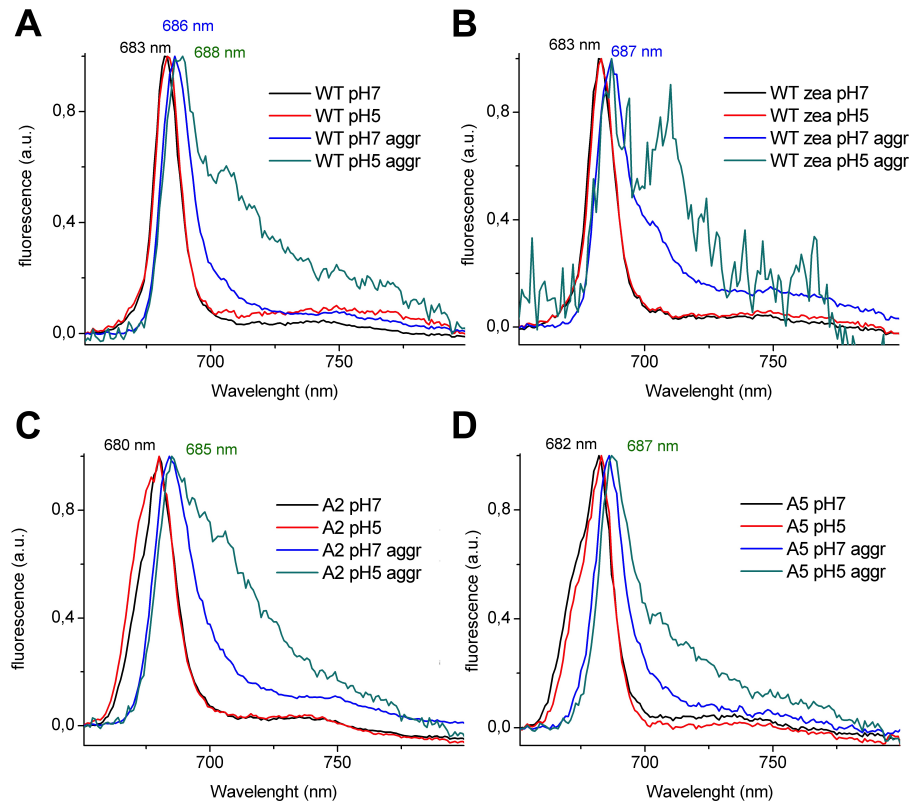
In this chapter, in order to understand the role of the different pigments bound to LHCSRs, we analyzed the *Physcomitrella patens* LHCSR1 protein, as a preliminary analysis to the corresponding *in vivo* work ongoing in the lab.

Previous work undertaken to identify the putative chlorophyll-binding sites (Girardon





**Figure 3.9 – Room temperature fluorescence spectra of LHCSR1 in aggregation.** Fluorescence emission spectra at room temperature of LHCSR1 WT (A) and mutants A2 (B) and A5 (C). Spectra were measured at pH 5 or pH 7 in presence of 0.003%  $\alpha$ -DM (aggregated state), exciting chlorophyll *a* (440 nm), chlorophyll *b* (475 nm) or carotenoids (500 nm). a.u. arbitrary units.



**Figure 3.10 – 77K fluorescence emission spectra of LHCSR1.** Fluorescence emission spectra at 77K of LHCSR1 WT protein reconstituted with (B) or without (A) zeaxanthin and of LHCSR1 mutants A2 (C) and A5 (D). Spectra were measured at pH 5 or pH 7 in presence of 0.03%  $\alpha$ -DM (solubilized state) or 0.003%  $\alpha$ -DM (aggregated state). a.u. arbitrary units.

(2013); Bonente et al. (2011)), led to the finding of eight conserved residues (figure 3.2). Two of these residues, coordinating chlorophylls B3 and B6, were not perfectly aligned with the ones present in CP29 and LHCII, and their actual role in pigment-binding was doubtful. With the 3D structural model of LHCSR1, built on the basis of the available LHC structures (Liu et al. (2004); Pan et al. (2011)) (figure 3.3), we obtained a strong confirmation of the conservation of six chlorophyll-binding residues (A1, A2, A3, A4, A5, B5), which maintain the same position and orientation as in LHCII and CP29. Site B3 and B6, however, are located differently with respect to the sites present in CP29 and LHCII, again questioning the conservation of their pigment-binding function.

We thus created mutated versions of LHCSR1, substituting one by one each putative chlorophyll-binding residue (Girardon (2013)), to express the proteins in *E. coli* and re-fold them *in vitro*, as already done for other LHC (Bonente et al. (2011); Ballottari et al. (2016); Bassi et al. (1999) and chapter 2). LHCSR1 WT protein and mutants lacking the sites A2, A3, A5, B3, B5 and B6 were able to properly re-fold *in vitro*, as shown by their circular dichroism and fluorescence spectra (figure 3.5 and figure 3.6). It was not possible instead to reconstitute mutants lacking sites A1 and A4, confirming that the removal of these sites cause a severe destabilization of LHC proteins *in vitro*, as already seen for other LHCs by Bassi et al. (1999); Formaggio et al. (2001) and Ballottari et al. (2009).

HPLC pigment analysis revealed a strong interconnection between the presence of some specific chlorophylls and the stability of the carotenoid bound to site L1 (figure 3.11). The mutant protein lacking chlorophyll A2 presents a carotenoid content lower than two, indicating a destabilization of one of the inner carotenoid binding sites. This site is likely the carotenoid-binding site L1, located close to Chl A2, and in fact the A2 mutant shows a strong reduction of lutein content.

Mutations near the L2 site, like the one removing chlorophyll A5, did not cause instead

the loss of any carotenoid. Site L2 in CP29 can bind violaxanthin or zeaxanthin, and CP29 and LHCII are stable even when no xanthophyll is bound to the site (Bassi et al. (1999); Formaggio et al. (2001)), probably to allow the exchange of pigments of the xanthophyll cycle. This may indicate a less strong interaction and structural stabilization of L2 site with the surrounding chromophores and protein residues.

In LHCSR proteins, one or two additional and more external xanthophyll-binding sites are predicted to be present (V1 and N1, Bonente et al. (2011)), so that xanthophyll exchange may also happen there. These extra carotenoid-binding sites are strongly destabilized in chlorophyll-binding site mutants, as mutants A3, B3 and B5 all lost one carotenoid with respect to the WT protein. This indicates that subtle protein conformation changes induced by mutations affect the less stable carotenoid-binding sites at the periphery of the protein.

The HPLC analysis pointed to the presence of functionally conserved binding-sites for chlorophylls B3 and B6, confirming that at least eight porphyrins are bound to LHCSR1, as already proposed by Pinnola et al. (2015). Normalization of pigment content to eight chlorophylls per protein also revealed the presence of one Chl *b* bound to each LHCSR1, in contrast to what was previously detected by over-expressing this protein in tobacco (Pinnola et al. (2015)); this difference is probably due to the different folding environment, and it remains to be established whether LHCSR1 really binds Chl *b* in *P. patens*. However, in no mutant we observed the loss of Chl *b*, suggesting that Chl *b* is bound to LHCSR1 not specifically to a chlorophyll-binding site or that it is bound to external sites.

Chlorophylls bound to photosynthetic proteins can absorb different wavelengths, depending on the protein environment in which they are buried (Bassi et al. (1999)). We determined the  $Q_y$  absorption of the missing pigments in the reconstituted LHCSR1 mutants by comparison with the WT protein (figure 3.8). Chlorophylls in sites B5 and B6 both absorb at 680nm, but are probably interacting one with the other

(or with another chromophore, as for example chlorophyll A1). This is suggested by the excitonic interaction that produce the smaller peak at 665 nm (figure 3.11). The similarity of the absorption spectra of B5 and B6 would also be indicative of a similar positions of the two chlorophylls, again pointing to an incorrect prediction of the three-dimensional model for the location of site B6.

An excitonic interaction is also probably presents between chromophores in sites A3 and B3, as the major peak of A3 has the same absorption wavelength of the minor peak of B3, and vice-versa (figure 3.11).

These interaction are in contrast with what has been shown in CP29 (Bassi et al. (1999)), where each pigment was responsible for only one absorption form. This is probably caused by a tighter coupling of the pigments present in LHCSR1, or to the presence of unidentified chromophores that increase the pigment to protein ratio in LHCSR1.

Conversely, the removal of pigments bound to sites A2 and A5 lead to the loss of a single peak at 682 and 681 nm respectively. These were the most red-shifted chlorophylls identified in LHCSR1, in agreement with previous observations on other LHC proteins (Bassi et al. (1999); Remelli et al. (1999); Ballottari et al. (2009)).

The A5 mutant also had a blue-shift in its fluorescence emission compared to WT (figure 3.6). These facts seemed to point to chlorophylls A2 or A5 as possible lowest energy level pigments in LHCSR1, and thus also the possible traps for excitons. In addition to that, A2 and A5 sites are predicted to be close by to sites L1 and L2 respectively, where xanthophylls are bound, and molecular dynamic simulations suggested a high flexibility of chlorophyll bound to these sites (Liguori et al. (2015)). According to both the quenching models of the radical cation (Ahn et al. (2008)) and of the carotenoid quenching with the population of the  $S_1$  excited state (Ruban et al. (2007)), the establishment of an interaction between a chlorophyll and a carotenoid is essential for the dissipation of the absorbed energy. LHCSR proteins have already been shown to produce high amounts

of carotenoid radical cation, up to ten times more with respect to CP29 (Bonente et al. (2011)). For these reasons, we decided to investigate more deeply the quenching properties of LHCSR1 in mutants A2 and A5.

As already seen for LHCSR3 (chapter 2), quenching properties of *in vitro* refolded proteins are difficult to study when LHCs are solubilized with high concentrations of detergents, such as  $\alpha$ -DM. By lowering the detergent concentration below the critical micelle concentration, the formation of small aggregates of membrane proteins is favored, mimicking more closely the *in vivo* protein-protein interactions present in the thylakoid membranes (Miloslavina et al. (2008)). In this way, proteins assume a conformation that resemble the quenched state, increasing pigment-pigment interaction (Pandit et al. (2013); Belgio et al. (2013); Petrou et al. (2014)). LHCSR proteins in particular have been shown to form dimers *in vivo* (Bonente et al. (2011); Pinnola et al. (2013, 2015)), as also PSBS does (Fan et al. (2015)), thus a lower concentration of detergent may also allow to mimic this native condition.

LHCSR1 is supposedly activated by the acidification of thylakoid lumen, since it conserved the key pH-sensing residues identified in LHCSR3 from *C. reinhardtii* (chapter 2).

Fluorescence emission upon aggregation in LHCSR1 WT at pH 7 showed a peak at 684 nm (figure 3.9 A). A similar emission was measured for mutant A2 at pH 7 (683 nm), while mutant A5 was strongly blue-shifted to 680,5 nm (figure 3.9 C). This is an indication that the lack of chlorophyll A5 leads to an increase in the lowest energy level of the protein also in the aggregated state, as fluorescence emission happens from shorter wavelength compared to the WT.

But upon acidification to pH 5, WT and mutant proteins all shifted their emission to around 685 nm (figure 3.9). This could indicate that, even if at pH 7 the lack of chlorophyll A5 affected the final emitter of fluorescence, the decrease in pH changed the conformation of the protein, leading to a low energy state independent on the presence of chlorophyll A2 or A5.

As low temperature fluorescence measure-

	WT	A1	A2	A3	A4	A5	B3	B5	B6
Mutation	-	E209V	N212F	Q226L	R213L	H99F	H237L	E159V	E149Q
Stable for <i>in vitro</i> refolding	✓	✗	✓	✓	✗	✓	✓	✓	✓
Closest carotenoid-binding site		L1	L1	L1 V1	L2	L2	L1	?	?
Missing pigments	-		Chl a Luteins Viola	Chl a Lutein Viola		Chl a	Chl a Lutein	Chl a Lutein	Chl a
Missing Chl spectral forms									
peak wavelength (shoulder wavelength)		?	682nm	667nm (667nm)	?	681nm	677nm (667nm)	680nm (665nm)	680nm (665nm)

**Figure 3.11 – Summary of the properties of LHCSR1 pigment-binding mutants.** Properties of *P. patens* LHCSR1 pigment-binding mutants, including the mutation to remove each chromophore and the stability upon *in vitro* refolding of the proteins. Missing pigments are derived from HPLC data (table 3.2), lack of violaxanthin in mutants A2 and A3 is only partial (as the WT protein only binds 0.3 violaxanthin molecules for each polypeptide). Closest carotenoid-binding site to each of the mutated chlorophyll-binding site is derived from figure 3.4 and from the data of Formaggio et al. (2001); B5 and B6 have question marks because they are predicted to be next to site N1 (for neoxanthin), whose presence in LHCSR1 is unclear. The spectra of the missing chlorophylls are taken from figure 3.8, wavelength of major and minor peaks are indicated.

ments *in vivo* have shown a correlation between far-red emission and quenching activity (Baltotari et al. (2010); van Oort et al. (2007); Miloslavina et al. (2008)), we determined the emission of WT and mutant proteins at 77K. Measurements were done with the WT protein reconstituted with both violaxanthin or zeaxanthin, as zeaxanthin was reported to be crucial for LHCSR quenching activity in *P. patens* (Pinnola et al. (2013)), and for all the proteins, we tried two different pH, 7 or 5.

Fluorescence emission at 77K on solubilized proteins showed no response of LHCSR1 to acidification (figure 3.10). WT LHCSR1 emitted at 683 nm at both pH 7 and 5, even when reconstituted with zeaxanthin, while mutants A2 and A5 emitted at 680 and 682 nm, respectively.

In aggregated samples, fluorescence emission at 77K at pH 7 was red-shifted in all samples, to approximately 686 nm. Upon acidification to pH 5, a far-red (>700 nm) peak emerged. This was more pronounced in the WT sample reconstituted with zeaxanthin, where fluorescence was also strongly decreased, indi-

cating an higher quenching activity.

Mutants A2 and A5 had a divergent behavior at 77K: LHCSR1 without chlorophyll A2 increased the far-red emission at pH 5 in aggregation compared to the WT protein, likely indicating a stronger quenching activity. On the contrary, mutant A5 had a lower far-red emission peak, pointing to a slightly reduced thermal dissipation with respect to WT (figure 3.10 C and D).

The reported results are still incomplete an unclear, as many questions remain without an answer. For example, absorption spectra and fluorescence emissions (figure 3.6 and figure 3.8) indicated chlorophylls A2 and A5 as the lowest energy level pigments in LHCSR1, but fluorescence in aggregation at room temperature or 77 K (figure 3.9 and figure 3.10) shows little difference in emission between the WT and the mutant proteins, especially at pH 5. In addition do that, A5 mutant seems to be more affected than A2 by acidification in aggregation at room temperature (figure 3.9), as its emission is strongly shifted between pH 7 and pH 5. But the opposite is true at 77K, when

mutant A2 had a stronger far-red emission fluorescence than mutant A5 at pH 5, even though at pH 7 both the proteins had similar spectra (figure 3.10).

To better understand these puzzling results, fluorescence lifetime measurements are needed, as they allow a much better characterization of quenching capacity (Moya et al. (2001)). We are currently reconstituting all the pigment-binding mutants with both violaxanthin and zeaxanthin, to test their fluorescence decay kinetics. In addition to that, complementation of LHCSR1 WT and mutant proteins are on going, in both *P. patens* or *Arabidopsis thaliana*. Results from these experiments will hopefully allow us to uncover the role of the different

pigments and to identify where the quenching actually happens in LHCSR1.

It is worth to notice that, recently, an excitonic model of LHCSR3 for *C. reinhardtii* was developed, indicating Chl A3 as the chlorophyll with the lowest energy, and suggesting that this may act as a possible quenching site (Liguori et al. (2016)). Our results indicate that, in LHCSR1 of *P. patens*, Chl A3 is not the reddest chlorophyll, at least in absence of zeaxanthin. Future work is required in order to assess if the peculiar zeaxanthin dependent activation of LHCSR1, observed in *P. patens*, relies on a conformational changes that leads to the formation of a new quenching site in Chl A3.

## Chapter 4

# Multisignal control of expression of the LHCX protein family in the marine diatom *Phaeodactylum tricornerutum*

### Summary of the work

Moving away from the green lineage, soon after the discovery of LHCSR3 in *Chlamydomonas reinhardtii* by Peers et al. in 2009, similar antennae were identified also in the diatoms *Thalassiosira pseudonana* (Zhu and Green 2010) and *Phaeodactylum tricornerutum* (Bailleul et al. 2010), where they were called LHCX. Diatoms are marine photosynthetic microalgae that dominate phytoplanktonic communities, particularly in turbulent coastal waters, where environmental conditions are very dynamic. They have a different evolutionary history compared to green algae and mosses, as their chloroplast derives from a series of secondary endosymbiotic events between and ancient heterotroph and red and green algae.

In *P. tricornerutum*, four isoforms of LHCX have been identified, but only for LHCX1 a direct proof of an involvement in photoprotection and NPQ was demonstrated, by characterizing knock-down and over-expressing lines (Bailleul et al. 2010). The other LHCX isoforms show a complex and sometimes different regulation than LHCX1 in the various transcriptomic studies performed in the last years (Allen et al., 2008; Nymark et al., 2009; Dyhrman et al., 2012; Thamtrakoln et al., 2012; Ashworth et al., 2013; Nymark et al., 2013; Keeling et al., 2014; Valle et al., 2014; Alipanah et al., 2015). However, none of these studies allowed to correlate LHCXs expressions with a possible function in the regulation of photosynthesis or photoprotection. Thus when I moved to Paris, I decided to directly address this issue with a more narrowed approach, together with another Ph.D. student in the laboratory, Lucilla Taddei.

The investigation of the available genome sequences from different diatom species allowed us to characterize the impressive expansion of the LHCX gene family in these algae, where up to 17 members were found in certain species. To better understand if these different isoforms have a specific function in the regulation of chloroplast physiology, we decided to study not only LHCXs gene expression but also protein accumulation and photosynthetic parameters in *P. tricornerutum* cells exposed to different light and nutrient stress conditions known to affect chloroplast activity.

We found that light, darkness, iron and nitrogen limitation all influenced LHCXs transcript and protein contents, and at the same time major readjustments in the regulation of the photosynthetic apparatus took place. The presence of specific LHCXs under different stresses allowed to hypothesize their involvement in chloroplast acclimation processes. As an example, LHCX2 is the only isoform to be strongly induced following iron deficiency, and it could play a key role in chloroplast regulation under iron starvation. On the contrary, LHCX2 and LHCX3 are both strongly expressed under high light, and they could act with LHCX1 in the high light acclimation responses.

LHCX4 is the most different isoform, for its characteristic induction upon darkness and rapid inhibition of its expression following dark to light shift. We hypothesized that LHCX4 could contribute to the capacity of *P. tricornutum* to survive to long periods in the dark, and its repression could be needed for a rapid acclimation following re-illumination. However, a direct role in NPQ has been questioned for LHCX4, as also suggested by the lack of conserved pH-sensing residues in the luminal region of this protein, that in LHCSR3 from *Chlamydomonas* are involved in NPQ onset when the lumen acidifies. These residues are instead conserved in LHCX1, LHCX2 and LHCX3.

This work allowed us to establish that multiple abiotic stress signals converge to regulate the LHCX content of cells, providing a way to fine-tune light harvesting and photoprotection. Moreover, our data indicate that the expansion of the LHCX gene family reflects functional diversification of its members, which could benefit cells responding to highly variable ocean environments.

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I was responsible of the analysis of conserved pH-sensing residues with respect to LHCSR3 from *Chlamydomonas reinhardtii* (see chapter 2), for the 3D modeling of LHCX proteins and of all the characterization of photosynthetic and photoprotective parameters. I participate to the analysis of the data and to the writing and correction of the manuscript.



RESEARCH PAPER

# Multisignal control of expression of the LHCX protein family in the marine diatom *Phaeodactylum tricornutum*

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## Abstract

Diatoms are phytoplanktonic organisms that grow successfully in the ocean where light conditions are highly variable. Studies of the molecular mechanisms of light acclimation in the marine diatom *Phaeodactylum tricornutum* show that carotenoid de-epoxidation enzymes and LHCX1, a member of the light-harvesting protein family, both contribute to dissipate excess light energy through non-photochemical quenching (NPQ). In this study, we investigate the role of the other members of the LHCX family in diatom stress responses. Our analysis of available genomic data shows that the presence of multiple *LHCX* genes is a conserved feature of diatom species living in different ecological niches. Moreover, an analysis of the levels of four *P. tricornutum* *LHCX* transcripts in relation to protein expression and photosynthetic activity indicates that LHCXs are differentially regulated under different light intensities and nutrient starvation, mostly modulating NPQ capacity. We conclude that multiple abiotic stress signals converge to regulate the LHCX content of cells, providing a way to fine-tune light harvesting and photoprotection. Moreover, our data indicate that the expansion of the *LHCX* gene family reflects functional diversification of its members which could benefit cells responding to highly variable ocean environments.

**Key words:** Dark, gene expression, iron starvation, LHCX, light, marine diatom, nitrogen starvation, non-photochemical quenching.



## Introduction

The perception of environmental signals and the activation of appropriate responses to external stimuli are of major importance in the growth and survival of all organisms. At the cellular level, this requires the presence of complex signal perception and transduction networks, triggering changes in nuclear gene expression (Lee and Yaffe, 2014). External cues such as light, temperature, and nutrient availability strongly affect the physiology and metabolism of photosynthetic organisms, so acclimation mechanisms are needed to cope efficiently with short- and long-term environmental changes to maintain photosynthetic performances (Walters, 2005; Eberhard *et al.*, 2008). In eukaryotic phototrophs, chloroplast biogenesis and activity are integrated in broader regulatory programmes, requiring coordination between the nucleus and chloroplast genomic systems (Rochaix, 2011; Jarvis and Lopez-Juez, 2013). The nucleus responds to stimuli inducing the synthesis of regulatory proteins that modulate chloroplast responses. In turn, molecules originating from the chloroplast activity (e.g. redox state of the photosynthetic electron carriers, reactive oxygen species, plastid gene transcription, tetrapyrroles, and other metabolites) provide a retrograde signal feeding back to the nucleus (Woodson and Chory, 2008).

Marine photosynthesis is dominated by unicellular phytoplanktonic organisms, which are passive drifters in the water column and often experience drastic changes in their surrounding environment (Falkowski *et al.*, 2004; Depauw *et al.*, 2012). Diatoms are among the most abundant and diversified groups of photosynthetic organisms. They are particularly adapted to growing in very dynamic environments such as turbulent coastal waters and upwelling areas, as well as in polar oceans (Margalef, 1978; Field *et al.*, 1998; Kooistra *et al.*, 2007; Arrigo *et al.*, 2012). Several species can survive for long periods at depths where light is limiting for growth, and quickly reactivate their metabolism after returning to the photic zone (Sicko-Goad *et al.*, 1989; Reeves *et al.*, 2011). The adaptive capacity of such algae suggests that they have sophisticated mechanisms to perceive and rapidly respond to environmental variations. Consistent with this notion, genome sequence information of representative diatom model species such as *Thalassiosira pseudonana* and *Phaeodactylum tricorutum* (Armbrust *et al.*, 2004; Montsant *et al.*, 2007; Bowler *et al.*, 2008; Rayko *et al.*, 2010), and the availability of transcriptomic and proteomic data in various species exposed to different stimuli and stresses (Nymark *et al.*, 2009; Dyhrman *et al.*, 2012; Thamatrakoln *et al.*, 2012; Ashworth *et al.*, 2013; Nymark *et al.*, 2013; Keeling *et al.*, 2014; Valle *et al.*, 2014; Alipanah *et al.*, 2015; Muhseen *et al.*, 2015) have highlighted the existence of some diatom-specific adaptive strategies, pinpointing molecular regulators of environmental change responses. Several photoreceptors for efficient light colour sensing (Huysman *et al.*, 2013; Schellenberger Costa *et al.*, 2013; Fortunato *et al.*, 2015, 2016) have been identified in diatoms. Peculiar iron acquisition and concentration mechanisms are also known (Allen *et al.*, 2008; Marchetti *et al.*, 2012; Morrissey *et al.*, 2015), which contribute to their

survival in iron-limited waters and to their rapid proliferation when iron becomes available (de Baar *et al.*, 2005). Diatoms have peculiar gene sets implicated in nitrogen metabolism, such as a complete urea cycle, that could be used as temporary energy storage or as a sink for photorespiration (Allen *et al.*, 2011). Eventually, diatoms optimize their photosynthesis via extensive energetic exchanges between plastids and mitochondria (Bailleul *et al.*, 2015).

The ecological dominance of diatoms also relies on their capacity to cope with light stresses, thanks to very efficient photoprotective mechanisms. Diatoms possess a high capacity to dissipate excess light energy as heat through high energy quenching (qE) that, together with the photoinhibitory quenching (qI), can be visualized via the non-photochemical quenching (NPQ) of Chl *a* fluorescence (Lavaud and Goss, 2014; Goss and Lepetit, 2015). The xanthophyll diatoxanthin (Dt) pigment, synthesized from the de-epoxidation of diadinoxanthin (Dd) during illumination (Goss and Jakob, 2010; Lavaud *et al.*, 2012), and the LHCX1 protein, a member of the light-harvesting protein family (Bailleul *et al.*, 2010), have been identified as key components of the qE process in diatoms. *P. tricorutum* cells with deregulated LHCX1 expression display a significantly reduced NPQ capacity and a decreased fitness, demonstrating a key role for this protein in light acclimation (Bailleul *et al.*, 2010), similarly to the light harvesting complex stress-related (LHCSR) proteins of green algae and mosses (Alboresi *et al.*, 2010; Ballottari *et al.*, 2016).

Multiple nuclear-encoded and plastid-localized LHCX family members have been identified in the genomes of the diatoms *P. tricorutum* and *T. pseudonana*. Scattered information derived from independent gene expression analyses indicated that some LHCX isoforms are constitutively expressed while others are expressed in response to stress (Becker and Rhiel, 2006; Allen *et al.*, 2008; Nymark *et al.*, 2009; Zhu and Green, 2010; Bailleul *et al.*, 2010; Beer *et al.*, 2011; Lepetit *et al.*, 2013), similarly to what is observed for the two LHCSR proteins in *Physcomitrella patens* (Gerotto *et al.*, 2011). In this study, we have extended the characterization of the four *P. tricorutum* LHCXs, by combining detailed gene expression analysis in cells exposed to different conditions with *in vivo* analysis of photosynthetic parameters. The result of this analysis revealed a complex regulatory landscape, suggesting that the expansion of the LHCXs reflects a functional diversification of these proteins and may contribute to the regulation of the chloroplast physiology in response to diverse extracellular and intracellular signals.

## Materials and methods

### Analysis of the LHCXs in the diatom genomes

*Phaeodactylum tricorutum* and *T. pseudonana* LHCX gene model identifiers were retrieved from the diatom genomes, respectively, on *P. tricorutum* Phatr2 and *T. pseudonana* Thaps3 in the JGI database (<http://genome.jgi.doe.gov/>). *Phaeodactylum tricorutum* LHCX proteins were used as query to perform BlastP searches on the *Pseudo-nitzschia multiseriata* (<http://genome.jgi.doe.gov/Psemu1/Psemu1.home.html>) and *Thalassiosira oceanica* ([Downloaded from <http://jxb.oxfordjournals.org/> at Università degli studi di Verona on May 26, 2016](http://protists.</a></p>
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ensembl.org/Thalassiosira\_oceanica/Info/Index) genome portals. Best hit sequences were tested on Pfam (<http://pfam.xfam.org/>) to assess the presence of the Chloroa\_b-bind domain (PF00504), characteristic of light-harvesting proteins. Protein alignments were performed with MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>).

#### Diatom growth conditions

The *P. tricornutum* (Pt1 8.6, CCMP2561) cultures, obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, were used for the gene expression and photophysiology analyses. Cells were grown in ventilated flasks in *f/2* medium (Guillard, 1975) at 18 °C, in a 12h light/12h dark photoperiod using white fluorescence neon lamps (Philips TL-D 90), at 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (low light). High light treatments were performed by irradiating the cells with 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 5h, 2h after the onset of light, with the same light sources. Dark adaptation treatments were performed for 60h. Blue light (450 nm, 1  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) was applied for 10 min, 30 min, and 1 h on dark-adapted cells in the absence and presence of 2  $\mu\text{M}$  DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea]. In the iron starvation experiments, *P. tricornutum* cells at an initial concentration of  $2 \times 10^5$  cells  $\text{ml}^{-1}$  were grown in *f/2* artificial sea water medium (Allen *et al.*, 2008) modified to contain either 11  $\mu\text{M}$  iron (iron-replete) or 5 nM iron with the addition of 100  $\mu\text{M}$  of the  $\text{Fe}^{2+}$  chelator FerroZine™ (iron-limited) (Stookey, 1970). Cells were harvested after 3 d to perform the analyses. Nitrogen starvation was achieved by diluting *P. tricornutum* cells to  $2 \times 10^5$  cells  $\text{ml}^{-1}$  in *f/2* medium containing 1 mM nitrate ( $\text{NO}_3$ -replete) or 50  $\mu\text{M}$  nitrate ( $\text{NO}_3$ -limited). When cells attained a concentration of  $1 \times 10^6$  cells  $\text{ml}^{-1}$ , they were re-diluted to  $2 \times 10^5$  cells  $\text{ml}^{-1}$  in their respective media and harvested after 3d, 2h after the onset of light, and then used for experiments.

#### Generation of transgenic lines overexpressing the *LHCX* proteins

Vectors for *LHCX* overexpression were generated by cloning the full-length cDNA sequences of the four *LHCX* genes in the pKS-FcpBpAt-C-3HA vector (Siaut *et al.*, 2007), using the *EcoRI* and *NotI* restriction sites. The *LHCX* cDNAs were amplified by PCR using the primers described in Supplementary Table S1 at *JXB* online. Each vector was co-transformed with the pFCFPp-Shble vector for antibiotic selection into *P. tricornutum* Pt4 cells (DQ085804; De Martino *et al.*, 2007) by microparticle bombardment (Falciatore *et al.*, 1999). Transgenic lines were selected on 100  $\mu\text{g ml}^{-1}$  phleomycin (Invitrogen) and screened by PCR using primers specific for the four *LHCX*s (Supplementary Table S1). Transgenic lines overexpressing the *LHCX4* isoform in the Pt1 ecotype were also generated, as for the Pt4 ecotype.

#### RNA extraction and qRT-PCR analysis

Total RNA was isolated from  $10^8$  cells with TriPure isolation reagent (Roche Applied Science, IN, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed on wild-type cells and on the *LHCX*-overexpressing clones as described in De Riso *et al.* (2009). The relative quantification of the different *LHCX* transcripts was obtained using *RPS* (ribosomal protein small subunit 30S; ID10847) and *H4* (histone H4; ID34971) as reference genes, and by averaging of two reference genes using the geometric mean and the fold changes calculated with the  $2^{-\Delta\Delta\text{Ct}}$  Livak method (Livak and Schmittgen, 2001). Primer sequences used in qRT-PCR analysis are reported in Supplementary Table S1.

#### Protein extraction and western blot analysis

Western blot analyses were performed on total cell protein extracts prepared as in Bailleul *et al.* (2010), and resolved on 14% LDS-PAGE gels. Proteins were detected with different antibodies:

anti-LHCSR (gift of G. Peers, University of California, Berkeley, CA, USA) (1:5000); anti-D2 (gift of J.-D. Rochaix, University of Geneva, Switzerland) (1:10 000); anti-PsaF (1:1000) and anti- $\beta\text{CF1}$  (1:10 000) (gift of F.-A. Wollman, Institut de Biologie Physico-Chimique, Paris, France); and anti-HA primary antibody (Roche) (1:2000). Proteins were revealed with Clarity reagents (Bio-Rad) and an Image Quant LAS4000 camera (GE Healthcare, USA).

#### Chlorophyll fluorescence measurements

Light-induced fluorescence kinetics were measured using a fluorescence CCD camera recorder (JTS-10, BeamBio, France) as described (Johnson *et al.*, 2009) on cells at  $1-2 \times 10^6$  cells  $\text{ml}^{-1}$ .  $F_v/F_m$  was calculated as  $(F_m - F_0)/F_m$ . NPQ was calculated as  $(F_m - F_m')/F_m'$  (Bilger and Bjorkman, 1990), where  $F_m$  and  $F_m'$  are the maximum fluorescence emission levels in the dark and light-acclimated cells, measured with a saturating pulse of light. All samples, except the 60h dark-adapted cells, were adapted to dim light (10  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 15 min at 18 °C before measurements. The maximal NPQ response was measured upon exposure for 10 min to saturating green light of 950  $\mu\text{mol m}^{-2} \text{s}^{-1}$ . The relative electron transfer rate ( $\text{rETR}_{\text{PSII}}$ ) was measured with a JTS-10 spectrophotometer at different light intensities (20, 170, 260, 320, 520, and 950  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), by changing light every 4 min to minimize the photodamage.  $\text{rETR}_{\text{PSII}}$  was calculated as:  $Y2 \times \text{light intensity}$ , where  $Y2$  is the efficiency of PSII.

#### In silico analysis of the *LHCX* non-coding sequences

Determination of motif occurrence and *de novo* search of over-represented motifs in the 5'-flanking regions (1000 bp or the entire intergenic sequence between the coding gene of interest and the upstream gene) and the introns of the Pt*LHCX* genes were performed by the use of the FIMO (v4.11.1) and MEME Suite (v4.9.1) tools (Bailey *et al.*, 2009), with a *p*-value cut-off of 0.0001. The Tomtom tool (v4.11.1) on the MEME Suite was used to compare motifs with known transcription binding sites. Microarray data from Alipanah *et al.* (2015) (accession GSE58946) were downloaded from the GEO database (<http://www.ncbi.nlm.nih.gov/pubmed/23193258>) using the GEO query package (<http://www.ncbi.nlm.nih.gov/pubmed/17496320>). Data were loaded and analysed in the R environment using the Limma package (<http://www.ncbi.nlm.nih.gov/pubmed/25605792>). Selection of transcripts categories: (i) up-regulated,  $\log_2$  fold change (Fc) >3, adjusted *p*-value <0.01 (122 genes); (ii) down-regulated, Fc <-3, adjusted *p*-value <0.01 (200 genes). The *P. tricornutum* genome sequences and gff mapping of filtered gene models were downloaded from the JGI website and refer to Phat2 (<http://genome.jgi.doe.gov/Phat2/Phat2.home.html>). The significance of motif enrichment was evaluated using the binomial test with a *p*-value cut-off of 0.05. All analyses were performed using custom scripts in Perl and R.

## Results

### *LHCX* family expansion in the diatom genomes

Several genes belonging to the *LHCX* family have already been identified in the genome of the pennate diatom *P. tricornutum* (Bailleul *et al.*, 2010) and the centric diatom *T. pseudonana* (Zhu and Green, 2010), the most established model species due to the availability of molecular toolkits for genetic manipulations (Apt *et al.*, 1996; Poulsen and Kroger, 2005; De Riso *et al.*, 2009; Trentacoste *et al.*, 2013; Daboussi *et al.*, 2014; Karas *et al.*, 2015). The recently available genome sequences of the pennate diatom *P. multiseriis*, belonging to a widely distributed genus also comprising toxic species (Trainer *et al.*, 2012), and the centric diatom

*T. oceanica*, a species adapted to oligotrophic conditions (Lommer *et al.*, 2012), opened up the possibility to extend this investigation to other ecologically relevant species. As summarized in Table 1, comparative analysis indicates an expansion of the *LHCX* family in diatoms, compared with the green algae: four members are present in *P. tricornutum*, five in *P. multiseriis*, *T. pseudonana*, and *T. oceanica*, and up to 17 members have been found in the genome of the polar species *Fragilariopsis cylindrus* (B. Green and T. Mock, personal communication). Analysis of the intron–exon structure of all the available diatom *LHCX* genes revealed a variable number of introns (from zero to three) as well as variable intron and exon lengths (Table 1).

#### Light versus dark regulation of expression of the *LHCX* genes

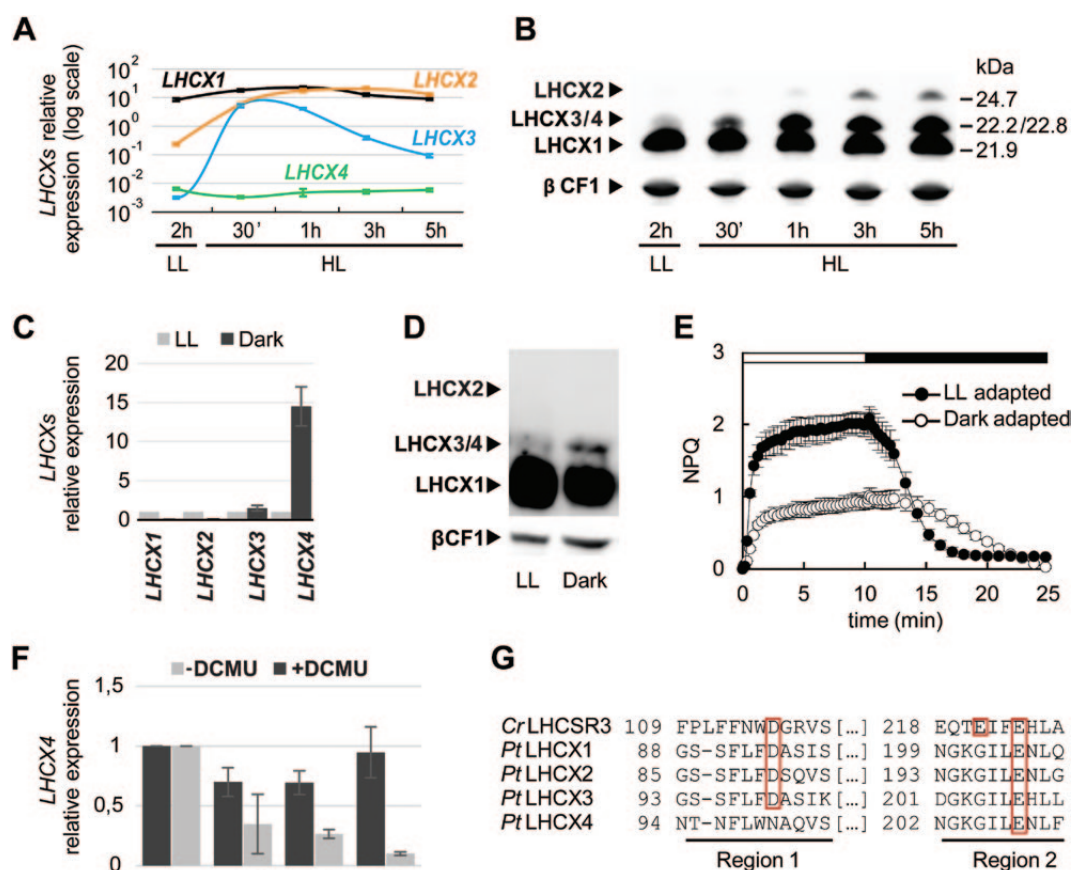
Independent gene expression studies performed in *P. tricornutum* cells suggest that the *LHCX* gene family is regulated by light via multiple regulatory pathways. To explore the mechanisms controlling the light responses of the *LHCX* genes further, we analysed mRNA and protein contents in cells exposed to different light conditions. We first monitored the expression of the *LHCX* genes in cells grown in low light (LL) and then exposed to high light (HL). In line with previous studies (Bailleul *et al.*, 2010; Lepetit *et al.*, 2013), qRT-PCR and western blot analyses (Fig. 1A and B, respectively) showed that *LHCX1* is expressed at very high levels in LL-adapted cells, and that HL treatment slightly increases the *LHCX1* content. Conversely, the isoforms 2 and 3 showed different responses to the LL to HL shift.

The *LHCX2* transcripts, which are significantly less abundant than that of *LHCX1* in LL, rapidly increased following HL stress, reaching levels comparable with those of *LHCX1* after 1 h HL exposure (Fig. 1A). This translated into an increase of the *LHCX2* protein observed by western blot (Fig. 1B). However, the increase in the protein content was lower than that of the transcript, possibly because of a low affinity of the LHCSR antibody (Peers *et al.*, 2009) for the *LHCX2* isoform. The *LHCX3* transcripts that were expressed at very low levels in LL quickly rose upon HL treatment, peaking after 30 min and starting to decrease after 1 h of light stress. Conversely, a different mRNA expression profile was found in the case of *LHCX4*, which, unlike the other isoforms, was barely detectable in both LL and HL conditions (Fig. 1A). The *LHCX3* and *LHCX4* proteins, having very similar molecular weights (22.8 kDa and 22.2 kDa, respectively), cannot be discriminated by western blot analysis. Based on the different transcriptional regulation of *LHCX3* and *LHCX4* by light, it is tempting to propose that the light-induced protein of ~22.8 kDa reflects the accumulation of the *LHCX3* isoform (Fig. 1B). However, in contrast to the transient induction of the *LHCX3* mRNAs, this protein is gradually accumulated during the LL to HL shift and it remains stable over the treatment. This discrepancy between transcript and protein expression profiles could be explained assuming that: (i) some post-transcriptional modifications regulate the accumulation of *LHCX3* in the light; or (ii) the light-induced protein isoform at 22.8 kDa also comprises the *LHCX4* protein, which could be present in HL-exposed cells, along with *LHCX3*.

**Table 1.** List of the *LHCX*s identified in the diatom genomes

Species	Name	ID	Chromosomal localization	Length (no. of amino acids)	No. of introns
<i>Thalassiosira pseudonana</i>	LHCX1	264921	chr_23:365603–366232 (-)	209	0
	LHCX2	38879	chr_23:368273–368902 (+)	209	0
	LHCX4	270228	chr_5:1446306–1447125 (-)	231	0
	LHCX5	31128	chr_1:2849139–2850176 (-)	236	3
	LHCX6	12097	chr_23:366611–367378 (+)	255	0
	<i>Phaeodactylum tricornutum</i>	LHCX1	27278	chr_7:996379–997300 (+)	206
LHCX2		56312	chr_1:2471232–2472170 (+)	238	2
LHCX3		44733	chr_5:76676–77606 (+)	206	1
LHCX4		38720	chr_17:53010–53733 (+)	207	1
<i>Pseudo-nitzschia multiseriis</i>	–	66239	scaffold_189:181982–182948 (+)	201	1
	–	238335	scaffold_95:121459–122306 (-)	202	1
	–	257821	scaffold_246:124909–125745 (+)	197	1
	–	264022	scaffold_1353:8720–9877 (+)	206	1
<i>Thalassiosira oceanica</i>	–	283956	scaffold_38:284133–284828 (-)	231	0
	–	Thaoc_09937	SuperContig To_g10869: 4.331–5.040 (-)	210	1
	–	Thaoc_12733	SuperContig To_g15184: 10.800–11.435 (-)	172	1
	–	Thaoc_28991	SuperContig To_g41561: 2.777–3.105 (+)	81	1
	–	Thaoc_31987	SuperContig To_g45669: 1–1.025 (-)	205	2
–	Thaoc_32497	SuperContig To_g46152: 5.664–6.285 (-)	180	1	

For *T. pseudonana* (Thaps3), *P. tricornutum* (Phatr2), and *P. multiseriis* (Psemu1), ID numbers refer to the genome annotation in the JGI database (<http://genome.jgi.doe.gov/>). For *T. oceanica* (ThaOc\_1.0), ID refers to the Ensembl Protist database ([http://protists.ensembl.org/Thalassiosira\\_oceanica/Info/Index?db=core](http://protists.ensembl.org/Thalassiosira_oceanica/Info/Index?db=core)). (+) and (-) indicate the forward and reverse chromosomal or scaffolds, respectively. The protein length and intron numbers are also indicated.



**Fig. 1.** Light and dark regulation of *P. tricornutum* LHCXs. Analysis of the four *LHCX* transcripts by qRT-PCR (A) and of LHCX proteins (B) by western blotting in cells adapted to low light (LL) (12L/12D cycles), after exposure to LL for 2 h then to high light (HL) for 30 min, 1 h, 3 h, or 5 h. mRNA levels were quantified by using *RPS* as the reference gene (A). Proteins were detected using the anti-LHCSR antibody which recognizes all the *PtLHCX*s (arrowheads) and the anti- $\beta$ CF1 antibody as loading control (B). Cells adapted to darkness for 60 h were compared with those grown in LL for the analysis of LHCX transcripts (C), proteins (D), and NPQ (E). Relative transcript levels were determined using *RPS* as a reference, and values were normalized to gene expression levels in LL. LHCX proteins were detected as in (B). The horizontal bar in (E) indicates when the actinic light was on (white) or off (black). (F) *LHCX4* mRNAs in 60 h dark-adapted cells (Time 0) and in response to 10 min, 30 min, or 1 h of blue light ( $1 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), in the presence (black) or absence (grey) of the inhibitor DCMU. Transcript levels were quantified by using *RPS* as the reference, and normalized to gene expression levels in the dark. Error bars represent  $\pm$ SD of three technical replicates from one representative experiment in (A), and  $\pm$ SD of three biological replicates in (C), (E), and (F). (G) Alignment of regions 1 and 2 of the *Chlamydomonas reinhardtii* LHCSR3 and *P. tricornutum* LHCX1, 2, 3, and 4 protein sequences. The boxes indicate the pH-sensing residues conserved between the LHCXs and LHCSR3. (This figure is available in colour at JXB online).

Previous reports (Lepetit *et al.*, 2013; Nymark *et al.*, 2013) indicate that the *LHCX4* transcript is induced in dark-adapted cells. Therefore, we extended the analysis of the expression of the four *LHCX* genes to cells adapted to prolonged darkness (60 h). In these conditions, we observed a significant increase only of the *LHCX4* mRNAs (Fig. 1C). For the same reason as described above, we attributed the band of  $\sim$ 22 kDa observed in the dark-adapted cells to the LHCX4 protein, although LHCX3 (Fig. 1D) could also be present. In the dark, cells were also showing a decreased NPQ capacity (Fig. 1E) and a slightly reduced PSII maximal quantum yield and overall photosynthetic electron flow capacity (Table 2). Other studies have revealed that blue light photoreceptors (Coesel *et al.*, 2009; Juhas *et al.*, 2014) and the redox state of the chloroplast (Lepetit *et al.*, 2013) could both contribute to the light regulation of *LHCX1*, 2, and 3 gene expression. Thus, we tested the possible role of these processes in the inhibition of *LHCX4* expression upon light exposure. We irradiated dark-adapted cells with low

intensity blue light ( $1 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) during 1 h, in the presence or absence of the PSII inhibitor DCMU (Fig. 1F). The analysis revealed that the *LHCX4* expression is repressed even at such low light irradiance. Moreover, this repression is lost by poisoning photosynthesis with DCMU, suggesting that this process plays an active role in the light-induced repression of *LHCX4*.

A recent study in *Chlamydomonas reinhardtii* showed that the activity of the protein LHCSR3 is regulated by the reversible protonation of three specific amino acidic residues following luminal pH acidification in the light (Ballottari *et al.*, 2016). In order to assess if this mechanism is conserved in diatoms, we analysed the *P. tricornutum* LHCX protein sequences. We found that LHCX1, 2, and 3 possess two of the three amino acids identified in LHCSR3 in conserved positions (Fig. 1G; Supplementary Fig. S2), suggesting that the pH-triggered activation of qE could be conserved in diatoms. On the other hand, only one of these protonatable residues was found in LHCX4.

## LHCX expression in iron starvation

Besides light, nutrient availability also affects chloroplast activity (Wilhelm et al., 2006; Gross, 2012). In many oceanic regions, iron is a major limiting factor for diatom distribution. A general down-regulation of photosynthesis has been reported in iron starvation in several diatom species (Laroche et al., 1995; Allen et al., 2008; Hohner et al., 2013), with a consequent decrease of the carbon fixation reactions, growth rate, and cell size. Since increased NPQ was previously observed in iron-starved *P. tricornutum* cells (Allen et al., 2008), we compared the expression of the different LHCX isoforms in cells grown under Fe-replete and Fe-limited conditions. We found that while a slight induction of the other LHCX proteins was seen, the *LHCX2*

**Table 2.** Photosynthetic parameters of the *P. tricornutum* wild type and transgenic lines

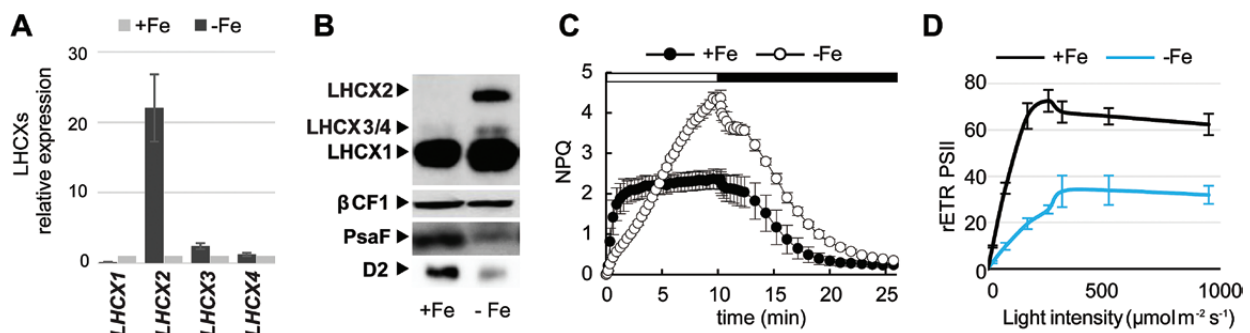
Strain	Conditions	$F_v/F_m$	rETR <sub>PSII</sub>	NPQ max
Pt1	LL	0.66±0.03	74.4±1.2	2.1±0.1
Pt1	Dark	0.60±0.02	67.2±4.0	1.0±0.1
Pt1	+Fe	0.65±0.003	72.6±4.8	2.2±0.3
Pt1	-Fe	0.20±0.004	25.7±1.8	4.4±0.3
Pt1	+N	0.65±0.006	74.4±2.4	2.1±0.1
Pt1	-N	0.40±0.008	27.5±3.2	3.2±0.4
Pt4	WT	0.68±0.01	79.3±2.6	0.83±0.04
Pt4	EVL	0.66±0.01	72.5±3.5	0.82±0.03
Pt4	OE1	0.67±0.01	70.7±2.6	1.00±0.1
Pt4	OE2.5	0.67±0.01	70.0±1.6	1.06±0.05
Pt4	OE2.20	0.66±0.01	69.3±1.6	1.02±0.08
Pt4	OE3.12	0.66±0.01	75.8±4.9	1.04±0.07
Pt4	OE3.33	0.68±0.03	71.0±3.1	1.00±0.1
Pt4	OE4.11	<b>0.59±0.01</b>	68.7±1.9	1.03±0.01
Pt4	OE4.13	<b>0.58±0.02</b>	69.4±1.3	1.07±0.04

PSII efficiency ( $F_v/F_m$ ) and relative electron transport rate (rETR<sub>PSII</sub>) in different growth conditions are reported. rETR<sub>PSII</sub> was measured at 260 μmol photons m<sup>-2</sup> s<sup>-1</sup> light intensity and calculated as: rETR<sub>PSII</sub>= $\phi$ PSII $\times$ actinic light intensity. Non-photochemical quenching (NPQ) was measured with an actinic light intensity of 950 μmol photons m<sup>-2</sup> s<sup>-1</sup> and calculated as in Maxwell and Johnson (2000). Data are the average of three biological replicates  $\pm$ SD.

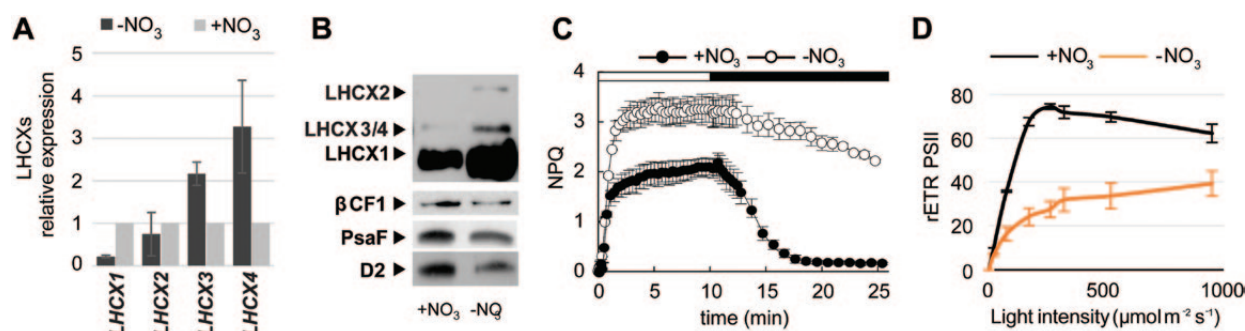
transcript was greatly induced in iron-limited cells (Fig. 2A), leading to a significant accumulation of the LHCX2 protein (Fig. 2B). Fe limitation also enhanced NPQ, while slowing down its kinetics (Fig. 2C), possibly because of a slower diadinoxanthin de-epoxidation rate. We also observed a severe impairment of the photosynthetic capacity in iron limitation as indicated by the decrease in  $F_v/F_m$  (Table 2) and in the PSII maximal electron transport rate (rETR<sub>PSII</sub>) (Fig. 2D; see also Allen et al., 2008). The decreased maximal rETR<sub>PSII</sub> was probably caused by a diminished capacity for carbon fixation. Moreover, in agreement with previous studies (Allen et al., 2008; Thamtrakoln et al., 2013), we observed a decrease in the amount of PSI (PsaF), which is the complex with the highest Fe content. This complex has been already shown to represent the first target of Fe limitation (Moseley et al., 2002). We also found a significant decrease in PSII (D2 protein), which was probably degraded because of sustained photoinhibition (see also Allen et al., 2008) (Fig. 2B).

## LHCX expression in nitrogen starvation

Besides iron, nitrogen (N) is also a limiting resource for diatoms (Mills et al., 2008; Moore et al., 2013; Rogato et al., 2015). Recent transcriptomic and proteomic analysis highlighted important metabolic modifications under N starvation, such as the up-regulation of nitrogen assimilation enzymes, the recycling of intracellular nitrogen-containing compounds from the photosynthetic apparatus and other sources, and the increase in lipid content as a consequence of remodelling of intermediate metabolism (Allen et al., 2011; Palmucci et al., 2011; Hockin et al., 2012; Alipanah et al., 2015; Levitan et al., 2015; Matthijs et al., 2016). We found that N limitation also has a significant effect on the expression of the LHCXs. In particular, N limitation triggered the induction of *LHCX3* and *LHCX4* mRNAs (Fig. 3A) and of LHCX3/4 proteins (Fig. 3B). The increase of the LHCX1 and 2 isoforms was only visible at the protein level (Fig. 3B). Up-regulation of the LHCX proteins in N limitation correlated with an increase of the NPQ capacity



**Fig. 2.** Effect of iron starvation on *P. tricornutum* LHCX expression and photophysiology. Experiments were performed on cells grown in iron-replete (11 μM, +Fe) or iron-limited (5 nM iron+100 μM FerroZine™, -Fe) conditions: (A) qRT-PCR analysis of *LHCX* transcripts in -Fe, normalized against the +Fe condition and using *RPS* and *H4* as reference genes. (B) Immunoblot analysis of the LHCX, D2, and PsaF proteins, using  $\beta$ CF1 as loading control. NPQ capacity (C) and relative electron transfer rates (rETR<sub>PSII</sub>) (D) of cells grown in +Fe or -Fe. The horizontal bar in (C) indicates when the actinic light was on (white) or off (black). rETR<sub>PSII</sub> was measured at different light intensities (20, 170, 260, 320, 520, and 950 μmol m<sup>-2</sup> s<sup>-1</sup>). In (A), (C), and (D), error bars represent  $\pm$ SD of three biological replicates. (This figure is available in colour at JXB online).



**Fig. 3.** Effect of nitrogen starvation on *P. tricornutum* LHCX expression and photophysiology. Experiments were performed on cells grown in nitrogen-replete (1 mM, +NO<sub>3</sub><sup>-</sup>) or nitrogen starvation (50 μM, -NO<sub>3</sub><sup>-</sup>) conditions: (A) qRT-PCR analysis of *LHCX* transcripts in -NO<sub>3</sub><sup>-</sup>, normalized against the values in the +NO<sub>3</sub><sup>-</sup> condition, and using *RPS* and *H4* as reference genes. (B) Immunoblot analysis of the LHCX, D2, and PsaF proteins, using βCF1 as loading control. NPQ capacity (C) and relative electron transfer rates (rETR<sub>PSII</sub>) (D) of cells grown in +NO<sub>3</sub><sup>-</sup> and -NO<sub>3</sub><sup>-</sup> conditions. The horizontal bar in (C) indicates when the actinic light was on (white) or off (black). rETR<sub>PSII</sub> was measured at different light intensities (20, 170, 260, 320, 520, and 950 μmol m<sup>-2</sup> s<sup>-1</sup>). In (A), (C), and (D), error bars represent ±SD of three biological replicates. (This figure is available in colour at JXB online).

(Fig. 3C). We note that NPQ was slowly relaxing upon dark exposure of N-limited cells, possibly reflecting the repression of the genes encoding the xanthophyll cycle enzymes including the zeaxanthin epoxidase (see Supplementary Fig. S3), when analysing available microarray data from N-depleted cells (Alipanah *et al.*, 2015). The N limitation also led to a drastically reduced  $F_v/F_m$  (Table 2) and a lower maximal rETR<sub>PSII</sub> (Fig. 3D). We also detected a reduced content of PSII and PSI proteins (Fig. 3B), in line with previous omic studies pointing to a general decrease of the photosynthetic capacity.

#### Analysis of the LHCX non-coding regions

Due to the observed transcriptional responses of *LHCX* genes in different light and nutrient conditions, we searched for known and potentially novel regulatory motifs in the 5'-flanking regions and the intronic sequences of the four isoforms (see Table 3; Supplementary Fig. S1). Because of their involvement in integrating light signals with CO<sub>2</sub>/cAMP-induced transcriptional responses, we searched for the three CO<sub>2</sub>/cAMP-responsive *cis*-regulatory elements (CCREs) identified in Ohno *et al.* (2012) and further characterized in Tanaka *et al.* (2016). Interestingly, we found CCRE-1 in the 5'-flanking sequences of *LHCX1* and 4, CCRE-2 in 1, 2, and 4, and CCRE-3 in *LHCX4*. These *cis*-regulatory elements may participate in the light-mediated regulation of the four *LHCX* genes.

In contrast, the two *P. tricornutum* iron-responsive elements identified in Yoshinaga *et al.* (2014) are not present in the analysed non-coding regions, suggesting that a different transcription factor should be involved in modulating the *LHCX2* transcriptional response to iron availability. Similarly, we could not find the two *P. tricornutum* motifs identified as responsive to short-term nitrogen deprivation (from 4 h to 20 h) in Matthijs *et al.* (2016), suggesting that distinct regulatory circuits may act in the short- and long-term acclimation to nitrogen deprivation.

To pinpoint possible novel regulatory motifs, we also scanned the non-coding sequences of the four isoforms

**Table 3.** The identified regulatory motifs and their occurrence in the *P. tricornutum* LHCX non-coding sequences

	Sequence	LHCX1	LHCX2	LHCX3	LHCX4
MOTIF 1	TCA[CT][AT]GTCA	2	2	1	–
MOTIF 2	CGAACCTTGG	–	–	2	–
MOTIF 3	CCT[GC]TCCGTA	–	–	2	–
MOTIF 4	GAGTCCATCG	–	–	–	2
MOTIF 5	CGATCACGGC	–	–	–	2
MOTIF 6	[TA]TGACTG	–	1	1	1
CCRE-1	TGACGT	1	–	–	1
CCRE-2	ACGTCA	1	1	–	1
CCRE-3	TGACGC	–	–	–	1

using the MEME Suite program (Bailey *et al.*, 2009). The analysis revealed six motifs repeated at least twice in each isoform and/or shared by more than one isoform (Table 3; Supplementary Fig. S1). None of the identified motifs corresponds to a known transcription factor-binding site. This suggests that these motifs could represent novel diatom-specific *cis*-regulatory elements. In order to examine the potential involvement of the identified motifs in the long-term nitrate deprivation transcriptional responses of *LHCX* genes, we analysed a published microarray data set performed on 48 h and 72 h nitrogen-deprived *P. tricornutum* cells (Alipanah *et al.*, 2015). We compared the frequency of the six identified motifs in the 5'-flanking sequences of responsive and unresponsive transcripts. Interestingly, motif 6 ([T-A]TGACTG) was significantly enriched ( $p=0.035$ ) in the 5'-flanking sequences of genes up-regulated in response to nitrogen starvation compared with down-regulated genes. The result suggests that motif 6 may be involved in gene transcriptional regulation in cells exposed to prolonged nitrogen starvation.

#### Modulation of LHCX gene expression in *P. tricornutum* transgenic lines

A role in the regulation of the NPQ in *P. tricornutum* has been proven for the LHCX1 protein by characterizing transgenic

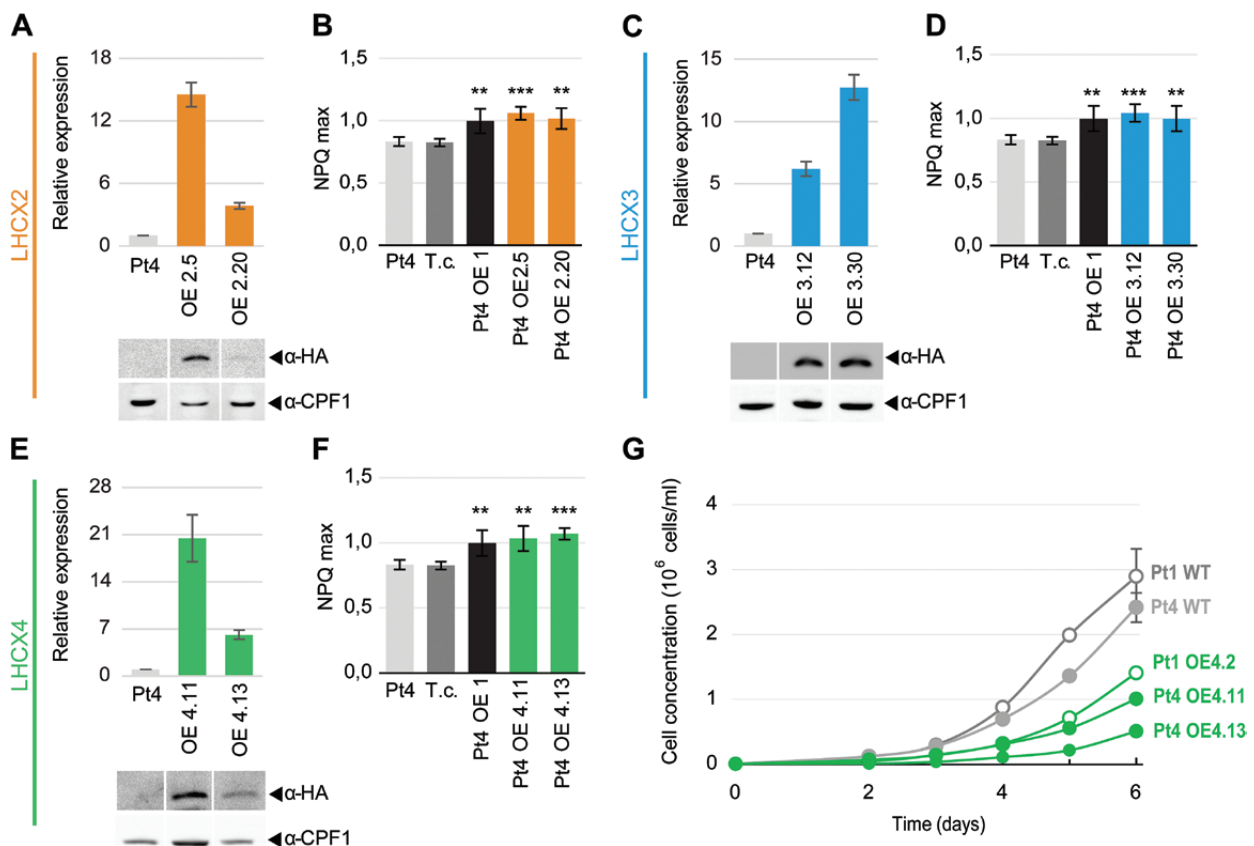
lines with a modulated content of LHCX1 by either gene silencing or gene overexpression (Bailleul *et al.*, 2010). Unfortunately, all the attempts to down-regulate the expression of *LHCX2*, 3, or 4 have been unsuccessful. Therefore, to explore their function, we opted for the strategy used in Bailleul *et al.* (2010), and tried to rescue the intrinsically lower NPQ capacity of the Pt4 ecotype. To this end, independent transgenic Pt4 lines were generated, bearing a vector in which the *LHCX2*, 3, or 4 genes were expressed under the control of the *P. tricornutum* *FCPB* (*LHCF2*) promoter. A HA-tag was fused to the C-terminal end of the *LHCX* transgenes to allow the specific detection of the transgenic proteins. qRT-PCR and western blot analyses (Fig. 4A, C, E) on independent transgenic lines confirmed the expression of the transgenic LHCX isoforms. NPQ analyses (Fig. 4B, D, F) showed that the overexpression of each LHCX isoform generated a modest, but statistically significant, increase in the NPQ capacity compared with the Pt4 wild type as well as compared with a transgenic line transformed only with the antibiotic resistance gene and used as control. Strikingly, we found that all the transgenic lines showed a similar NPQ

increase, regardless of which isoform was overexpressed and the different overexpression levels.

We also checked the possible effect of LHCX overexpression on growth and photosynthetic capacity. For the lines overexpressing the *LHCX2* and *LHCX3* proteins, we did not observe any altered phenotype (Table 2). In contrast, the Pt4 lines overexpressing *LHCX4* showed a reduced PSII efficiency (Table 2). By performing a growth curve analysis, we also observed that these overexpressing lines showed a lag phase lasting 2–3 d (Fig. 4G), which was not the case in wild-type cells. A similar effect on growth was also observed in transgenic lines in which the *LHCX4* gene was overexpressed in the Pt1 ecotype (Fig. 4G).

## Discussion

The presence of multiple *LHCX* genes in all the diatom genomes analysed to date strongly suggests that the expansion of this gene family is a common feature of these algae and may represent an adaptive trait to cope with highly variable environmental conditions. To investigate this scenario, in



**Fig. 4.** *Phaeodactylum tricornutum* Pt4 ecotype lines overexpressing the *LHCX* genes. (A), (C), (E) LHCX transcript (upper panels) and protein (lower panels) analyses in the Pt4 wild type and transgenic strains overexpressing HA-tagged *LHCX2* (A), *LHCX3* (C), or *LHCX4* (E). Transcript abundance was measured by qRT-PCR using *RPS* as the reference gene and normalized to the wild type expression value. Tagged proteins were detected by immunoblot using an anti-HA antibody, and an anti-CPF1 antibody as loading control. Bands are taken from the same blots but from non-adjacent lanes. (B), (D), (F) NPQ max capacity in the Pt4 wild type, in a transgenic strain expressing the vector for antibiotic resistance (transformation control, T.c.), and in independent transgenic lines overexpressing *LHCX1* (OE1), *LHCX2* (OE2), *LHCX3* (OE3), and *LHCX4* (OE4) genes. Asterisks indicate the results of two-tailed Student *t*-tests: \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . (G) Growth curves of Pt4 and Pt1 wild-type strains and Pt4 and Pt1 transgenic lines overexpressing the *LHCX4* (OE4) gene, grown in 12L/12D cycles ( $50 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). In all the experiments,  $n \geq 3$ , and bars represent  $\pm$ SD. (This figure is available in colour at JXB online).

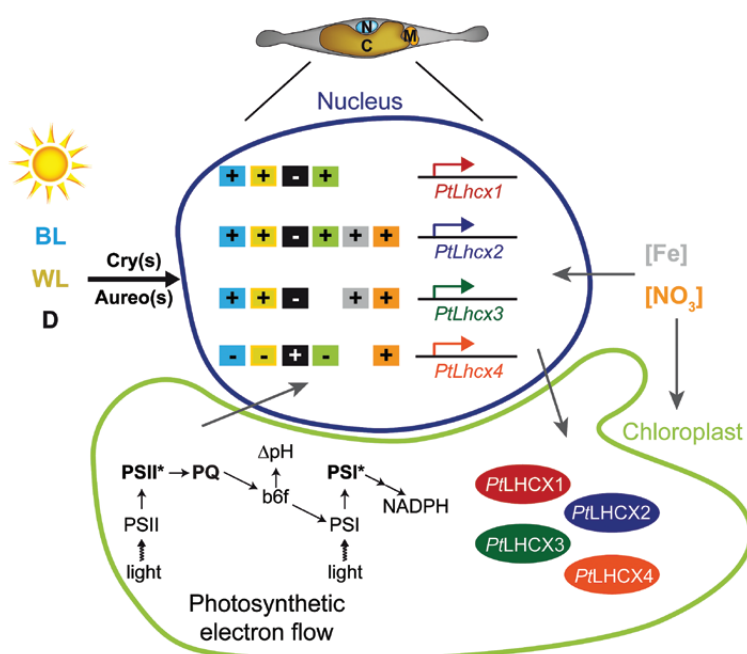
this work we correlated LHCX expression profiles with the photosynthetic and photoprotective performances in variable experimental conditions, including changes in light irradiance and nutrient availability. These analyses revealed that the four *P. tricornutum* LHCX genes respond differently to various environmental cues, as summarized in Fig. 5.

The analyses of the mRNA and protein responses indicate that amounts of the different LHCXs are tightly regulated at the transcriptional, and probably also the post-translational level. As LHCX3 and LHCX4 have a similar size, it was not possible to quantify the amount of these two proteins under the different stresses using one-dimensional electrophoresis. However, considering the transcript and biochemical analyses together (in the case of LHCX2 and LHCX1), it seems that LHCX1 is always expressed at high levels even in non-stress conditions, which is consistent with it having a pivotal role in NPQ regulation and light acclimation as proposed previously (Bailleul *et al.*, 2010).

LHCX2 and 3 are induced following high light stress, where they may contribute to increase the diatom photoprotection capacity. Their induction, as well as the accumulation of LHCX1, may result from the integration of different signals. Two members of the blue light-sensing cryptochrome photolyase family, CPF1 (Coesel *et al.*, 2009) and CRYP (Juhás *et al.*, 2014), modulate the light-dependent expression of LHCX1, LHCX2, and LHCX3. Also, the recently identified Aureochrome 1a blue light photoreceptor, which regulates *P. tricornutum* photoacclimation (Schellenberger Costa *et al.*, 2013), may affect how much of each LHCX there is in a cell. Moreover, chloroplast activity, through the redox state

of the plastoquinone pool, may also regulate *LHCX1* and *LHCX2* gene expression in HL (Lepetit *et al.*, 2013).

A different regulation pattern is seen in the case of LHCX4, the only isoform which is induced in the absence of light. The amount of *LHCX4* mRNA rapidly decreases following a dark to light transition, and this repression is lost when photosynthesis is halted with the PSII inhibitor DCMU. This suggests that chloroplast-derived signals could participate in inhibiting gene expression, even at very low light irradiance, by an as yet unknown process. The peculiar trend observed in the LHCX4 light response suggests a possible role for this protein in *P. tricornutum* photoacclimation. The increased LHCX4 transcript and possibly protein content is mirrored by a decrease in NPQ capacity and a slightly reduced  $F_v/F_m$  in the dark-adapted cells, compared with cells grown in the light (Fig. 1E; Table 2). Moreover, reduced PSII efficiency and slightly altered growth were observed in cells overexpressing LHCX4 in the light (Fig. 4G), suggesting that LHCX4 could have a negative impact on chloroplast physiology. Indeed, a comparative analysis of the *P. tricornutum* LHCX protein sequences indicates that LHCX4 lacks key protonatable residues that in *Chlamydomonas* are involved in NPQ onset when the lumen acidifies (Ballottari *et al.*, 2016). These residues are, however, conserved in the LHCX1, 2, and 3 isoforms. According to the model established in green algae for the protein LHCSR3, these residues diminish their electrostatic repulsion upon protonation, allowing a rearrangement of the protein structure and pigment orientation and enhancement of the quenching capacity (Ballottari *et al.*, 2016). The substitution in LHCX4 of the acidic residues (aspartate and



**Fig. 5.** Model of the *P. tricornutum* LHCX regulation. Scheme summarizing the multiple external signals and stresses that differentially regulate the expression of the four LHCXs. The LHCX genes are shown in the nucleus and the LHCX proteins in the chloroplast. + and - boxes indicate positive and negative transcriptional regulation, respectively, in response to white light (yellow), blue light (blue, through the cryptochromes, Cry, and aureochromes, Aureo, photoreceptors), darkness (black), chloroplast signals (green), iron starvation (grey), and nitrogen starvation (orange). In the *P. tricornutum* cell: N, nucleus; C, chloroplast; M, mitochondrion. In the chloroplast: PSI and PSII, photosystem I and II, respectively; PSI\* and PSII\*, excited photosystems; PQ, plastoquinone pool;  $b_6f$ , cytochrome  $b_6f$  complex;  $\Delta pH$ , proton gradient; NADPH, redox potential.



glutamate) with non-protonatable residues (asparagine and glycine) would prevent such regulation. Instead, LHCX4 could contribute to the observed capacity of *P. tricornutum* to survive long periods in the dark and its repression could be needed for a rapid acclimation following re-illumination (Nymark *et al.*, 2013). Consistent with this, high *LHCX* gene expression has also been observed in sea-ice algal communities dominated by diatoms that have adapted to the polar night (Pearson *et al.*, 2015).

Besides the light and redox signals discussed above, our study also shows that differences in the availability of iron and nitrogen strongly affect the expression of the different LHCXs. The signalling cascades controlling these responses are still largely unknown, but they probably involve multiple regulatory pathways into the nucleus and chloroplast, considering that these nutrients are essential for diatom photosynthesis and growth (Table 2; Fig. 5). Nitrogen starvation induces a general increase of all the LHCX isoforms, including LHCX4 that is normally repressed in light-grown cells (Fig. 3). We can hypothesize that the general increase of the LHCX content is needed to protect the photosynthetic apparatus, which is strongly affected by nitrogen deprivation, as shown by the drastically reduced  $F_v/F_m$  (Table 2), the lower maximal  $rETR_{PSII}$  (Fig. 3D), and the reduction of PSI and PSII protein content. Interestingly, an opposite trend is observed for the main enzymes of the xanthophyll cycle, which are either not induced or are repressed in cells grown in similar nitrogen stress conditions (Supplementary Fig. S3). Thus, in nitrogen starvation, the LHCXs could represent the major contributors to the observed NPQ increase (Fig. 3C).

At variance with nitrogen starvation, iron starvation has a more specific effect on LHCX expression. A strong induction of the LHCX2 mRNA and protein levels (Fig. 2) compared with the other isoforms was observed, pinpointing this isoform as the most likely regulator of the increased NPQ capacity observed in iron stress (Fig. 2C). NPQ in iron-limiting conditions is characterized by a slow induction and a complete relaxation in the dark. These slow induction kinetics might reflect either lower concentrations of the pH-activated de-epoxidase enzyme or its cofactor ascorbate (Grouneva *et al.*, 2006) or slower acidification of the thylakoid lumen due to a reduced photosynthetic activity. Indeed, the photosynthetic capacity is severely impaired when iron is limiting, as demonstrated by the reduction in PSI and PSII subunits (Fig. 2B), but also the lower  $F_v/F_m$  (Table 2) and  $rETR_{PSII}$  (Fig. 2D). The decreased electron flow per PSII could also reflect a decrease in the iron-containing cytochrome  $b_6f$  complex, as previously shown for other iron-limited diatoms (Strzpek and Harrison, 2004; Thamtrakoln *et al.*, 2013).

The observations made in this and in previous studies about the complex LHCX regulation in response to different signals prompted us to explore their possible functions in *P. tricornutum*, by modulating their expression in a natural Pt4 strain characterized by constitutive lower NPQ levels (Fig. 4). We observed that the increased expression of all the tested isoforms generates a small but still consistent increase in the NPQ levels, suggesting a potential involvement of the diverse proteins in NPQ modulation, as previously shown for

LHCX1 (Bailleul *et al.*, 2010). However, we also noticed that different overexpressing lines with different transcript and protein levels showed a similar NPQ increase. It is difficult to interpret these first results, especially in the case of lines overexpressing LHCX4, whose endogenous expression is inhibited by light (Fig. 1F). They probably reflect the complexity of NPQ regulation in diatoms, where the presence of multiple players (e.g. several LHCXs and enzymes of the xanthophyll cycle) possibly tend to reduce the consequences on NPQ of genetic modifications of the qE machinery.

Finally, the exploration of the 5'-flanking regions and intronic sequences of the *LHCX* genes revealed the presence of known and potentially novel *cis*-regulatory elements that may contribute to the transcriptional regulation of the different isoforms in stress conditions. We revealed an uneven distribution of the CCREs (Ohno *et al.*, 2012; Tanaka *et al.*, 2016) in the four *LHCX* genes that may be linked to their different light-mediated transcriptional responses. In addition, we identified a 7 bp motif in the non-coding sequences of *LHCX2*, 3, and 4. Using genome-wide transcriptomic data, we found this motif specifically enriched in long-term nitrogen starvation-induced genes, suggesting a possible involvement in the regulation of gene expression in response to nitrogen fluctuations. Although additional studies are required to demonstrate the functionality of these motifs, their discovery may represent a starting point for the identification of the *LHCX* regulators in the diatom acclimation mechanisms to stress.

### Outlook

Here we discovered that the four *P. tricornutum* LHCXs are regulated in a sophisticated way (Fig. 5). Different and probably interconnected regulatory pathways activated by different signals and stresses tightly control the amount of each LHCX isoform in the cell. By narrowing down the specific growth conditions in which the different LHCXs are required, our results set the basis for future work to define the function of each isoform in the regulation of chloroplast physiology. The generation of new transgenic lines in which the content of each LHCX isoform is specifically modulated will be instrumental in assessing whether they act with the NPQ regulator LHCX1, or play other specific roles. Considering the robustness of LHCX1 expression in all the conditions tested, future studies will probably require the use of new LHCX1 loss-of-function diatom strains. Additional information about the association of LHCXs with photosynthetic complexes and pigments will also be necessary to understand the role played by the expanded *LHCX* gene family in the efficient acclimation of diatoms to environmental changes.

### Supplementary data

Supplementary data are available at *JXB* online.

**Figure S1.** Localization of the enriched motifs in non-coding regions of *P. tricornutum* *LHCX* genes.

**Figure S2.** Alignment of the LHCX proteins and three-dimensional model of LHCX1.

**Figure S3.** Expression of the *P. tricorutum* xanthophyll cycle genes in nitrogen starvation.

**Table S1.** List of the oligonucleotides used in this work.

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## Supplementary Data

*Journal of Experimental Botany*

**Multi-signal control of the expression of the LHCX protein family in the marine diatom *Phaeodactylum tricornutum***

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## PtLhcx1

### 5'-flanking sequence

TCAAAGGAAGGAATGTAGAGTAAGCAAACCATCATTTGCGCGCTTCCATTACCAAGAGAACCGACCAAGAGCCCTACCTGTATGTCATTCGTATAAAC  
GTGCACCTGAATCGCCCAGCATGTGAACGCACCATCTCGAATGGCCATCGACAATTCCTTCGACCGAAAAAGCAAACACCACAAGCTTTCGTAGAGGG  
CTTATTCCTCACATCCGTACATGACATCTTCGTTGAAGTCTACTAGGACAAGAAGCCACCGAGTCTGTGCGGAACCATCGTGTAGCAAATCGGCGAGGC  
GAAGCTATTACAACCTTAACCTGGTTCAGTACAGCGTGTGATTGTGAACAAAACCAATGGCCAGACGGATTTCGCAAGTTCAGACAGCTTTCGACGGTG  
AGAATGATGGTATCGCTTCGGGTTCGGTTCGGTACTTTCGCGGACTGGCAACGTTCGGCAGAGGGAC **TCACGGTCA**CTTCACTGTCTGTATCGACTGACGA  
CTGACTCGTCTGAT **TCACTGTCA**GGTTCTGATCTAAGTTTATCAAATGCTACTACTTGAGGTAGTCGTATCATTGAACTGCAACTTACAAAATCGA  
AGGTCCTTGAACATTTCTGACATGGGTAATTTCAATAGGATGTACAATGTCGAGCTGTATCACTCGGATACAACAGACCCTTTAGGAAAGGGATTT  
TTTTTTGTGCGAAAATAGGATGACATCAACAGGAAGTACGTTTATAGCCCTTTATTTATAATTGATGGGTCAATCCGACGTACGCATGTGCTTTTGAC  
CTTGTATCCATAACAGATGACCCGTCCTGTGCAAT **ACGTCA**CGCCAAATGAAAGACCCCGGATAAAGGGCTAAAAATTCAGTCTCGTGCAAAACA  
CGCAGGATGATGCACCTGAACAG **TCACGT**GTTTCGCGCAGTCGAAATGAGTTCCGAAGCCATCAGAGGCGAATTTATTTTGCAACTGTTTGACCCCATAA  
AACCATTCTT

## PtLhcx2

### 5'-flanking sequence

ATCACGTGAGATATCACTGATATCTC **ACGTCA**ATAGACAAGCTGCTTTACAATGGGTGAAAACAACCACCACAGAAACAAGTTCCTGTAACCTCTAAAT  
ATTGATGTAAATGGTGAAGAAAGCATTTTCCTGACTGTGGGTACATGGAAATACATCCTTTTTTGATAAATGTACAAACAAAGACTCATTTACGTGTAG  
**TCATAGTCA**AGCGCAGTGCCTAC **TTGACTG**TGAAAAAATCGAGCTCTGAAAGGGTTAAAGTTCAAGAGGTACAAGGTAGCTGGTTTCGCGTGTGTACAGG  
CATGGCCTACCTGACTCATGCATTCGCGGTGCCACAAAAC **TCACAGTCA**GAGAGCCACTCCGAGAATCCTCCAGAAATTCGTGGAGATTTTTTCGTCT  
ATCTTTTTCCAGTCTCCGCATAGCTCTCATAGTTTCGTTTGGTTCCTCGTCAACACACCCGAAAAAGATAACGTCCAATTCAGCCACTACGTAC  
ACC

## PtLhcx3

### 5'-flanking sequence

AGAAATGTCAAGATCATGGATGGTTTCAAGGACACATGACATGTAAATCCTTACGGCTATTTTCGATCCGAACCCAGAGTATGGTAGGTAGGTAGTGAC  
ACGAGCGAGTCATAGAATTGTGTTTTGATCAGACAATCTGTACTTATTCGACCATGCTATTTATTACACACGTGTTTGTACCGCCAAACAGATT  
CTTTCGCTAGAATCCGACTGATCGACGACCACACAGAGAGGGACTGACGAAGTACAACACGCGACGAAACACCAGGCAACACAACATACCGTTA  
ATTTTACAATTAGTGCTGACTAACAGCGTATACTGTCAACAGAGTCCG **ATGACTG**GC TGACAATGACTTGGAAACGCAATTTGCATTTGATAGTGACTAT  
GAGTT **CCAAGCTTCG**ATCGGGCCGAACCAAGTAGAAAGATACATGTAAACGACTACCATATACTGGCTGATTTCAATCCGACCCAGCAGAGTAACCCCAT  
CTGGTGTGCGATTCCTCTGCGAATACTCTAGAGGTATCACGGTATGTATATAGTATATAGTACTCGTCCGCCACGGTTGTCTTCGATTTCCATCCATCCT  
GT **TACGGACAGG**AACCAGACACGACACGGCCCTA **CCTCTCCGTA**TCCGATCCTTGCCATCACTTTTGGGGTACTTTTCGAAAAAGACTGAT **TCATAGTCA**  
AATGCCTTCTGACGGATCCAGAGGGTACGAGAGTCTGCTGACGAACGTCTCGTACACTCATTTCTGGTTGTGGAAATCATCTCCCTACACACTACG  
CTCCAAAATGATAACTCTGCACAATTCACACAATACTCTAAACAGTACTTTCGATGCACTTGCAACCCACGTACCACCACCAGTACCAAACACAAT  
CATTACACC

### intron1

GTACGTCTACG **CGAACCTTGG**CATTTTTCCAGGTACCGCAGTGTTCGGTGTGTACAGTTAGTGTGTCGTCGCGATTGCAATTCCTACGAACCACACTCGTT  
CCGCTTTGTCCGGCAG

## PtLhcx4

### 5'-flanking sequence

GAAAATCTTAGCCCGGCACGGCAGGCCGTCTCTGCACCTGTAGTGAAGCGAATGTGCAATGTCCATGTGCAAGTTCGTAGGAGACGCCGACAGAGAAAC  
CACGGCTATCATGTAAACAATATGCAAGAGTACCTTACTTATCGAACATGCTGGAGTTATTTGCTGTTGATTTAAGTATAAATTAAT **TGACGG**AGTATA  
TATAAATCAGTGCACATAAAGTTTGACGAAACCTCATAGTTTCGAGTTTGTATGCACTACTGAAATTTCCAGAATCGGCAATCCTGTAACACCTATT  
TTTACACTTACATCGATAAATGGTTACAATCAAGCCTTGGCCCAAGCACCAGCTAACATAAGTGGTTCACAGCAATTTAGTGGTTCGTGTCATACTC  
AAGCAGCAATCACCAATTCGGAAGTAAACCGAACT **CGATCACGGC**GATGCAAGTCCCTTTAATTCAC **GAGTCCATCGA**CT **CGATCACGGC**GAT  
GCAAGTCCCTTTTCTCAG **GAGTCCATCG**AGCTCGATAACAACGAAAGCAGGTCGACGCCACGTGTTCAGAAAATGGTTGCCTTCTGTGACTAACTCGTTC  
TTTTCGATCTCGAATGAATTCCTTTGCTTTCCATACTTGT **TCACGT**CGTCGAGGGGAGTCTGTGAGAGCAGGCAGCGAGTCTGTTACTCCTCGGATTTGT  
TCATTC **CAGTCA**TGCAAGCAGTGGTCTTTGACTCTCACCTTACAATTTGAGATCCTTTCGGTTTTCGAGAGAGTGAGAC **ACGTCA**AACGCACTCAGC  
CAGT

Motif 1: **TCA[CT][AT]GTCA**

Motif 2: **CGAACCTTGG**

Motif 3: **CCT[GC]TCCGTA**

Motif 4: **GAGTCCATCG**

Motif 5: **CGATCACGGC**

Motif 6: **[TA]TGACTG**

CCRE 1: **TGACGT**

CCRE 2: **ACGTCA**

CCRE 3: **TGACGC**

Fig. S1: Localization of the enriched motifs in *LHCXs* non-coding regions.

**A**

CrLHCSR3	-----MLANVVS RKASGLRQT- PARATVAVKSVSGRR---TTAAEPQTAAFPVAEDVFA	50
PtLHCX1	MKFAATILALI-G-SAAAFAPA-----QTSRA--STSLQY	31
PtLHCX2	MKLSLAILALC-ASTNAAFAPSVSQRTSVSVRESLDPTE SMSEVEGAVKDAAPKVSDPFD	59
PtLHCX3	MKCIAAIALLA-T-TASAFNAF-----GAAKAA--PKKPVF	33
PtLHCX4	MKLFITIFLPLVLVGTAA GFAS-----GPF SK--KASPSPEV	34

CrLHCSR3	YTKNLPGV TAPFEGVFD PAGFLATAS IKDVRWRRESE I THGRVAMLAALGFVWGEQLQDF	110
PtLHCX1	AKEDLVGAI PP-VGFFDPLGFADKADSP TLKRYREAE L THGRVAMLA VVGFV LGEAVEGS	90
PtLHCX2	SPRDLAGV VAP-TGFFD PAGFAARADAG TMKRYREAE V THGRVGMMAVVGFLAGEAVEGS	118
PtLHCX3	SIETIPGALAP-VGIFDPLGF AAKADESTL KRYREAE L THGRVAMLA TVGFLVGEAVEGS	92
PtLHCX4	SIESMPGIVAP-TGFFDPLRFAERAPSNTL KRYRECE L THGRVAMLA TVGFLAGEAVQNT	93

CrLHCSR3	PLFFNW <sup>*</sup> GRVSGPAIYHFQQIGQGFWEPLLI AIGVAESYRVAVGWATPTGTGF--NSLK	167
PtLHCX1	S--FLF <sup>D</sup> ASISGPAITHLSQVPAFWVLLT I AIGASEQTRAVIGWVDPADAPVDKPGLLR	148
PtLHCX2	S--FLF <sup>D</sup> SQVSGPAITHLNQIP SIFWILLTVGIGASEVTRAQIGWVEPENVPPGKPGLLR	176
PtLHCX3	S--FLF <sup>D</sup> ASIKGPAISHLAQVPTPFWVLLT I FIGAAEQTRAVIGWRDPSDVPFDKPGLLN	150
PtLHCX4	N--FLV <sup>D</sup> MAQVSGPAITHI PQIPATFWVLLT L FIVAE LSRAQTAMVPPSDI PVGKAGRMR	151

Region 1

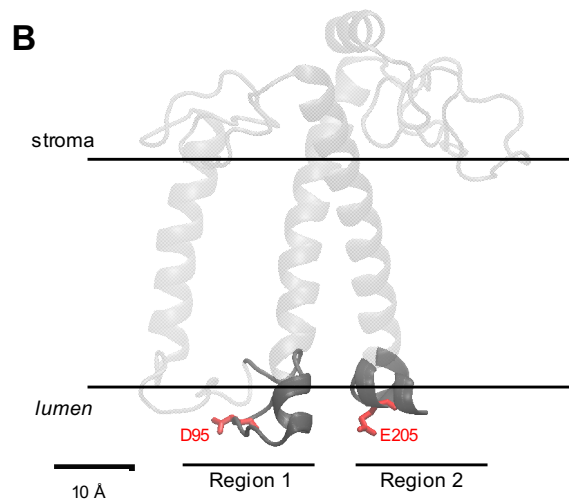
  

CrLHCSR3	DDYEPGDLGFDFLGLKPTDPEELKVMQTKELNNGRLAMIAIAAFVAQELVEQI <sup>*</sup> IE <sup>*</sup> HLA	227
PtLHCX1	DDYVPGDLGFDFLGLKPSDPEELITLQTKELQNGRLAMLAAAGFMAQELVNGKGI <sup>*</sup> ENLQ	208
PtLHCX2	DDYVPGDIGFDFLGLKPSDAQALSKIQTKE LQNGRLAMLAAAGCMAQELANGKGI <sup>*</sup> ENLG	236
PtLHCX3	EDYTPGDIGFDFLGLKPTDAEELRVLQTKELQNGRLAMLAAAGFMAQELVDGKGI <sup>*</sup> ENLL	210
PtLHCX4	EDYNPGDIGFDFLNLMPESSEEFYRLQTKELQNGRLAMLGAAGFLAQEA VNGKGI <sup>*</sup> ENLF	211

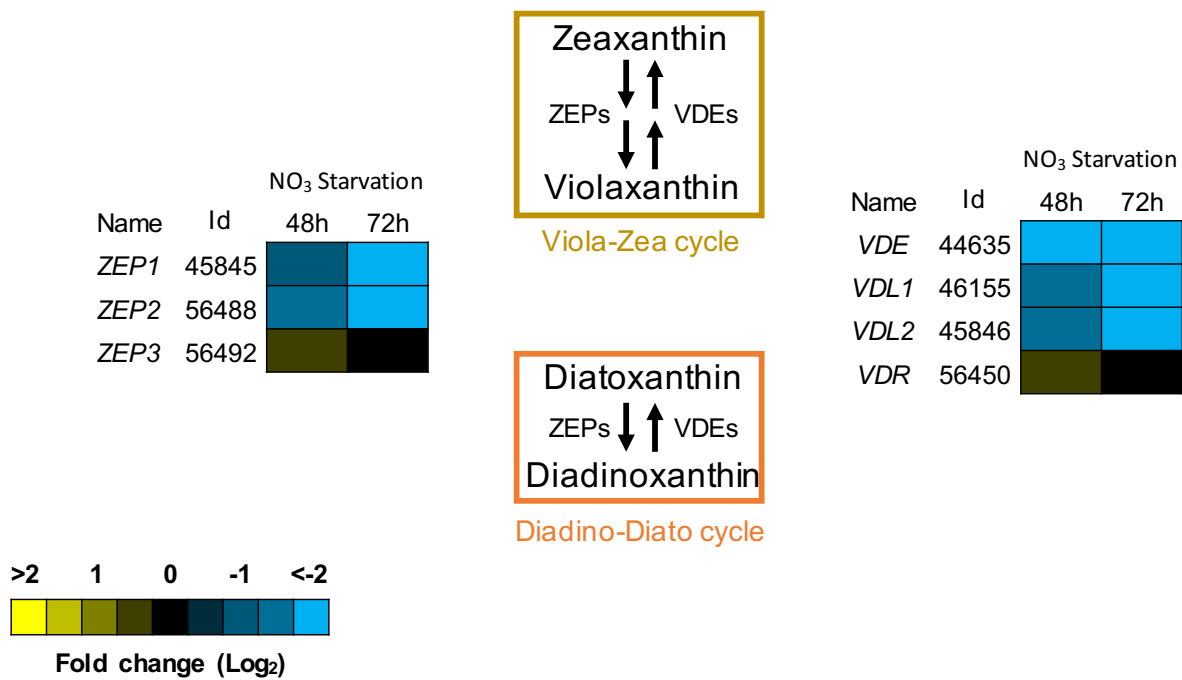
Region 2

CrLHCSR3	LRF EKEAILELDDIERDLGLPVTPLPDNLKSL	259
PtLHCX1	G-----	209
PtLHCX2	L-----	237
PtLHCX3	-----	210
PtLHCX4	G-----	212



**Fig. S2:** Protein alignment of the LHCX proteins and three-dimensional model of the LHCX1. (A) Multiple sequence alignment of *Chlamydomonas reinhardtii* LHCSR3 and the four *Phaeodactylum tricornutum* LHCX proteins. Putative protonatable aminoacids in LHCX proteins conserved with respect to *Chlamydomonas reinhardtii* LHCSR3 (Ballottari et al. 2016) are indicated in red. (B) LHCX1 homology-based model from LHCII and CP29 crystallographic structures. Putative protonatable aminoacids conserved with respect to *Chlamydomonas reinhardtii* LHCSR3 are indicated in red (D95 and E205). Region 1 and 2 refers to part of LHCSR3 putatively exposed to thylakoydal lumen, where pH sensitive residues are located.



**Fig. S3:** Expression of *P. triconutum* xanthophyll cycle genes in nitrogen starvation. VDE, VDL1-2 and VDR are the enzymes putatively catalyzing the de-epoxidation reactions active in high light to form Zea- and Diatoxanthin from Viola- and Diadinoxanthin, respectively. ZEP1, 2 and 3 enzymes putatively catalyse the epoxidation reaction, active in low light, to form Viola- and Diadinoxanthin from Zea- and Diatoxanthin. Microarray data of cells after 48h and 72h of nitrogen starvation were taken from Alipanah *et al.*, 2015.



Species	Name	Id	Chromosomal localization	Length (aa)	N° Intron
<i>Thalassiosira pseudonana</i>	LHCX1	264921	chr_23:365603-366232 (-)	209	0
	LHCX2	38879	chr_23:368273-368902 (+)	209	0
	LHCX4	270228	chr_5:1446306-1447125 (-)	231	0
	LHCX5	31128	chr_1:2849139-2850176 (-)	236	3
	LHCX6	12097	chr_23:366611-367378 (+)	255	0
	<i>Phaeodactylum tricornutum</i>	LHCX1	27278	chr_7:996379-997300 (+)	206
LHCX2		56312	chr_1:2471232-2472170 (+)	238	2
LHCX3		44733	chr_5:76676-77606 (+)	206	1
LHCX4		38720	chr_17:53010-53733 (+)	207	1
<i>Pseudo-nitzschia multiseriis</i>	-	66239	scaffold_189:181982-182948 (+)	201	1
	-	238335	scaffold_95:121459-122306 (-)	202	1
	-	257821	scaffold_246:124909-125745 (+)	197	1
	-	264022	scaffold_1353:8720-9877 (+)	206	1
	-	283956	scaffold_38:284133-284828 (-)	231	0
<i>Thalassiosira oceanica</i>	-	Thaoc_09937	SuperContig To_g10869: 4.331-5.040 (-)	210	1
	-	Thaoc_12733	SuperContig To_g15184: 10.800-11.435 (-)	172	1
	-	Thaoc_28991	SuperContig To_g41561: 2.777-3.105 (+)	81	1
	-	Thaoc_31987	SuperContig To_g45669: 1-1.025 (-)	205	2
	-	Thaoc_32497	SuperContig To_g46152: 5.664-6.285 (-)	180	1

**Table S1.** List of LHCX genes identified in diatom genomes. For *T. pseudonana* (Thaps3), *P. tricornutum* (Phatr2) and *P. multiseriis* (Psemu1), ID numbers refer to the genome annotation on JGI database (<http://genome.jgi.doe.gov/>). For *T. oceanica* (ThaOc\_1.0), ID refers to the Ensembl Protist database ([http://protists.ensembl.org/Thalassiosira\\_oceanica/Info/Index?db=core](http://protists.ensembl.org/Thalassiosira_oceanica/Info/Index?db=core)). (+) and (-) indicate the forward and reverse chromosomal or scaffolds, respectively.

**qPCR/PCR Oligos**

Gene Id (Phatr2)	Oligo Name	Sequence (5' – 3')	Lenght
27278	Lhcx1Fw	CCT TGC TCT TATCGGCTCTG	20
	Lhcx1_Rv	ACGGTATCGCTTCAAAGTGG	20
56312	Lhcx2Fw	CAGCAC TAA TGCCGCTTTCG	20
	Lhcx2_Rv	CGT GAG TAACT TCCGCTTCC	20
44733	Lhcx3Fw	TCC CGT TGG TA TCT TTGATCC	21
	Lhcx3_Rv	GAA GAT CCT TC CACGGCTTC	20
38720	Lhcx4Fw	TCT TTGATC CACTCCGCTTC	20
	Lhcx4_Rv	GGC GTT CCA TAGAAAGTTCG	20
10847	Rps F	CGA AGT CAA CCAGGAAACCAA	21
	Rps R	GTG CAA GAG ACCGACATAACC	21
34971	H4 Fw	AGG TCC TTC GC GACAATATC	20
	H4 Rv	ACG GAA TCA CGAATGACGTT	20

**Cloning Oligos**

Construct	Oligo Name	Sequence (5' – 3')	Lenght	Restriction Site
27278	L1OE_Fw	acg tGC GGC CGCAT GAAGT TCGCTGCCACCATC	33	NotI
	L1OE_Rv	acg tGAATT Ct taACCC TGAAGATTCTCAAGGA	33	EcoRI
56312	L2OE_Fw	acg tGC GGC CGCAT GAAAT TAT CCT TGGCTATCC	34	NotI
	L2OE_Rv	acg tGAATT Ct taGAGCCCAAGGTTTTCGAGGAT	34	EcoRI
44733	L3OE_Fw	acg tGC GGC CGCAT GAAGT GCA TCGCCGCTATC	33	NotI
	L3OE_Rv	acg tGAATT Ct taGAGGAGGTGTTCCAAGATTCC	34	EcoRI
38720	L4OE_Fw	acg tGC GGC CGCAT GAAAT TGT TCACCATC TTC	33	NotI
	L4OE_Rv	acg tGAATT Ct taGCCAAA CAAATTCTC AAAAT	34	EcoRI

**Table S2:** List of the oligonucleotides used in this work.



## Chapter 5

# The Xanthophyll Cycles in *Phaeodactylum tricornutum*

### Summary of the work

The study of photoprotection in diatoms couldn't be considered complete without a careful look at the xanthophyll cycles. The classic violaxanthin cycle, with the conversion of violaxanthin (Vx) to anthera- and zeaxanthin (Zx), was first discovered by Yamamoto et al. in 1962 in plants and green algae. Soon after, a similar cycle, converting diadinoxanthin (Ddx) into diatoxanthin (Dtx) in high light conditions, was found in brown algae and in diatoms (Stransky and Hager (1970)). The involvement in photoprotection of these cycles in the different organisms had been established, either with genetic approaches or using inhibitors of the violaxanthin de-epoxidase enzyme, even though the debate is still open on the exact mechanisms that allow Zx and Dtx to enhance quenching.

More recently, Lohr and Wilhelm (1999) discovered that diatoms, which use the Ddx cycle, also possess the Vx cycle (typical of plants). This raised the question if the two cycles had both an impact on photoprotection or if it was just a fortuity of evolution (*i.e.* the leftover of a common biosynthetic pathway). Moreover, once the complete genome sequence of the diatom *Phaeodactylum tricornutum* became available, multiple genes with high similarity to the ones involved in the xanthophyll cycle in plants were identified (Bowler et al. (2008); Coesel et al. (2008)), pointing to a possible specialization of the various putative enzymes either in the Vx or in the Ddx cycle.

Recently, the involvement of the violaxanthin de-epoxidase (VDE) protein of *P. tricornutum* in the de-epoxidation of Ddx has been already established (Lavaud et al. (2012)). To understand the specific function of the other six genes putatively involved in the xanthophyll cycles in *P. tricornutum*, I generated knock-down lines for each of them (the zeaxanthin epoxidases *ZEP1*, *2* and *3* and the VDE-like *VDL1* and *VDL2*, and the VDE-related *VDR*). I then performed a screening of the putative knock-down lines based on NPQ activity, either in low light or high light treated cells, using information on the gene expression profiles (Nymark et al. (2009)) as a blue print to carry on the screening.

In this way, during my second year of Ph.D., when I moved to the UPMC in Paris, I identified different knock-down lines for *VDL2* and *VDR* proteins, showing a lower photoprotection capacity compared to wild-type strain when exposed to prolonged high light stress. During the rest of my doctoral training, I decided to focus my efforts on the characterization of the *VDL2* and

VDR proteins, whose function was completely unknown. Analyses of pigment contents revealed that both these mutants accumulated more pigments of the Vx cycle (mainly Zx) and less of the Ddx cycle, maintaining or even increasing the level of the de-epoxidation reactions. This indicated that VDL2 and VDR proteins were not involved in the de-epoxidation reactions but rather in the synthesis of xanthophylls and/or in the regulation of the two xanthophyll cycle pools. Interestingly, the higher accumulation of Vx cycle pigments correlated with a lower NPQ capacity. This suggests that, in *P. tricornutum*, Zx is not involved in NPQ and that the accumulation of this pigment may even interfere with the photoprotective action of Dtx.

To obtain complementary information with respect to the knock-down mutants, I also produced transgenic lines over-expressing all the genes putatively involved in the xanthophyll cycles. To this aim, I cloned the full length coding sequences of the xanthophyll cycle genes under the control of a strong *P. tricornutum* promoter (Siaut et al. (2007)). Unfortunately, we had some long lasting problems with our biolistic transformation apparatus, so it took long time before obtaining any transformants. During this last year I finally got some colonies with higher content of VDR protein (and only one clone over-expressing VDL2). The analysis of these lines so far indicate that gene over-expression has no significant effect on NPQ or pigment content. This may indicate that VDR needs a partner to be active (for example to form a heterodimer), which would be in agreement with the lack of *in vitro* activity for VDR (Martin Lohr, personal communication). Even though not conclusive, these results are present as supplementary data in the next chapter (supplementary figure 5.15), which is organized as a paper in preparation.

Since no antibody was available for VDR and VDL2, I also expressed the proteins in an heterologous system (*Escherichia coli*), in order to purify the proteins and produce antibodies. In particular, *VDR* and *VDL2* coding sequences were cloned in different vectors (pDEST17 and pET28a) for bacterial expression and used to transform three *E. coli* strains (Rosetta 6, Rosetta 7 and Origami). The expression of the proteins were tested in various conditions, and I finally got the best results for both proteins with the pDEST17 expression vector, inducing Rosetta 6 *E. coli* strain with 500 $\mu$ M IPTG at 18°C over-night. Both the proteins accumulated in non soluble inclusion bodies, which I purified as in Bonente et al. (2011) and used to immunize different rabbits (using external services from Agrisera for VDR and in house at the University of Padua, thanks to a collaboration with Prof. Tomas Morosinotto). The obtained antibodies for VDR were tested and used in this work, while the production of the antibody for VDL2 has just finished and I am currently testing it.

When I started my Ph.D. in Paris, at the private company Collectis S.A., the study of the xanthophyll cycle(s) was already in our minds. In fact, of the various TALENs™ I made, six of them had putative xanthophyll cycle genes as targets, namely *VDE*, *VDL2* and *ZEP1* (two TALENs™ for each gene, targeting different loci). These targets were chosen i) either because we already knew their function (de-epoxidation of Ddx in the case of *VDE*, Lavaud et al. (2012)) and we wanted to obtain an “*npq1*” knock-out mutant for *P. tricornutum* (*i.e.* lacking de-epoxide xanthophylls); ii) or because their transcription profiles, measured in high light (Nymark et al. (2009)), suggested a possible role in photoprotection (for *VDL2* and *ZEP1*). Due to the decision of Collectis to stop the research on algae in 2014, this project was put in standby for quite some time and a material transfer agreement between Collectis and the UPMC was signed only in 2015. In the meantime we initiated the work with the reverse genetic approaches described above. Transgenic lines with the TALENs™ I produced were only recently obtained, and we are still in the screening process of putative knock-outs. Therefore, in this chapter, I focused on the description of the data obtained with the RNAi approach. However, as discussed in the conclusion chapter, I am confident that the new knock-out mutants generated with the TALENs™ will represent powerful resources in the understanding of the functions of these proteins and, more in general, of diatoms photoprotective mechanisms.

# Characterization of VDR and VDL2 proteins in *Phaeodactylum tricornutum* provides new insights on the function of the two xanthophyll cycles in marine diatoms

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I was responsible of all the experiments reported.

**Abstract** Diatoms are marine microalgae that inhabit a variety of different niches and are dominant in turbulent environments with dynamic light regimes. They show very efficient photoprotective mechanisms, as higher Non-Photochemical Quenching (NPQ) when compared to land plants.

The xanthophyll cycle, with the conversion of violaxanthin (Vx) to zeaxanthin (Zx) via the antheraxanthin intermediate, in high light conditions, is one of the main enhancer of NPQ in plants. Diatoms also possess a second xanthophyll cycle, converting diadinoxanthin (Ddx) into diatoxanthin (Dtx) in high light conditions. Dtx concentration is strictly correlated to NPQ level, but the actual role of the Vx-Zx cycle has not been established yet. Consistent with the additional xanthophyll cycle, more copies of the genes encoding violaxanthin de-epoxidase (VDE) and zeaxanthin epoxidase (ZEP) enzymes have been found in the diatom genomes, compared with other photosynthetic eukaryotes.

To understand the regulation and function of these two xanthophyll cycles, we knocked-down two of the four genes putatively involved in the conversion of Vx to Zx and/or of Ddx to Dtx in the diatom *Phaeodactylum tricornutum*, VDE-like 2 (VDL2) and VDE-related (VDR). These genes are up regulated in response to prolonged high light stress, and we found that the VDR and VDL2 knock-down lines have a lower NPQ, accumulate more xanthophylls of the Vx-Zx pool and less of the Ddx-Dtx pool respect to wild-type, but are not affected in the de-epoxidation

reactions. This indicates that VDL2 and VDR proteins are involved in the biosynthetic pathway of xanthophylls and that the higher amount of Vx and Zx had a negative effect in the development of NPQ. Thus, Zx does not participate in the enhancing of NPQ in diatoms, and its accumulation may even interfere with Dtx photoprotective action.

Abbreviations: Ax, antheraxanthin. DD, diadinoxanthin-diatoxanthin pool. Ddx, diadinoxanthin. DES, de-epoxidation state. Dtx, diatoxanthin. Fx, fucoxanthin. Nx, neoxanthin. VAZ, violaxanthin-antheraxanthin-zeaxanthin pool. VDE, violaxanthin de-epoxidase. VDL, violaxanthin de-epoxidase like. VDR, violaxanthin de-epoxidase related. Vx, violaxanthin. XC, xanthophyll cycle. ZEP, zeaxanthin epoxidase. Zx, zeaxanthin.

## 5.1 Introduction

Photosynthesis is a biochemical process that produces oxygen and organic compounds from atmospheric CO<sub>2</sub> and water. It uses solar light energy to drive its reactions, and most life on Earth depends on this process. In stressful light conditions, when absorbed photons exceed the capacity for light utilization, photosynthetic organisms must dissipate the excess of energy. This is possible by using different protective mechanisms, including Non-Photochemical Quenching (NPQ), a short-term response that dissipates this excess excitation energy as heat (Niyogi (1999); Eberhard et al. (2008)).

Organisms with different evolutionary histories have developed different strategies to adapt their photosynthetic apparatus to changing light conditions and to optimize photosynthetic yield (Goss and Lepetit (2014); Demmig-Adams et al. (2014)). Diatoms are very successful marine microalgae that account for 40% of the marine photosynthesis and inhabit a variety of ecological niches, from the poles to tropical seas. They represent a major contributor to global carbon fixation and to the biogeochemical cycles of nitrogen, phosphorus, iron and silica (Falkowski (1998); Smetacek (1999)). Diatoms are also dominant in turbulent water, where the amount and quality of light is very unpredictable, and are capable to reach higher levels of NPQ compared to most land plants (Lavaud and Goss (2014); Goss and Lepetit (2014); Ruban et al. (2004)).

Diatoms chloroplast derives from a series of secondary endosymbiotic events between an ancient heterotroph and red and green algae

(Howe et al. (2008); Frommolt et al. (2008); Moustafa et al. (2009)). Because of their evolutionary history, diatoms show distinct metabolic and cellular features compared to plants (Bowler et al. (2008)), and use different pigments for light harvesting and photoprotection (Kuczynska et al. (2015)). Diatoms do not synthesize lutein, since they lack the enzyme lycopene  $\epsilon$ -cyclases that convert lycopene to  $\alpha$ -carotene (Dambek et al. (2012); Grossman et al. (2004); Bertrand (2010), figure 5.1), but accumulate other xanthophylls that are not present in the green lineage. Diatoms show high levels of fucoxanthin, which give them their characteristic brown color, but also of diadinoxanthin and diatoxanthin (Kuczynska et al. (2015)).

This set of pigments can help diatoms to deal with stressful light conditions. When exposed to an excess amount of light (high light, HL), green algae and higher plants convert violaxanthin (Vx) to antheraxanthin (Ax) and zeaxanthin (Zx) via two de-epoxidation reactions; the opposite occurs when the organisms are shifted from high light to low light (LL) (Demmig et al. (1987)). The whole process is called the xanthophyll cycle (XC) (figure 5.1). Diatoms, along with the conventional Vx-Zx cycle, also use diadinoxanthin (Ddx, accumulated in LL conditions) and diatoxanthin (Dtx, produced in HL), converting them via epoxidase and de-epoxidase reactions (Lohr and Wilhelm (1999)). The Ddx-Dtx cycle (which is absent in plants and green algae) is much more active in diatoms than the Vx-Zx cycle, and its role in photoprotection is of primary impor-

tance (Jakob et al. (2001); Lohr and Wilhelm (1999); Wilhelm et al. (2014)). NPQ in diatoms is, in fact, positively correlated with Dtx accumulation, even when the  $\Delta\text{pH}$  is abolished (Goss et al. (2006)).

In plants, Zx has various functions: it is important to increase NPQ response, in particular the fast  $\Delta\text{-pH}$  dependent component qE (Niyogi et al. (1998)) and the long-lasting component qZ (Nilkens et al. (2010)), but can also reduce chlorophyll triplet yield in LHC proteins (Dall'Osto et al. (2012)) and scavenge dangerous reactive oxygen species (Havaux and Niyogi (1999); Havaux et al. (2007); Dall'Osto et al. (2010)). The role of Zx in diatoms, however, is less clear, since Dtx seems to be the main xanthophyll involved in photoprotection (Goss and Lepetit (2014)).

In the genome of the diatom model species *Phaeodactylum tricornutum*, seven genes putatively involved in the two XCs have been found: three for the epoxidase reactions (the zeaxanthin epoxidases *ZEP1*, *2* and *3*) to convert Zx to Vx or Dtx to Ddx, and four for the opposite reactions (violaxanthin de-epoxidase *VDE*, *VDE*-like *VDL1* and *VDL2*, and the *VDE*-related *VDR*) (Coesel et al. (2008); Dambek et al. (2012); Eilers et al. (2016)). The expansion of the XC gene family is in contrast with what found in plants, where only one *VDE*, one *VDR* and one *ZEP* genes are present (Coesel et al. (2008); Frommolt et al. (2008)).

*VDE*, *VDLs* and *VDR* are all part of the lipocalin family, since they all possess a lipocalin domain (figure 5.2), which is important for the binding to the substrate (*i.e.* the xanthophylls). They also have a cysteine-rich N-terminal domain, essential for the folding of the protein (Hieber et al. (2001)), but only *VDE* possesses a glutamic acid-rich C-terminal domain, which is supposed to be important for the attachment of the protein to thylakoid membranes when the luminal pH drops (Hieber et al. (2001); Morosinotto et al. (2002)).

*VDR* probably derive from a gene duplication of *VDE* and is present in all eukaryotic phototrophs (with the exception of red algae, Coesel et al. (2008)), which suggests that this protein is not specifically related to or-

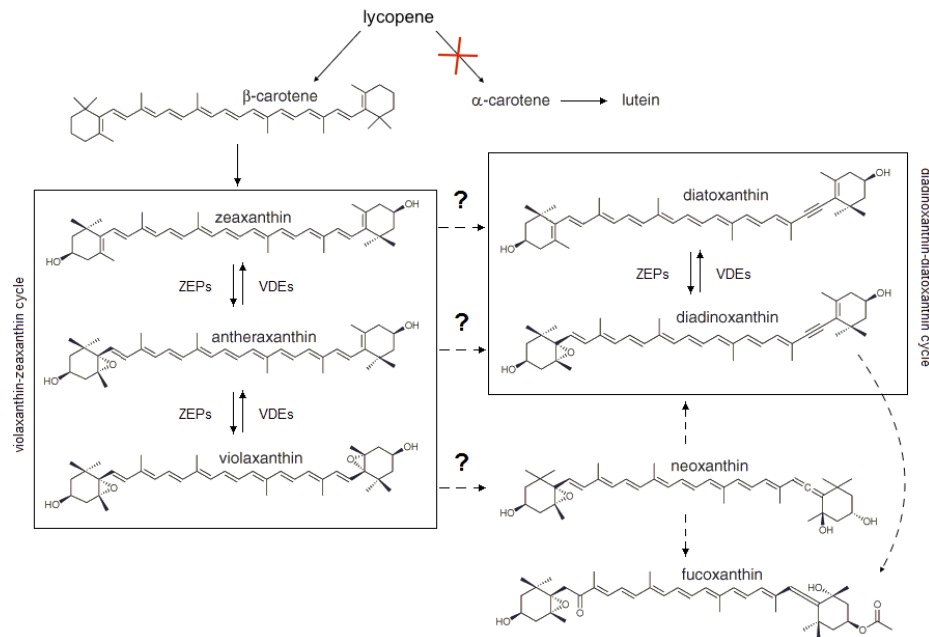
ganisms that use the Ddx-Dtx XC. The green alga *Chlamydomonas reinhardtii* lacks a classical *VDE* in its genome, and *VDR* may have taken its role in the XC. *VDLs* are instead present only in chromalveolates, like diatoms and coccolithophores (Coesel et al. (2008)), which possess the Ddx-Dtx XC. *VDLs* probably originated from gene duplication after the secondary endosymbiotic event or were lost during evolution by plants and green algae (Coesel et al. (2008)).

In plants, *VDE* is a soluble protein in the thylakoid lumen, that is active and interacts with the membranes (where its substrate is located) in acidic pH conditions (Morosinotto et al. (2002)). Of the four *VDEs* present in diatoms, only the *VDE* protein has been characterized *in vitro*. Differently from the plant *VDE*, the diatom enzyme is active not only in acidic environments but already at neutral pH (Jakob et al. (2001)), and it has a higher  $K_M$  for the co-factor ascorbate (Grouneva et al. (2006)). With the characterization of knock-down mutants, *VDE* in *P. tricornutum* has also been demonstrated to be involved in the conversion of Ddx to Dtx *in vivo* (Lavaud et al. (2012)).

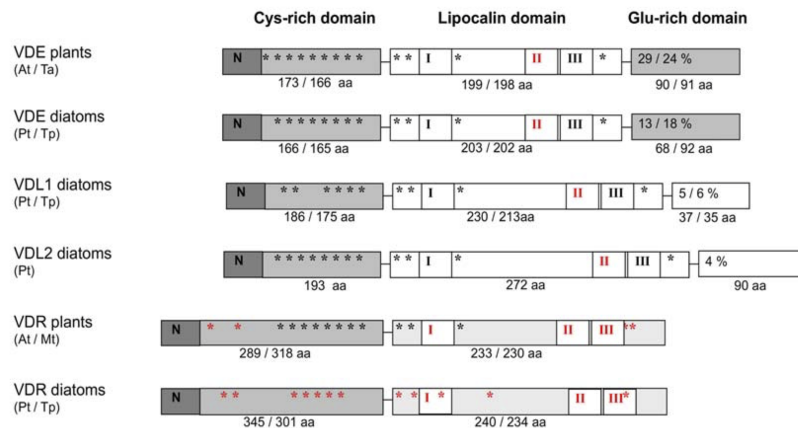
Despite the ecological relevance of diatoms and the fact they accumulate large amounts of xanthophylls, their carotenoids biosynthetic pathway and their xanthophyll cycles are still not fully characterized (Dambek et al. (2012); Mikami and Hosokawa (2013)). Moreover, the reactions leading to the formation of diadinoxanthin and fucoxanthin, the main xanthophylls involved in the XC and light harvesting respectively, are only hypothetical, since no putative catalyzing enzyme could be found in *P. tricornutum* genome (Dambek et al. (2012); Coesel et al. (2008)). The involvement of *VDL2* and *VDR* in the xanthophyll cycles also lacks experimental proofs, even though both genes are strongly induced by prolonged high light (Nymark et al. (2009)), suggesting a possible involvement in photoprotection.

Here, we show that knock-down mutants for *VDR* and *VDL2* are not impaired in de-epoxidase reactions but rather in the conversion of pigments from the Vz-Zx pool to the





**Figure 5.1 – Putative carotenoids biosynthetic pathway in diatoms.** The schema represents the two diatom xanthophyll cycles and the putative biosynthetic pathway. Due to the lack of the enzyme lycopene  $\epsilon$ -cyclases, that convert lycopene to  $\alpha$ -carotene, diatoms do not possess  $\alpha$ -carotene and lutein. Putative reactions are represented by dashed arrows. Abbreviations correspond to: ZEPs, zeaxanthin epoxidase 1, 2 and 3; VDEs, violaxanthin de-epoxidase, -like and -related proteins.



**Figure 5.2 – Domain structure of violaxanthin de-epoxidases and related proteins.** Schematic representation of domains in VDE, VDL and VDR proteins. The N-terminal contains the chloroplast target sequence and a cysteine-rich domain, essential for the folding of the proteins. Conserved and divergent cysteine residues are indicated with black and red asterisks, respectively. The central lipocalin domain, which is important for the binding to the substrate, is indicated with a white rectangle; conserved or divergent lipocalin motifs are represented as black or red roman numbers, respectively. In the C-term domain, the percentage of glutamic acid residues is indicated. Abbreviations indicate: At, *Arabidopsis thaliana*. Ta, *Triticum aestivum*. Pt, *Phaeodactylum tricornutum*, Tp, *Thalassiosira pseudonana*. Mt, *Medicago truncatula*. Image from Coesel et al. (2008).

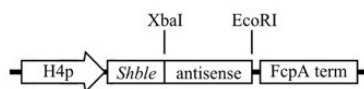
Ddx-Dtx pool, suggesting that these proteins are involved in the biosynthetic pathway of xanthophylls. The accumulation of Vx and Zx in the knock-down mutants leads to a decreased

NPQ capacity, indicating that Zx, contrary to what happens in plants, does not enhance qE in diatoms, which is solely regulated by Dtx content.

## 5.2 Materials and Methods

**Diatom growth conditions:** The *P. tri-cornutum* (Pt1 8.6, CCMP2561) cultures, obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, were used for the gene expression and photo-physiology analyses. Cells were grown in ventilated flasks in f/2 medium (Guillard (1975)) at 18 °C, in continuous light using white fluorescence neon lamps (Philips TL-D 90), at 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  (low light). High light treatments were performed by irradiating the cells with 500  $\mu\text{mol m}^{-2} \text{s}^{-1}$  for 24h, in continuous light regime, always using the same light sources.

**Generation of transgenic knock-down lines:** Antisense vectors for *VDL2* and *VDR* gene silencing were generated by amplifying their 3'UTR sequences, using the primers described in supplementary table 5.2. An EcoRI restriction site was added to forward primes and an XbaI site to the reverse ones and the PCR products were then cloned in the diatom silencing vector. The final constructs, contained the antisense sequence, cloned downstream to the *Shble* gene for phleomycin resistance and under the control of the same promoter and terminator (figure 5.3).



**Figure 5.3 – Antisense construct.** Antisense construct used for gene silencing. H4p: histone 4 promoter; *Shble*: gene for phleomycin resistance. XbaI and EcoRI: restriction sites used in the cloning process; FcpA term: FcpA terminator. Antisense: short antisense sequence for gene silencing.

**Heterologous protein expression in *Escherichia coli*:** *VDR* and *VDL2* coding sequences were cloned in pDEST17 vectors for bacterial heterologous expression and used to transform Rosetta 6 strain of *Escherichia coli*. The expression of the proteins was induced with 500  $\mu\text{M}$  of IPTG, at 18°C over-night. Both the proteins accumulate in the non-soluble inclusion bodies, which were then purified as in Bonente et al. (2011) and used to immunize different rabbits. The antibodies for VDR were produced by Agrisera, and checked for their specificity both with total diatom proteins extracts and against the recombinant proteins from *E. coli*. The VDL2 antibody has been produced at the University of Padua and the tests to verify its activity is currently on-going.

**RNA extraction and qRT-PCR analysis:** Total RNA was isolated from  $10^8$  cells with TriPure isolation reagent (Roche Applied Science, IN, USA) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) was performed on wild-type cells and on the knock-down clones as described in De Riso et al. (2009). The relative quantification of the different VDEs and ZEPs transcripts was obtained using RPS (ribosomal protein small subunit 30S; ID10847) as reference gene, and fold changes were calculated with the  $2^{-\Delta\Delta C_t}$  Livak method (Livak and Schmittgen (2001)). Primer sequences used in qRT-PCR analysis are reported supplementary table 5.3.

**Protein extraction and western blot analysis:** Western blot analyses were performed on total cell protein extracts prepared as in Bailleul et al. (2010), and resolved on 10% or 14% LDS-PAGE gels. Proteins were detected with different antibodies: anti-LHCSR (gift of G. Peers, University of California, Berke-

ley, CA, USA) (1:5000); anti-D2 (gift of J.-D. Rochaix, University of Geneva, Switzerland) (1:10 000); anti-PsaF (1:1000) and anti- $\beta$ CF1 (1:10 000) (gift of F.-A. Wollman, Institut de Biologie Physico-Chimique, Paris, France); anti-HA primary antibody (Roche) (1:2000). Antibodies anti-VDR were produced by Agrisera, anti-VDL2 were produced at the University of Padua (Italy), both using proteins expressed and purified from *E. coli*. Proteins were revealed with Clarity reagents (Bio-Rad) and an Image Quant LAS4000 camera (GE Healthcare, USA).

#### Electron Transport Rate and Non-Photochemical Quenching measurements:

Light-induced fluorescence kinetics were measured using a fluorescence CCD camera recorder (JTS-10, BeamBio, France) as described (Johnson et al. (2009)) on cells at  $1-2 \times 10^6$  cells  $\text{ml}^{-1}$ .  $F_v/F_m$  was calculated as  $(F_m - F_0)/F_m$ . NPQ was calculated as  $(F_m - F_m')/F_m'$  (Bilger and Bjorkman, 1990), where  $F_m$  and  $F_m'$  are the maximum fluorescence emission levels in the dim light and high light-acclimated cells, measured with a saturating pulse of light. All samples were adapted to dim light ( $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ ) for 15 min at 18 °C before measurements. The maximal NPQ response was measured upon exposure for 10 min to saturating green light of  $950 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Actinic lights of 320, 520 and  $950 \mu\text{mol m}^{-2} \text{s}^{-1}$  were used to obtain different levels of NPQ for the NPQ vs diatoxanthin plots. The relative electron transfer rate (rETRPSII) was measured with a JTS-10 spectrophotometer at different light intensities (20, 170, 260, 320, 520, and  $950 \mu\text{mol m}^{-2} \text{s}^{-1}$ ), by changing light every 4 min. rETRPSII was calculated as:  $Y2 \times \text{light intensity}$ , where Y2 is the efficiency of PSII.

#### Absorption and Fluorescence measurements:

Room temperature absorption spec-

tra were recorded using an SLM-Aminco DK2000 spectrophotometer, in F/2 medium. The wavelength sampling step was 0.4 nm. Fluorescence emission spectra were measured using a Jobin-Yvon Fluoromax-3 device, exciting cells at 475 nm. Antenna size was measured as in Ferrante et al. (2012). Fast light-induced chlorophyll fluorescence OJIP curves (Kautsky effect) were measured *in vivo* using a homemade fluorimeter (Antal and Rubin (2008); Stirbet and Govindjee (2011)). Cells in exponential phase of growth were collected, 10X concentrated and dark adapted for 30 minutes. A light intensity of  $1100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  was used and the measurement was stopped after 1 second of treatment.

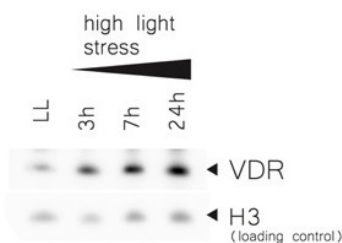
**Pigment Analysis:** Pigments from WT and mutant cells were extracted using 96% ethanol, buffered with  $\text{Na}_2\text{CO}_3$ . Cells were either taken from growing light, quickly filtered and frozen in liquid nitrogen, or collected during the NPQ measurement (for pigment vs NPQ correlation plots) and immediately frozen in liquid nitrogen. Pigment extraction was performed in ice, at the dark, for 30 minutes and centrifuged. The supernatant was loaded in a HPLC Thermo with a detector Diode array for analyze the visible region with a C18 spherisorb column (7.3 x 30mm) and ran using an aqueous mixture of acetonitrile/methanol/0.1 M Tris-HCl buffer (pH 8.0) (72:8:3, buffer A) and a methanol hexane mixture (4:1, buffer B). The runs were done at a flux of 1.5 mL, starting with 100% buffer A: 0-5 min 97% A, 5-17 min a gradient to 80% A, 17-18 min to 100% of buffer B, 18-23 min 100% B, 23-27 min to 85% buffer A, 27-29 min to 100% A. Pigments are distinguishable by the retention time and by their absorption spectrum. De-epoxidation state was calculated as  $(Zx + \frac{1}{2}Ax)/(Zx + Ax + Vx)$  or as  $(Dtx)/(Dtx + Ddx)$  (Ruban et al. (2004); Bonente et al. (2011)).

## 5.3 Results

### Regulation of VDR and VDL2 by light:

In *P. tricornutum*, four proteins putatively involved in de-epoxidation have been identified (Coesel et al. (2008)), but, with the exception of VDE (Lavaud et al. (2012)), their function is still unknown. Transcriptomic analysis of *P. tricornutum* cells exposed to high light shows that *VDR* and *VDL2* are both induced after prolonged (12-24h) light stress (Nymark et al. (2009)), suggesting their possible involvement in photoprotection. We thus decided to characterize these two proteins, in order to obtain novel information of their function and on the regulation of the xanthophyll cycles.

To confirm VDR and VDL2 induction also at the protein level, we produced antibodies to recognize these two proteins. We then analyze protein expression in cells adapted to low light (LL, 30  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) or following a LL to high light shift (HL, 500  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for different time periods. In accordance with the transcriptomic data, we found that VDR is expressed to low levels in LL and it is accumulated upon continuous HL exposure, steadily increasing over time (figure 5.4)<sup>1</sup>.



**Figure 5.4 – VDR induction upon high light exposure.** Immunoblot analysis of VDR accumulation. Protein content was measured in low light adapted cells (LL) and after exposure to high light for 3h, 7h and 24h. Immunoblot analysis of the H3 (histone 3) is shown as a control for loading.

**mRNA and protein quantification in knock-down lines:** To knock-down VDL2 and VDR, we transformed *P. tricornutum* cells with antisense constructs that target the 3'UTR region of the transcript of each gene (fig-

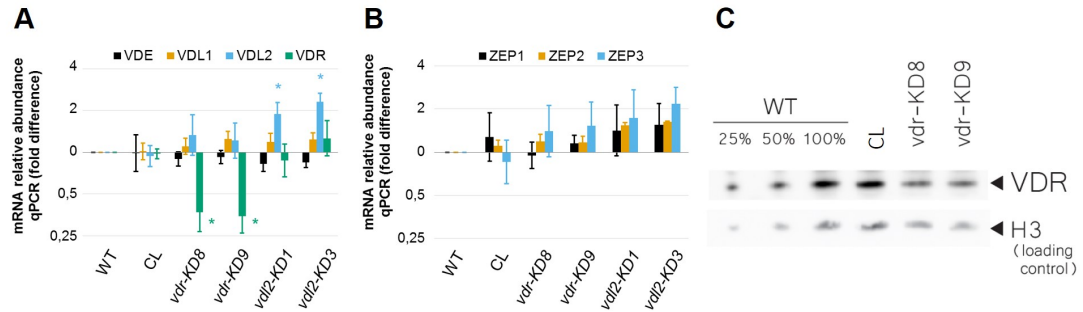
ure 5.3). The obtained mutants were screened for their NPQ capacities, and approximately half of the *vdr* and *vdl2* putative knock-down lines screened were affected in photoprotection (lower NPQ and PSII efficiency) compared to the WT after 24h of HL (supplementary 5.10). No statistically significant difference was detected in LL adapted cells or after short exposure to HL.

The two lines with the strongest NPQ phenotype were further characterized at molecular level, together with the wild-type strain and an aspecific transgenic line carrying only the antibiotic resistance gene (figure 5.6). Analysis of the mRNA levels by qRT-PCR showed that both *vdr* knock-down lines had a lower level of VDR mRNAs after 24h of HL stress, indicating successful gene silencing (figure 5.5 A). Reduction of VDR after HL exposure was also confirmed at the protein level for the two knock-down lines (figure 5.5 C). On the contrary, *vdl2* knock-down lines showed an up-regulation of *VDL2* (figure 5.5 A), and a slight increase of the ZEP mRNA (figure 5.5 B). An increase in the mRNA level of the target gene, when using gene silencing techniques, is not uncommon in diatom lines expressing the antisense constructs (De Riso et al. (2009); Lavaud et al. (2012)). However, since the test of anti-VDL2 antibody is still on going, it has not been possible for us to assess if the putative *vdl2* knock-down lines show a reduced content of VDL2 protein.

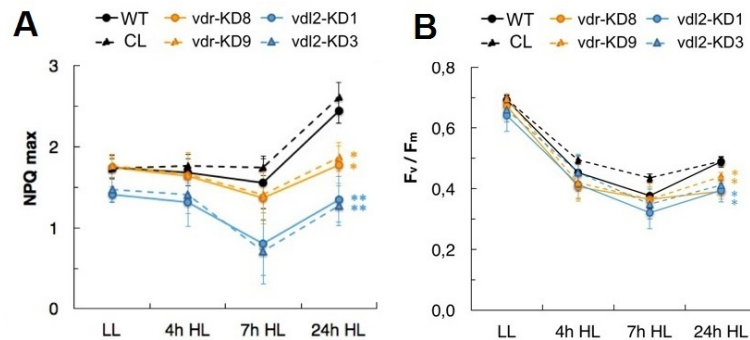
### Characterization of photosynthetic proteins, antenna size and growth:

To check if the misregulation of VDR and VDL2 have any adverse effect on photosystems (PS), we measured the accumulation of proteins of the PSI core (PsaF), PSII core (D2), antenna proteins LHCFs and the photoprotective antennae LHCXs, both for cells acclimated to LL or after 24h of HL (supplementary figure 5.11). None of the knock-down lines showed differences in

<sup>1</sup>Since I'm currently testing the antibody for VDL2, only the result regarding VDR are shown.



**Figure 5.5 – mRNA and protein levels of xanthophyll cycle genes in *P. tricornutum* knock-down lines.** mRNA relative abundance of genes putatively involved in the de-epoxidase (A) and epoxidase (B) reactions of the xanthophyll cycles in *P. tricornutum* in response to 24h of high light stress. (A) *VDE*, *VDL1*, *VDL2* and *VDR* transcript levels in control line (CL) and knock-down mutants for *vdr* and *vdl2*. (B) *ZEP1*, *ZEP2* and *ZEP3* transcript levels in control line (CL) and knock-down mutants for *vdr* and *vdl2*. Transcript abundance was measured by qRT-PCR using RPS as the reference gene and normalized to the wild type expression value (log<sub>2</sub> scale). (C) Immunoblotting analysis of VDR protein accumulation in *P. tricornutum* WT, control line and *vdr* knock-down lines in cells exposed to 24h of high light stress; immunoblot analysis of the H3 (histone 3) is shown as a control for loading. qRT-PCR measurement are the average of three biological replicas and error bars represent standard error. Asterisks indicate the results of two-tailed Student t-tests: \**p*<0.05. Immunoblots were reproduced three times.



**Figure 5.6 – NPQ and PSII efficiency in *P. tricornutum* wild-type and knock-down lines.** NPQ maximal level (A) and PSII efficiency ( $F_v/F_m$ ) (B) of wild-type (WT), control line (CL) and two independent knock-down transgenic lines for *VDR* (orange lines) and *VDL2* (blue lines). Each NPQ and  $F_v/F_m$  measurement is the average of at least three times, error bars represent standard error. Asterisks indicate the results of two-tailed Student t-tests: \**p*<0.05; \*\**p*<0.01.

photosynthetic proteins accumulation with respect to WT and control line, both in LL or HL conditions. In all genotypes, PsaF protein (of the PSI core) was unaffected by LL to HL shift, while D2 protein (PSII core) was reduced in HL with respect to LL, in agreement with PSII photoinhibition (Adir et al. (2003); Nyman et al. (2009)). Accumulation of LHCF antenna proteins was only slightly affected by HL treatment, with an increase of lower molecular weight isoforms, as already shown by Lepetit et al. (2012). HL exposure caused the accumulation of the photoprotective antennae LHCX2 and 3 (see chapter 4), while LHCX1 was already present in LL conditions (Bailleul et al. (2010)).

Antenna size of PSII can be estimated from chlorophyll fluorescence kinetics in the presence of the PSII inhibitor DCMU (Ferrante et al. (2012)): the antenna size of *vdr* and *vd12* knock-down mutants was not altered in LL or HL, with respect to WT and control line (supplementary figure 5.12). Also the plastoquinone pool was not influenced in the knock-down mutants (supplementary figure 5.12), as shown by the area above the JI-phase of the OJIP curve, which is proportional to the size of the plastoquinone pool (Toth et al. (2007); Grouneva et al. (2009)).

Energy transfer of photosystems reaction centers, measured by fluorescence spectra at 77K on entire cells exposed to 24h of HL, was also not affected, since no direct fluorescence emission FCP antennae was detected (supplementary figure 5.13).

No difference was found in growth rate, as well as in electron transport rates of photosystem II, nor in LL neither in HL conditions (supplementary figure 5.14).

**Pigment content in knock-down mutants:** Since VDR and VDL2 have been proposed to be enzymes involved in the XC (Coesel et al. (2008)), in order to understand their specific function we measured the pigment content in the knock-down lines, both in continuous LL and HL conditions.

HPLC analysis allowed the detection of chlorophyll *a* and *c*,  $\beta$ -carotene, fucoxanthin

(Fx), diadinoxanthin (Ddx) and diatoxanthin (Dtx), violaxanthin (Vx), antheraxanthin (Ax) and zeaxanthin (Zx), and the putative intermediate neoxanthin (Nx), as identified by Dambek et al. (2012). Chlorophyll *c1* and *c2* were detected as a single peak.

Pigment content in knock-down lines for *vdr* and *vd12* was identical to that of WT and control line both in LL and after 3h and 7h of HL treatment (data not shown), in agreement with NPQ analysis. Significant differences were instead detected after 24h of HL (figure 5.7): in particular, total content of Ddx and Dtx (DD pool) was decreased in knock-down mutants by around 19 and 30% in *vdr* and *vd12* respectively, while the content of Vx, Ax and Zx (VAZ pool) was increased by 3 to 4 times, so that the total amount of pigments involved in the two XCs was not statistically different (figure 5.7 A).

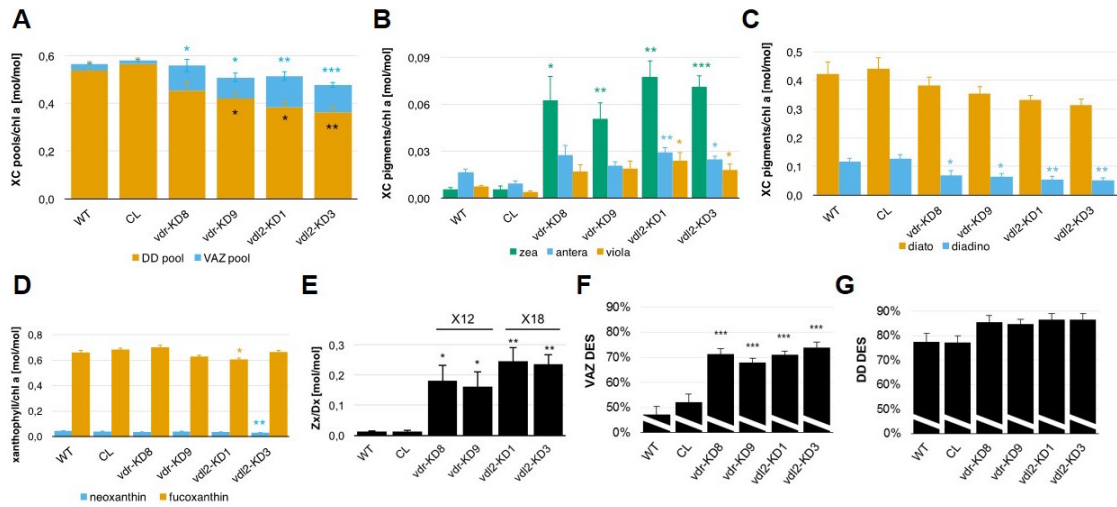
Regarding the pigments of the VAZ pool, Zx was accumulated to high extent (8 to 10 times more) in knock-down mutants compared to WT, while Ax and Vx had a more moderate increase (figure 5.1 B), thus resulting in a much higher de-epoxidation state in *vdr* and *vd12* (DES, figure 5.1 F).

In the DD pool, Ddx was more reduced than Dtx in all the knock-down mutants, also here resulting in a higher DES (figure 5.1 C and G).

The imbalance between the DD and VAZ pools and the higher DES suggest that both VDR and VDL2 proteins may not be involved in the de-epoxidation reactions of the XCs (*i.e.* the conversion of Vx in Zx and Ddx in Dtx), but rather in the biosynthetic pathway of xanthophylls, in particular in the hypothetical reactions that connect the two cycles (figure 5.1).

The increase of Zx and reduction of Dtx lead to an imbalance of the “photoprotective” xanthophylls in all the knock-down mutants: while, in WT and control line, for every molecule of Zx there were around 75 molecules of Dtx, in *vdr* this ratio was 12 times lower (1:6) and in *vd12* almost 18 times lower (1:4) (figure 5.1 E).

Xanthophylls that are putative intermediate between the two XCs or derived from Ddx were also identified, but nor Fx neither Nx showed a strong and coherent change in the



**Figure 5.7 – HPLC pigment analysis.** Pigment content of cells exposed to 24h of HL, measured via HPLC and normalized for chlorophyll *a* content. (A) Total level of diadino-diatoxanthin (DD) and viola-anthera-zeaxanthin (VAZ) pools. (B) Levels of the pigments involved in the viola-zeaxanthin cycle. (C) Levels of the pigments involved in the diadino-diatoxanthin cycle. (D) Levels of neoxanthin and fucoxanthin. (E) Ratio between zeaxanthin and diatoxanthin content in WT, control and knock-down mutant lines. (F) De-epoxidation states (DES) of the viola-anthera-zeaxanthin xanthophyll pool (VAZ) calculated as  $(Zx + (Ax/2)) / (Zx + Ax + Vx)$ . (G) De-epoxidation states (DES) of the diadino-diatoxanthin xanthophyll pool (DD) calculated as  $Dtx / (Dtx + Ddx)$ . Vertical axes in (F) and (G) are cutted between 0 and 50%. Asterisks indicate the results of two-tailed Student t-tests: \* $p < 0.05$ . \*\* $p < 0.01$ . \*\*\* $p < 0.001$ . Data are the average of six biological replicas, error bars represent standard error.

knock-down mutants (figure 5.1 D).

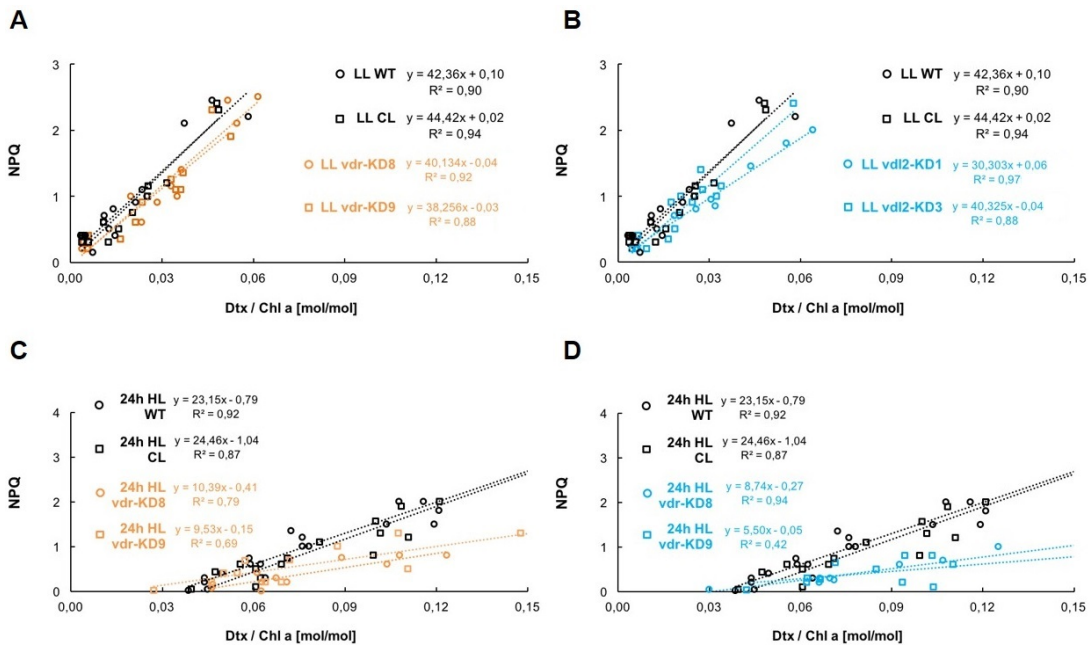
**Correlation between pigment content and NPQ:** Non-Photochemical Quenching (NPQ) in diatoms is positively correlated with Dtx accumulation, even if the delta pH is abolished (Goss et al. (2006)), indicating the fundamental importance of this xanthophyll in photoprotection. On the contrary, the role of Zx in organisms containing the Ddx-Dtx cycle is not clear (Lohr and Wilhelm (1999)). To address this question, we correlated the pigment content of the knock-down mutants, characterized by an high Vx and Zx content, with NPQ, measured with different actinic light intensities.

LL adapted cells of WT, control line, *vdr* and *vdl2* knock-down mutants all shown a linear correlation between Dtx content and maximal NPQ (figure 5.8 A and B), with no difference between genotypes. In our growth conditions, the slope of the correlation curve is similar to what was previously measured (Goss

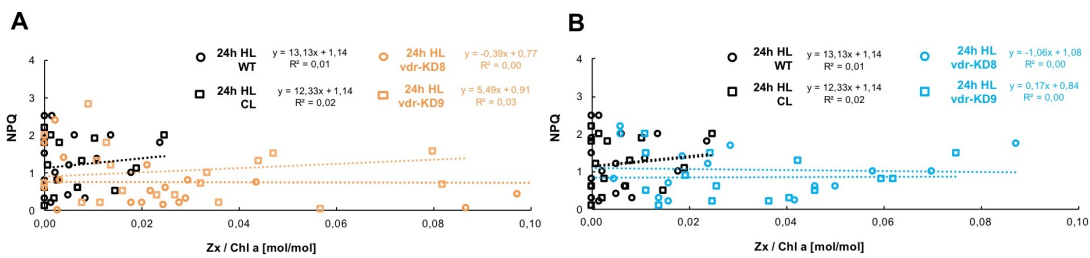
et al. (2006); Lavaud and Lepetit (2013); Schumann et al. (2007)). After 24h of HL stress, WT and control line both decrease the slope of the correlation between Dtx and NPQ, indicating that in HL more pigments were needed to develop the same NPQ capacity, in agreement with Schumann et al. (2007). We found that early Dtx accumulation, up to approximately 0.03 mol of Dtx/mol of Chl *a*, did not lead to any NPQ activity (figure 5.8 C and D), suggesting that this pool of Dtx is not involved in quenching.

Both the knock-down lines for *vdr* and *vdl2* showed a much stronger decrease of the quenching efficacy per Dtx molecule with respect to WT (figure 5.8 C and D), suggesting that the imbalanced pigment content present in these lines had an impact on the ability of Dtx to induce NPQ.

Zx was only detectable after the HL treatment and, contrary to Dtx, it did not correlate to NPQ (figure 5.9).



**Figure 5.8 – NPQ diatoxanthin correlation.** Correlation between diatoxanthin (Dtx) content and NPQ levels in low light adapted cells (A and B) and cells exposed to 24h of high light (C and D). WT (circles) and control line (squares) are indicated in black, *vdr* knock-down mutants in orange, *vdI2* knock-down mutants in light blue. Coefficients of regression lines and R<sup>2</sup> parameter are indicated for each plot. Diatoxanthin level is normalized over chlorophyll *a*. Data represent four biological replicas.



**Figure 5.9 – NPQ zeaxanthin correlation.** Correlation between zeaxanthin (Zx) content and NPQ level for cells exposed to 24h of high light. WT (circles) and control line (squares) are indicated in black, *vdr* knock-down mutants in orange (A), *vdI2* knock-down mutants in light blue (B). Coefficients of regression lines and R<sup>2</sup> parameter are indicated for each plot. Zeaxanthin level is normalized over chlorophyll *a*. Data represent four biological replicas.



## 5.4 Discussion

Diatoms show great photoprotective capacities, and in particular higher NPQ levels with respect to most land plants (Ruban et al. (2004); Brunet and Lavaud (2010)). These photoprotective mechanisms likely contribute to their ecological success in highly dynamic and turbulent environments (Margalef (1978); Field (1998); Kooistra et al. (2007) and chapter 4). Due to their particular evolutionary history, and notably their secondary endosymbiotic derived plastid, diatoms accumulate different pigments with respect to the green lineage, like fucoxanthin, diadinoxanthin (Ddx) and diatoxanthin (Dtx) (Kuczynska et al. (2015)), but neither the biosynthetic pathways generating these pigments nor their role in the photoprotection is completely understood.

Here we studied the role of VDR and VDL2, two of the enzymes putatively involved in the de-epoxidation reactions of Vx and Ddx in *P. tricornutum*. *VDR* gene is widely spread among all eukaryotic phototroph (with the exception of red algae), while *VDL2* seems to be specific to chromalveolates (Coesel et al. (2008)), which possess both the Vx and Ddx cycles. Remarkably, no information regarding the functions of VDR and VDL2 has been reported so far in any photosynthetic organism.

Both *VDR* and *VDL2* transcripts are up regulated in prolonged HL stress (Nymark et al. (2009)), and we observed a similar induction also at the protein level for VDR (figure 5.4). This indicates a possible involvement of these two proteins in long-term light acclimation responses (Eberhard et al. (2008)), and motivated us to further characterize their functions.

After the generation of independent transgenic lines containing antisense constructs specific for *VDL2* and *VDR* genes, we screened the putative knock-down lines for their photoprotective capacities. We found several transgenic lines for both genes that showed a reduced NPQ capacity after a 24h continuous high light (HL) stress, while their NPQ levels were equal to that of the WT in LL conditions (figure 5.6 A).

These knock-down mutants showed no ma-

ajor defect in photosynthetic proteins accumulation (supplementary 5.11), in antenna size and in photosystem assembly state (supplementary figure 5.13). No significant growth difference or change in electron transport rate was also found (supplementary figure 5.14).

Pigment content analysis did instead reveal a strong difference between *vdr* and *vdl2* knock-down and WT lines, with the formers accumulating high amounts of Vx-Ax-Zx pigments (VAZ pool) and lower level of Ddx and Dtx (DD pool). Reduction of VDR and VDL2 did not negatively impact de-epoxidation (figure 5.7 F and G), differently for what reported for *vde* knock-down lines (Lavaud et al. (2012)). These results suggest that VDR and VDL2 proteins might be involved in the reactions that connect the two XC (figure 5.1) rather than in the de-epoxidation of Vx and Ddx in excessive light conditions. In both the knock-down mutants, in fact, the reduced content of VDR and VDL2 decreased the conversion of the VAZ pigments into DD, and preliminary biochemical analysis indicates that VDR and VDL2 are indeed not able to catalyze Vx or Ddx de-epoxidation *in vitro* (Martin Lohr, personal communication).

Dambek et al. (2012) recently proposed neoxanthin as the intermediate molecule that would connect the two XCs, with fucoxanthin also deriving from either neoxanthin or Ddx (figure 5.1). In our *vdl2* and *vdr* knock-down lines, no significant difference in the accumulation of neoxanthin or fucoxanthin was detected, and three possible explanations can account for this: 1) VDR and VDL2 might be involved in reactions that directly convert Zx into Dtx or Ax into Ddx (figure 5.1), and neoxanthin and fucoxanthin could derive from Vx. In this case, VDR function in diatoms would be different than in plants and green algae (that do not possess Dtx and Ddx); 2) Other intermediate pigments, which were not detected in the HPLC analysis, could be involved in alternative reactions connecting the two cycles, which we currently ignore. 3) the involvement of neoxanthin as an intermediate between Vx and Ddx, proposed by Dambek et al. (2012), may be in-

correct.

Together with the increased VAZ pool, *vdr* and *vdl2* knock-down mutants had two other visible phenotypes: i) an increased de-epoxidation state (DES) of VAZ pool (*i.e.* an higher accumulation of Zx with respect to Vx-figure 5.7 F) and ii) the already mentioned lower NPQ max level with respect to WT.

The higher DES of the VAZ pool in the knock-down mutants indicates again that VDR and VDL2 are not involved in the de-epoxidation reactions. This higher DES can have different explanations: on one hand, it can be due to the action of the violaxanthin de-epoxidase (VDE) protein. Since this enzyme can use both Vx and Ddx as a substrate *in vitro* (Yamamoto and Higashi (1978)), the accumulation of Vx in the knock-down mutants may increase the portion of Vx dissolved in thylakoid membranes available for de-epoxidation. On the other hand, VDR and VDL2 may be involved in reactions that directly convert Zx into Dtx (figure 5.1), thus the decrease of these proteins content would lead to an accumulation of their substrate.

To find an explanation for the lower NPQ in mutants accumulating Zx, we correlated pigment content to NPQ level at different light intensities. Dtx content has been already shown to linearly correlate with NPQ (Goss et al. (2006)), with the slope of the regression line (representing the efficacy of quenching per Dtx molecule) lowering in HL conditions (Schumann et al. (2007)). Data in figure 5.8 are in agreement with previous findings for *P. tricornutum*, with the exception that in HL treated cells, early Dtx accumulation (up to approximately 0.03 mol of Dtx/mol of Chl *a*) did not lead to any NPQ activity. This may either be due: i) to incomplete epoxidation of Dtx during dim light incubation, before NPQ measurement; ii) or to the different light treatments used with respect to previous studies (7 $\mu$ E in LL and 100  $\mu$ E in HL for Schumann et al. (2007), 30 $\mu$ E in LL and 500  $\mu$ E in HL in this work), which created a distinctive pool of Dtx not bound to FCPs, and thus not involved in thermal dissipation (Schumann et al. (2007)).

The higher Zx content in *vdr* and *vdl2* was

instead not active in NPQ, as demonstrate by the lack of any correlation between Zx accumulation and NPQ (figure 5.9). This finding is in strong contrast to the role of Zx in plants, where higher de-epoxidation and increased VAZ pool augment NPQ capacities (Goss et al. (2006); Goss and Lepetit (2014); Dall'Osto et al. (2005)).

A possible reason for the lower quenching efficiency of diatoxanthin in *vdr* and *vdl2* knock-downs in HL (figure 5.8), could be that the abnormal accumulation of VAZ pigments leads to the binding of Vx and Zx to carotenoid-binding sites in the FCP proteins that should be occupied by Ddx and Dtx, thus reducing the quenching capacity. A similar substitution of carotenoids in LHC proteins was found in the case of Zx-deficient mutants, where lutein occupies the site where usually Zx is present (Li et al. (2009)), and in lutein-deficient mutants, where Vx takes its place (Pogson et al. (1998); Pogson and Rissler (2000); Dall'Osto et al. (2006)). This indicates some flexibility in the selectivity of carotenoid-binding sites.

According to the most recent data, FCP monomers in diatoms bind 18 pigments in total, notably eight Chl *a*, eight Fx and two Chl *c* (Premvardhan et al. (2010)). In HL conditions, one Dtx molecule is also probably bound per FCP proteins, where it can act as direct quencher or allosteric modulators (Lavaud and Lepetit (2013)), even though its not known if it occupies a V1-like or L2-like carotenoid-binding site, has what happens in plants (Morosinotto et al. (2002, 2003)). The presence of Zx in the hypothetical V1 or L2 sites would not lead to a switch in the conformation of the antenna protein, thus keeping the FCPs in the light harvesting state and not in the dissipative state (Liguori et al. (2015); Schaller-Laudel et al. (2015)).

In addition to that, FCP proteins are usually surrounded by a lipid shield of monogalactosyldiacylglycerol (MGDG), where Ddx can be much better solubilized than Vx (Goss et al. (2005, 2009); Lepetit et al. (2010)). In *vdr* and *vdl2* knock-down mutants, the higher amount of Vx could be thus not completely solubilized in the MGDG shield, leading to the binding

of Vx to antenna proteins. On the contrary, Ddx could be completely dissolved in the lipid phase, where it would not be involved in NPQ (Schumann et al. (2007)).

It is in fact known that Ddx and Dtx (DD pool) can be found in four distinctive pools, not all of which are active in NPQ (Lavaud and Lepetit (2013); Lepetit et al. (2010)). DD can be 1) free in the thylakoid membrane; 2) in the lipid MGDG shield around PSII and FCP proteins; 3) bound to FCP proteins involved in light harvesting, notably to antennae of PSI; 4) bound to FCPs involved in quenching.

The presence of high concentration of VAZ pigments would thus change the balance of the DD pigments between the above mentioned four pools, reducing the amount of Dtx in the last one and changing the slope of the relationship NPQ vs Dtx.

Pigments dissolved in thylakoid membranes and in MGDG domains have two main functions, the first being a reservoir for de-epoxidation and/or fucoxanthin synthesis (in HL and LL conditions respectively) (Lohr and Wilhelm (1999)), the second preventing lipid peroxidation and scavenging of singlet oxygen. In plants, Zx is not only an enhancer of NPQ, since it also has an antioxidant role in detoxifying reactive oxygen species (ROS) (Havaux et al. (2007); Dall'Osto et al. (2010)), and a similar double function has also been proposed for Dtx in diatoms (Lepetit et al. (2010)).

Our data suggest that Zx is not involved in NPQ in *P. tricornutum*, and yet we cannot completely exclude a role of this pigment in photoprotective mechanisms, since Zx can still retain an anti-oxidative function. The comparative analysis of reactive oxygen species (ROS) and scavenging activity in wild-type and the transgenic lines with an increased Zx content generated in this work could help to address this important function.

Dtx molecules active in NPQ are probably bound to LHCX and LHCR proteins, as these nuclear-encoded antennae are the only LHC expressed in HL (Nymark et al. (2009)), and newly synthesized Dtx does not increase NPQ if nuclear gene expression is blocked (Lepetit et al. (2012); Nymark et al. (2009)). Activation

of LHC stress-related proteins (LHCSR) upon binding of de-epoxidated pigments have been demonstrated for LHCSR1 in mosses (Pinnola et al. (2013)). Knock-down of *vdr* and *vdl2* have no effect on LHCX and other antenna proteins (supplementary 5.11), and the lower NPQ is thus likely caused by competitive binding of Zx to the sites destined to Dtx, which would not allowed the switch of LHCs to the quenching state.

An interesting and unexpected result of our analysis is that the *vdr* and *vdl2* knock-down lines show very similar phenotypes. Both these proteins belong to the lipocalin family, and possess a conserved lipocalin domain likely implicated in the binding to xanthophylls (Arnoux et al. (2009)). We could hypothesized that the two proteins might be involved in sequential reactions (*e.g.* one catalyzing A→B and the other B→C) or parallel reactions with similar substrates or products (*e.g.* one catalyzing A→C and the other B→C). To this stage, we can not confidently chose any of these alternative explanations, and further *in vitro* and *in vivo* experiments are needed to finally asses VDR and VDL2 functions. However, the presence of *VDR* genes in all eukaryotic phototrophs (excluding red algae) likely support a function not directly related to the presence of a double xanthophyll cycle. On the contrary, the specific appearance of *VDLs* in secondary endosymbionts suggests that these proteins might be involved in photoprotective mechanisms or biosynthetic pathways typical of these species.

Finally, the results of this chapter show how the fine regulation of xanthophyll content, and in particular the balance between VAZ and DD pigment pools, is important for the photoprotection in highly light stress conditions in *P. tricornutum*. In particular Zx, which is an important enhancer of NPQ in plants, does not increase the quenching in knock-down mutants with increased pigment content. VAZ pool was instead negatively correlated with NPQ, indicating that Dtx is the essential pigment that allows antenna proteins to switch to the dissipative conformation in *P. tricornutum*. Further studies are needed to better understand the biosynthetic pathway of xanthophylls in di-

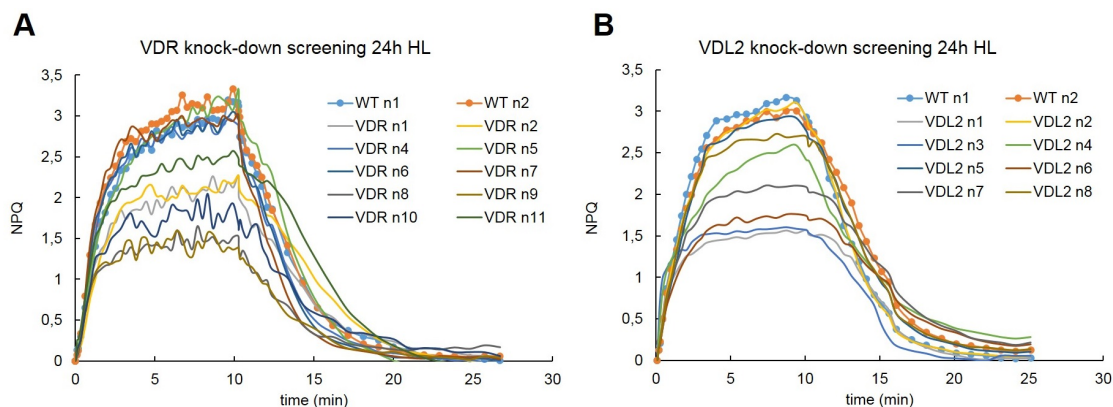
atoms, and in particular the reaction that connect the two xanthophyll cycles. We are currently working to isolate knock-out mutants for VDL2, generated with the TALEN™ technology (Daboussi et al. (2014)), which will hopefully help us unveiling its precise function.

**Acknowledgments** We thank Alessandro Alboresi and Tomas Morosinotto (University of Padova) for the help in the production of the anti-VDL2 antibody, Soizic Cheminant Navarro (UPMC) for the transformation with over-expressing vectors, Marianne Jaubert (UPMC) and Benjamin Bailleul (IBPC) for fruitful discussions.

## 5.5 Supplementary information

	Name	Restriction site added	Sequence
	VDE_DraI_ok_f	DraI	ACCTTTAAAAATGAAGTTTCTCGGTGTACCAGC
	VDL1_DraI_ok_f	DraI	ACCTTTAAAAATGCGATTTCGTTGGGTGGT
	VDL2_DraI_ok_f	DraI	ACCTTTAAAAATGAAGCGAGCCACGAGGAA
	VDR_DraI_ok_f	DraI	ACCTTTAAAAATGAAGCTCCACCGGAAAGG
	ZEP1_DraI_ok_f	DraI	ACCTTTAAAAATGAAGTTTCTACCACGGTGTCA
	ZEP2_SalI_f	SalI	TTCAGTCGACAATGGGTCTTTCGTTTCTATCATTAT
	ZEP3_DraI_ok_f	DraI	ACCTTTAAAAATGAAAAGATCTGCAATAGTCACAATCC
with STOP	VDEXhoIStR	XhoI	GTACTCGAGTTATTGCTGGGAGGTTTCTCG
	VDL1NotIStR	NotI	GTAGCGGCCGCTTAGCGTTTCGCCTTGTATTCC
	VDL2NotIStR	NotI	GTAGCGGCCGCTTAGTTCCTTGACATCTTCTGC
	VDRXhoIStR	XhoI	GTACTCGAGCTAAGGATGAGGACTACTAGCC
	ZEP1XhoIStR	XhoI	GTACTCGAGTTAAACTCTGGCGGTATAG
	ZEP2XhoIStR	XhoI	GTACTCGAGTTAGTCTTCTTTCGTAGCTG
	ZEP3XhoIStR	XhoI	GTACTCGAGTTACAAACCGGCTGCGCCACC
without STOP	VDE_XhoI_r	XhoI	GTACTCGAGTTTGTGGGAGGTTTCTCG
	VDL1_NotI_r	NotI	GTAGCGGCCGCAAGCGTTTCGCCTTGTATTCC
	VDL2_NotI_r	NotI	GTAGCGGCCGCAAGTTCCTTGACATCTTCTGC
	VDR_XhoI_r	XhoI	GTACTCGAGGAAGGATGAGGACTACTAGCC
	ZEP1_XhoI_r	XhoI	GTACTCGAGGAAAACCTCTGGCGGTATAG
	ZEP2_XhoI_r	XhoI	GTACTCGAGGAGTTCTTCTTTCGTAGCTG
	ZEP3_XhoI_r	XhoI	GTACTCGAGGACAAACCGGCTGCGCCACC

**Table 5.1** – List of primers used to amplify complete coding sequence of the genes putatively involved in the xanthophyll cycle in *P. tricornutum*. Two different versions of reverse primers were used, one just adding the restriction site for the cloning in pENTR3c vector (with STOP), the other that also mutated the STOP codon to remove it (without STOP) and add to the coding sequence a C-term HA-tag, present in the destination vector.



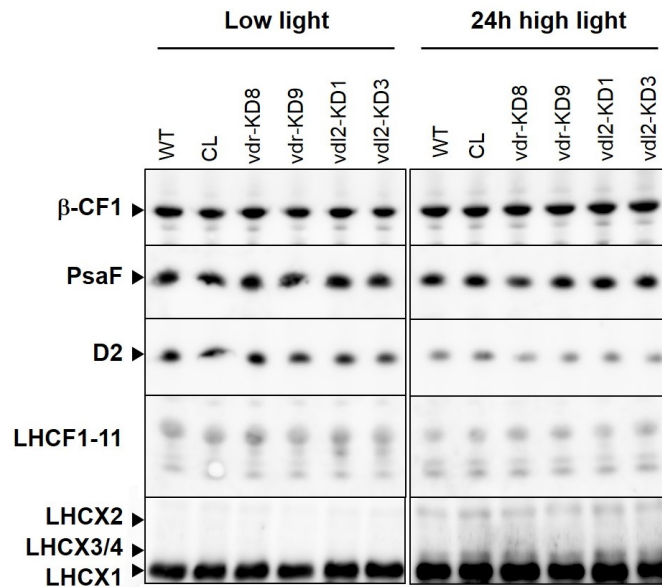
**Figure 5.10** – NPQ screening of *vdr* and *vdl2* knock-down transgenic lines. NPQ screening of *vdr* (A) and *vdl2* (B) knock-down transgenic lines exposed to 24h of high light stress. Measurements were done using an actinic light of  $950 \mu\text{mol m}^{-2} \text{s}^{-1}$ .

Name	Restriction site added	Sequence
VDE_AS_EcoRI_f	EcoRI	CCGGAATTCTTCGTCTACTATCGCGGCAA
VDE_AS_XbaI_r	XbaI	GCTCTAGACAGAGACTCAGGTAGCTGGG
VDL1_AS_EcoRI_f	EcoRI	CCGGAATTCAATGGAGCAATCTGACGTCG
VDL1_AS_XbaI_r	XbaI	GCTCTAGACACCTTTGATTCCACGC
VDL2_AS_EcoRI_f	EcoRI	CCGGAATTCGGTTACCAATCAGCTACGC
VDL2_AS_XbaI_r	XbaI	GCTCTAGACTTCGCCGCTGACAAGAATT
VDR_AS_EcoRI_f	EcoRI	CCGGAATTCITTCCTGAAAAGTCGCTTGC
VDR_AS_XbaI_r	XbaI	GCTCTAGAGTACCACAAATCTCGACCGC
ZEP1_AS_EcoRI_f	EcoRI	CCGGAATTCCTACGGTGGACCCATACAG
ZEP1_AS_XbaI_r	XbaI	GCTCTAGATGCTTGCCGAGTCTAGAAA
ZEP2_AS_EcoRI_f	EcoRI	CCGGAATTCGTCTTTGCGGGAGAAACAA
ZEP2_AS_XbaI_r	XbaI	GCTCTAGATACCACTGAATCGGCCAT
ZEP3_AS_EcoRI_f	EcoRI	CCGGAATTCAAAACGCCGGCAGAAAATC
ZEP3_AS_XbaI_r	XbaI	GCTCTAGACTTAACGGTTCCTTTGGCA

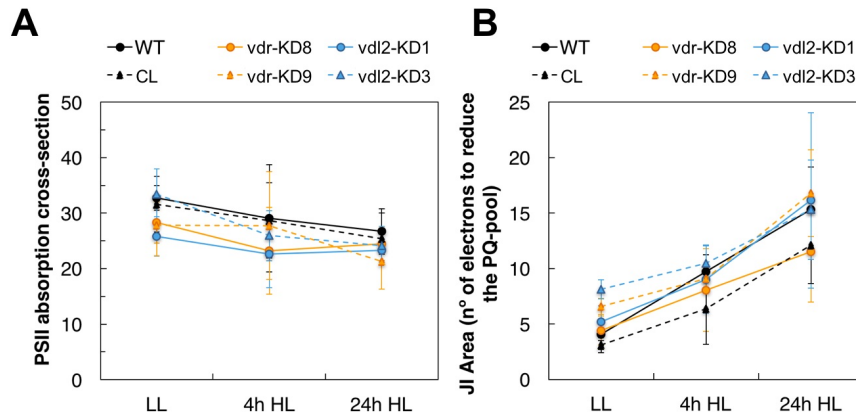
**Table 5.2** – List of primers used for amplification of antisense fragments of the genes putatively involved in the xanthophyll cycle in *P. tricornutum*. The restriction sites used for cloning are indicated.

Name	Sequence
VDE_qPCRf	ACAGCATTGGCACTAACGATT
VDE_qPCRr	TCGTCCCGTACAGGTGTTAATG
VDL1_qPCRf	TCTACTAGGAGGGACCCCGTTAC
VDL1_qPCRr	CTCGTTCTTGTTTCACCCATACC
VDL2_qPCRf	TGTTTGGATTAAAGTTCTGGGAGA
VDL2_qPCRr	GCGCCTTCGTAGGTGTTTTG
VDR_qPCR3f	GAAGCCGTTTCGTATCTTGC
VDR_qPCR3r	CGTCTTGCTCTTCGATGGGA
ZEP1_qPCRf	CCAGATTCTACTCGGCAAGGAC
ZEP1_qPCRr	CATTCTGATCTCCTGGCTCCTC
ZEP2_qPCRf	TCCGCCGATGTTCTAGTAGGAT
ZEP2_qPCRr	TTGCATAGTAGTCCGGGGTCTT
ZEP3_qPCRf	TCACCACATCCTCAGGGCTA
ZEP3_qPCRr	CCAATGACAAAAGCATCTTCGAT

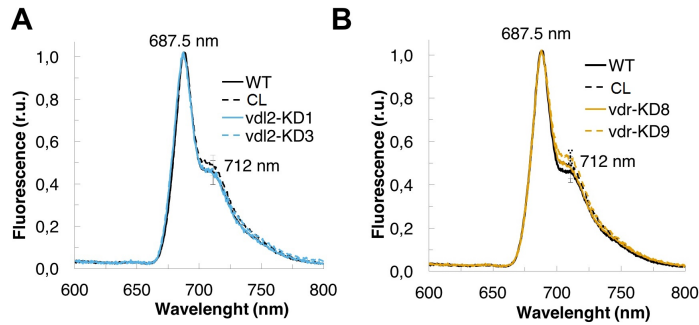
**Table 5.3** – List of primers used for qPCR amplification of the genes putatively involved in the xanthophyll cycle in *P. tricornutum*.



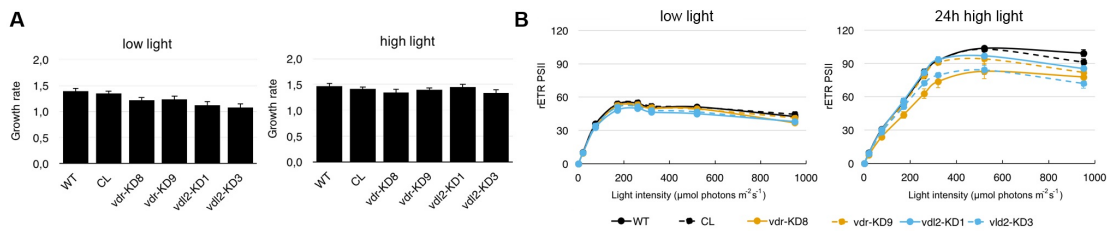
**Figure 5.11 – Immuno blot analysis of photosynthetic proteins.** Immuno blot analysis of photosynthetic proteins of the PSI core (PsaF), PSII core (D2), antenna proteins LHCfs (LHCf1-11) and the photoprotective antennae LHCxs in wild-type (WT), control line (CL), *vdr* and *vdl2* knock-down lines. Proteins total extracts were extracted from cells adapted to continuous low light or after 24h of high light stress. The subunit of the chloroplast ATPase  $\beta$ -CF1 is shown as a representative loading control.



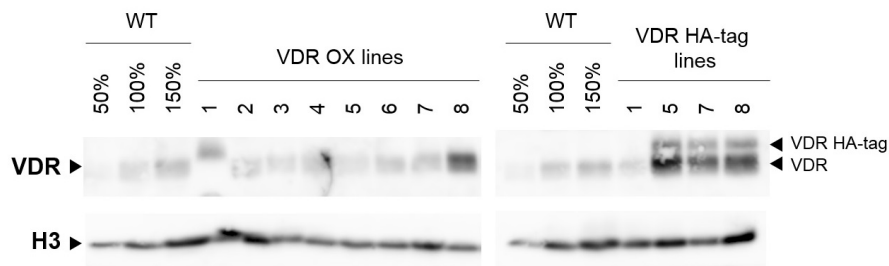
**Figure 5.12 – Antenna size, plastoquinone pool and assembly state of PSII supercomplexes.** (A) Antenna size of PSII (PSII absorption cross-section), (B) size of the plastoquinone pool (JI Area of the OJIP kinetic) of wild-type, control line, *vdr* and *vdl2* knock-down mutants for low light adapted cell and after 4h or 24h of continuous high light exposure.



**Figure 5.13 – 77K fluorescence emission spectra.** Fluorescence emission spectra of whole cells of wild-type, control line, *vdl2* (A) and *vdr* (B) knock-down mutants after 24h of continuous high light exposure. Fluorescence wavelength emission of PSII (687.5 nm) and PSI and red antennae (712 nm) is indicated. Direct emission of FCP proteins detached from the core would emit fluorescence at 675 nm (Lepetit et al. (2007)). Spectra are normalized over PSII emission. The experiments were reproduced two times. Error bars represent standard error.



**Figure 5.14 – Growth rate and electron transport rate.** (A) Growth rate of WT and knock-down mutants for cells grown in continuous low light or high light conditions. (B) Relative electron transport rate of PSII (rETR PSII) of WT and knock-down mutants for cells adapted to continuous low light or after 24h of high light stress. rETR PSII was measured at different light intensities (20, 170, 260, 320, 520, and 950  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ). Error bars represent standard error of at least three biological replicates.



**Figure 5.15 – Immunoblot analysis of VDR in over expressing lines.** Immunoblot screening of overexpressing (OE) lines for VDR. Different protein quantities of wild-type (WT) were loaded, OE mutant lines with VDR sequence (VDR OX) and OE lines with VDR with HA-tag (VDR HA-tag) are shown. VDR OX8, VDR HA-tag 5, 6, and 7 were selected as true overexpressing lines and screened for their photoprotective capacities. In VDR HA-tag 5, 6, and 7 lines, the tagged version of VDR is also visible as the upper band. Proteins were extracted from cells adapted to low light conditions. The protein histone 3 (H3) is shown as loading control.





## Chapter 6

# Conclusion and perspectives

The aim of my thesis was to provide novel information about photosynthesis and its dynamic regulation, with a focus on photoprotection in green algae, mosses and diatoms. If you arrived at this final chapter of the thesis, I'm pretty sure that by now you know very well that proteins and pigments used in photoprotection can differ quite a lot, but that they also share some interesting characteristics and mechanisms of action, even in distantly related organisms. The three classes of organisms I studied diverged hundreds of millions of years ago, and they offer a great opportunity to study how photoprotective mechanisms were conserved, evolved or disappeared in different lineages.

One *fil rouge* of the thesis are certainly the Light Harvesting Complexes Stress-Related (LHCSR) proteins, a family that I studied in *Chlamydomonas* (chapter 2), *Physcomitrella* (chapter 3) and *Phaeodactylum* (chapter 4).

LHCSR proteins were first identified in *C. reinhardtii* (Peers et al. (2009)) where they seemed to substitute the well characterized PSBS protein, present in higher plants (Li et al. (2000b)), in the induction of NPQ. PSBS is a pH sensor, and the protonation of two glutamic acid residues activate photoprotection when pH in the thylakoid lumen drops. A similar mechanism was proposed also for LHCSR3, since *in vitro* the protein responds to acidification by lowering its fluorescence lifetime (Bonente et al. (2011)), an indication that the conformation switches from an unquenched to a thermal

dissipation state.

Bailleul et al. (2010) and Liguori et al. (2015) indicated the C-terminus domain of the *C. reinhardtii* LHCSR3 and *P. tricornutum* LHCX1 proteins as the possible pH-sensor. However, my analysis of LHCSR/LHCX proteins alignment shows that the C-term domain is present only in a few algal species (chapter 2). Since LHCSR/LHCXs are present in pretty much all algae and mosses (with the exception of red algae), I hypothesized that the pH-sensing domain (if it's really present) should be conserved, to guarantee that these proteins detect the excess of light and induce NPQ. Indeed the new protonatable residues I identified in chapter 2, which are in the loops between the trans-membrane  $\alpha$ -helices, are present not only in *Chlamydomonas*, but also in other green algal species, in diatoms, and in mosses. We showed that these protonatable residues are extremely important for the induction of NPQ *in vivo* in *C. reinhardtii*, and that the recombinant protein without these aminoacids do not respond to pH changes *in vitro*. Thanks to these results, we now know that the pH-sensing function of LHCSR/LHCX proteins, that allows the organism to perceive the excess of light by the acidification of thylakoid lumen, is probably a characteristic that was maintained through evolution, since the identified protonatable residues are conserved in green algae, diatoms and mosses. A notable and interesting exception is LHCX4 in *P. tricornutum*, since this protein does not have the conserved pH-

sensing residues identified in the other proteins, suggesting a different function in diatoms photoacclimation, as I will discuss below.

Current understanding of NPQ suggests that quenching reactions need pigments, with either the formation of a carotenoid radical cation (Ahn et al. (2008)), the transfer of energy from chlorophylls to the  $S_1$  state of carotenoids (Ruban et al. (2007)) or a strongly coupled chlorophylls dimer that can perform charge separation (Muller et al. (2010)). PSBS in higher plants acts as a pH sensor, but it can't be the quenching site of excitation energy since it doesn't bind pigments (Dominici et al. (2002); Bonente et al. (2008)), and thermal dissipation takes place in LHC antennae (Gerotto et al. (2015)).

On the contrary, LHCSR/LHCXs do bind pigments, they can form carotenoid radical cation (Bonente et al. (2011)) and enhance their quenching capacity after the binding of zeaxanthin (Pinnola et al. (2013)). It was therefore hypothesized that LHCSR/LHCXs can be not only pH-sensors, as PSBS, but also an actual quenchers of excess energy.

Most of the pigment-binding residues I identified in chapter 3 are present not only in mosses (*Physcomitrella*), but also in the proteins from *C. reinhardtii* (chapter 2) and *P. tricornutum* (unpublished data). This, once again, highlights that these proteins might share a common mechanisms of action, not just for pH-sensing but also for energy transfer between chromophores. Nevertheless, carotenoid-dependent activation of thermal dissipation in LHCSRs is different, since the protein from *Physcomitrella* strongly enhance its quenching activity in the presence of zeaxanthin, while the protein from *Chlamydomonas* does not. This is in line with the fact that, in *C. reinhardtii*, lutein contributes more to qE than zeaxanthin does, as suggested by the fact that mutants lacking lutein are more affected in NPQ than those lacking zeaxanthin (Niyogi et al. (1997)). Thus, xanthophyll-dependent photoprotection seems to have changed during the evolution of these organisms, and the specific role of each pigment in LHCSRs may be different as well.

We are currently continuing the analysis of

pigment-binding mutants of LHCSR1 protein from *P. patens* both *in vitro* and *in vivo*, and a similar analysis is also on going with LHCSR3 from *C. reinhardtii*. The future results of these studies, will hopefully lead to asses whether the energy transfer and quenching properties of LHCSRs in these two organisms rely on similar mechanisms or if the role of their chromophores has changed during evolution.

In diatoms, nothing is known on LHCXs pigment binding properties and on the possible dependence of their activity upon interaction with diatoxanthin (or zeaxanthin). These remain important open questions for future investigation. Unfortunately, preliminary attempts to reconstitute *in vitro* LHCX1 to study its characteristics have failed (Pool (2011)). Further work is thus needed to obtain *in vitro* re-folded diatoms photoprotective antennae, since they would be invaluable for the understanding of both their pigment-binding properties, as well as for the functional characterization of the pH-sensing residues identified in LHCSR proteins of the green lineage.

A third feature that distinguishes LHCSR/LHCXs from PSBS is the expansion of the members of the LHCSR protein family in algae and mosses: the plant *A. thaliana* has in fact only one PSBS protein, and the knock-out of its gene completely abolish any qE activity (Li et al. (2000b)). Also in the moss *P. patens* only one PSBS protein is present, but here *two* LHCSR proteins can support NPQ even in the absence of PSBS (Alboresi et al. (2010)). In the green alga *C. reinhardtii*, *three* genes encoding LHCSRs are present, even though two of these genes encode for the same aminoacid sequence (Bonente et al. (2011)). Finally, in the marine diatom *P. tricornutum* the situation is even more extreme, with *four* different LHCX isoforms present in *P. tricornutum*, and an even more important expansion in other diatoms species (chapter 4).

The presence of different members of the LHCSR/LHCX family in the same organism suggests a diversification of the role of each isoform: in *C. reinhardtii*, for example, LHCSR1 is always present and can induce only a moderate qE (Thuy Binh Truong (2011)). LHCSR3, on

the contrary, is induced under excessive light conditions or low CO<sub>2</sub> (Peers et al. (2009); Correa-Galvis et al. (2016)), but can lead to a much stronger NPQ. This might indicate that the extent of photoprotection in different light conditions is regulated by the abundance of these two isoforms.

In mosses, differential regulation of the two LHCSR proteins seems to be also related to stress responses, since LHCSR1 and 2 are induced by excessive light conditions and cold stress respectively (Gerotto et al. (2011)).

I found in diatoms a similar scenario: the four LHCX isoforms in *P. tricornutum* are in fact differently regulated by both light and stress conditions, such as nutrient starvation. One protein, LHCX1, is always present and can induce high level of NPQ, but high light exposure induce the expression of other isoforms as well, namely LHCX2 and LHCX3, while nutrients starvation can lead to the accumulation of either LHCX2, 3 or 4. Lastly, cold stress impact only the expression of LHCX1 (unpublished data). The role of all these proteins on photoprotection is yet still mysterious, since knock-down mutants of LHCX2, 3 and 4 do not have a clear NPQ phenotype, likely because of the strong expression of LHCX1 in these lines (Lucilla Taddei, personal communication).

A notable outlier in the LHCX family is LHCX4: this protein, in fact, is accumulated after dark incubation, and its mRNA level quickly drops upon light exposure. In addition to that, LHCX4 has most of the pigment-binding sites found in the protein from *P. patens* (unpublished data) but does not conserve the pH-sensing residues identified in *C. reinhardtii*. As suggested in chapter 4, LHCX4 may be involved in the regulation of chloroplast physiology upon sudden re-illumination after a period of darkness (*e.g.* during day to night transition, when cells are transported at the surface or in cells that experience long periods of darkness at high latitudes).

Once again, tuning of LHCSR/LHCX protein abundance seems to be a regulative mechanisms “of higher level” than lumenal acidification or redox potential (since without protein no other regulation can occur). In general,

the complex regulation suggests a diversification of LHCXs function, and the presence of multiple proteins can be an advantage to finely regulate photoacclimation responses in highly variable environments, like in rapidly mixing water bodies or in the underbrush. However, further characterization of the function of each LHCX isoform will be needed to support this hypothesis, and hopefully TALENs™ will help us to reach this goal (see later).

Regulation of photosynthesis and photoprotection in diatoms can't really be understood without also looking at xanthophyll composition, since these pigments are so important for light harvesting, triplet quenching, ROS scavenging and thermal dissipation. Diatoms brownish color is in fact due to their high content in xanthophylls (in particular fucoxanthin) with respect to chlorophylls. Diadino/diatoxanthin content in *P. tricornutum* can be as high as 1/7 of total chlorophyll content (Lavaud (2002); Ruban et al. (2004)), and high accumulation of these pigments allows *P. tricornutum* to reach NPQ values up to 10 (Lavaud (2002)).

The importance of xanthophylls in photoprotection in diatoms is clearly shown by the strict correlation between diatoxanthin content and NPQ level in different species (Goss et al. (2006)). In plants, zeaxanthin alone induces very little NPQ (the qZ component, Nilkens et al. (2010)), as protonation of LHC is needed to maintain an active quenching. Upon the collapse of the proton gradient throughout thylakoidal membranes in fact, NPQ is also instantly reduced in plants (Goss et al. (2006)), as the major qE component is dependent on  $\Delta$ pH. In diatoms, on the contrary, diatoxanthin alone can sustain NPQ, even after the addition of an uncoupler that destroys the proton gradient (Goss et al. (2006)), a fact that questions if the division between qE and qZ is applicable to these microalgae.

As diadino- and diatoxanthin are accumulated to such high levels, little attention has been given to viola- and zeaxanthin in diatoms, which have been ignored in most of the recent publications (Lavaud (2002); Goss et al. (2006); Lepetit et al. (2010); Lavaud et al. (2012); Lavaud and Lepetit (2013); Schellen-

berger Costa et al. (2013)). Zeaxanthin involvement in photoprotection in diatoms (either via NPQ or ROS scavenging) was, to my knowledge, never tackled before.

With my research, I discovered that a correct balance between the two xanthophyll pools is necessary for efficient NPQ in diatoms. I found that two proteins, VDR and VDL2, with a hypothetical role in the de-epoxidation reactions, are instead implicated in the regulation of the reactions that connect the two xanthophyll pools. These proteins may be involved in sequential reactions (*e.g.* one catalyzing  $A \rightarrow B$  and the other  $B \rightarrow C$ ), or parallel reactions with similar substrate or products (*e.g.* one catalyzing  $A \rightarrow C$  and the other  $B \rightarrow C$ ), as the knock-down lines for VDR and VDL2 have very similar phenotypes.

My study indicates that, at the photoprotective level, the reduction of diatoxanthin is not compensated by the over-accumulation of zeaxanthin in the VDR and VDL2 mutants. This suggests that that zeaxanthin in diatoms is not involved in thermal dissipation, while in plants and green algae this xanthophyll regulates and enhances NPQ. Zeaxanthin accumulation in *P. tricornutum* did not correlate with NPQ level, and unusual amounts of violaxanthin and zeaxanthin are apparently deleterious for thermal dissipation, as suggested by the lower correlation between diatoxanthin and NPQ in knock-down lines. The hypothesized substitution in FCP antennae of violaxanthin instead of diadinoxanthin and diatoxanthin could have a negative impact on the ability of diatoxanthin to perform NPQ in *P. tricornutum*, as diatoxanthin quenching efficiency decreases. This may indicate that zeaxanthin binding to FCPs does not allow the antennae to aggregate or to change their conformation, to transit from a light harvesting to a heat dissipation state, as they do when diatoxanthin is bound.

It would be now interesting to isolate the different antennae of knock-down mutants in their native conformation (using sucrose gradient ultra centrifugation or native gels) and analyze their pigment content to understand if zeaxanthin substitution happens in specific proteins. Another possible future study with *vdr*

and *vd12* knock-down lines would be to analyze their resistance to oxidative stress, as zeaxanthin role in diatoms could still be related to ROS scavenging, as it is in plants (Havaux et al. (2007); Dall'Osto et al. (2010)). But we can't rule out the hypothesis that this pigment may just be an unavoidable biosynthetic intermediate in the pathway that leads to the formation of diadinoxanthin and fucoxanthin. The response to oxidative stress in *vdr* and *vd12* knock-down lines, together with the TALEN™ knock-out mutants for VDL2, will hopefully give us some directions to follow to fully comprehend zeaxanthin role in marine diatoms.

In conclusion, such a diversification in the function of xanthophylls clearly separates the organisms I studied, each one having its "favorite" xanthophyll. We span from *P. patens*, where zeaxanthin enhance NPQ (Azzabi et al. (2012); Pinnola et al. (2013)), to *C. reinhardtii*, where zeaxanthin and lutein cooperate in thermal dissipation (Niyogi et al. (1997)), to *P. tricornutum*, that lacks lutein and uses diatoxanthin to regulate NPQ instead of zeaxanthin. Thus, photoprotection performed with de-epoxidated xanthophylls is far more variable between organisms than the component controlled by LHCSR/LHCXs, which appears more conserved for both the pH-sensing and the pigment-binding residues.

The understanding of how photoprotective mechanisms were either conserved or diverged during evolution is of major interest for basic biology. Nevertheless, the study of photosynthesis and photoprotection is not only important from an ecological and evolutionary point of view, but also from an applied one: photoautotrophic organisms are in fact of great interest for the biotechnological industry, for the production of both low (*e.g.* biofuels) and high (*e.g.* bioactive molecules, as some pigments) value added products. Growth of microalgae inside photobioreactors (PBR) is currently the cultivation technique that allows to reach higher biomass yields (Chisti (2007)), but major problems persist today. One of the most important one is the efficient use of light inside PBRs (Chisti (2007); Bozarth et al. (2009); Radakovits et al. (2010)), as mixing of dense

cultures continuously moves cells from the surface of the PBR (where light is abundant) to the center of the culture (where cells are self-shading one with the other), stressing the photosynthetic apparatus. A better understanding of photosynthesis, and of the mechanisms that triggers and regulate photoprotection, could thus lead to higher yields thanks to increase in growth, resistance to oxidative stress and optimization of light use in photobioreactors.

One limit of the genetic techniques used in my works in *P. tricornutum* is that knock-down or over-expressing mutants can only give us partial information on protein function, especially in the case of expanded genes families. The constant presence of the photoprotective antenna LHCX1 in all the conditions examined in chapter 4, for example, may prevent us from detecting the contribution of the other (probably) less abundant isoforms. We faced a similar problem in chapter 5, where four proteins with potentially similar functions in the xanthophyll de-epoxidation reactions are present in *P. tricornutum* (VDE, VDL1 and 2, and VDR). In addition to that, knock-down lines of VDL2 and VDR showed only a partially reduced protein content, thus making more difficult to understand their role in pigments conversion.

More recent studies demonstrated the feasibility of gene knock-out in diatoms using site-specific endonucleases (Daboussi et al. (2014); Weyman et al. (2014)). At the beginning of my Ph.D. I worked at the private company Collectis S.A. to engineer some nucleases to knock-out genes involved in photoprotection. In particular I obtained TALENs<sup>TM</sup> (transcription activator-like effector nucleases) to target the genes for LHCX1, LHCX2, VDE, VDL2 and ZEP1 (zeaxanthin epoxidase).

LHCX1 knock-out mutants will allow us to better study the functions of the other isoforms in the absence of this constitutively expressed antenna, and LHCX2 knock-out will be important to study high light acclimation and chloroplast response to iron starvation, as this isoform

has the strongest induction under these stress conditions (chapter 4).

A knock-out strain for VDE could prove if this is the only protein that perform the de-epoxidation reactions in *P. tricornutum* (Lavaud et al. (2012)). VDL2, with its characteristic induction after prolonged light stress (Nymark et al. (2009)) and its connecting function between the two xanthophyll cycles (chapter 5) will help us to better understand light acclimation, the biosynthetic pathway of xanthophylls and the role of zeaxanthin in diatoms. Finally, ZEP1 was chosen as a target for its strong repression after high light exposure (Nymark et al. (2009)), and, recently, it has been demonstrated that this protein can't perform the epoxidation reaction in *A. thaliana* (while ZEP2 and ZEP3 can, Eilers et al. (2016)), thus rising interesting questions on its function.

TALENs<sup>TM</sup> technology not only allows to knock-out genes, but also to perform gene replacement (Weyman et al. (2014)), and in this context it would be extremely interesting to substitute LHCX genes in *P. tricornutum* with mutated versions, lacking either the putative pH-sensing residues or the pigment-binding aminoacids. Similarly, also the substitution of endogenous LHCX sequences with LHCSR genes from the green lineage could be performed.

Unfortunately, I didn't have the possibility to explore these topics during my Ph.D., due to the adjustments that we had to made on my project. But after an agreement between UPMC and Collectis, the TALENs<sup>TM</sup> I developed are now available, and we are currently screening the putative knock-out mutants of *P. tricornutum*. Hopefully these new tools will allow us to reveal even more information on the regulation of photoprotective antennae and pigments in diatoms, trying to close the knowledge gap we currently have with the green lineage.

But this is probably the topic for another thesis. Thank you for your attention, I hope you enjoyed the reading.



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*Bibliography*

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## Light stress and photoprotection in Green algae, Mosses and Diatoms

**Thesis summary (English):** The molecular bases of responses to light excess in photosynthetic organisms having different evolutionary histories and belonging to different lineages are still not completely characterized. Therefore I explored the functions of photoprotective antennae in green algae, mosses and diatoms, together with the role of the two xanthophyll cycles present in diatoms.

I studied the Light Harvesting Complex Stress-Related (LHCSR) proteins in different organisms. In the green alga *Chlamydomonas reinhardtii*, LHCSR3 is a protein important for photoprotection. I used site-specific mutagenesis *in vivo* and *in vitro* and identified three residues of LHCSR3 that are responsible for its activation.

With the moss *Physcomitrella patens* I studied the *in vitro* spectroscopic and quenching characteristics of different pigment-binding mutants of the protein LHCSR1, focusing in particular on chlorophylls A2 and A5.

LHCSRs in diatoms are named LHCXs, and in *Phaeodactylum tricorutum* I found that multiple abiotic stress signals converge to regulate the LHCX content of cells, providing a way to fine-tune light harvesting and photoprotection.

The other main driver of photoprotection in diatoms is the xanthophyll cycle. Here I found that the accumulation of viola- and zeaxanthin in *P. tricorutum* have a negative effect in the development of NPQ, showing that zeaxanthin does not participate in the enhancing of NPQ in diatoms.

Thanks to these studies done on different organisms, we gained a deeper knowledge on the shared characteristics and on the peculiar features about photoprotection in green algae, mosses and diatoms.

Keywords: algae, mosses, diatoms, photosynthesis, photoprotection, antenna proteins, xanthophyll cycle.

**Résumé de la thèse (Français):** Les bases moléculaires des réponses aux excès de lumière chez les organismes photosynthétiques appartenant à des lignées évolutives distinctes ne sont toujours pas complètement caractérisées. Par conséquent, j'ai caractérisé des antennes photoprotectrices dans les algues vertes, les mousses et les diatomées et j'ai exploré la fonction de deux cycles de xanthophylles chez les diatomées.

J'ai étudié les protéines Light Harvesting Complex Stress-Related (LHCSR) dans tous ces organismes. Chez l'algue verte *Chlamydomonas reinhardtii*, j'ai identifié par mutagenèse dirigée, complémentation fonctionnelle et par une approche biochimique les acides aminés responsables de l'activation de LHCSR3, une protéine importante pour le NPQ.

Dans le modèle de mousse *Physcomitrella patens*, j'ai étudié *in vitro* les caractéristiques spectroscopique ainsi que le quenching de différents mutants de liaison de pigment sur la protéine LHCSR1.

Les protéines LHCSR dans les diatomées sont nommées LHCXs, et dans *Phaeodactylum tricorutum* j'ai montré que l'expansion de la famille des gènes LHCX reflète une diversification fonctionnelle de ces protéines permettant de répondre à des environnements marins très variables.

L'autre acteur principal de la photoprotection dans les diatomées est le cycle des xanthophylles. J'ai trouvé que l'accumulation d'une grande quantité de viola- et zéaxanthin a un effet négatif sur le NPQ montrant que la zéaxanthin ne participe pas au NPQ chez diatomées.

Grâce à ces études effectuées, nous avons acquis une connaissance plus approfondie sur les caractéristiques communes et les spécificités de la photoprotection chez différents organismes.

Mots clés : algues, mousses, diatomées, photosynthèse, photoprotection, protéines d'antenne, cycle des xanthophylles.

**Riassunto della tesi (Italiano):** Le basi molecolari delle risposte all'eccesso di luce negli organismi fotosintetici con differenti storie evolutive e appartenenti a diverse linee non sono ancora completamente caratterizzati. Ho perciò esplorato le funzioni delle antenne fotoprotettive in alghe verdi, muschi e diatomee, insieme al ruolo dei due cicli delle xantofille presenti nelle diatomee.

Ho studiato le proteine Light Harvesting Complex Stress-Related (LHCSR) in diversi organismi. In *Chlamydomonas reinhardtii*, LHCSR3 è una proteina importante per la fotoprotezione. Ho usato mutagenesi sito-specifica *in vivo* ed *in vitro* e identificato tre residui di LHCSR3 che sono responsabili della sua attivazione.

Con il muschio *Physcomitrella patens* ho studiato *in vitro* le caratteristiche spettroscopiche di diversi mutanti per il legame dei pigmenti della proteina LHCSR1, concentrandomi in particolare sulla clorofilla A2 e A5.

Le LHCSR nelle diatomee sono chiamate LHCX, ed in *Phaeodactylum tricorutum* ho trovato che più segnali di stress abiotici convergono per regolare il contenuto di LHCX delle cellule, fornendo un modo per regolare la raccolta di luce e la fotoprotezione.

L'altro principale motore della fotoprotezione nelle diatomee è il ciclo delle xantofille. Qui ho scoperto che l'accumulo di viola- e zeaxantina in *P. tricorutum* hanno un effetto negativo nello sviluppo di NPQ, dimostrando che la zeaxantina non partecipa nell'NPQ nelle diatomee.

Grazie a questi studi condotti su organismi differenti, abbiamo acquisito una conoscenza più approfondita sulle caratteristiche condivise e su quelle peculiari nella fotoprotezione di alghe verdi, muschi e diatomee.

Parole chiave: alghe, muschi, diatomee, fotosintesi, fotoprotezione, proteine antenna, ciclo delle xantofille.