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CICLO XXVI

Optimization of light use efficiency in *Nannochloropsis* for biodiesel production

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Fragte man einen wahrhaften Men schen, einen,
der aus seinem eigenen Grundewirkt:
«Warum wirkst du deine Werke?»,
wenn er recht antwortete,
würde er auch nur sagen: «Ich wirke, um zu wirken»

Se si chiedesse a un vero uomo,
ad uno che opera sul proprio terreno:
«perché operi le tue opere?»,
se egli rispondesse rettamente,
direbbe solo «opero per operare»

Eckhart

Sono nato, casualmente, così come si accende casualmente una candela.
Ho vissuto precariamente per almeno 76 anni, così come una candela accesa arde con un fuoco assai facile a spegnere.
Morirò casualmente fra non molto, così come una candela si spegne casualmente quando un soffio porta via la sua fiamma o quando il lucignolo si annega nella cera fusa che ne circonda la base.
Lo stesso mistero avvolge l'umanità intera, la quale possiede e trasmette di generazione in generazione la voglia di vivere costruendo, e sa tuttavia di essere apparsa senza plausibile ragione in un momento qualsiasi; di poter sparire senza ragione plausibile in un momento qualsiasi; e che certamente sparirà ad un certo momento.

A. Spinelli - Come ho tentato di diventare saggio

Ai miei genitori

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Abstract

At present energy demand is largely fulfilled by fossil fuels, and it is easy to suppose that also in next decades they will still provide a large fraction of the energy supply. Since oil reserves are finite and the cost for its extraction will continuously raise, it is necessary to find alternatives source of energy. Renewable energy sources represent a good solution to the energy-problem and for this reason they are receiving a strong attention from industry and research centers. Photosynthetic organisms can represent a promising alternative to oil because they can be exploited for the production of different molecules exploitable as fuels such as ethanol, hydrogen and lipids. Fuels produced exploiting living organisms are called biofuels. Currently most of biofuel from photosynthetic organisms, is obtained by plant crops which present many limitations like the seasonality production and a low oil fraction that constitutes only 5% of the biomass. To overcome this limitation, an interesting alternative is the exploitation of photosynthetic microrganisms such as microalgae.

Some algae species have, in fact, the ability of accumulating large amount of lipids within their cells which can be exploited as feedstock for the production of biodiesel. Microalgae is a wide group comprehending thousand of different algae species, so one fundamental step is the choice of a suitable organism. A suitable candidate should have a fast growth rate in a large range of light intensities and the ability to accumulate large amount of lipids. Although there is no ideal organism which will be suitable for all, we focused on the seawater alga *Nannochloropsis gaditana*.

The general aim of this work is to investigate photosynthetic apparatus of *Nannochloropsis gaditana* in order optimize the light use efficiency in this microalga to increase the productivity.

In fact, as wide describe in the second chapter, light provides the energy supporting algae metabolism and consequently lipids production. Then available radiation must be exploited with the highest possible efficiency to optimize productivity and make microalgae large scale cultivation energetically and economically sustainable. In this chapter the molecular and the factors influencing light use efficiency in algal biomass production bases were investigated. Moreover exempla focused on how algae genetic engineering and control of light environment within photobioreactors can improve the productivity of large scale cultivation systems were given.

In the third chapter photosynthetic apparatus of *Nannochloropsis* was studied. *Nannochloropsis*'s photosynthetic apparatus is characterized by the presence of only chlorophyll a, with violaxanthin and vaucheriaxanthin esters as the most abundant carotenoids. The photosynthetic apparatus was analyzed by purifying the thylakoids and isolating the different pigment-binding complexes upon mild solubilization. The results from the biochemical and spectroscopic characterization showed

that the photosystem II antenna is loosely bound to the reaction center, whereas the association is stronger in photosystem I, with the antenna-reaction center super-complexes surviving purification. Such a supramolecular organization was found to be conserved in photosystem I from several other photosynthetic eukaryotes, even though these taxa are evolutionarily distant.

Obtained result suggests the presence of a selective advantage for a stable antenna (called VCP, Violaxanthin Chlorophyll binding Protein) and core complex association in the case of PSI but not in the case of PSII. A possible explanation was done considering that PSII supercomplexes are involved in several regulatory mechanisms which require a flexible binding of the antenna complexes to the reaction center along with the possibility of modulating this association according to environmental stimuli. On the contrary PSI reaction center is known to be stable with regard to light stress and to undergo a very low turnover although the PSI antenna have also shown to experience some regulation, the present knowledge suggests that the mechanisms affecting PSI antennae are less extensive and do not require the continuous modulation of its interactions with PSI and are thus compatible with a stronger association with the reaction center.

After having investigated photosynthetic apparatus of *Nannochloropsis*, in the fourth chapter, sequences and proteomic analysis on antenna protein were studied. A phylogenetic analysis of LHC sequences, identified in *Nannochloropsis* genome, allowed their classification into six different subgroups of antenna proteins. The preliminary proteomic analysis on different sucrose gradients fractions corresponding to antenna proteins, PSI and PSII fraction showed the existence of specific antenna proteins bound to PSI forming PSI-supercomplex.

In fifth chapter we focused out attention on different strategies for the biotecnological optimization of *Nannochloropsis* strain. Despite the fact that *Nannochloropsis* is considered the model organism for the production of biofuels, tools for the genetic manipulation of this species are still under development and only recently a few genome sequences become available, making possible to design specific protocol for the biotechnological improvement of this microalga. In this chapter two different methods, homologous recombination transformation and random mutagenesis, were applied on *Nannochloropsis* to generate and isolate possible valuable mutants. Obtained results highlighted that *Nannochloropsis* is not able to perform homologous recombination with high efficiency and chemical mutagenesis, is now, a more valuable methods to manipulate *Nannochloropsis* strain.

Photosynthesis is a complex phenomenon which is finely tuned according to environmental conditions which are far from being completely understood. This understanding is also seminal in the perspective of algae biofuels production in order to make genetic manipulation efforts more effective. One of the main target in this field is the xanthophyll cycle, a photoprotection mechanism which is activated under strong irradiation, present in most photosynthetic

eukaryotes including *Nannochloropsis*. In the sixth chapter was investigated a possible further level of regulation, depending from the redox state, of this cycle. The study was performed on *A. thaliana* VDE N-terminal domain enriched in cysteines, but as demonstrated in the chapter, residues analyzed are well conserved in all other species and thus all conclusions are most likely valid for *Nannochloropsis* protein as well. Results revealed that cysteines are fundamental for VDE enzymatic active and all of these residue, except one, are involved in disulfide bridge. Redox titration showed a steep dependence from redox potential of protein activity, drawing a scenario where redox regulation is not suitable for a fine tuning of VDE activity.

In appendix 1 isolated Violaxanthin Chlorophyll binding Protein (VCP) was studied thanks to advanced EPR techniques in order to investigate the presence of the photoprotective mechanism based on triplet-triplet energy transfer (TTET). Results of the data showed a strong similarity in terms of triplet state populations between VCP, FCP from diatoms and LHC-II from higher plants. Even if these antenna proteins have differentiated sequences and binds different pigments, the results suggest that in all members of the LHC superfamily there is a core represented by two central carotenoids surrounded by five Chlorophyll *a* molecules. This conserved structural organization plays a fundamental photo-protective function in Chl triplets quenching.

Riassunto

Attualmente la richiesta mondiale di energia viene in gran parte soddisfatta utilizzando combustibili fossili, primo tra tutti il petrolio. Nonostante sia facile supporre che anche nei prossimi decenni la produzione energetica derivante da fonti fossili sarà preponderante, già ora sono necessarie delle alternative: le riserve di petrolio sono limitate ed i costi di estrazione sono in continua crescita. Le fonti energetiche rinnovabili rappresentano una buona soluzione al problema energetico e per questo motivo stanno ricevendo una forte attenzione da parte dell'industria e dei centri di ricerca. Esistono diverse fonti di energia rinnovabile, tra queste gli organismi fotosintetici possono rappresentare un'alternativa promettente perché possono essere sfruttati per la produzione di diverse molecole utilizzabili come combustibili potendo generare etanolo, idrogeno e lipidi. I combustibili prodotti utilizzando organismi viventi sono chiamati biocarburanti e per la maggior parte sono ottenuti da coltivazioni di piante oleaginose come la soia, la palma e la colza. Queste coltivazioni tuttavia presentano anche molti lati negativi sia perché la loro produzione è esclusivamente stagionale sia perché il loro contenuto lipidico è pari al 5% della biomassa totale il che abbassa notevolmente la redditività di queste coltivazioni. È possibile superare queste limitazioni sfruttando altri tipi di microrganismi fotosintetici come microalghe.

Alcune specie di alghe hanno, infatti , la capacità di accumulare grandi quantità di lipidi al loro interno e tali lipidi possono essere impiegati come materia prima per la produzione di biodiesel. Le microalghe sono gruppo molto vasto comprendente migliaia di differenti specie: la scelta dell'organismo adatto è un passo fondamentale. La microalga ideale dovrebbe essere capace di mantenere un elevato tasso di crescita sotto differenti intensità luminose ed avere la capacità di accumulare elevate quantità di lipidi. Parte di queste caratteristiche ideali sono state rilevate in *Nannochloropsis gaditana*, una microalga marina, diventata, non solo per il nostro gruppo di ricerca, l'organismo modello per il biodiesel.

L'obiettivo generale di questo lavoro è indagare l'apparato fotosintetico di *Nannochloropsis gaditana* per ottimizzare l'efficienza nell'utilizzo della luce al fine di aumentare la produttività di lipidi.

Infatti, come ampiamente descritto nel secondo capitolo, la luce fornisce l'energia a sostegno del metabolismo delle alghe e, di conseguenza, supporta anche la produzione di lipidi. Quindi l'energia luminosa disponibile deve essere sfruttata con la massima efficienza possibile per ottimizzare la produttività e rendere la coltivazione di microalghe su larga scala energeticamente ed economicamente sostenibile all'interno di fotobioreattori. In questo capitolo sono state studiate le basi molecolari e i fattori che influenzano l'efficienza della luce e il suo utilizzo per la produzione di biomassa. Vengono inoltre forniti esempi di come sia possibile migliorare la

produttività nei fotobioreattori lavorando in modo sinergico sia a livello biotecnologico, modificando geneticamente l'organismo, sia a livello ingegneristico con il miglioramento del design del fotobioreattore e con il controllo della luce all'interno dello stesso.

Nel terzo capitolo, viene studiato in dettaglio l'apparato fotosintetico di *Nannochloropsis*, il quale risulta caratterizzato dalla presenza della sola clorofilla *a*, mentre violaxantina e vaucheriaxanthina-estere sono invece i carotenoidi più abbondanti. L'apparato fotosintetico è stato analizzato purificando i tilacoidi e isolando i diversi complessi attraverso una blanda solubilizzazione. I risultati della caratterizzazione biochimica e spettroscopica hanno dimostrato che le antenne (chiamate VCP, Violaxanthin Chlorophyll binding Protein) del fotosistema II (PSII) sono debolmente legate al centro di reazione, mentre questa associazione risulta più forte nel caso del fotosistema I (PSI), dove i supercomplessi centro di reazione-antenne non vengono intaccati dalla purificazione. Questo tipo di organizzazione del fotosistema I non è presente solo in *Nannochloropsis*, ma risulta essere conservata anche in diversi altri eucarioti fotosintetici, anche se questi taxa sono evolutivamente distanti. Il risultato ottenuto suggerisce una forte pressione evolutiva per la presenza di un'associazione stabile tra il complessi antenna e il centro di reazione nel caso di PSI ma non nel caso di PSII. Una possibile spiegazione viene data dal fatto che il PSII è coinvolto in diversi meccanismi regolatori che richiedono un legame flessibile tra complessi antenna e il centro di reazione, lasciando quindi la possibilità di modulare questa associazione secondo gli stimoli ambientali. Il centro di reazione del PSI, al contrario, si caratterizza per essere stabile per quanto riguarda lo stress da alta luce e subire un turnover molto basso. Nonostante sia stato dimostrato che anche il complesso antenna del PSI subisce delle forme di regolazione in presenza di stimoli ambientali molto forti, le attuali conoscenze suggeriscono che i meccanismi che incidono sui complessi antenna del PSI siano meno invasivi rispetto a quelli del PSII e non richiedano la continua modulazione della sua interazione con il centro di reazione del PSI e siano quindi compatibili con una forte associazione con il centro di reazione.

Dopo aver indagato l'apparato fotosintetico di *Nannochloropsis*, nel quarto capitolo, sono state studiate le sequenze delle proteine antenna ed è stata fatta un'analisi del proteoma dei complessi antenna, del PSI e del PSII. L'analisi filogenetica delle sequenze antenna, identificate nel genoma di *Nannochloropsis*, ha permesso la loro classificazione in sei diversi sottogruppi. L' analisi preliminare del proteoma ha evidenziato l'esistenza di specifiche proteine antenna legate al PSI che formano i supercomplessi del PSI.

Il quinto capitolo è dedicato alle diverse strategie impiegate per l'ottimizzazione biotecnologica di *Nannochloropsis*. Nonostante *Nannochloropsis* sia considerato l'organismo modello per la produzione di biocarburanti, i protocolli per la manipolazione genetica di questa specie sono ancora in via sviluppo e solo recentemente sono state rese note le sequenze del genoma, rendendo possibile progettare protocolli specifici per il miglioramento biotecnologico di questa microalga. In questo capitolo sono stati testati due metodi differenti, la ricombinazione omologa e la mutagenesi

chimica, su colture di *Nannochloropsis* per generare e isolare possibili mutanti. I risultati ottenuti hanno evidenziato che *Nannochloropsis* non è in grado di effettuare la ricombinazione omologa con alta efficienza e la mutagenesi chimica, rappresenta per ora il metodo più efficace per migliorare i ceppi di *Nannochloropsis*.

La fotosintesi è un fenomeno complesso, finemente regolato sulla base delle condizioni ambientali e ben lungi dall'essere completamente compreso. Tale comprensione è però fondamentale nella prospettiva della produzione di biocarburanti da alghe al fine di rendere le strategie di manipolazione genetica più efficaci. Uno dei target principali in questo campo è il ciclo delle xantofille, un meccanismo di fotoprotezione che viene attivato in condizioni di forte illuminazione, presente nella maggior parte eucarioti foto sintetici, compresa *Nannochloropsis*. Nel sesto capitolo viene considerata l'ipotesi che questo meccanismo sia regolato in base allo stato redox. Lo studio è stato eseguito sul dominio N-terminale ricco in cisteine della VDE di *A. thaliana*; com'è dimostrato nel capitolo, i residui analizzati sono ben conservati in tutte le altre specie e quindi le conclusioni tratte sono molto probabilmente valide anche per la VDE di *Nannochloropsis*. I risultati hanno rivelato che le cisteine sono fondamentali per l'attività enzimatica della VDE e tutti questi residui, tranne uno, sono coinvolti in ponti disolfuro. La titolazione redox, inoltre, ha mostrato una forte dipendenza dell'attività dal potenziale redox, disegnando uno scenario in cui la regolamentazione redox non risulta particolarmente adatta per una fine regolazione dell'attività della VDE.

Nell'appendice 1, le tecniche avanzate di EPR sono state impiegate al fine di indagare la presenza del meccanismo fotoprotettivo basato su triplet-triplet energy transfer (TTET) nelle antenne (VCP) di *Nannochloropsis*. I risultati ottenuti mostrano una forte somiglianza in termini di popolazione dello stato di tripletto tra VCP, FCP di diatomee e LHCII di piante superiori. Nonostante queste proteine antenna abbiano sequenze diverse e leghino pigmenti differenti, i risultati suggeriscono che in tutti i membri della superfamiglia LHC sussiste un *core* costituito da due carotenoidi, il quale è circondato da cinque molecole di clorofilla *a*. Questa organizzazione strutturale conservata gioca una particolare funzione fotoprotettiva fondamentale nel quenching dei tripletti di Chl.

CHAPTER 1

INTRODUCTION

1.1 Biodiesel: a new source of clean energy

According to BP Energy Outlook, energy demand will increase by 60% (Ahmad A. L. et al. 2011) in the next twenty years and the largest fraction (65%) of this growth will be accounted by China and India (BP Energy Outlook; 2013). At present energy request is largely fulfilled by fossil fuels which now account for 86% of global production. It is probably that in next decades fossil fuels will still provide a large fraction of the energy supply but alternatives are necessary since oil reserves are finite and the cost for its extraction will continuously raise (Rodolfi L. et al. 2009 and Hannon M. et al. 2010). In addition to issues with supply, it is widely recognized that fossil fuels are the largest contributors of greenhouse gas emissions (GHG)(NRC-2010) due to large scale use of these fuels for transport, electricity and thermal energy generation. With the increase awareness that anthropogenic factors have a role climate change it has become important to develop abatement techniques and adopt policies to reduce emissions.

Renewable energy sources represent a solution to both to the energy supply and reduction of greenhouse gas emissions, for this reason they are receiving a strong attention from industry and research centers. Renewable energy is generally defined as energy originating from resources which are continually replenished on a human timescale such as sunlight, wind and geothermal heat. It is clear that no one single renewable energy source can support all global energy demand, but it is necessary to exploit different sources which all cover a fraction of the global demand. Solar, wind and geothermal represent sources of clean energy are all already exploited on large scales with positive results. However, all these renewable sources produces electricity which only represents around 30% of global energy consumption. The remaining 70% is instead represented by liquid fuels for which production there are, at present, no viable renewable alternatives. Liquid fuels are particularly important for their use for transportation where the use of electricity either requires large investments in infrastructures or is inapplicable with present technology as in the aviation sector (Gouveia L. and Oliveira A. C. 2009).

Photosynthetic organisms can represent a promising alternative in this direction since they can be exploited for the production of different molecules exploitable as fuels such as ethanol, hydrogen and lipids. Fuels, produced exploiting living organisms, are called biofuels and they represents a valuable alternative for replacing fossil fuels because they are renewable and they do not contribute to the liberation of CO_2 in the atmosphere (Gouveia L. and Oliveira A. C. 2009). The term biofuels includes bioethanol, biohydrogen, biogas and biodiesel. The present work is focused on the latter and thus on the possibility of producing a fuel, biodiesel, from the transesterification of triacilglicerols. Biodiesel represents an environmentally friendly alternative to diesel fuel being non-toxic fuel, biodegradable and with lower emission of GHG upon combustion (Demirbas A., 2009)

At present crops like soy, palm, rapeseed and sunflower are used as a feedstock for biodiesel production but this approach shows several limitations. First of all the production is limited by seasonality since plants in temperate climates are not productive for a large fraction of the year. Also oil represents at most the 5% of the biomass in these crops (Chisti Y. 2008). Because of all these reasons the replacement of a significant fraction of fossil fuel by biodiesel, produced from plants, will require unrealistic areas of cultivation. It is also difficult to hypothesize a dramatic increase on yields for these species (Chisti Y. 2007, Jeong G. T. et al. 2004) since they have been intensively cultivated for a long time and already underwent genetic improvements and agricultural practices (Doebley J. F. et al. 2006). Another major issue is that all these crops are normally cultivated for food or feed production and their exploitation as a feedstock for biofuels will cause a undesired competition for cultivation areas and potable water and a consequent raise in prices (Singh A. et al. 2011).

An interesting alternative to plants is the exploitation of other photosynthetic microrganisms such as microalgae which show several advantages respect to food crops (Hannon M. et al. 2010, Rodolfi L. et al. 2009). As reported in Malcata F. X. (2011) a great number of microalgae species are able to accumulate large amount of lipids within their cells, which can be extracted and trans-esterified into biodiesel. In some species lipids content can go beyond 50% of total dry weight, thus a fraction at least ten times higher than any crop plant.

Microalga species	Lipid content (%, w/w_{DW})
Botryococcus spp.	25.0–75.0
Chaetoceros calcitrans	14.6–39.8
Chaetoceros muelleri	33.6
Chlorella emersonii	25.0–63.0
Chlorella protothecoides	14.6–57.8
Chlorella pyrenoidosa	2.0
Chlorella sorokiniana	19.0–22.0
Chlorella vulgaris	5.0–58.0
Chlorella spp.	10.0–57.0
Chlorococcum spp.	19.3
Dunaliella primolecta	23.1
Dunaliella salina	6.0–25.0
Dunaliella tertiolecta	16.7–71.0
Dunaliella spp.	17.5–67.0
Ellipsoidion spp.	27.4
Haematococcus pluvialis	25.0
Isochrysis galbana	7.0–40.0
Isochrysis spp.	7.1–33.0
Nannochloris spp.	20.0–56.0
Nannochloropsis oculata	22.7–29.7
Nannochloropsis spp.	12.0–53.0
Neochloris oleoabundans	29.0–65.0
Pavlova salina	30.9
Pavlova lutheri	35.5
Phaeodactylum tricornutum	18.0–57.0
Scenedesmus obliquus	11.0–55.0
Scenedesmus quadricauda	1.9–18.4
Scenedesmus sp.	19.6–21.1
Spirulina platensis	4.0–16.6

Table 1.1 Typical ranges of lipid content and productivity of selected marine and freshwater microalga species. (Malcata F. X. 2011).

Furthermore plants have several tissues, like roots or stems, that are not photosynthetically active and thus act as energy sinks. On the contrary in microalgae the entire cell is photosynthetically active, so all the solar energy harvested by photosynthesis is used for biomass production. Microalgae cultivation can be also combined with bio-fixation of waste CO_2 (Rodolfi L. et al. 2009) reducing greenhouse gas emission (Lam K. M. and Lee T. K. 2012) and bioremediation of wastewaters to reduce their nitrogen and phosphorus content (Jiang L. L. et al. 2011, Sivakumar G. et al. 2012) cutting down in this way the production costs. Finally microalgae do not need to exploit arable land since they can be cultivated in land not suitable for agriculture.

Summarizing the production of biodiesel from microalgae shows several potential point of strength. However, the high cost and inefficiencies of algae large scale cultivation systems prevents it from becoming a serious competitor for petroleum fuels at present. As petroleum fuel costs rise and supplies dwindle, biodiesel will become more attractive to both investors and consumers if also new more efficient systems for their cultivation are developed (Campbell M.N., 2008) by improved design of photobioreactors and microalgae genetic engineering (Beer L. L. et al. 2009, Demirbas A. 2009, Radakovits R. et al. 2010).

1.2 *Nannochloropsis gaditana*

As discussed above, biodiesel from microalgae represents one promising possibility to reduce energetic dependence from fossil fuels, even if strong efforts are necessary to make these applications economically sustainable on the industrial scale. One fundamental step for these kind of applications is the choice of a suitable organism among the thousands of different algae species. A suitable candidate should have a fast growth rate in a large range of light intensities and the ability to accumulate large amount of lipids. Although there is no ideal organism which will be suitable for all conditions several groups, including ourselves, focused on the seawater alga *Nannochloropsis gaditana* and other species of the same genus (Rodolfi L. et al. 2009, Sforza E. et al. 2010, Simionato D. et al. 2011, Radakovitz R. et al. 2012, Vieler A. et al. 2012). For this reason we use this species as model organism for this work, although several of the conclusions will be valid also if other species are investigated.

N. gaditana belongs to the kingdom of Chromista (Cavalier-Smith T. 2004) which includes organisms presenting a chloroplast enveloped by four membranes, originated from a secondary endo-symbiotic event where an eukaryotic host cell engulfed a unicellular ancestor of a red alga (Archibald, J. M. and Keeling, P. J. 2002). Within the kingdom of Chromista, *Nannochloropsis* genus belongs to the infrakingdom of Heterokonta (Cavalier-Smith T. 1995), which includes all eukaryotic motile biflagellate cells having typically a forward directed flagellum (Fig. 1.1). The variety among Heterokonta is striking including both multicellular seadweeds and unicellular

algae present in fresh and marine water (diatoms, brown algae, chrysophite). Due to this large diversity this infrakingdom is divided into three phyla and *Nannochloropsis* genus belongs to the Ochrophyta, which includes mainly autotrophic organisms but also a few heterotrophs (Riisberg, I. et al. 2009). This phylum is further divided into the subphyla of Khakista (Cavalier-Smith T. and Chao E.E.Y., 2006) and Phaeista (Cavalier-Smith and Chao E.E.Y., 2006); the latter comprises seven classes including the Eustigmatophyceae where *Nannochloropsis* are found (Riisberg, I. et al. 2009).

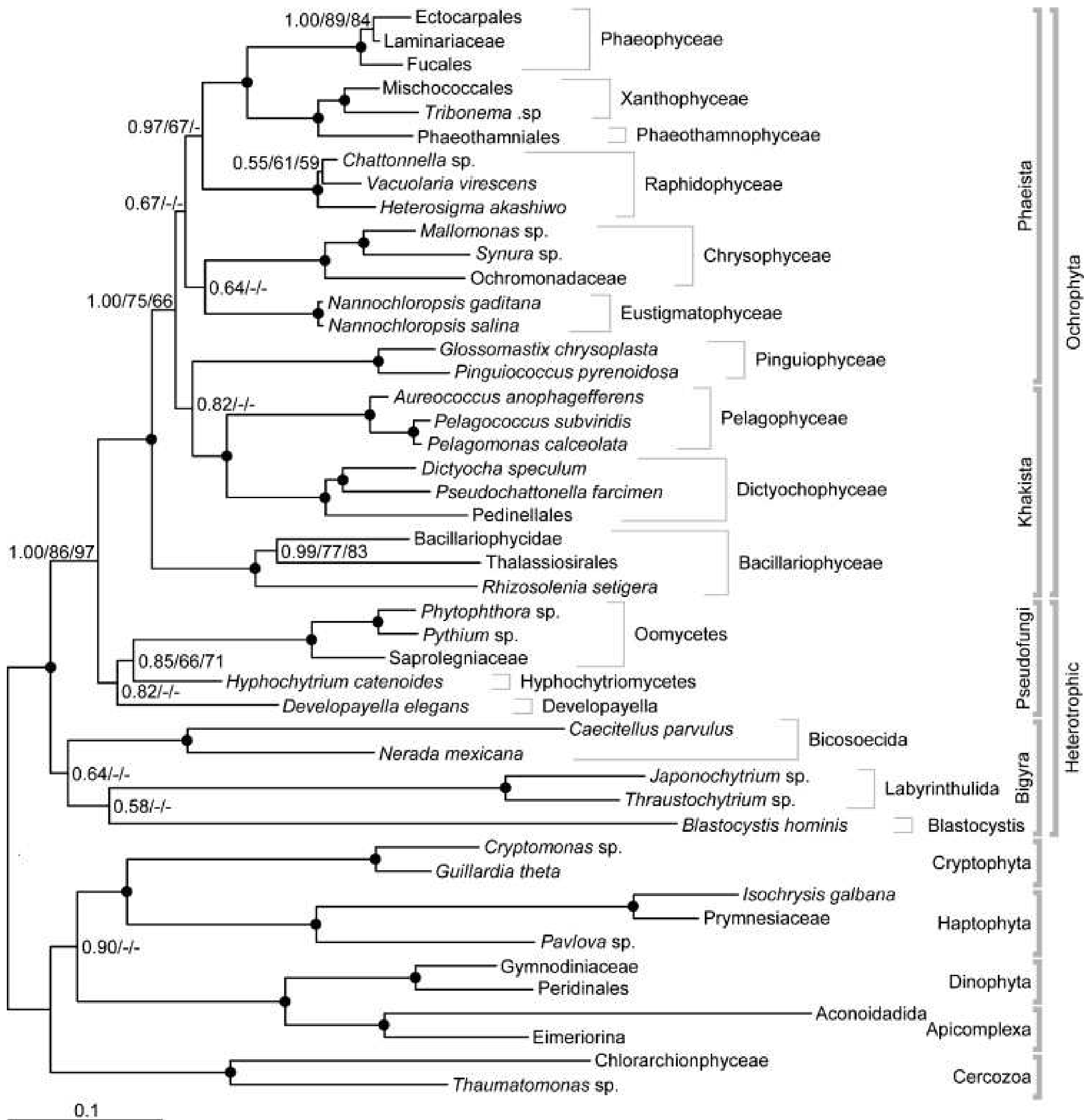

Fig. 1.1: rDNA phylogeny of heterokonts (Riisberg 2009).

The name of this class derived from the *eustigma* a particular vesicular structure in the cytoplasm which normally enables an oriented movement response with respect to the direction and intensity of incident light on the cell in flagellated algae (Kreimer G. 2009). Despite the name the presence of an eustigma in *Nannochloropsis* is still debated. Also being not flagellated and the biological role of this eventual eustigma is also unclear (Lubiàn L. M., 1982, Santos L.M.A., 1995).

Nannochloropsis genus includes six species, one growing in fresh water, while others are all found in marine environment. *N. gaditana* has a reduced cell size (2-5 μm) (Hibberd et al. 1981), and most of the cell volume is occupied by the chloroplast (Lubian L. M. 1982, Simionato D. et al. 2013). *Nannochloropsis* has a unique pigments content with only chlorophyll (Chl) *a*, lacking completely of other accessory chlorophyll such as *b* or *c*. As described in (Lubian L. M. et al. 2000) violaxanthin and vaucheriaxanthin are the major carotenoids detected in the cell, but also ketocarotenoids such as astaxanthin and cantaxanthin have been detected in aged cultured grown in high light or in nutrient deprivation (Simionato D. et al. 2013).

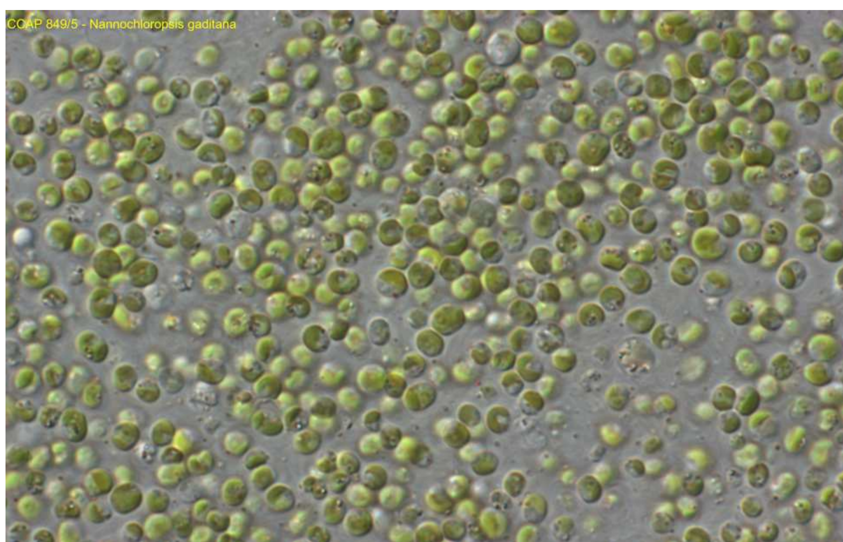

Fig.1.2 *Nannochloropsis gaditana* Culture Collection of Algae and Protozoa (www.ccap.ac.uk)

Nannochloropsis is an autotrophic organisms that grows at pH value around 7.5-8 (Rocha J.M.S. et al. 2003) and salinities from 3.6 to 44 g/l. (Radakovits R. et al. 2012) even if the best condition of this parameter was estimated at 31 g/l (Hu H. and Gao K. 2006). It is able to utilize glycerol for mixotrophic growth (Sforza E. et al. 2012).

Nannochloropsis is well known as a source of different valuable pigments, such as Chl *a*, zeaxanthin, canthaxanthin and astaxanthin (Lubian L. M. et al. 2000), produced at high levels; it is also recognised as a good potential source of EPA (20:5ω3), an important polyunsaturated fatty

acid with important activity in human health for prevention of several diseases (Sukenik, A. et al. 1998).

Currently *Nannochloropsis* is used in aquaculture for its a high nutritional value and a protein content (Rocha J.M.S. et al. 2003) but in recent years it started receiving an increased attention for biofuels production due to its very high content in lipids, in the form of triacylglycerides (TAG) which are the best substrate to produce biodiesel. In particular *Nannochloropsis* under abiotic stress such as nitrogen or phosphorous starvation and high light (Rodolfi L. et al. 2009, Damiani M. C. et al. 2010 Sukenik A. et al. 2009) was shown to increase lipid production. In several algal species it has been observed that under nutrient shortage, cells accumulate lipids but the stress also decrease biomass production (Rodolfi L. et al. 2009). *Nannochloropsis* in this sense appears to be peculiar since the induction of lipid synthesis, during N-starvation, does not reduce biomass productivity, at least for the first few days (Rodolfi L. et al. 2009). Lipids produced, thus, are synthesized from newly fixed carbon thanks to a reorganization of photosynthetic apparatus and metabolism which allow *Nannochloropsis* to maintain a sufficient photosynthetic activity (Simionato D. et al. 2013).

1.3 The importance of photosynthetic efficiency for biofuels production

Nannochloropsis is a photosynthetic organism and thus light is the only source of energy supporting the entire metabolism. Both biomass and lipids production are thus strongly light-dependent. The efficiency for the use of this resource is thus seminal to determine the productivity. This is exemplified in fig. 1.3 where the area needed to produce a ton of biomass per year is represented depending on the energy conversion efficiency.

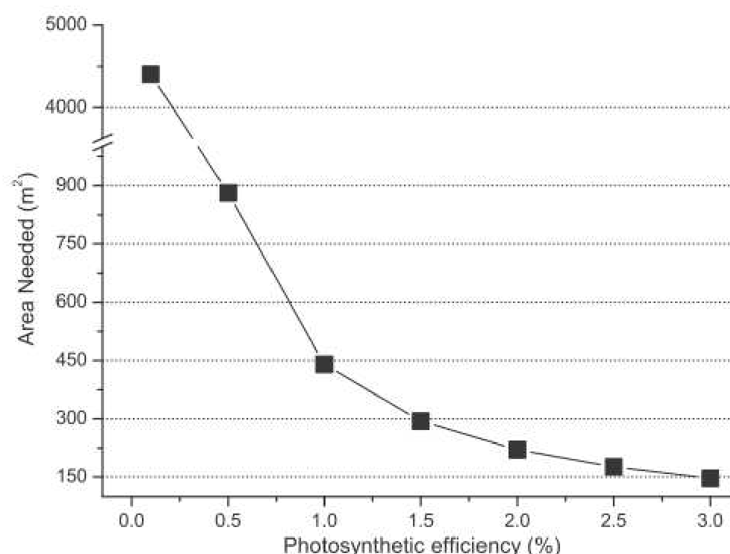

Fig. 1.3: Estimation of area needed for alga production. The area occupied by an alga cultivation system producing 1 ton of dry biomass per year is shown in dependence of the light use efficiency. Light input is assumed as the one reaching Padova every year (Simionato et al. 2013)

As shown in Fig. 1.3 if efficiency conversion is very low, around 0.1% as for crop plants (Heaton E. A. et al. 2008) , then the area needed for the cultivation is huge. While large areas are normally cultivated with crops it should be underlined that algae cultivation systems are at present much more expensive and labor intensive. Therefore if the efficiency is too low and the area needed too high, then the algae cultivation would be energetically and economically unsustainable. However, microalgae in laboratory conditions can achieve photosynthetic efficiencies around 3% (Blankenship R. E. et al. 2011), if similar values can be approaches in large scale systems this would mean that the area needed is much smaller and then an industrial cultivation of algae can become competitive.

These considerations clearly support the need to investigate the parameters influencing algae photosynthetic efficiency in order to approach the values measured in the lab in a large scale photobioreactor. This objective, if achieved, could drastically push the competitiveness of the technology.

A further interesting consideration would be that the maximal theoretical efficiency of photosynthesis should be around 10-12% in the conversion of light into biomass (Melis A. 2009 Hambourgher M. et al. 2008). The study of light use efficiency in algae is seminal for two objectives: first it can also contribute to move algae photosynthetic efficiency from the low value of the industrial scale to the 3% reached in a lab scale condition, and second, once the first goal is achieved, move algae photosynthetic efficiency to higher values in order to reduced the gap with maximum theoretical efficiency.

In Stephenson P.G. et al. (2011) the causes responsible of the low performance in photobioreactors were identified as:

- *Light-saturation of photosynthesis*: photosynthesis rate increases linearly with light intensity up to a saturation point. Once this is reached, antennae proteins continue to absorb light but the energy cannot be used for photochemistry. In these conditions energy excess can drive to generation of reactive oxygen species and inhibition of photosynthetic apparatus. The activation of a photoprotection process, safely dissipates this energy via heat and fluorescence and is helpful in preventing photodamage (Niyogi K.K. 2000) but energy dissipation still causes a strong reduction of cells light use efficiency.

- *Mass culture self-shading*: when algae growth in thick layers in mass culture, cells on the surface are often light-saturated. These cells on the external layer absorbs most of the energy but use it with low efficiency. The rest of the culture is instead exposed to very low illumination reducing their productivity. The combination of a subpopulation of cell in a condition of photoinibition and other subpopolations in a light limited state can heavily limit energy conversion efficiency by as much as 95% (Melis A. 2009, Polle J.E. et al. 2003).

Other factors are instead responsible of the gap between the observed and theoretical efficiencies (Stephenson P.G. et al. 2011):

- *Excitation energy transfer*: the primary photochemical reactions proceed from relatively low-energy, long wavelengths of light (680 nm PSII and 700 nm PSI), so much of the energy of shorter, high-energy wavelengths of photons absorbed by the antennae is re-emitted as heat and fluorescence. The process of photosynthesis is therefore unable to utilize the additional energy of blue photons of light (approximately 75% greater than red photons), resulting in a loss of energy from incident solar light (Barber J. 2009).

-*Photorespiration*: the enzyme RUBISCO, which is required for the carboxylation of ribulose-1,5-bisphosphate (RuBP), has a low specific affinity for CO_2 and can also catalyze an oxygenation reaction (Whitney S.M.et al. 2010) leading to light-dependent consumption of oxygen, ATP and NADPH in a process called photorespiration, which leads to a decrease photosynthesis efficiency due to consumption of ATP and the release of recently fixed carbon.

- *Respiration*: as well as performing photosynthesis, all marine microalgae respire fixed carbon, a necessary life process. This consumption of fixed carbon is difficult to measure and is species specific, but can account for up to 30% of fixed carbon(Zhu X.G. et al. 2007).

These negative factors can be overcome with the integration of both the engineering and the biological approach: in fact the development of an optimize photobioreactor (Zou N. et al. 2001 Campbell M.N. 2008) with an optimized mixing, in order to guarantee the correct amount of light for every cell, has the same importance than the design of mutant strains of microalgae with an improving pigments composition that could be obtained with a manipulation of carotenoids pathway (Brenner M.P. et al. 2006) or the development of mutants with different light absorption capacity (Melis A. 2009), or the generation of microalgae with modified light independent reaction increasing RUBISCO catalytic rate or reducing its oxygen affinity (Andrew J.T. and Whitney S.M. 2003).

It is thus possible imagine that, in the next years, the improvement of technologies on biofuels would be increase the biomass productivity through the photosynthesis reaction reaching the optimization of solar energy conversion. This probably represent the main goal to obtain a sustainable and economically advantageous biodiesel.

This subject will be further discussed on chapter 2.

1.4 Oxygenic photosynthesis

Oxygenic photosynthesis is the process supporting life on earth: photosynthetic organisms harvest solar light and convert it to ATP and NADPH which are used as energy sources to reduced inorganic carbon and produce carbohydrates and molecular oxygen as secondary product.

The entire process is divided into two phases called respectively light and a dark phase. The light phase occurs first and solar light is used to oxidize water molecules and produce ATP and NADPH. In the dark reaction ATP and NADPH, are exploited for CO_2 fixation in the Calvin-Benson cycle (Benson, A. A. and Calvin, M. 1950) where one glyceraldehyde 3-phospate (GAP) is synthesized from three CO_2, nine ATP and six NADPH. Ribulose 1,5-bisphospate (RuBP), the starting material for the cycle, is regenerated with the cost of one molecule of ATP to preserve the cyclic character of the process.

Photosynthesis in eukaryotes is performed in a specific organelle called chloroplast characterized by two membranes (together called envelope). The presence of these membranes takes origin from a primary endosymbiotic event between a heterotrophic organism and a cyanobacterium. Some organisms, like heterokonts, show a chloroplast with four membranes due to their origin from a secondary endosymbiosis .

The envelope divides a compartment called *stroma* where plastidial DNA, RNA, ribosome and enzymes with a role in the dark phase are located. Inside the *stroma* there are the thylakoids membranes forming a physically continuous three-dimensional network which surrounded the thylakoids *lumen*; while in plants electron microscopy shows thylakoids organized in stacked piles forming *grana* in *Nannochloropsis* this organization is not clearly defined as shown in Fig. 1.4 (Simionato D. et al. 2013). Here thylakoids are formed by three overlapped membrane layers with no clear distinction between grana and stroma lamellae. In thylakoid membranes all protein complexes involved in light phase reaction of photosynthesis are found.

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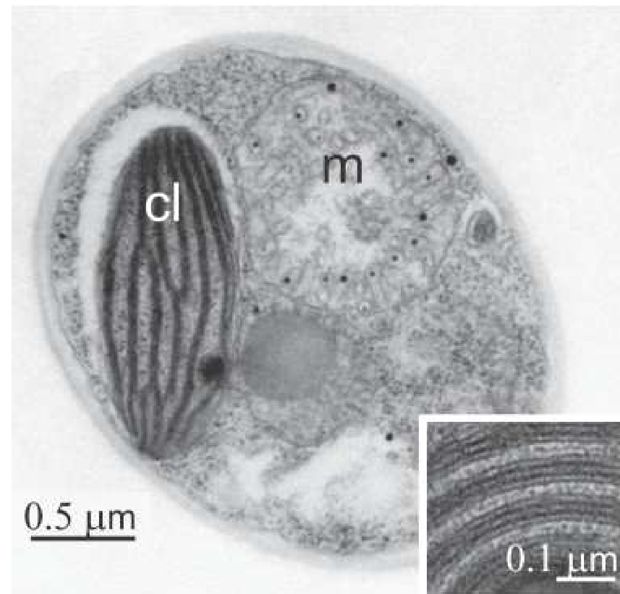


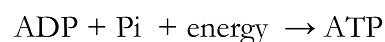
Fig. 1.4 Transmission electron microscopy of *Nannochloropsis* cells. m, mitochondria; cl, chloroplast; adapted from Simionato et al. 2013

1.4.1 Light phase

The first step of light phase is the harvesting of solar energy by chlorophylls and carotenoids bound to light harvesting complexes (LHC). The light energy, once harvested, is transferred to the reaction center where it is exploited for a charge separation. Here two specific Chl *a* are able to use excitation energy to transfer one electron to a series of electron transfer processes, yielding in the formation of a proton gradient across the thylakoid membrane and the generation of free energy and reducing power in the form of ATP and NADPH + H^+. The primary electron donor is water: Oxygen Evolving Complex (OEC) oxidized H_2O and splitted into 1/2 O_2+ $2H^+$.

The entire process can be summarized with these equations:

$$2\,H_2O + 2\,NADP^+ + 8\text{ photons} \rightarrow O_2 + 2\,NADPH + 2H^+$$



$$ADP + Pi + \text{energy} \rightarrow ATP$$

As described in Hill R. and Bendall F. 1960 two photosystems called PSI and PSII act in series following the Z scheme to provide the energy required for the production of NADPH.

While the primary charge separation creates an electrical potential, the difference in proton concentration between the stromal and the lumenal side of the membrane is developed by a two main process: the so called Q-cycle and the oxidation of water (Fig.1.5).

In the latter 4 protons are released in the lumen for each O_2 molecule produced, while during the Q-cycle, Cyt b_6f catalyzes the transfer of electron from a reduced plastoquinone (PQH_2) to plastocyanin, a soluble copper protein. The result of this cycle is the pumping of protons across the membrane; from the stromal compartment to the thylakoid lumen 3 H^+ are translocated in the lumen for every 2 electrons transported to PSI. The formation of an electrochemical potential across the thylakoid membrane is exploited to synthesize ATP starting from ADP + Pi in a process called photosphororilation. The accumulation of proton in thylakoid lumen acts as a feedback control on light harvesting, as further discusses in paragraph 1.5 of this chapter. On the stromal side protons are involved in the reduction of NADP by an iron containing protein: the ferrodoxin.

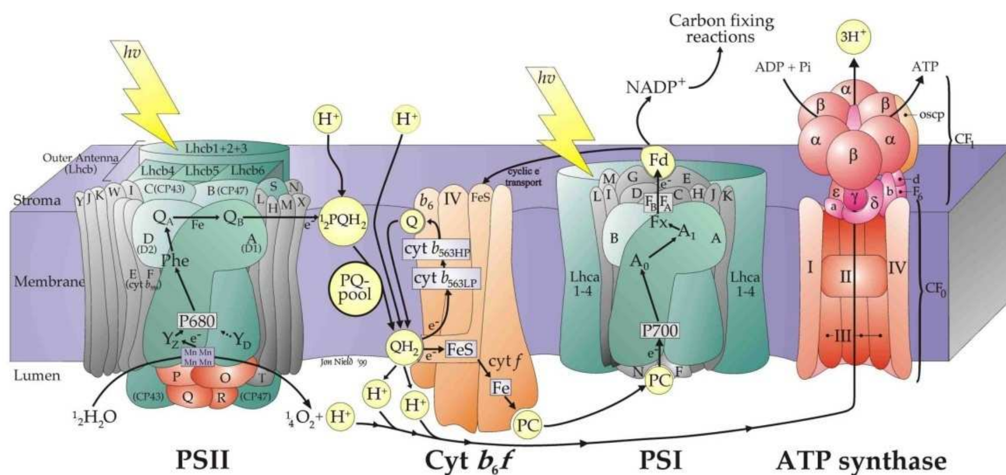


Fig. 1.5. Electron transport chain in thylakoids (Nield 1999). PSII and PSI photosystem II and I, PQ plastoquinone, Cyt *b6f* cytochrome *b6f* complex, OEC oxygen evolving complex, PC plastocianin, FD ferredoxin, LHC light harvesting complex www.queenmaryphotosynthesis.org/nield

It is worth to underlining that during the evolution, in relation to the different availabilities of Cu and Fe in the environment, plastocyanin was substituted with Cyt*c6* (Howe J. C. et al. 2006, Peers G. and Price N. M. 2006) in photosynthetic bacteria and eukaryotic algae where it is widely distributed among green, red, and brown (Sandmann G. et al., 1983; Kerfeld C.A. and Krogmann D.W. 1998). While plastocyanin seems absent in *Nannochloropsis* genome, the gene for *Cytc6* was identified (Radakovits, R. et al. 2012, Corteggiani, Carpinelli E. et al. 2013); the presence of *Cytc6* instead of plastocyanin is probably due to the higher concentration of Fe than Cu on the coast (Peers G. and Price N. M., 2006, Boubunari T. et al. 2009), the typical habitat of *Nannochloropsis*.

1.4.2 Proteins complexes involved in the light phase of photosynthesis: reaction centers

1.4.2.1 Photosystem II

Photosystem II catalyzes the electron transfer from water to PQ. The complex contains four large membrane-intrinsic subunits (called PsbA–D), three membrane-extrinsic subunits and a large number of small subunits, most of which span the membrane once. PsbA (D1) and PsbD (D2) constitute the photochemical reaction center where the charge separation and primary electron transfer reactions take place, while PsbB (CP43) and PsbC (CP47) have a light-harvesting function (Dekker J.P and van Grondelle 2000).

1.4.2.2 Photosystem I

PSI is the second photosystem in photosynthetic light reaction; is a light dependent plastocyanin-ferrodoxin oxidoreductase. In higher plants the core is composed of 14 subunits (Jensen, P. E. et al. 2007), among these subunits 8 are conserved and found also in diatoms PSI (Grouneva I . et al. 2011). PsaA and PsaB bind the Chl a responsible for the charge separation while PsaC, D and E are involved in connection with plastocyanin or Cyt*c6* in the lumenal side of the membrane or with ferrodoxin in the stromal one.

1.4.3 Proteins involved in the light phase of photosynthesis: light harvesting complex

All photosynthetic eukaryotes share the presence of Light Harvesting Complex superfamily which is responsible of the first step of photosynthesis (Green B.R. and Durnford D.G., 1996). Recently Engelken J. et al. (2010) and coworkers demonstrated that LHC evolved from an internal gene duplication/unequal crossing-over of tandem genes copying in this way a putative carotenoid-binding motif together with the chlorophyll binding motif. The genes encoding for LHCs are nuclear, are translated in the cytosol, and their products are then post-translationally directed, thanks to a signal peptide at N-terminal, to the chloroplasts where they associate with pigments and insert into the thylakoid membrane (Koziol A.G. et al. 2007).

LHCs possess three α -helical transmembrane regions (TMR), connected by stroma and lumen-exposed loops (Fig. 1.6). Two of these helixes, A and B, are homologous and share a "generic LHC motif" constituted by an highly hydrophobic sequence that contains a glutamic acids involved in the chlorophylls (Chls) binding and in the stabilization of the folding through salt bridges with arginine in the other helix (Liu Z. et al. 2004, Bassi R. et al. 1999). This structure is conserved during the evolution and characterized LHCs among all photosynthetic eukaryotes (Green B.R and Pichersky E. 1994).

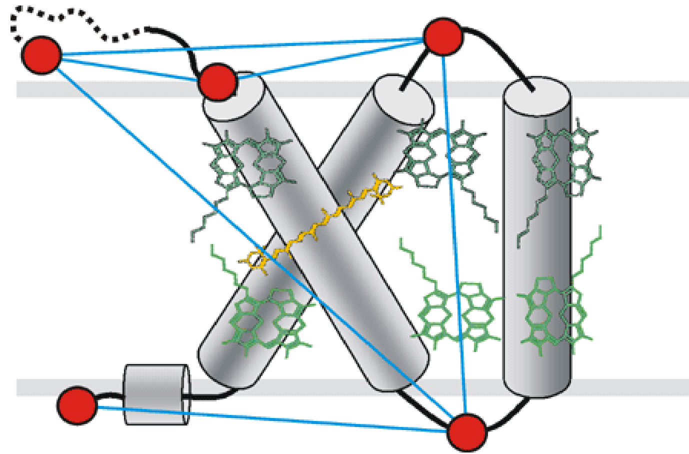


Fig 1.6 Structure of light harvesting protein. http://www.esr-group.org/conferences/Warwick/

Despite the fact that TMRs are conserved among photosynthetic organisms the specific binding site of pigments can be different thanks to the diverse pigment composition that LHCs members show; in fact LHCs comprehend several subfamilies that can be summarized by table 1.2.

Systematic name	Other names/subclades	Lineages
LHCA	LHC I	Plants
LHCB	LHC II, CP24,CP26,	Plants
LHCF	FCP, CAC	Haptophytes, heterokonts
LHCR	LhcaR	Rhodophytes
-	LhcZ	Cryptophytes, haptophytes, heterokonts
LHCSR	LhcX	Chlorarachniophytes, chlorophytes, fucoxanthin-containing dinoflagellates, haptophytes, heterokonts

Table 1.2: The systematic nomenclature was established by Jansson et al. 1999. The subfamily termed Lhcz does not have systematic names and were identified by Koziol et al. 2007 and Gagne and Guertin 1992, respectively. The genes in Lhca, Lhcb and related clades recently identified by Koziol et al. 2007 form a monophyletic outgroup. Adapted from Hoffmann et al. 2010

LHCA and LHCB include sequences only from the green lineage and contain the Chl *a/b* binding protein (CABs). LHCA and LHCB respectively indicate the antenna proteins bound to PSI and to PSII forming, respectiveley PSI and PSII supercomplex.

FCP or LHCF indicates the antenna proteins binding fucoxanthin, a xanthophyll found as an accessory pigment in the chloroplasts of brown algae and most other heterokonts, which is

responsible of their peculiar brown or olive-green color (Koziol A.G. et al. 2007, Neilson J.A.D. and Dunford D.G. 2010).
LHCR or LHCAR family comprehends antenna proteins found in red algae (Neilson J.A.D. and Dunford D.G. 2010 Pearson et al 2010 Koziol A.G et al. 2007) and in secondary endosymbiont originated like diatoms (Zhu S.H. and Green B.R. et al. 2008, Nymark M. et al. 2009); these antenna proteins bind Chl *a/c*, zeaxanthin and β-carotene (Wolfe G.R. et al. 1994).
LHCZ is a group originally highlighted by Koziol A.G. et al. 2007 and is composed of members from the cryptomonads, haptophytes, and chlorarachniophytes. It was named in absence of any indication on function or localization of this class of proteins.
LHCSR, also called LHCX in diatoms (Nymark M. et al. 2009), contains most of the stress-induced chlorophyll binding proteins (CPBs). In this family are also present the Li818 proteins that have been recently functionally characterized in *Chlamydomonas* and in *Physcomitrella*, with a function in Non Photochemical Quenching (NPQ)(Peers G. et al. 2009, Alboresi A. et al. 2010).
In *Nannochloropsis* the major antenna protein was identified as Violaxanthin Chlorophyll Binding protein (VCP) and characterized by Sukenik et al. 1992, and 2000: this protein binds only Chl *a* and violaxanthin. At present is not clear to which of the previously mentioned groups belongs VCP.

In green lineage the separation between antenna bound to PSI and to PSII is well established and also the different role of these two groups of antennae. PSII is more sensitive to photoinibition requiring an efficient mechanisms of repair (Nath K. et al. 2013) and its antenna complexes, which play a dual role, both light harvesting and photoprotection (Horton P. and Ruban A. V. 2005), have to be loosely bound to PSII in order to allow a easily and fast reparation. Instead PSI is less susceptible to light stress and does not undergo to a mechanism of repair and only recently photoprotection capacity of its antennae complexes were investigated (Alboresi A. et al. 2009). Differentiation between PSI and PSII antennae is not a peculiar trait of higher plants: thanks to the improvement in proteomic analysis, it was shown that red algae like *P. cruentum* (Wolfe G.R. et al. 1994) and *C. meriolae* (Bush A. et al. 2010) and diatoms like *C. meneghiniana* (Veith T. et al. 2009), *T. pseudonana* (Ikeda Y. et al. 2013) and *P. tricornutum* (Ikeda Y. et al. 2013, Lepetit B. et al. 2010, Veith T. and Buchel C. 2007) have specific antenna bound to PSI.

1.5 Photoprotection mechanism

Light is not constant in a natural environment, so from dawn to sunset photosynthetic organisms are exposed to continuous changes in irradiation conditions.

At early morning or if a cloud passes in front of the sun, photosynthetic organisms can be found in limiting light where all absorbed energy must be exploited for photochemistry. On the contrary, in other moments, the irradiation can be in excess with respect to the amount of energy used by photochemistry. In these conditions the photon absorption causes an accumulation of excitation energy in the antennae protein in the form of singlet state excitation of Chl molecules ($^1Chl^*$). These conditions can drive to the formation of triplet Chls ($^3Chl^*$) by intersystem crossing (Fig. 1.7). Chl triplets are stable enough to react with molecular oxygen O_2 forming singlet oxygen ($^1O_2^*$) and other reactive oxygen species (ROS). These molecules are harmful since they can oxidize protein, lipids, pigment inside chloroplast causing photo-oxidative damage and eventually cell death.

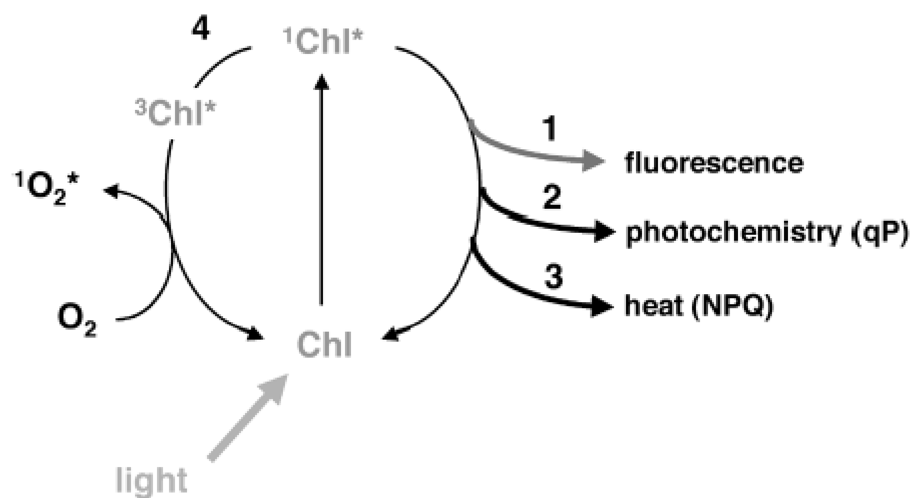


Fig. 1.7: $^1Chl^*$ singlet excited state has several ways to relax to the ground state. It can relax as fluorescence (1). Its excitation can be used for photosynthetic reactions (2), or it can de-excite by dissipating heat (3); By intersystem crossing, $^1Chl^*$ produces $^3Chl^*$ (4),which in turn is able to produce $^1O_2^*$, a very reactive oxygen species (Muller P. et al. 1992).

Photosynthetic organisms evolved different photoprotection mechanism to avoid the formation of reactive oxygen species. For instance carotenoids, bound to photosystems, constitutively protect the photosynthetic apparatus from excess energy by scavenging Chl triplets and ROS eventually formed. Other photoprotection mechanisms are instead activated in response to exposition to strong irradiation: this response is highly complex and various components can be distinguished based on their activation time scales (Niyogi K. K., 2000).

The fastest one is called NPQ in which excess of light energy can be eliminated by thermal dissipation by de-excitation of $^{1}Chl^{*}$. It is measured by quantifying light induced quenching of Chl fluorescence (Demmig-Adams, B. et al. 1995, Niyogi, K. K. 1999). NPQ is a complex phenomenon and involves several components: qE (Energy-dependent quenching) which is the fastest one, qI (as photoInibitory quenching) which relaxes in hours (Niyogi, K. K. 2000, Muller, P. et al. 2001).

1.5.1 The Xanthophylls cycle

Carotenoids are present in all living organisms and show a role protecting them from reactive oxidative stress: lutein and zeaxanthin can be found, for instance, also in the human eye, where they are involved in the protection of the retinal cells from light (Roberts, R. L. et al. 2009). For photosynthetic organisms such a protection is fundamental since pigments light harvesting are connected to the formation of excited states and ROS.

Carotenoid composition is not constant and the exposition to strong irradiation leads to an alteration of chloroplast pigments, thanks to the activation of xanthophyll cycle.

In this cycle the diepoxide xanthophyll violaxanthin is reversibly converted to the epoxide-free zeaxanthin under the action of the enzyme Violaxanthin De-Epoxidase (VDE). Anteraxanthin is the intermediate step of the reaction which is normally not accumulated *in vivo*. Ascorbate supplies the reducing power required for the reaction.

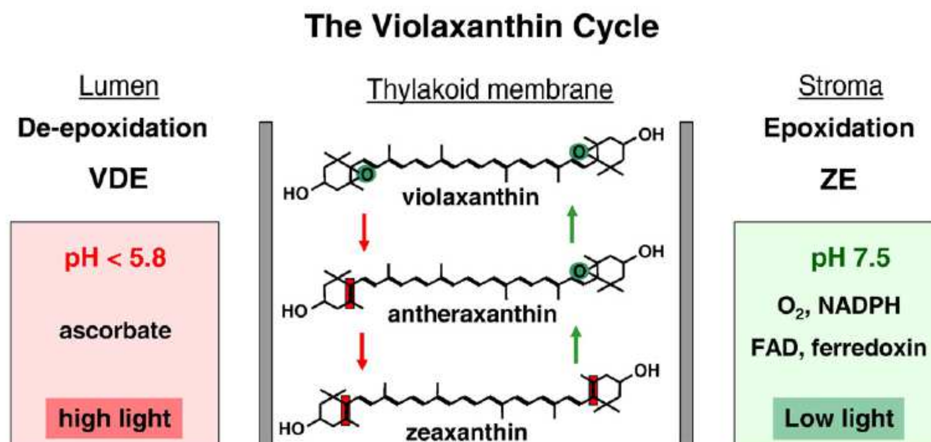


Fig 1.8: The xanthophylls cycle (from Jahns P. et al. 2009)

The xanthophylls cycle is one of the most important mechanisms to protect photosynthetic apparatus from high light: zeaxanthin increases the photoprotection capacity since it enhances

quenching of Chl excited states (both singlet and triplets) as well as acts as antioxidant for the scavenging of ROS in the membranes to avoid lipids peroxidation (Arnoux P. et al. 2009, Holt N. E. et al. 2005, Havaux M. and Niyogi K. K. 1999, Jahns P. et al. 2009). In low light conditions zeaxanthin is back converted to violaxanthin by a stromal enzyme, Zeaxanthin Epoxidase (ZE). Violaxanthin with respect to zeaxanthin increases light harvesting efficiency, an important ability when solar radiation is limiting. Regulation of the cycle is thus fundamental to dissipate energy only when this is in excess.

All photosynthetic eukaryotes show the presence of this cycle, although with different features depending on the organism. Three different violaxanthin cycles have been described in literature: the violaxanthin cycle (in all plants and green algae) (Siefermann-Harms, D. 1985), the diadinoxanthin cycle (in diatoms) (Hager A. and Stransky H. 1970) and the lutein-epoxide cycle (García-Plazaola J.I et al. 2007). In *Nannochloropsis* evidences of a xanthophyll cycle were described in (Gentile M.P and Blanch H.W. et al 2001) and recently the genome annotation confirms the presence of both ZE and VDE, with the latter showing two isoforms (Corteggiani Carpinelli E. et al. 2013).

VDE is a soluble monomeric enzyme belonging to the multigenic protein family called lipocalins whose members show a conserved structural organization with an 8 strands β-barrel and often bind little hydrophobic molecules (Hieber A.D. et al. 2002). VDE has two other domains with an unclear homology to any other known protein, and with a particular amino acid composition: the N-terminal domains is enriched with cysteines while the C-terminal domain is glutamates enriched (Hieber A.D. et al. 2002, Bugos R.C. and Yamamoto H.Y. 1996). The VDE lipocalin domain structure was recently crystallized both at acidic and neutral pH (Arnoux P. et al. 2009), and the results show that VDE undergoes to a pH dependent conformational change associated with protein activation. Analysis on the structure at pH 5 suggest that VDE dimerizes when active and binds to the membrane (Arnoux, P. et al. 2009, Saga, G. et al. 2010), probably in a regions enriched in a particular lipid called MGDG which forms inverted hexagonal structures in water, structures required for VDE activity (Latowsky D. et al. 2004). The hypothesis of dimerization is also confirmed by Fufezan C. et al. (2012) who defined a model where four of the five potential residues, involved in the activation, form a cooperative effect (Pfündel E.E. and Dilley R.A.,1993) on the activation of the enzyme.

The key role of VDE and ZE is demonstrated in different studies: *Arabidopsis* mutants *npq1,* depleted in VDE, shows increased light induced PSII damage and lipid peroxidation (Havaux, M. and Niyogi, K. K. 1999, Niyogi, K. K. et al. 1998); on the contrary *Arabidopsis* mutants *npq2,* depleted in ZE and with a constitutive high level of zeaxanthin, evidences a faster NPQ activation and a slower relaxation respect to the WT, also in low light condition. This leads to a reduced growth under dim light because a considerable fraction of energy is continuously

dissipated instead of being used to sustain growth (Dall'Osto, L. et al. 2005, Niyogi, K. K. et al. 1998).

These examples underline the importance of a precise regulation of the xanthophyll cycle, regulation that assumes a primary role in the case of an algae photobioreactor where the dissipation of energy and photodamage can affect considerably the productivity.

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CHAPTER 2

OPTIMIZATION OF LIGHT USE EFFICIENCY FOR BIOFUELS PRODUCTION IN ALGAE

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OPTIMIZATION OF LIGHT USE EFFICIENCY FOR BIOFUELS PRODUCTION IN ALGAE

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Highlights

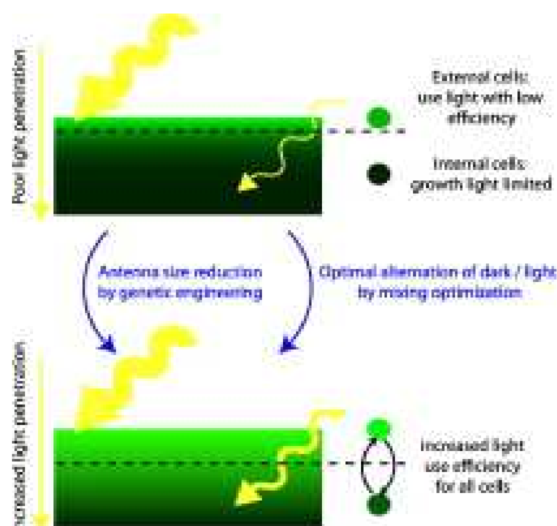
- Algae have interesting potential for the production of biofuels.
- Light use efficiency is one of the major factors influencing algae productivity.
- Investigation of molecular bases influencing photochemical efficiency is seminal to optimize algae productivity.
- Productivity can be improved by genetic engineering and optimization of photobioreactors operational parameters.

Abstract

A major challenge for next decades is development of competitive renewable energy sources, highly needed to compensate fossil fuels reserves and reduce greenhouse gas emissions. Among different possibilities, which are currently under investigation, there is the exploitation of unicellular algae for production of biofuels and biodiesel in particular. Some algae species have the ability of accumulating large amount of lipids within their cells which can be exploited as feedstock for the production of biodiesel. Strong research efforts are however still needed to fulfill this potential and optimize cultivation systems and biomass harvesting.

Light provides the energy supporting algae growth and available radiation must be exploited with the highest possible efficiency to optimize productivity and make microalgae large scale cultivation energetically and economically sustainable. Investigation of the molecular bases influencing light use efficiency is thus seminal for the success of this biotechnology. In this work factors influencing light use efficiency in algal biomass production are reviewed, focusing on how algae genetic engineering and control of light environment within photobioreactors can improve the productivity of large scale cultivation systems.

Graphical abstract



Keywords: Microalgae biofuels, *Nannochloropsis*; Photosynthesis; Photobioreactor; Pulsed light; Algae genetic engineering

Introduction

The largest fraction of world energy demand is presently met by the combustion of coal, oil and natural gas. Such a massive exploitation of fossil fuels leads to the release of large amounts of carbon dioxide and other pollutants in the atmosphere with detrimental effects on the environment. Also, because of this massive consumption, global reserves will be depleted in the future. It is thus evident that there is a strong need of alternative, renewable and environmentally compatible sources of energy in order to sustain our present lifestyle [1]. Among different possibilities, photosynthetic organisms are receiving growing attention for their potential exploitation in the production of biofuels [2], [3], [4], [5] and [6]. These bio-derived compounds in fact represent one of the most promising sources of liquid fuels, which are extensively used for transportation and in some cases, such as for jets, are not replaceable by electricity with the present technology.

In this direction, a major potential alternative to fossil fuels for transportation is biodiesel which can be produced from vegetal oil through a process of trans-esterification. Biodiesel production on a large scale, however, is at present strongly limited by the feedstock supply. Nowadays, most

biodiesel is produced from oils extracted from crops like soy and palm, which have a limited productivity and would demand unrealistic areas of cultivation in order to replace a substantial fraction of fossil fuels [7] and [8]. A further critical point is that crop plants are normally employed as food or feed and their exploitation for biodiesel will generate an undesirable competition for cultivation areas and fresh water [9].

One interesting alternative to crops is the exploitation of other photosynthetic organisms such as microalgae, which are capable of accumulating large amounts of lipids which can be extracted, processed and refined into transportation fuels [4] and [6]. Algae also have additional interesting features such as the ability to efficiently use CO_2[4] and, at least for some species, fast growth rate [10], [11], [12], [13] and [14]. The production of biofuels can also be combined with the use of algal systems for wastewater treatment to reduce the carbon, nitrogen and phosphorus content in industrial, municipal and agriculture wastes [15] and [16]. Furthermore, microalgae-derived high added value molecules can be used in the cosmetic or food industry such as astaxanthin, β-carotene, omega-3-fatty acids, vitamin E and other pigments [16], [17], [18] and [19].

While it is thus clear in the scientific community that algae are highly promising for biofuel production and other applications, intensive research efforts are still needed to exploit their potential in large scale cultivation systems [5], [6] and [20]. Many factors influence algae growth and productivity and deeper investigations are necessary to optimize operating parameters in large scale algae cultivation systems (photobioreactors, PBRs) and maximize their productivity (for a comprehensive review see [4] and [21]). One of the major factors affecting algae growth is light: as for all photosynthetic organisms, sunlight provides the energy supporting their metabolism and its efficient conversion into biomass has a major influence on productivity. The importance of this parameter is exemplified in Fig. 1, where the area needed to produce a ton of biomass per year is represented depending on the energy conversion efficiency. For values as low as 0.1%, the average value for most crop plants in field conditions [22], the requested area is very large, while this is drastically reduced if photosynthetic efficiency reaches 3%, a value experimentally obtained with algae in laboratory conditions [23]. Possible improvements could eventually further increase the biomass productivity, closing the gap with the maximal theoretical efficiency (77 ± 5 g dw m^{-2} d^{-1}, corresponding to ≈ 12% efficiency [24]).

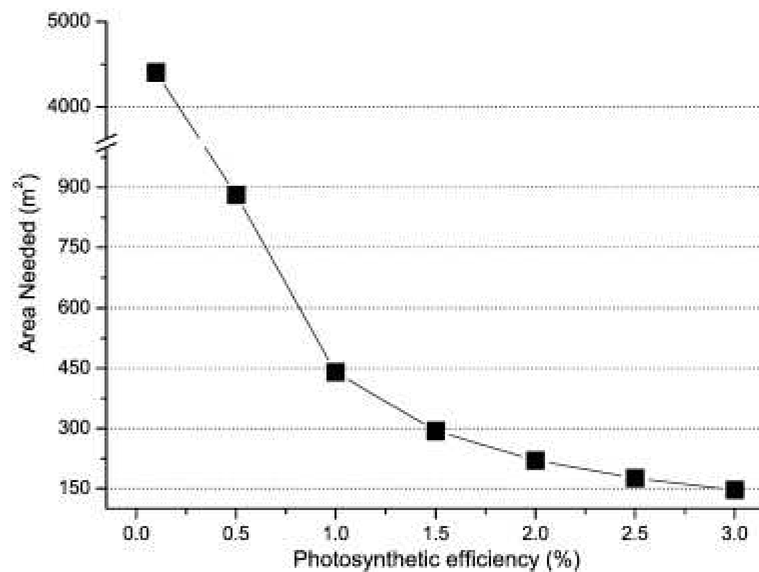


Fig. 2.1 Estimation of area needed for alga production. The area occupied by an alga cultivation system producing 1 ton of dry biomass per year is shown in dependence of the light use efficiency. Average radiation intensity was assumed to be 4541 MJ m^{-2} y^{-1} (data for Padova, Italy, according to Photovoltaic Geographical Information System, PVGIS Solar Irradiation Data, 2007, http://sunbird.jrc.it/pvgis/) and biomass energy content was assumed to be 20 kJ/g.

It should be underlined that, while crop plants are routinely cultivated in large areas, algae are cultivated in photobioreactors or ponds which have high energetic and monetary cost for building and maintenance. Therefore any increase in the area occupied by the alga cultivation makes the process less sustainable from the energetic and economic point of view. Therefore, a high photosynthetic efficiency is indispensable for a viable algae large scale cultivation system, even more than for crop plants. For this reason a deeper understanding of the molecular bases of the light use efficiency for these organisms is seminal to optimize their cultivation on a large scale and will be the focus of the present work.

Although it is unlikely that a single species will have all the optimal characteristics for biodiesel production in all conditions, the species belonging to the genus *Nannochloropsis* are receiving increasing attention for this kind of applications. In fact, they present several positive features such as good growth rates and the ability to accumulate large amounts of lipids, up to 60% of total dry weight [14], [25] and [26]. Recent availability of genome sequences and tools for their molecular modification is also contributing to make this species a model for the study of biofuel production from algae [27] and [28], complementing the studies on other model organisms such as the green alga *Chlamydomonas reinhardtii* which is better characterized but less efficient as lipids producer. For this reason, *Nannochloropsis* and *Chlamydomonas* will be used here as the main reference species, although major conclusions are most likely valid for other species as well.

Influence of light intensity on photosynthetic efficiency

Algae grown in large scale cultivation systems, such as PBRs, are exposed to a complex light environment. First of all sunlight is not constant but its intensity continuously changes during the day and the seasons. Illumination intensity has an important influence on alga productivity, as shown in Fig. 2.2 for the case of *Nannochloropsis salina*: up to 150 μmol of photons $m^{-2} s^{-1}$ an increase in illumination stimulates growth, showing that, in this range of intensities, available light is the limiting factor. Once this limit is surpassed, however, growth is not stimulated anymore by an increase in light intensity but, on the contrary, it has an inhibitory effect, causing reduction in duplication rate [29]. It is important to underline that, in the experiments reported here, *Nannochloropsis* cells were cultivated in a flat-bed PBR in order to expose all cells to the same irradiation, reducing as much as possible the cells' self-shading. Also, carbon dioxide and nutrients were provided in excess to highlight the influence of light regime on growth kinetics.

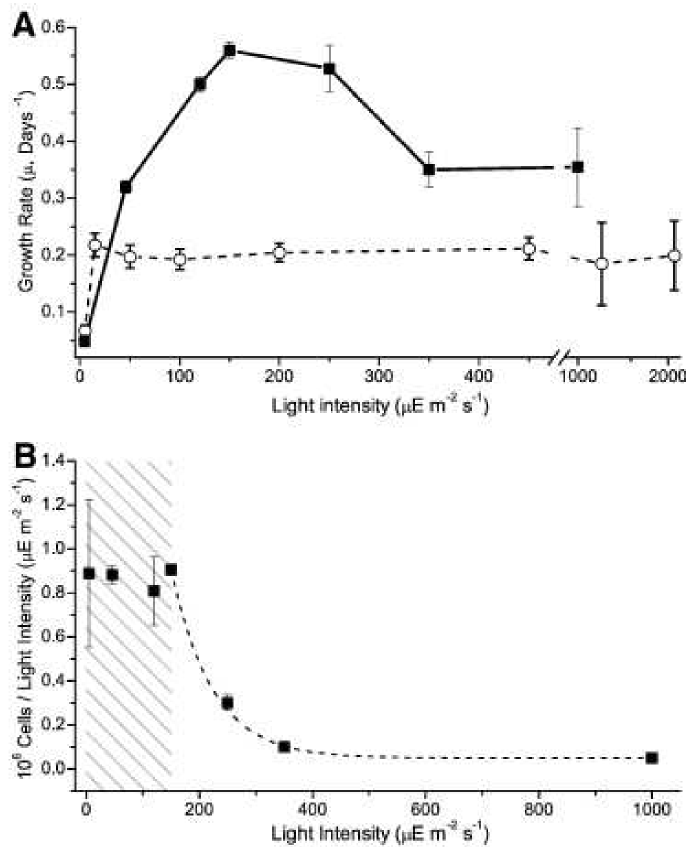


Fig. 2.2. Influence of light intensity on algae growth. A) *Nannochloropsis* growth, quantified as the specific growth rate calculated during the exponential phase under constant illumination is shown in dependence from light intensity (black squares). Light energy available increases linearly with the illumination intensity, as represented by the dotted line. CO_2 and nitrogen (as nitrate) were provided in excess to avoid growth limitation due to these nutrients and highlighting the influence of the light regime. B) cell concentration normalized to the light intensity, expressed as μE (μmol of photons) $m^{-2} s^{-1}$. Data reported are from [29].

Similar experiments, performed at atmospheric CO_2 concentration, showed different results and light was limiting in a much smaller interval, only below 15 μmol of photons m^{-2} s^{-1}[30]. In these conditions, irradiation between 15 and 150 μmol of photons m^{-2} s^{-1} has little influence on duplication rates suggesting that, in this case, growth is limited by CO_2 supply (Fig. 2.2). The importance of this key substrate for algae growth is well established and in fact all large scale PBRs are normally designed to provide cells with additional CO_2 supply. Actually, the ability of algae to exploit high carbon dioxide supplies represents a major advantage of these organisms and opens the possibility of cultivating algae in connection with industrial processes which produce large amounts of CO_2. Such a combination, in fact, while providing a low cost CO_2 source for algae cultivation allows fixing part of it into biomass, thereby reducing emissions in the atmosphere.

For the above mentioned reasons, the influence of light regime on algae performances in a large scale PBR should be studied under conditions where CO_2 is in excess. When the effect of different light intensities is considered, it is important to underline that the amount of energy available grows linearly with the radiation intensity. Algae are highly efficient in harvesting light and even when exposed to a strong irradiation the culture still absorbs most of the available photons, meaning that the energy absorbed by the cells is also following a similar linear growth. Over 150 μE m^{-2} s^{-1}, however, an increase in the light available to the cells does not correspond to faster growth, implying that cells absorb energy in excess which they cannot use for biomass accumulation. This difference can be visualized by normalizing the growth to light intensity which allows an estimation of light use efficiency of the cultures. As shown in the first part of the curve in Fig. 2.2B, up to 150 μmol of photons m^{-2} s^{-1} this value is roughly constant, suggesting that in this range cells use light with a similar efficiency. When light intensity is over the maximal growth value, however, light use efficiency rapidly decreases. As an example, a comparison of data from 120 vs. 250 μmol of photons m^{-2} s^{-1} shows that while growth rate is similar, light use efficiency in the latter is already ≈ 50% less [29]. While data shown are referred to a specific set of experiments, a similar trend is observed for *Nannochloropsis* grown in cultivation systems with different geometries [31] or for other algae species [32], [33], [34], [35] and [36], suggesting that the conclusions can be generalized.

Data reported above clearly show that light intensities over the saturation limit cause a drastic decrease in light use efficiency. Even if cells are still able to maintain a significant growth also under very intense illumination [29] and [30], in these conditions cells are highly inefficient in converting light into biomass. It is worth underlining that, while from the biological point of view there is no harm in using inefficiently an abundant resource, from the perspective of an alga large scale cultivation system any decrease in light use efficiency has a detrimental effect on system productivity, as shown in Fig. 2.2.

In order to devise strategies to keep algae productivity under a wider range of illumination, it is important to understand the molecular mechanisms responsible for the drop in light use efficiency under strong illumination. In photosynthetic eukaryotes most light is absorbed by pigments bound a family of proteins called antenna or light harvesting complex (LHC, [37]). Absorbed energy in the form of electronic excitation is transferred between nearby pigments and is eventually trapped by the special chlorophyll *a* contained in the reaction centers (RC) of the photosystems (PS). Here, electronic excitation drives a charge separation with one electron being transferred from the excited Chl *a* (Chl *a**) to a nearby acceptor molecule starting the electron transport chain which leads to ATP and NADPH synthesis.

Although light is indispensable to support algae metabolism, it may also become dangerous when in excess [32] and [33]. Light absorption and charge separation, in fact, take place in the presence of molecular oxygen. Toxic amount of ROS is formed in the thylakoid membrane when the absorption of light by chlorophylls exceeds the photosynthetic apparatus capacity of using excitation energy for electron transport, and photochemical reactions are saturated [38]. In particular, the very reactive singlet oxygen ($^1O_2$) can easily be created by light within the PSII complex in the presence of a photosensitizer such as chlorophyll, which is the main pigment of the photosynthetic apparatus [33] and [38]. Then, under conditions of intense illumination, excess energy leads to the production first of an increased amount of triplet excited state chlorophyll (^{3}Chl*) which in turn generates $^1O_2$[39] that can easily oxidize and degrade pigments, proteins and lipids.

This ROS production under strong illumination has been suggested to impair PSII efficiency by inducing the degradation of some components of this protein–pigment complex (see a recent review by [40]). In cells exposed to strong illumination, the Photosystem II protein subunit D1 is continuously degraded and re-synthesized [32], [41], [42] and [43]. Although the molecular mechanism of reparation in vivo is not completely clarified, the damaged D1 subunit appears to be first removed from a photoinactivated PSII center through the progressive action of FtsH proteases [44] and [45] which bind the N-terminus of damaged D1 to drive its removal from the Photosystem II and its subsequent complete degradation [46] and [47]. After the removal of damaged D1 a new copy of the polypeptide is synthesized and re-inserted in PSII [44]. This repair mechanism is found conserved in all organisms performing oxygenic photosynthesis, from cyanobacteria [44] to plants [48], indicating that it plays a fundamental role in protection from irreversible photoinhibition. It has been estimated that D1 turnover in cells under illumination is around 30 minutes [32], and considering the abundance of PSII complexes in alga cells this implies that a relevant part of energy is invested in resynthesizing this protein. Although these mechanisms are clearly important to ensure cells survival, in the context of alga biomass production such a massive turnover clearly impair the efficiency of light conversion into biomass [43].

An additional strategy to cope with a strong illumination is the thermal dissipation of part of the energy absorbed by the antenna so as to balance the light capture to the photochemical reactions rate. Antenna complexes are responsible for most of the light harvesting, but they are also involved in this dissipation of excess excitation [49] and [50]. Energy absorbed by LHC, in fact, can be dissipated as heat before it reaches the RC reducing the amount of Chl excited states and consequently decreasing the probability of reactive oxygen species generation. This process is called Non Photochemical Quenching (NPQ) and it can dissipate as heat up to 80% of the total absorbed energy [33]. The NPQ is activated by strong light and it is present in all photosynthetic organisms starting from cyanobacteria to land plants, although the molecular mechanisms are variable in different organisms [50].

The fastest component of NPQ is activated a few seconds after exposition to strong illumination, when the accumulation of protons in the thylakoids lumen causes the protonation of PSBS in plants and LHCSR in algae [50], [51], [52], [53], [54], [55] and [56]. When protonated, these proteins drive NPQ activation and the consequent decrease in excited state lifetime in pigment-binding subunits of the antenna system therefore reducing the possibility of generating high reactive singlet oxygen within PSII [57]. Carotenoids also play a major role in photoprotection and in all pigment–protein complexes of photosynthetic apparatus they are found close to chlorophylls, thus the sites for the potential production of triplet Chl and singlet oxygen. These carotenoid molecules act as quenchers for Chl excited states and also scavengers for reactive oxygen species eventually formed [58] and [59]. Carotenoids have different photo-protective abilities due to their specific chemical and structural properties, with zeaxanthin being particularly effective [58], [60] and [61]. After exposition to strong light, zeaxanthin is synthesized from violaxanthin thanks to the activity of the enzyme violaxanthin de-epoxidase (VDE). This enzyme, at neutral pH, is in its monomeric inactive form in the lumen but, in high light, when the pH of this compartment decreases, it dimerizes, associates to the thylakoid membrane and converts violaxanthin into zeaxanthin [62] and [63]. This conversion increases cells' ability to quench $^1Chl^*$, $^3Chl^*$ and $^1O_2$[58] and [61].

A further mechanism contributing to photo-protection is 'state transition' which consists in the migration of LHCII, the PSII major antenna complex, from PSII (state 1) toward PSI (state 2) in order to equilibrate an imbalance of light excitation in the two photosystems. LHCII migration from PSII to PSI is activated upon phosphorylation when PSII is saturated and PQ pool is over-reduced. This is a reversible phenomenon and LHCII can migrate back to PSII when the excitation energy balance between the two photosystems is restored [64]. Although state transitions are present in both green algae and plants, its physiological role seems to be more important in the former [64]. In fact, in *Chlamydomonas*, the ability of activating state transitions was shown to contribute to a better carbon assimilation and algal growth [65]. Also, it was recently demonstrated in *Chlamydomonas* that cells' exposure to high light induce a persistent state

2 which contributes to reducing PSII functional antenna size and consequently protects this photosystem from over-excitation and in particular decreases hydrogen peroxide formation [66].

All mentioned mechanisms allow cells to safely dissipate energy in excess, thereby reducing oxidative damage, so as to survive under intense illumination [51]. In the perspective of algae large scale cultivation, however, energy dissipation as heat still causes a reduction of light use efficiency and has therefore a negative effect on culture productivity. Energy dissipated for protection, as well that used to repair the photosynthetic apparatus, strongly reduces biomass productivity and should be minimized in a large scale cultivation system [24] and [42].

Increase of light use efficiency by algae genetic engineering

An additional factor to be considered is that algae are cultivated in photobioreactors at high concentration and, because of the pigments present in the cells, the medium has a high optical density. As a consequence, light distribution in the systems is highly inhomogeneous [67], [68] and [69] with the surface-exposed cells absorbing most of the available light, leaving only a residual part of the radiation for cells underneath (Fig. 2.3). For this reason, external layers are easily exposed to excess light and, as discussed above, to maintain their photosynthetic activity they need to dissipate energy and repair photoinhibited complexes with poor light use efficiency [24], [70] and [71] (Fig. 2.3). At the same time, most of the cells in the culture are instead exposed to a weak illumination limiting their growth. If light is below the compensation point, cells might even have a negative productivity since respiration can be faster than photosynthesis. The relevance of this inhomogeneous light distribution on algae cultivation productivity is underlined by the observation that the overall efficiency of photobioreactors increases when the light path through the culture is shorter, reducing the inhomogeneity of light distribution [67], [72], [73] and [74]. Unfortunately, short light paths are difficult to be implemented in large-scale structures because of practical and economic reasons.

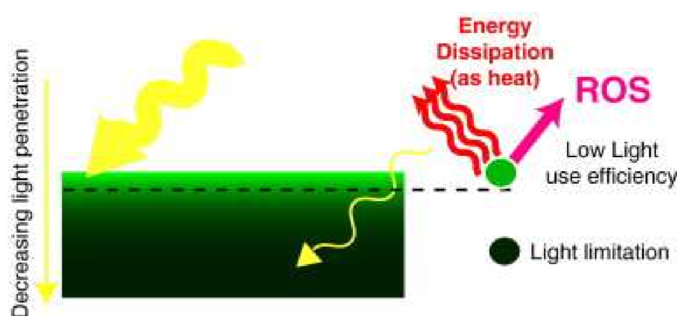


Fig. 2.3. Model of the light distribution in a photobioreactor. An algae photobioreactor has high optical density because of the high pigment concentration in the cells. A first layer of cells is thus exposed to full illumination and absorbs a major fraction of the light energy available. These cells likely have a saturated

photosynthesis, leading to energy dissipation as heat and ROS production. As a consequence they not only absorb a large fraction of light available but they also use it with low efficiency. The rest of the biomass is left exposed to limiting light.

One possibility to reduce this limitation and increase productivity in algae large scale outdoor cultivation systems is to genetically engineer these organisms and make them more suitable to grow in the light environment found in a photobioreactor. In a natural environment, cells need to harvest light efficiently to compete with others and have no evolutionary advantage in leaving radiation energy to underneath layers. On the contrary, in a photobioreactor each cell should ideally only harvest the amount of light it can efficiently use for photochemistry. Unfortunately even if algae are a very diverse group of organisms it is unlikely that species isolated from the environment can have all the ideal characteristics for large scale cultivation and therefore their genetic improvement will likely be fundamental to optimize productivity [27] and [75]. Wild type (WT) algae thus need to be "domesticated", similarly to what happened for crops where multiple traits which would have a negative effect in a natural environment were artificially selected because, in cultivated crops, they provided a positive influence on productivity.

One of the possible strategies under intense study to improve light distribution in a photobioreactor is to generate strains with a decreased photosystems antenna size [36]. As mentioned above, antennas (or LHC) are pigment binding proteins which harvest light and transfer energy to the reaction centers. These proteins are particularly important in a light limiting environment because they increase the cells' ability to efficiently harvest available radiation. They also bind most of the pigments in algae cells and therefore are the main responsible for the optical density of the culture. Algae cells normally have a large number of antenna proteins associated to both Photosystems I and II and, for instance for *Chlamydomonas*, it has been estimated that there are respectively 200–240 and 190–210 chlorophylls per reaction center [76], [77] and [78]. The presence of antenna proteins, however, is not necessary for electron transport reactions and their content can be reduced without affecting the photosystems' ability to perform photochemistry. It was estimated that the minimum number of Chl molecules is 37 for PSII and 95 for PSI, since below these limits the assembly of the photosystem core complexes is affected, impairing their photochemical activity [79]. However, these figures suggest that it is possible to strongly reduce the number of antenna proteins associated to each photosystem, which should have a positive effect on productivity in a photobioreactor by reducing the amount of pigments in each cell and improving light distribution, thereby increasing the energy available for all cells. Also cells with a small Chl antenna size would reach photosynthesis saturation at higher light intensity [71] reducing the amount of energy lost by cells at the surface of the photobioreactor.

However, as mentioned above, antenna proteins are not only involved in light harvesting but also play a fundamental role in the protection from high light. In fact, thermal dissipation of energy absorbed in excess (NPQ described above) requires antenna proteins to be activated and mutants

completely depleted of antenna proteins have been shown to be particularly sensitive to strong illumination. Thus, the desirable situation should not be a complete depletion of the antenna proteins but its selective reduction, which clearly requires a deeper knowledge of factor involved in photosystem biogenesis [80]. Following the domestication hypothesis, photosystems' antenna size must thus be re-optimized for photobioreactor conditions.

Different *C. reinhardtii* strains with reduced antenna size have already been isolated and characterized in the past few years using insertional mutagenesis and RNAi approaches [76], [77], [81], [82], [83], [84] and [85]. *tla1*[77], *tla2*[76] and *tla3*[85] are DNA insertional transformants carrying mutations in genes involved in the modulation of expression of genes encoding for light harvesting complexes. As a consequence, these mutants have a truncated light harvesting chlorophyll antenna size and show a lower chlorophyll per cell content with respect to the corresponding WT strains. One interesting consequence of the truncated antenna size is the difference in the saturation of photosynthesis which occurs in *tla1* mutant at about 2500 μmol of photons m^{-2} s^{-1} instead of 1000 μmol of photons m^{-2} s^{-1} measured for WT [76] and [85].

Mutants with reduced antenna size were also obtained by RNA interference, exploiting the similarity between different LHC proteins to ensure the simultaneous down regulation of multiple genes. One line developed with this approach, called *StmLR3*[82], presents reduced levels of both LHCI and LHCII mRNAs and proteins. *StmLR3* shows a higher photosynthetic quantum yield and a reduced sensitivity to photo-inhibition which led to a faster cell growth under strong illumination. In fact, under illumination at 1000 μmol of photons m^{-2} s^{-1}, *Stm3LR3* cultures reached peak density already after 26.5 h when WT cultures only were at 54% of their maximal cell densities.

In conclusion, these works showed that the advantage of mutants with reduced antenna size is twofold. On one side, cells with truncated antenna, when exposed to strong irradiation, harvest light less efficiently, reducing the damage on the photosynthetic apparatus and the need to thermally dissipate absorbed energy. On the other side, light absorption by single cells in a mass culture is minimized allowing a better transmission in the culture thereby increasing overall photosynthesis and biomass accumulation. Both contributions yield into a higher productivity which for Tla1 at 1500 μmol of photons m^{-2} s^{-1} was estimated to be twice that of WT [77]. Although all present studies were performed with the model alga *C. reinhardtii*, it is expected that strains with a truncated antenna isolated for other species, more suitable for industrial applications, will yield similar advantages.

Light use efficiency increase through optimal alternation of dark/light cycles

Considering data reported in Fig. 2.2, a possible solution to grow algae with a good light use efficiency would be to cultivate them under light intensity below the saturation limit. However, illumination under a full sunlight in summer is over 2000 μmol of photons $m^{-2} s^{-1}$, suggesting that, for a large fraction of time, algae exposed to direct sunlight use energy with low efficiency, especially those located at the more external layers. However, this first layer of cells absorbs most of the available radiation and therefore this has a major negative effect for the overall productivity.

An additional factor of complexity to be also considered is that cells in a photobioreactor are actively mixed and move between dark and fully exposed regions of the photobioreactor [69]. The kinetics of mixing cycles vary greatly according to cultivation systems and change between a millisecond time-scale in closed tubular reactors or optical fiber-based photobioreactors to longer times by several order of magnitude in open ponds [69]. These dark/light cycles can strongly affect algae photosynthetic efficiency depending on the frequency and intensity of light flashes [86], [87], [88], [89], [90] and [91]. An example of their influence is shown in Fig. 2.4 where the growth of *N. salina* cells is reported when exposed to square-wave light/dark cycles to simulate mixing [29]. All experiments were performed providing the same total amount of photons, corresponding to 120 μmol of photons $m^{-2} s^{-1}$ of continuous illumination, to evidence differences in light use efficiency due to frequency and intensity of light pulses [29]. In some conditions, the growth rate corresponds to that of cells exposed to constant moderate light (120 μmol of photons $m^{-2} s^{-1}$, Fig. 2.4A), suggesting that, in these conditions, cells were able to completely integrate the light absorption, exploiting intense light pulses as well as continuous illumination [92], [93] and [94]. It is important to stress that this result implies that cells not only were able to avoid photo-oxidation damage under saturating flashes, but they were also able to use energy from pulses with the same efficiency as dim continuous light, even if they were 8 times over the saturation limit. Also, it is worth underlining that the intensity of the pulses was not affecting the results, at least in the range tested, since flashes of 350 and 1200 μmol of photons $m^{-2} s^{-1}$ were exploited with the same efficiency.

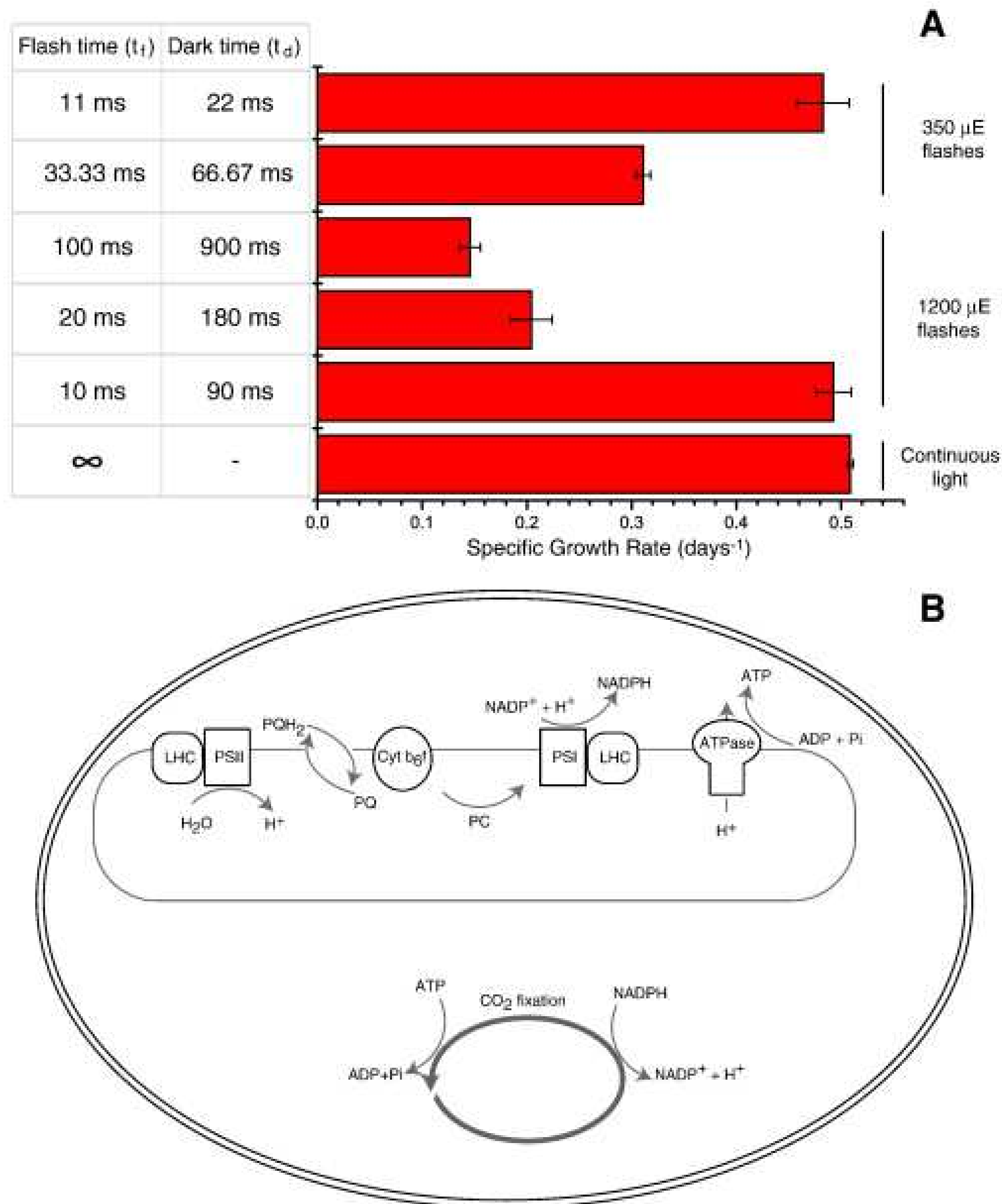


Fig. 2.4. Effect of pulsed light in algae growth. A) influence of alternation of light pulses and dark time on Nannochloropsis cell growth (data from [29]). All conditions provide a total amount of light corresponding to 120 µE m− 2 s− 1 of continuous light. CO2 and nitrogen (as nitrate) were provided in excess to avoid growth limitation due to these nutrients and highlighting the influence of the light regime. B) Schematic representation of a chloroplast and main reactions of photosynthesis. Above the thylakoid membranes is shown together with the four protein super-complexes involved in the light phase of photosynthesis, PSII, Cytb6f, PSI and ATPase. Some electron transport reactions are also indicated, water oxidation, diffusion of PQ between PSII and Cytb6f, plastocyanin (PC) reduction and oxidation and NADP+ reduction. Below is a schematic representation of Calvin–Benson cycle which consumes the products of light phase, ATP and NADP+, regenerating the required substrates.

The results with light flashes, however, were not always positive and in other cases growth was inhibited even if the integrated amount of photons provided was the same. The conditions

showing the best productivity (1200 μmol of photons $m^{-2} s^{-1}$ — 10 Hz and 350 μmol of photons $m^{-2} s^{-1}$ — 30 Hz) had in common the same length of the light pulse (Fig. 2.4), which thus appears to have a large influence on biomass productivity among the parameters considered here. The optimal duration of light pulses was found to be around 10 ms [29] while longer pulses were not efficiently exploited. Similar results have been described for other species of microalgae [89] and [95] and with the model green alga *C. reinhardtii* it was shown that the specific growth rate under flashing light increased with the rise in flash frequency. Algae grown at 1000 μmol of photons $m^{-2} s^{-1}$ — 100 Hz, again with 10 ms flash duration, presented similar growth rate and final biomass yield as the algae exposed to continuous 100 μmol of photons $m^{-2} s^{-1}$, confirming that the flash duration has a key influence on the light use efficiency [96] and [97]. These results suggest that a photobioreactor might well exploit with good efficiency even in highly intense light provided that mixing is optimized to that scope.

The timescale of the optimal duration of the light flashes, around 10 ms, is consistent with the suggested PSII turnover rate in whole cells Malcata F.X. 2011 and Dubinsky Z. et al. 1986, meaning that after charge separation by the photosystems, 10–15 ms are needed before the photosystem is ready to receive another photon [69]. In Fig. 2.4 a scheme providing a possible explanation for these observations is shown. One of the major rate limiting steps for photosynthesis is the Calvin–Benson cycle, which consumes ATP and NADPH produced by the light phase of photosynthesis for carbon fixation. Its activity has a fundamental influence also on the light phase because it re-generates the indispensable substrates ADP, Pi, and $NADP^+$. If light is too intense and Calvin–Benson cycle is not capable of fixing CO_2 at a sufficient rate, these substrates become limiting for the light phase of photosynthesis which, as a consequence, is not able to use all available energy for photochemistry, leading to the above discussed radiation damage and activation of heat dissipation mechanisms. In the case of experiments with pulsed light shown in Fig. 2.4A the total amount of energy provided is lower than the saturation point (120 vs. 150 μE $m^{-2} s^{-1}$) suggesting that the dark reactions should be able to use the energy with good efficiency and carbon fixation rate should not be limiting in these conditions.

Another limiting step for photosynthesis is electron transport between PSII and PSI via $Cytb_6f$ which requires the diffusion of plastoquinone in the membrane. PSII final acceptor is a plastoquinone molecule bound to Q_B site, which is reduced in around 1 ms. Once plastoquinol is formed, however, it must diffuse into the membrane and donate electrons to $Cytb_6f$ and another plastoquinone molecule has to take its place in the Q_B site. This step is known to be a rate limiting step for the light phase of photosynthesis and in fact under intense illumination PSII is saturated at the level of PQ pool. Under light flashes plastoquinone is reduced but, if light is switched off fast enough, it allows the time for re-oxidation of electron transporters, thus preparing the reaction centers for the following pulse. If the light exposure is longer, instead, it increases the probability that a second photon reaches the reaction center when this is still in the oxidized state, thus leading to the generation of ROS and photo-damage. If the illumination is

short enough, instead, not only the damage is reduced but also, since energy is "stored" as reduced PQ, the electron transport can proceed efficiently. In these conditions, the plastoquinone pool can act as a buffer which temporarily stores electrons allowing the efficient use of even very short light flashes.

These results suggest that very intense light can be harvested and exploited efficiently by cells growing in a photobioreactor, even if the total intensity is well beyond the saturation limit for that particular species. They also suggest that, provided that they are cultivated in optimal conditions, high photosynthetic efficiencies can be obtained also with algae growing in outdoor conditions exposed to very intense illuminations. However, for this to occur it is necessary that photobioreactor design is such that mixing is optimized and cells are exposed to short light pulses before moving back to the dark part of the photobioreactor. If this is possible, even very intense external light intensities could be harvested and used efficiently for photosynthesis.

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CHAPTER 3

CHARACTERIZATION OF THE PHOTOSYNTHETIC APPARATUS OF THE EUSTIGMATOPHYCEAN *NANNOCHLOROPSIS GADITANA*: EVIDENCE OF CONVERGENT EVOLUTION IN THE SUPRAMOLECULAR ORGANIZATION OF PHOTOSYSTEM I.

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Abstract

Nannochloropsis gaditana belongs to Eustigmatophyceae, a class of eukaryotic algae resulting from a secondary endosymbiotic event. Species of this class have been poorly characterized thus far but are now raising increasing interest in the scientific community because of their possible application in biofuel production.

Nannochloropsis species have a peculiar photosynthetic apparatus characterized by the presence of only chlorophyll *a*, with violaxanthin and vaucheriaxanthin esters as the most abundant carotenoids. In this study, the photosynthetic apparatus of this species was analyzed by purifying the thylakoids and isolating the different pigment-binding complexes upon mild solubilization. The results from the biochemical and spectroscopic characterization showed that the Photosystem II antenna is loosely bound to the reaction center, whereas the association is stronger in Photosystem I, with the antenna-reaction center super-complexes surviving purification. Such a supramolecular organization was found to be conserved in photosystem I from several other photosynthetic eukaryotes, even though these taxa are evolutionarily distant. A hypothesis on the possible selective advantage of different associations of the antenna complexes of photosystems I and II is discussed.

Introduction

Organisms that perform oxygenic photosynthesis are capable of converting light into chemical energy due to the activity of photosystem (PS[1]) I and PS II, two multi-protein supercomplexes located in the thylakoid membrane. In eukaryotes, both photosystems are composed of two moieties: (i) a core complex responsible for charge separation and electron transfer reactions and (ii) an antenna system with a role in light harvesting. The core complexes of both PSI and PSII are highly conserved among oxygenic photosynthetic organisms as a result of their common origin from an endosymbiosis event and strong selective pressure [1-3]. Conversely, antenna proteins show greater variability among different groups of organisms, possibly correlating with their colonization of different environments. In photosynthetic eukaryotes, the antenna system is mainly composed of membrane proteins belonging to the multigenic LHC (light-harvesting complex) family, which has diverged into different groups, such as the chlorophyll *a/b*-binding proteins found in *Viridiplantae* (LHCA/LHCB), fucoxanthin chlorophyll *a/c*-binding protein (called FCP or LHCF) in diatoms, LHCR in red algae/diatoms, and LHCSR/LHCX in diatoms and green algae [3-6]. All of these proteins share the same evolutionary origin and have a common structural organization, with three membrane-spanning regions connected by stroma- and lumen-exposed loops [6]. In the case of green algae and plants, it is well established that two distinct groups of proteins, LHCA and LHCB, are specifically associated with the two photosystems, PSI and PSII, respectively [7]. Some antenna proteins are also believed to be specifically associated with PSI or PSII in diatoms and red algae [4,8-14].

In addition to light harvesting, the different members of the multigenic family of LHC proteins are also involved in protection against excess illumination [5,15-18]. Indeed, LHC proteins are reportedly involved in several regulatory mechanisms in photosynthetic eukaryotes, including photosynthetic acclimation [19], state transition and heat dissipation of excess energy [20-21]. Considering this diversity in the function of antenna proteins, their investigation in different organisms should provide valuable information on their properties, roles, and adaptation to different ecological niches.

Nannochloropsis gaditana is a microalga belonging to the Eustigmatophyceae class within Heterokonta, which also includes diatoms and brown algae [22,23]. This group of algae originated from a secondary endosymbiotic event in which a eukaryotic host cell engulfed a red alga [1]. In the last few years, species belonging to the *Nannochloropsis* genus have gained increasing interest for their possible exploitation for biodiesel production due to their rapid growth rate and ability

[1]The abbreviations used are as follows: α(β)-DM, n-dodecyl-α(β)-D-maltopyranoside; Chl, chlorophyll; FCP, fucoxanthin chlorophyll-binding protein; LHC, light-harvesting complex; LHCA (B), light-harvesting complex of photosystem I (II) in plants and green algae; LHCR, red algal/cryptomonad LHCs; LHCF, fucoxanthin-LHCs; LHCSR, light-harvesting complex stress related; PSI (II), photosystem I (II); VCP violaxanthin–Chl *a*-binding protein.

to accumulate a large amount of lipids [24-27]. However, despite this growing attention, little molecular information is available on the photosynthetic apparatus of *Nannochloropsis* or other related species. *Nannochloropsis* species are known to have a unique property among Heterokonta of presenting only Chl *a* and lacking Chls *b* and *c* or any other accessory Chl [28-30], though, in the case of *N. gaditana*, a member of the LHC family was identified and named violaxanthin–Chl *a*-binding protein (VCP) because of its high violaxanthin content [31-33]. The carotenoid composition in *Nannochloropsis* is also atypical, with violaxanthin and vaucheriaxanthin esters as the most abundant species [34]. Therefore, the photosynthetic apparatus of these algae presents distinct features with respect to other Heterokonta, and its characterization can contribute to a better understanding of LHC variability in different photosynthetic organisms.

Accordingly, the aim of this work was to isolate and characterize the pigment-protein complexes comprising the *N. gaditana* photosynthetic apparatus. The results show a different association of antenna proteins to photosystems I and II, with the former stably associated with the core complex and the interaction being easily disrupted upon detergent treatment in the latter. A comparison with other photosynthetic eukaryotes showed that this stronger association of the PSI antenna with its reaction center is conserved and is likely a result of convergent evolution.

Materials and Methods

Cell growth

Nannochloropsis gaditana from the Culture Collection of Algae and Protozoa (CCAP), strain 849/5, was grown in sterile F/2 medium [35] using 32 g/l sea salts (Sigma-Aldrich), 40 mM TRIS-HCl (pH 8), and Guillard's (F/2) marine water enrichment solution (Sigma-Aldrich). The cells were grown with 100 µmoles of photons $m^{-2}s^{-1}$ (µE) of illumination and air enriched with 5% CO_2. The temperature was set at 22 ± 1°C.

Thylakoid isolation

After testing the different methods available in the literature, the isolation of thylakoid membranes was performed as follows. Cells in the exponential growth phase were harvested by 10 minutes of centrifugation at 4 °C with a Beckman Allegra 250 at 4000 x g, washed twice in B1 buffer (0.4 M NaCl, 2 mM $MgCl_2$, and 20 mM Tricine-KOH [pH 7.8]), and then split into 2-ml safe-lock capped tubes covering at a maximum ¼ of the tube. After centrifugation, a volume of glass beads (diameter of 150-212 µm) equal to the volume of the pellet and 150 µl of B1 with 0.5% milk powder and 1 mM PMSF, 1 mM DNP-ε-amino-n-caproic acid, and 1 mM

benzamidine were added, and cells were then disrupted using a Mini Bead Beater (Biospec Products) for 20 seconds at 3500 RPM. Immediately after rupture, 1 ml of B1 with 0.5% milk powder, 1 mM PMSF, 1 mM DNP-ε-amino-n-caproic acid, and 1 mM benzamidine was added to each tube, and the pellet was resuspended. The unbroken cells were then separated by a centrifugation step (2500 x g, 15 minutes, 4°C), and the supernatant was collected. The supernatant containing the broken cells and thus the thylakoids was centrifuged at 15000 x g for 20 min, and the pellet was washed twice with B2 buffer (0.15 M NaCl, 5 mM $MgCl_2$, and 20 mM Tricine-KOH [pH 7.8]). The thylakoids were resuspendend in B4 buffer (0.4 M sorbitol, 15 mM NaCl, 5 mM $MgCl_2$ and 10 mM HEPES-KOH [pH 7.5]), immediately frozen in liquid nitrogen and stored at -80°C until use. All steps were performed at 4°C and in dim light. The total pigments were extracted with 80% acetone, and the chlorophyll concentration of the samples was determined spectrophotometrically using specific extinction coefficients [36] and the acetone spectra fitting previously described [37], which were modified to account for the unusual pigment content.

Thylakoid solubilization and sucrose gradients.

Thylakoid membranes corresponding to 500 μg of Chl were washed with 50 mM EDTA and then solubilized for 20 minutes on ice in 1 ml of final 0.4% α-DM or 1% β-DM and 10 mM HEPES (pH 7.5) after vortexing for 1 min. The solubilized samples were centrifuged at 15000 x g for 20 min to eliminate any unsolubilized material, and the supernatant with the photosynthetic complexes was then fractionated by ultracentrifugation in a 0.1–1 M sucrose gradient containing 0.06% α-DM and 10 mM HEPES (pH 7.5) (280000 x g, 18 hours, 4°C). The green fractions of the sucrose gradient were then harvested with a syringe.

Spectroscopy

Absorption spectra were determined between 350 and 750 nm using a Cary Series 100 UV-VIS spectrophotometer (Agilent Technologies). The antenna absorption spectra were fitted with the spectra of the individual pigments in the protein matrix, as previously described for Chl red absorption [38] and for the Soret absorption region [39]. The 77 K fluorescence spectra between 650 and 800 nm were recorded in a buffer containing 60% w/v glycerol, 10 mM HEPES (pH 7.5), and 0.06% α-DM with an excitation at 440 nm (Luminescence Spectrometer LS 50, Perkin Elmer).

Pigment analysis

The chlorophyll and total carotenoids were extracted from the gradient fractions using 80% acetone, and the pigment content was determined by fitting the acetone spectra from 350 to 750 nm [37]; the content of individual carotenoids was determined using HPLC (Beckman System Gold), as described [40]. The peaks of each sample were identified through the retention time

and absorption spectrum [41]. The vaucheriaxanthin retention factor was estimated by correcting that of violaxanthin for their different absorption at 440 nm.

Electrophoresis and western blotting

A 12% SDS–PAGE analysis for both the sucrose gradient fractions and thylakoids extracts was performed using a TRIS–glycine buffer system, as described [42]. The samples were solubilized for 20 minutes at RT in 10% glycerol, 45 mM TRIS (pH 6.8), 0.03 M dithiothreitol, and 3% SDS. After solubilization, the samples were centrifuged for 15 minutes at 15000 x g, and the supernatant was loaded onto the gel. The gels were silver stained as previously described [43,44]. Western blot analyses were performed after transferring the proteins to nitrocellulose (Bio Trace, Pall Corporation). An anti-PsaA antibody (raised against the *Chlamydomonas reinhardtii* protein) was purchased from Agrisera. The antibody against D2 was generated by immunizing New Zealand rabbits with the spinach protein, whereas the recombinant protein was used for the antibody against VCP, which was obtained by cloning the cDNA (GenBank: U71602.1) into pETite N-HIS (Lucigen-Expresso T7 Cloning and Expression System), expressing the protein in *Escherichia coli* BL21 (DE3, Invitrogen), and purifying as inclusion bodies. Non-denaturing Deriphat-PAGE was performed as described in [45] by loading 3 μg of Chl relative to sucrose gradient green bands of PSI-LHC purified from *N. gaditana*, *C. reinhardtii*, and *Physcomitrella patens*.

Sequence analysis

The protein sequences and nomenclature of the light-harvesting protein from Phaeodactylum tricornutum and Thalassiosira pseudonana and Lhcx from C. reinhardtii were reported elsewhere [5], as were those from Arabidopsis thaliana and P. patens [45]. The light-harvesting protein sequences from N. gaditana [46] were retrieved from www.nannochloropsis.org. The sequences were aligned using the ClustalW algorithm in Bioedit 7.1.3.0. The phylogenetic trees were generated using Neighbor Joining and UPGMA, with 100 iterations, in CLC Sequence Viewer 6.9.

Results

Isolation of pigment-binding complexes from Nannochloropsis gaditana.

The first requirement for characterizing pigment-binding protein complexes from *Nannochloropsis gaditana* is the ability to isolate intact thylakoid membranes. The different protocols for algal thylakoids purification available in the literature were tested, and additional modifications were necessary to ensure the absence of residual intact cells or debris in the thylakoids preparation, which can impair efficient detergent solubilization. After isolation, the thylakoids were solubilized with mild detergents to further isolate the pigment-binding complexes in a state as close as possible to their native state in the membrane. Starting from the different methods described in the literature, multiple detergent and solubilization conditions were tested to select a combination (0.4% α-DM with 0.5 mg/ml Chl *a*) that yielded good solubilization using the smallest possible amount of detergent to ensure minimal alteration of the protein properties.

After solubilization, the different pigment-binding complexes were separated by ultracentrifugation in a sucrose gradient. Five different fractions were separated with distinct migration rates in the sucrose gradient (fractions F1-5 in Figure 3.1A), with a sixth fraction collected as the pellet at the bottom of the tube. The sucrose gradient protein migration was compared to other species for which a good characterization of the photosynthetic apparatus was available: one diatom, *Phaeodactylum tricornutum*, and one plant, *Physcomitrella patens* (Figure 3.1A). The comparison allowed for a tentative identification of F1 as a free pigment, F2-F3 as monomeric-oligomeric antenna (LHC, hereafter called VCP) complexes, F4 as the PSII core complex, and F5 as a PSI supercomplex (PSI-LHC).

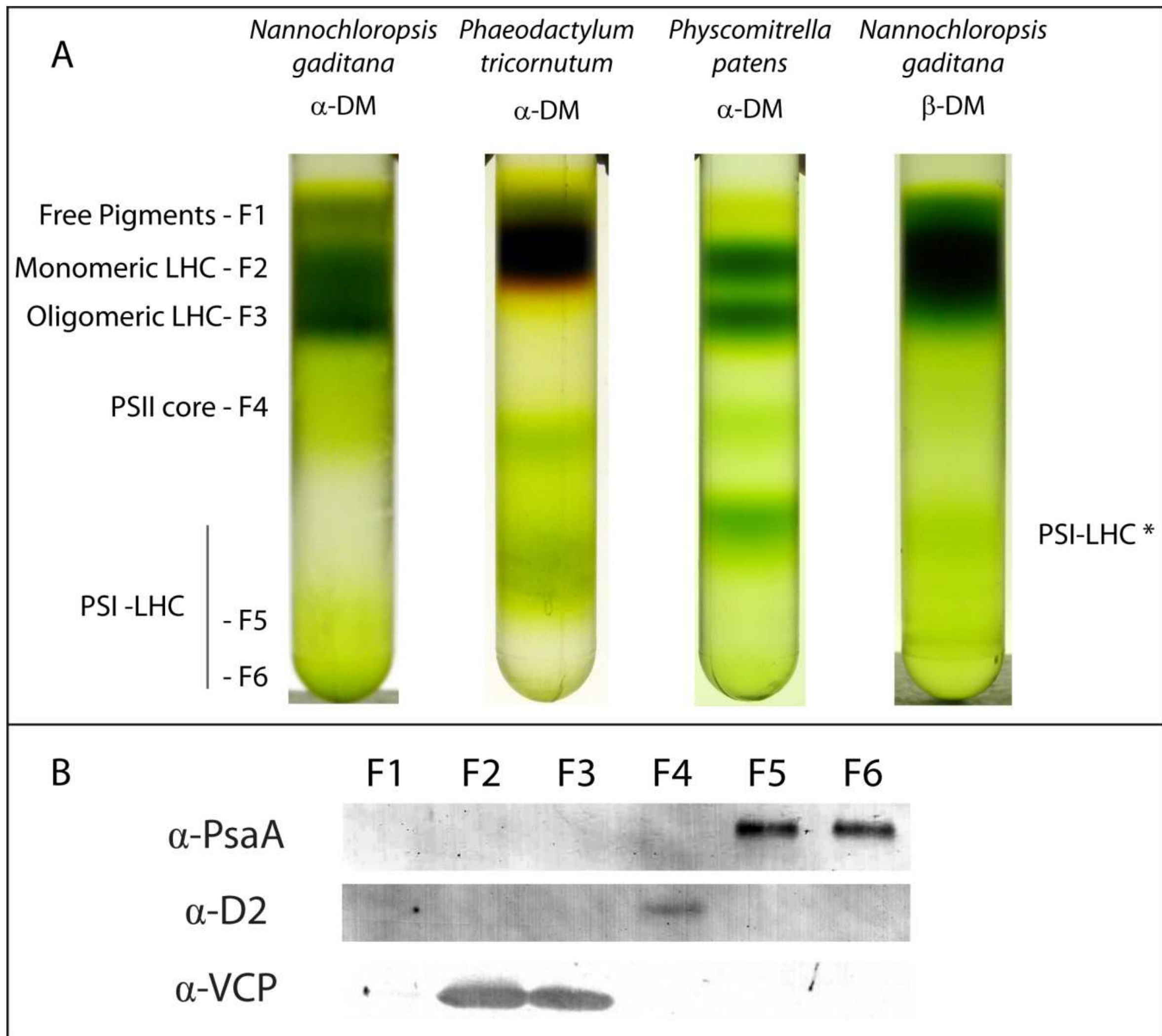

FIGURE 3.1. Isolation of pigment binding proteins from *Nannochloropsis gaditana* by sucrose gradient ultracentrifugation. A) *Nannochloropsis* sucrose gradient after mild solubilization with 0.4 % α-DM. Five distinct bands are distinguishable (F1-F5) while a sixth is present at the bottom of the tube (F6). Mobility of sucrose gradients after solubilization is compared with other species, a diatom (*Phaeodactylum tricornutum*) and a plant (*Physcomitrella patens*). Bands identification is reported according to mobility, western blotting and spectroscopic analysis. A similar sucrose gradient after 1% β-DM solubilization is also shown, which caused the appearance of a lighter PSI band (PSI-LHC*). B) western blotting analysis of bands protein composition using antibodies against subunits of the Photosystem I and II core complexes, PsaA D2 and VCP respectively. In order to assess the protein distribution in the gradient, equal volume of the bands (40 µl for PsaA and D2, 20 µl for VCP) was loaded for each band.

As shown by the sucrose gradient migration, the latter supercomplexes have an apparently larger size with respect to the PSI-LHC complexes of plants and diatoms. This result was confirmed by non-denaturing electrophoresis in which PSI-LHC from *Nannochloropsis* (F5) showed a migration similar to the *Chlamydomonas* complex, with a clearly larger size than that purified from plants (Figure 3.S1). This finding suggests that the number of antenna subunits associated with PSI in *Nannochloropsis* is closer to the nine of *Chlamydomonas* that the four of plants [47,48].

The identification of the sucrose gradient fractions was corroborated by other biochemical and spectroscopic evidence. An anti-VCP antibody showed a clear cross-reacting band at approximately 22 kDa in F2 and F3, consistent with the Coomassie-stained SDS–PAGE (Figure 3.2A), which showed a major band at this size, confirming the identification of F2 and F3 as antenna fractions. Interestingly, antibodies against LHC proteins from diatoms, green algae, or plants failed to recognize any band, likely because of the sequence variability among the different species. In contrast, antibodies against the core complex subunits of PSII (D2) and PSI (PsaA) were successful in identifying photosystem reaction center bands, even though they were produced using the plant/green algae isoforms, likely because of the conservation of these protein complexes among eukaryotes [49]. Western blotting showed that the PSII core subunits are indeed found only in F4, whereas the PSI core is present in both F5 and F6; thus, according to the results (Figure 3.1B), the latter contains PSI but not PSII particles. PSI from plants has previously been shown to form artificial detergent-induced aggregates upon α-DM solubilization [50], and our results may reflect a similar phenomenon. Nonetheless, it is possible that this fraction contains residual membrane particles that were not completely solubilized but enriched in PSI and depleted of PSII.

With the aim of eliminating these PSI aggregates, sucrose gradients were repeated after a stronger solubilization with 1% β-DM (Figure 3.1A). In this case, the pellet disappeared, and most PSI-LHC was found as a new fraction (PSI-LHC*) composed of smaller particles with lower mobility in sucrose gradients. The stronger solubilization also caused a dissociation of antenna oligomers, and all of the antenna proteins migrated in a single, thicker band.

SDS–PAGE of the PSI-LHC particles (from both solubilization) did not show any band with apparent weight of 22 kDa, as was the case for the monomeric/oligomeric VCP bands (Figure 3.2B). Bands attributable to the antenna proteins in the PSI particles were instead found at a lower apparent MW, approximately 20 kDa, similar to those observed in PSI from diatoms [9-11] (Figure 3.2B).

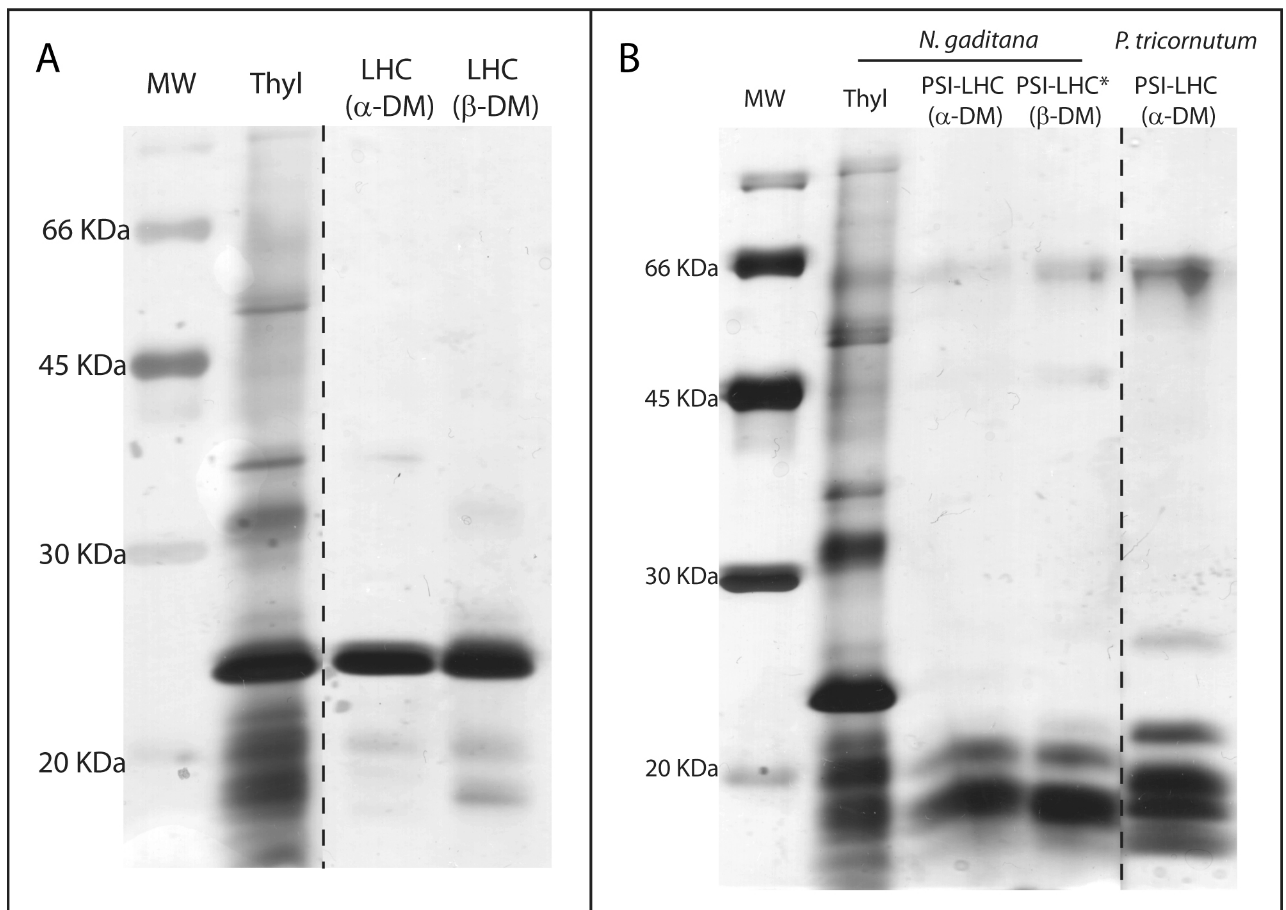

FIGURE 3.2. Analysis of sucrose bands polypeptide composition. A) SDS-PAGE of N*annochloropsis* thylakoids and antenna bands from α and β-DM gradients (F3 and F2 respectively). 4 and 2 Chl μg were loaded for thylakoids and LHC bands respectively. B) SDS-PAGE of PSI bands, comparing PSI from *Nannochloropsis* purified either form α and β-DM gradients and PSI from a diatom (*P. tricornutum*). 2 and 4 μg of Chl were loaded for PSI bands and thylakoids respectively.

Biochemical and spectroscopic characterization of different complexes

Absorption spectra of different sucrose gradient fractions can provide further information on purified pigment-binding complexes. As shown in Figure 3.3A, fraction F1 exhibited a maximum in the Qy region at 670 nm, which is typical for free Chl *a* in a detergent solution, supporting the identification of this band as free pigments liberated by the thylakoid solubilization. However, the Chl maximum in the monomeric and oligomeric antennas (F2 and F3) was at 675 nm, indicating that Chls are coordinated to a protein and therefore in a different electronic environment. Both the F2 and F3 bands show identical spectra, suggesting that oligomerization exerts little influence on pigment coordination. Band F4, identified by western blotting and migration in the sucrose gradient as consisting of PSII core complexes, showed spectra very similar to the analogous band from plants or diatoms (Figure 3.S2), in agreement with the strong conservation of this complex among different photosynthetic organisms. Bands F5 and F6, identified as the PSI-LHC

supercomplexes, showed the presence of a typical red-shifted absorption over 700 nm (Figure 3.3B); the spectra of these two bands are very similar, supporting the identification of F6 as PSI-LHC aggregates or PSI-enriched membrane particles.

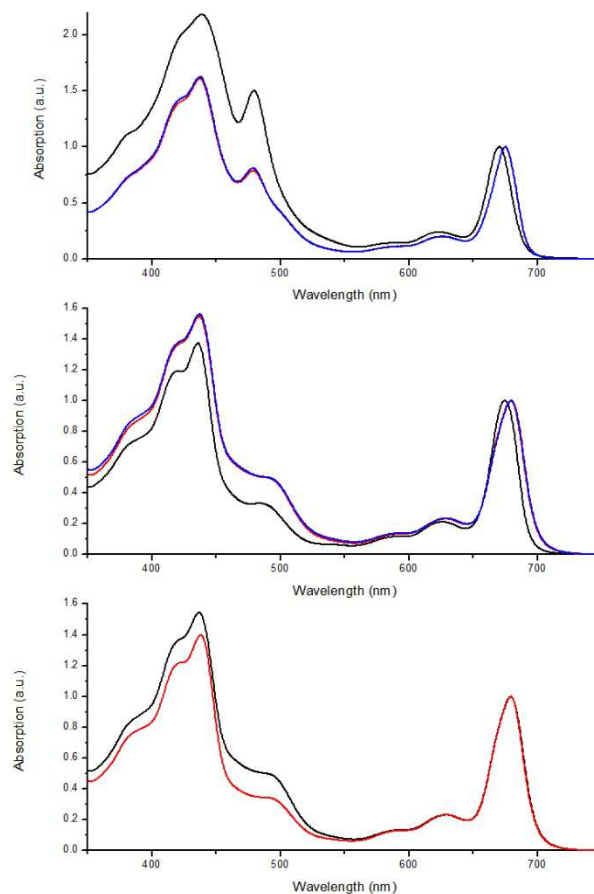

FIGURE 3.3. Absorption spectra of sucrose gradient bands. A) Free pigments (F1, black), monomeric and oligomeric antennas (F2, red and F3, blue). F1 has a maximum in the Qy region at 670, typical for free Chl *a* in a detergent solution, Chls maximum in monomeric and oligomeric antennas is at 675 nm. B) PSII core complex (F4, black), PSI-LHC (F5, red and F6, blue). PSI-LHC present a typical absorption over 700nm. C) comparison between PSI-LHC (black) and PSI-LHC* (from β-DM gradient, red). PSI-LHC* has a reduced xanthophyll contribution in the absorption spectra, indicating a smaller amount of antenna proteins associated with PSI. All spectra are normalized to the Chl *a* maximum in the red part of the absorption spectra.

The presence of antenna complexes in the different sucrose fractions of *Nannochloropsis* is not as easily detectable by the absorption spectra as it is in plants and diatoms for which they are marked by the signal of such accessory pigments as Chl *b* or *c*. However, the comparison of the F2 and F4 spectra with that of the PSII core complex (Figure 3.S2) shows that the antenna complexes display large signals from carotenoids in the 470-510 nm range. In the case of PSI-LHC* (fraction F5), an intermediate signal was observed in the same 470-510 nm range,

suggesting an association of PSI with the antenna complexes (Figure 3.3A and B), in agreement with its identification. In Figure 3.3C, the comparison between PSI-LHC purified with α- and β-DM solubilization reveals a reduced xanthophyll contribution in the absorption spectra of PSI-LHC*, indicating a lower amount of antenna proteins in the latter.

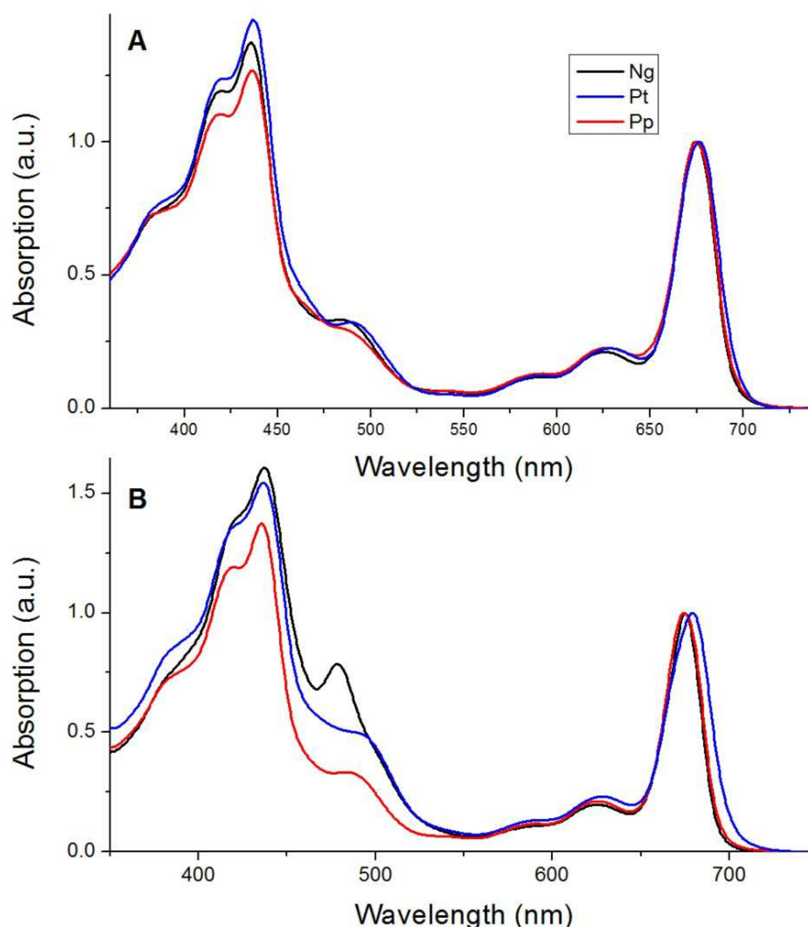

FIGURE 3.S2. Comparison of PSII core absorption spectra from different species. A) Sucrose gradient band corresponding to PSII core complex from *Nannochloropsis gaditana, Physcomitrella patens* and *Phaeodactylum tricornutum* respectively in black, red and blue. B) Comparison of absorption spectra of bands named F2, F4 and F5 containing respectively monomeric antennas, PSII core and PSI-LHC.

Nannochloropsis has a peculiar carotenoid composition, with violaxanthin, β-carotene, and vaucheriaxanthin as the major pigments (Table 3.1). The xanthophylls violaxanthin and vaucheriaxanthin were found to be associated with the antenna complexes (F2-F3), whereas β-carotene was strongly enriched in the PSII core. In contrast, PSI-LHC presents substantial amounts of both xanthophylls and β-carotene, confirming the presence of antenna complexes associated with the PSI core. PSI-LHC* purified with β-DMs still shows the presence of both xanthophylls and β-carotene, though the relative content of xanthophylls is lower, confirming

that a stronger detergent solubilization induces the dissociation of some antenna proteins from the supercomplex.

Fractions	Name	Violaxanthin	Vaucheriaxanthin	Anteraxanthin	Zeaxanthin	β-car	Chl / Car
F1 α-DM	*Free Pigments*	48.4 ± 5.8	19.8 ± 4.9	6.5 ± 2.2	7.7 ± 3.6	5.6 ± 2.3	1.1 ± 0.1
F2 α-DM	*Monomeric antenna*	28.1 ± 2.5	18.8 ± 2.5	4.2 ± 1.4	5.0 ± 2.2	< 1	1.8 ± 0.1
F3 α-DM	*Oligomeric antenna*	29.3 ± 3.8	18.2 ± 2.5	4.3 ± 1.4	4.9 ± 2.3	<1	1.7 ± 0.2
F4 α-DM	*PSII Core complex*	5.1 ± 1.2	1.2 ± 0.5	< 1	1.4 ± 0.5	12.8 ± 2.2	4.7 ± 0.7
F5 α-DM	*PSI-LHC*	14.2 ± 2.3	3.2 ± 0.9	2.8 ± 0.7	3.5 ± 1.5	10.8 ± 0.9	2.9 ± 0.1
F6 α-DM	*PSI-LHC*	14.2 ± 3.5	2.6 ± 0.3	2.8 ± 0.8	4.2 ± 1.8	11.1 ± 1.6	2.9 ± 0.3
F5 β-DM	*PSI-LHC**	7.2 ± 2.2	1.4 ± 0.6	< 1	1.3 ± 0.7	12.5 ± 2.1	4.3 ± 0.7

Table 1. Pigment data of sucrose gradient fractions. Bands content in the different carotenoids is reported expressed as mol/100 Chls. Values are reported as mean ± Standard Deviation, (n > 4 for α-DM samples and 3 for β-DM

Fluorescence spectra at 77 K are a valuable tool to highlight the presence of red-shifted Chls, which are typical of photosystem I. Indeed, Chls emitting at 720 nm were found in bands F5-F6, also confirming the presence of red-shifted forms in photosystem I of *N. gaditana.* However, PSI fluorescence in this species was not as red shifted as in higher plants, being more similar to what is observed in other algae, such as *Chlamydomonas* [47,51], also showing that this property can be variable between different organisms. No red-shifted Chls were found in the isolated LHCs (F2, F3), which showed a narrow emission peak at 683 nm, suggesting the presence of predominantly PSII antennas. It is worth emphasizing that a broad emission spectra was observed for PSI-LHC, with a clear contribution at approximately 675 nm. At low temperature, such an emission is only expected if some chlorophylls are unable to efficiently transfer excitation to the reaction center and red forms. The most likely explanation is that some antenna proteins were disconnected from PSI during the purification and therefore were impaired with regard to efficient transfer energy to the reaction center, as has also been observed in *Chlamydomonas* [47].

It is interesting to observe the same spectra from the β-DM solubilization (Figure 3.4B): the PSI-LHC* samples still showed a red-shifted emission, though the ratio between the peaks at 720 and 680 was decreased, with less red-shifted forms. Furthermore, we observed an alteration of the

fluorescence in the free antenna fraction (F2, Figure 3.4C), with an emerging red-shifted contribution at approximately 690 nm, suggesting some antenna disconnection from PSI-LHC by the stronger solubilization. Both these observations clearly indicate that some red-shifted chlorophylls are found associated with PSI antenna complexes in *Nannochloropsis*.

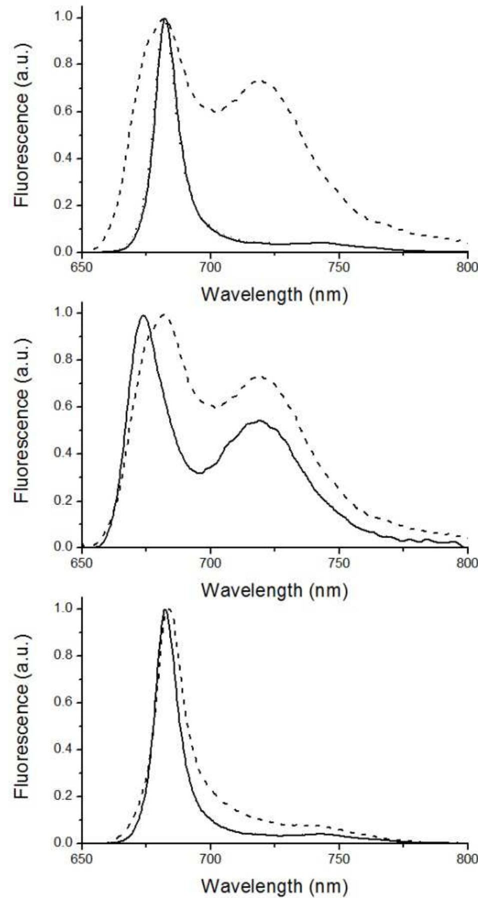

FIGURE 3.4. Fluorescence LT spectra. A) Comparison of 77 K Fluorescence spectra of monomeric and trimeric antennas (F2, solid, F3, dotted) and PSI-LHC (F5, dashed). Fluorescence at 720 nm, due to red shifted Chl is detected only in PSI-LHC. B) Spectra of PSI-LHC solubilized with α-DM (dashed) and β-DM (PSI-LHC*, solid), both show a fluorescence peak at 720 nm due to Red- shifted Chl. C) Spectra of monomeric antennas after α-DM and β-DM solubilization (F2, respectively in solid and dashed).

Discussion

Algae are a very diverse group of organisms, and their photosynthetic apparatus shows variable protein and pigment compositions. Exploration of this diversity provides valuable information on the structure and function of these pigment-binding complexes and on how their properties have adapted to different environmental niches. In the case of *Nannochloropsis* and other species with a potential application in biofuel production, a detailed characterization of the photosynthetic apparatus is also seminal for genetic engineering efforts aimed at optimizing the productivity of algal photobioreactors. In fact, the manipulation of the antenna complex content has been shown to improve the light-use efficiency of cultures, making these proteins a major target for genetic improvement efforts [52,53].

Antenna complexes with violaxanthin as a major carotenoid

According to the recently sequenced genome of *Nannochloropsis oceanica*, the antenna complexes of *Nannochloropsis* species belong to the LHCF, LHCR, and LHCSR/LHCX groups, similar to those found in diatoms ([54], Figure 3.S3). A notable difference with diatoms and any other known heterokonta, however, is that the *Nannochloropsis* antenna has the unusual property of binding only Chl *a*, with no accessory Chls.

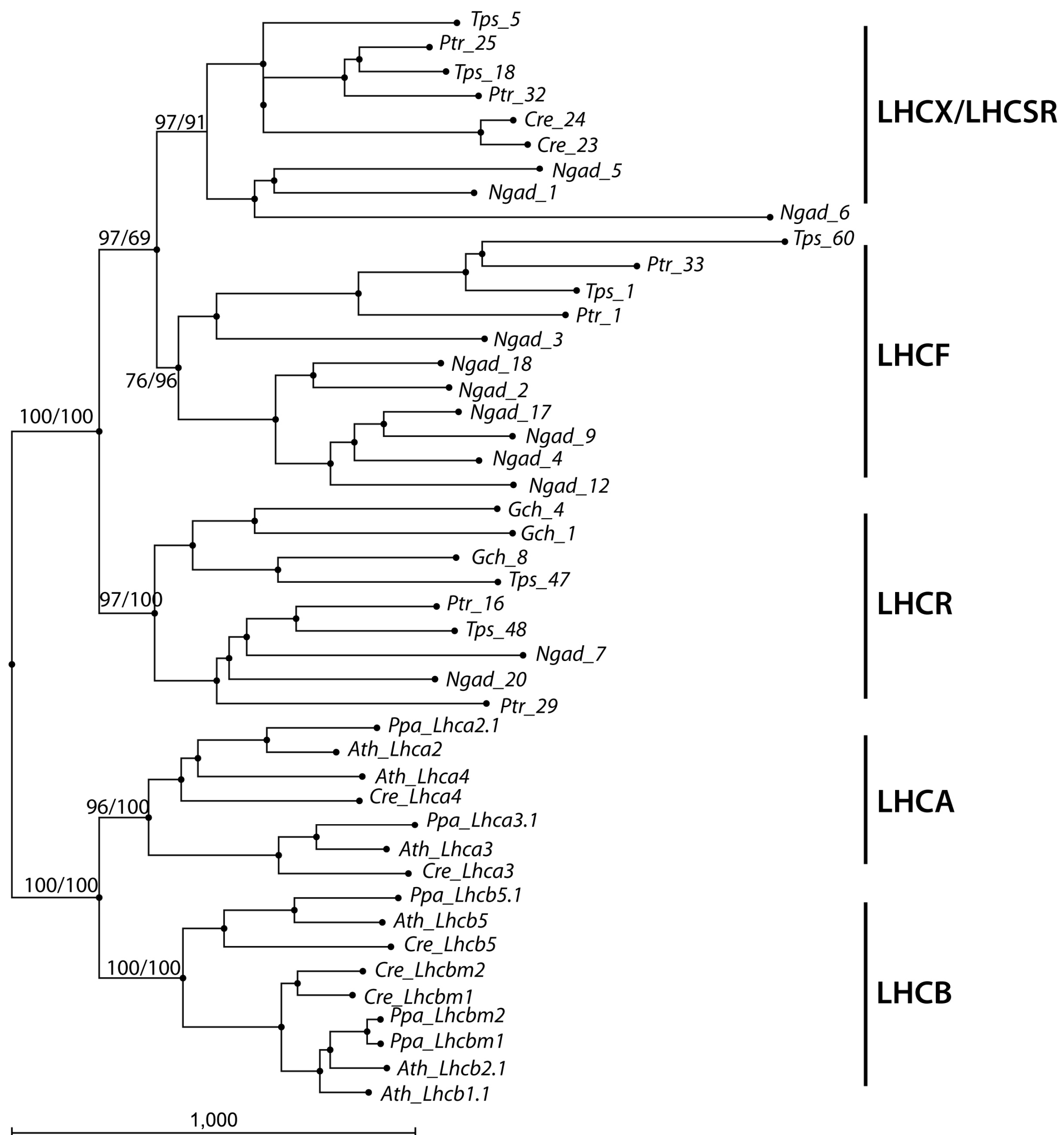

FIGURE 3.S3. Phylogenetic tree of LHC proteins from different photosynthetic eukaryotes. Phylogenetic tree of LHC proteins identified in Nannochloropsis gaditana compared with other light harvesting groups LHCSR/LHCX, LHCR, LHCF, LHCA and LHCB from selected photosynthetic eukaryotes. Ath, Arabidopsis thaliana; Cre, Chlamydomonas reinhardtii; Ppa, Physcomitrella paten; Tps, Thalassiosira pseudonana; Ptr, Phaeodactylum tricornutum, Gch, Gracilaria changii; Ngad, Nannochloropsis gaditana. In significant nodes bootstrap values calculated using respectively NJ and UPGMA algorithms are reported.

Each Chl molecule within an antenna complex has different absorption properties depending on its binding site and specific electronic environment. The pigment-protein complex spectrum can thus be described as the sum of contributions from several Chl molecules with slightly different absorption bands, as described for the plant proteins [38,55]. The absorption spectrum of *Nannochloropsis* VCP was reconstructed using a similar procedure, and a good fitting was found

using the sum of four major forms, with maxima between 668 and 682 nm (Figure 3.5A). Chls bound to plant antenna complexes have similar absorption peaks, as demonstrated by site-directed mutagenesis [56,57]. This finding suggests that, although the polypeptide sequences of antenna polypeptides are differentiated between different photosynthetic groups, the electronic environment of most Chls is similar in the *Nannochloropsis* and plant proteins.

The *Nannochloropsis* antenna complexes also display an unusual carotenoid composition, with violaxanthin as the major carotenoid. Violaxanthin is widespread in different organisms from diatoms to plants, but this xanthophyll always represents a minor component with respect to other carotenoids, such as fucoxanthin or lutein, and its presence as a major carotenoid is thus unusual. It is also worth mentioning the detection of a significant amount of antheraxanthin and zeaxanthin bound to both the PSI and PSII antennas, consistent with the presence of an active xanthophyll cycle in this species [58], which was likely activated even in the relatively dim growth light employed in our study.

An even more atypical feature is the abundance of the xanthophyll vaucheriaxanthin in the form of 19' deca/octanoate esters [59-61]. Although LHCs are known to possess a large flexibility in accommodating different carotenoid molecules [62], the presence of the extra aliphatic chain of vaucheriaxanthin raises the question about how this carotenoid can be accommodated into an antenna complex. The analysis of the absorption spectra in the Soret region and their reconstruction as the sum of contributions from the individual pigments can provide information on their electronic environments and association with proteins (Figure 3.5B, [38,39]). Although multiple solutions can be found to describe each absorption spectrum, any good fitting requires the presence of at least two different carotenoid spectral forms, one with a peak at approximately 480 nm and another at 495-503 nm. However, no accurate description of the shape of the spectrum could be achieved without employing absorption forms at these wavelengths, suggesting that carotenoids with distinct electronic environments are found in *Nannochloropsis* antennas. For plant antenna complexes, for which more information is available due to structural data and extensive mutational analyses, the carotenoid absorption wavelength has been correlated with the binding to different sites: those found buried in the protein structure (sites L1-L2, also called 620-621, [63]) absorb in the 490-500 range [64], whereas those bound to more external sites (V1, N1) are more exposed to the solvent and have a less red-shifted absorption at approximately 485 nm [65,66]. Although it is not possible to speculate in detail on the possible conservation of carotenoid-binding sites between *Nannochloropsis* and plants antenna complexes based on the present knowledge, the spectral analysis suggests the presence of some binding sites buried in the structure and others external and exposed to the solvent. The latter would be the most likely candidates for binding vaucheriaxanthin esters because of the increased possibility of accommodating the extra chain.

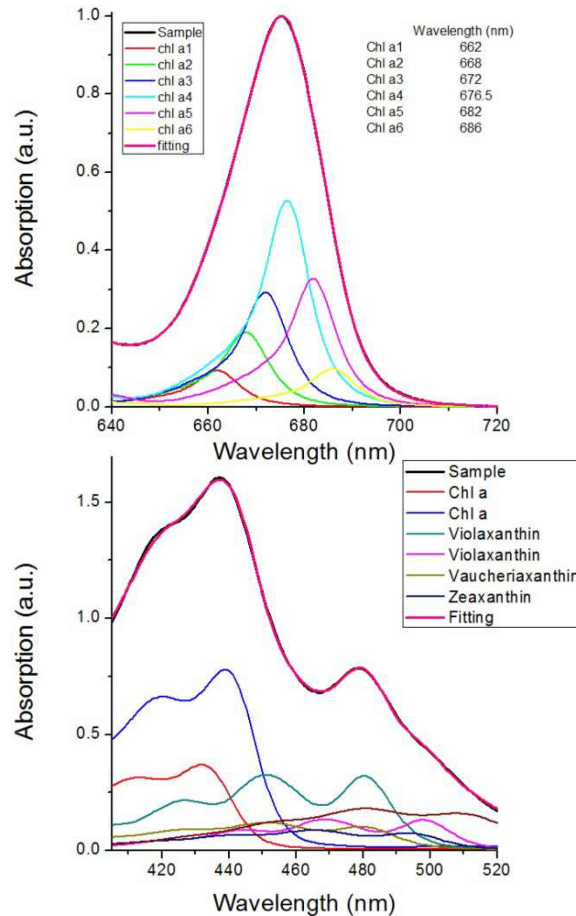

FIGURE 3.5. Analysis of LHC complexes absorption spectra from *N. gaditana*. A) Distribution of Chl spectral forms in Qy region of the spectrum. In order to describe the absorption spectrum four major Chl forms absorbing at 668, 672, 676.5 and 682 nm were employed together with two minor forms at 662 and 686 nm. Original spectrum is shown in black and fitting result in pink. B) fitting of the Soret region spectrum using Chl *a* and different carotenoid forms (violaxanthin vaucheriaxanthin, zeaxanthin). Among different solutions the one more consistent with relative ration of different carotenoids according to HPLC analyses was retained. Two different spectral forms for violaxanthin were employed together with one for vaucheriaxanthin and zeaxanthin. Original spectrum is shown in black and fitting result in pink. Analyses were performed as described in [39,40].

PSII and PSI supramolecular organization in Nannochloropsis

PSII in *Nannochloropsis* is easily dissociated from its antenna moiety upon detergent solubilization. Furthermore, no PSII core protein was detected in the heavier bands of the sucrose gradient, even when further reducing the detergent concentration or using other mild detergents, such as digitonin. Such a labile association between PSII and its antenna has been commonly observed in several other photosynthetic eukaryotes, both plants and algae, even if in some cases PSII-LHC super-complexes have been successfully purified [65,67,68].

In *Nannochloropsis*, the isolated PSII antenna complexes are found both as monomers and oligomers, most likely also trimers, as the sucrose gradient migration was similar to those of trimeric LHCB from plants (Figures 3.1-2). Monomers and oligomers have remarkably similar features, with indistinguishable absorption, fluorescence emission, and pigment composition, as in diatoms [10,69]. Conversely, subtle but detectable differences between monomeric and trimeric antenna complexes are found in plants [70].

Different from PSII, PSI-LHC is found as a stable supercomplex between the core complex and antenna proteins (Figure 3.1). This was shown by the sucrose gradient migration and SDS–PAGE analysis and confirmed by the presence of a large amount of xanthophylls in the PSI-LHC fractions, consistent with the presence of antenna polypeptides in the complex (Table 3.1). This finding was also confirmed by examining the PSI-LHC band by non-denaturing gel electrophoresis, whereby *Nannochloropsis* PSI showed a size comparable to that from *Chlamydomonas*, which is known to contain up to nine antenna subunits and is significantly larger than that from plants, with only four subunits (Figure 3. S1)[47,48].

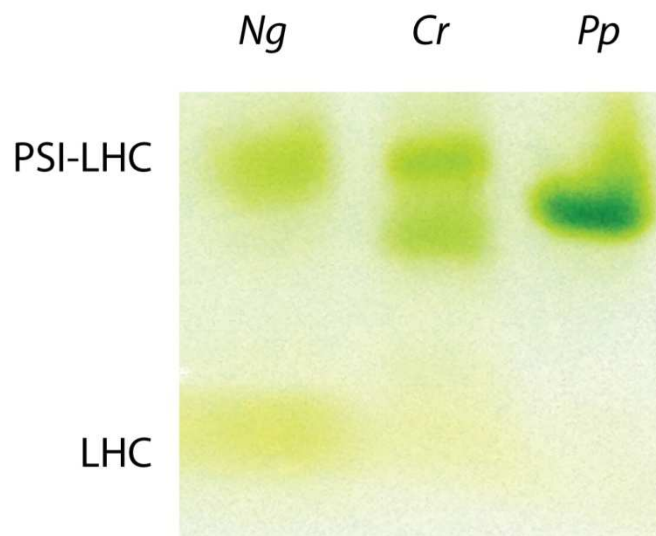

Figure S1. Comparison of PSI-LHC from different species. **PSI-LHC complexes purified form *Nannochloropsis gaditana (Ng)*, *Chlamydomonas reinhardtii (Cr)*, *Physcomitrella patens (Pp)* was compared using non denaturing electrophoresis (Deriphat-PAGE). During separation some antennas are dissociated from *Nannochloropsis* and *Chlamydomonas* complexes.**

PSI-LHC* isolated upon stronger β-DM solubilization showed slower migration in the sucrose gradient and a reduced xanthophyll content, consistent with the partial dissociation of the antenna complexes. Interestingly, SDS–PAGE showed that the antenna complexes associated with PSI have an apparent molecular weight of approximately 20 kDa (Figure 3.2B), distinct from the PSII antenna complexes in which the major band is larger (Figure 2A). This difference clearly

suggests that a distinct set of LHC proteins is preferentially associated with PSI and PSII in *Nannochloropsis gaditana*.

Convergent evolution in PSII and PSII supramolecular organization.

The presented characterization of the *N. gaditana* photosynthetic apparatus shows that PSI forms a stable association with its antenna subunits, whereas PSII-LHC supercomplexes were not detectable. Although PSII-LHC supercomplexes have been isolated from different species, in all cases described to date, the antenna interaction with the core complex appears to be more easily dissociated than in PSI [71-73]. This diverse organization of PSI and PSII is found to be conserved in many different photosynthetic eukaryotes, such as plants, green algae, diatoms, and red algae [8,10,51]. Additionally, this difference between the two photosystem appears to be correlated with different antenna proteins specifically associated with either PSI or PSII. In green algae and plants, two groups of antennas, called LHCA and LHCB, are well known to be associated to PSI and PSII, respectively. The presence of antenna complexes specifically associated with PSI and PSII has also been suggested for diatoms and red algae [14] and is likely also present in *Nannochloropsis*, as demonstrated by the bands of different molecular weight identified in the PSII and PSI fractions (Figure 3.2). The above-mentioned strong association between the antenna and PSI is thus achieved by the presence of specialized LHC proteins having specific interactions with the core complex.

It is however interesting to verify whether these specialized LHC subunits are conserved in photosynthetic eukaryotes. The phylogenetic tree shown in Figure S3 shows the distribution of different LHC proteins from plants, green algae, diatoms, red algae, and *Nannochloropsis*. As illustrated, LHCA proteins were found in all Viridiplantae (green algae and plants) but were not conserved in red algae, diatoms, or even *Nannochloropsis* [54]. Conversely, LHCR subunits, which are suggested to be associated with PSI in diatoms and red algae [8,9], were not found in plants or green algae. Consistently, the LHCA/LHCB proteins have a common ancestor that diverged from the LHCF/LHCR found in red algae, diatoms, and *Nannochloropsis* prior to their differentiation as PSI and PSII antenna complexes [5]. This finding suggests that the specific association of some antennae with PSI evolved after the separation of the green and red lineages and appeared independently in the two phylogenetic groups.

Thus, the observed conserved organization of the PSI supercomplex is not the result of the conservation of specific subunits but rather the results of "convergent" evolution, which in all groups selected for PSI antenna subunits to be more strongly associated with the reaction center relative to those interacting with PSII. This result suggests the presence of a selective advantage for a stable antenna and core complex association in the case of PSI but not in the case of PSII. A possible explanation can be found by considering how the PSII supercomplexes are involved

in several regulatory mechanisms. In fact, the number of antenna complexes associated with PSII reaction center is known to change in response to illumination conditions [19]. PSII is also known to undergo continuous repair, the mechanism of which requires a multistep process involving the reversible phosphorylation of the PSII core proteins in the granum stacks, PSII monomerization, migration to the granum margins, and partial disassembly to allow the degradation of damaged D1 and the insertion a new copy [74]. Lastly, non-photochemical quenching has recently been proposed to regulate heat dissipation by modulating the dissociation of antenna complexes from the reaction center [75]. For all of these mechanisms to be effective, a flexible binding of the antenna complexes to the reaction center is required, along with the possibility of modulating this association according to environmental stimuli. The presence of a strong and stable association of antennae with the PSII reaction center would likely hinder the possibility of the antenna to participate to these important regulations. In contrast, the PSI reaction center is known to be stable with regard to light stress and to undergo a very low turnover [76,77]. The PSI supercomplex was also proposed to have limited regulation of its antenna size and pigmentation under different light conditions, as observed in plants, *Chlamydomonas reinhardtii*, and the diatom *Cyclotella meneghiniana* [78]. Although the PSI antenna has also been shown to experience some regulation, for instance, in the case of iron deficiency [79], the present knowledge suggests that the mechanisms affecting PSI antennae are less extensive and do not require the continuous modulation of its interactions with PSI and are thus compatible with a stronger association with the reaction center.

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CHAPTER 4

SEQUENCE AND PROTEOMIC ANALYSIS OF ANTENNA PROTEINS IN *NANNOCHLOROPSIS GADITANA*

SEQUENCE AND PROTEOMIC ANALYSIS OF ANTENNA PROTEINS IN *NANNOCHLOROPSIS GADITANA*[1]

Abstract

In the previous chapter we described the photosystems supramolecular organization in *N. gaditana* which was further investigated here. On one side a phylogenetic analysis of LHC sequences, identified in *Nannochloropsis* genome, allowed their classification into different isoforms and comparison with other species, providing new information on antenna complexes from heterokonta. Also we performed a proteomic analysis on different sucrose gradients fractions corresponding to antenna protein, PSI and PSII fractions clarified their protein composition, identifying some antenna complexes associated with PSI and others to PSII.

Introduction

Photosynthetic organisms exploit solar light to drive oxygenic photosynthesis: the first step of the process is the light harvesting which is performed by proteins belonging to a multigenic family called Light Harvesting Complexes (LHC). LHC are encoded in the nucleus by a multigene family and targeted to the chloroplast thanks to transit peptide at N-terminal recognized by the import apparatus (Stengel A. et al. 2007). After the transit peptide is cleaved, the apoprotein is assembled with chlorophyll and carotenoids and inserted into the thylakoid membranes (Hoober J.K. et al. 2007).

LHCs are characterized by the presence of three membrane-spanning α-helices regions (TM) connected by both stroma and lumen-exposed loops. A and B helices show a clear similarity and Green B.R. and Pichersky E. in 1994 attributed this to a common origin from an internal duplication. These regions present the characteristic LHC motif (ExxxxRxAM) where the Glu (E) from one helix forms a salt bridge with the Arg (R) of the other (Kühlbrandt W. et al.1994, Engelken J. et al. 2010) stabilizing the central two helices.

Antenna proteins can be divided into different subfamilies with a likely different function. They thus represent an excellent example of how the duplication and divergence led to the functional

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specialization of groups of antenna proteins vital for acclimating to dynamic environments (Ganeteg U. et al. 2004), as reported in Table 1.2., Chapter 1.

Recently *Nannochloropsis gaditana* photosynthetic apparatus has been characterized (Basso S. et al. 2014) showing a photosystems supramolecular organization conserved with respect to several other photosynthetic eukaryotes, even if evolutionarily distant. In all organisms characterized so far, in fact, antenna proteins are loosely associated to photosystem II (PSII), while they are strongly bound to photosystem I (PSI). In the case of *N. gaditana*, the major PSII antenna complex have been named Violaxanthin-Chlorophyll protein (VCP) due to presence of violaxanthin as the main carotenoid. This complex also binds smaller amounts of vaucheriaxanthin in the form of 19' deca/octanoate esters (Britton G. et al. 2004, Hager A. and Stransky H. 1970, Mangoni O. et al. 2011). VCP is a member of LHC superfamily as FCPs and LHCA/B, although the pigment binding properties are different, with FCP binding Chl *a*, Chl *c*, and fucoxanthin, and LHCA/B binding Chl *a*, Chl *b*, and various xanthophylls. The VCP fractions has a major 22-KDa principal polypeptide component and according to sequence analysis it shows similarity with LHCF from diatoms (Sukenik A. et al. 1992, Basso S. et al. 2014).

In this work thanks sequences encoding for antenna proteins were identified in *Nannochloropsis* genome (Corteggiani Carpinelli E. et al. 2013) and classified into six different groups: LHCX, LHCF, LHCF-like, LHC-like, LHCR and LHCR-like. Proteomic analyses showed that some of these antennas are specifically associated with PSI or PSII.

Materials and Methods

Cells growth

Nannochloropsis gaditana from CCAP, strain 849/5, was grown in sterile F/2 medium (Guillard R.R.L. and Ryther J.H., 1962), using sea salts 32 g/l from Sigma Aldrich, 40 mM TRIS/HCl pH 8, Sigma Aldrich Guillard's (F/2) marine water enrichment solution. Cells were grown with 100 μmoles photons $m^{-2}s^{-1}$ (μE) of illumination and mixed with air enriched with 5% CO_2. Temperature was set at 22±1°C.

Thylakoids isolation

Thylakoids were extracted according to Basso et al. 2014. Briefly cells in exponential growth phase were harvested by centrifugation (4000 g, 10 minutes, 4° C), washed twice in B1 buffer (0.4 M NaCl, 2 mM $MgCl_2$, 20 mM Tricine /KOH pH 7.8) and then splitted into 2 ml safe lock cap tubes. A volume of glass beads (diameter 150-212 μm) equal to the volume of the pellet was added and cells then disrupted with a Mini Bead Beater (Biospec Products) for 20 seconds at 3500 RPM. Immediately after rupture, 1 ml of B1 with 0.5% milk powder, 1mM PMSF, 1mM DNP-ε-amino-n-caproic acid and 1mM Benzamidine was added in each tube and the pellet

resuspended. Unbroken cells were then separated by a centrifugation step (2500g, 15 min, 4°C) and the supernatant collected. The supernatant was centrifuged at 15000 g for 20 min and the pellet washed twice with B2 buffer (0.15 M NaCl, 5 mM MgCl2, 20 mM Tricine /KOH pH 7.8). Finally thylakoids were resuspendend in B4 buffer (0.4 M Sorbitol, 15 mM NaCl, 5 mM MgCl2, 10 mM Hepes/KOH pH 7.5) and immediately frozen in liquid nitrogen and stored at -80°C until use. All steps were performed at 4°C and in dim light. The total pigments were extracted with 80% acetone and the chlorophyll concentration of the samples was determined spectrophotometrically using specific extinction coefficients (Porra R.J. et al. 1989) and the acetone spectra fitting as in (Croce R. et al. 2002), modified to account the peculiar pigment content.

Thylakoids solubilization and sucrose gradients.

Thylakoid membranes corresponding to 500 μg of Chl were washed with 50 mM EDTA and then solubilized 20 minutes in ice in 1 ml of final 0.4% α–DM (n-dodecyl-α-D-maltopyranoside) or 1% β-DM, 10 mM HEPES pH 7.5 after vortexing for 1 min. The solubilized samples were centrifuged at 15000 g for 20 min to eliminate unsolubilized material and the supernatant with the photosynthetic complexes was then fractionated by ultracentrifugation in a 0.1–1M sucrose gradient containing 0.06% α-DM and 10mM HEPES, pH 7.5 (280000 g, 18 hours at 4 °C). The green fractions of the sucrose gradient were then harvested with a syringe.

Protein composition

N. gaditana protein composition was determined by SDS-PAGE using a precast 12% polyacrylamide SDS gel (C.B.S. Scientific) and stained with Coomassie Brilliant Blue or by silver stain. Apparent molecular weights were estimated by co-electrophoresis of a low molecular weight protein standard (Fermentas).

Mass Spectrometry analysis (MS)

In-gel tryptic digestion was performed as described in (Shevchenko A. et al. 2007), with minor modification with acetonitrile as the organic phase. The MS measurements were performed as described by Terashima et al. (2010) using an Ultimate 3000 nanoflow HPLC system (Dionex) coupled with an LTQ Orbitrap XL mass spectrometer (Thermo Finnigan) device for autosampling, column switching and nano-HPLC.

For the identification of peptides, OMSSA (version 2.1.4) (Geer L. Y. et al. 2004). A new database was created downloading *N. gaditana* sequences from www.nannochloropsis.org/page/ftp to assign the peptides resulting from MS analysis to *N. gaditana* protein.

Phylogenetic analysis

Phaeodactylum tricornutum, Thalassiosira pseudonana, Ostreococcus tauris, Cyclotella cryptica, Chlamydomonas reinhardtii, Mesostigma viride, Physcomitrella patens, Arabidopsis thaliana, Gracilaria changii genome databases were accessed online via the National Center for Biotechnology Information (NCBI) portal using TBLASTN and BLASTP. Additional data were collected from http://bioinformatics.psb.ugent.be/genomes/view/Ectocarpus-siliculosus for *Ectocarpus silicolosus* genome, *Department of Energy Joint Genome Institute (JGI) for Emiliania huxley* sequences.
Nannochloropsis gaditana antennas sequences were retrieved from www.nannochloropsis.org (Corteggiani Carpinelli E. et al. 2013) using TBLASTN, and reference sequences representing the major clades in our phylogeny. *Nannochloropsis* sequences were named in the order they were found. All hits (e-value < 12) were submitted to InterProScan (Punta M. et al. 2012) using default parameters, and considered only if the PFAM00504/IPR022796 motif was detected.
Signal peptides were identified using ChloroP 1.1. Alignment analysis were performed using T-coffee (Di Tommaso P. et al. 2011, Notredame C. et al. 2000) and manually modified with Bioedit 7.1.3.0.
Bootstrap values (100 replicates) for the Neighbor-joining and UMPGA analyses were obtained in CLC Sequence Viewer 6.8.

Results and Discussion

Protein sequence analysis

Nannochloropsis antenna sequences were retrieved from www.nannochloropsis.org (Corteggiani Carpinelli E. et al. 2013) using a blast search with different LHC from different organisms. Retrieved sequences were then compared with others from different eukaryotes, including all Heterokonta with a sequenced genome: *E. huxley, E. silicolosus, G. changii P. tricornutum, T. pseudonana, C.criptica, G.changii, M. viride, C. reinhardtii, P.patens, A.thaliana, O. tauri.* For the alignment N-terminal peptide and residues after A-helix were cutted.

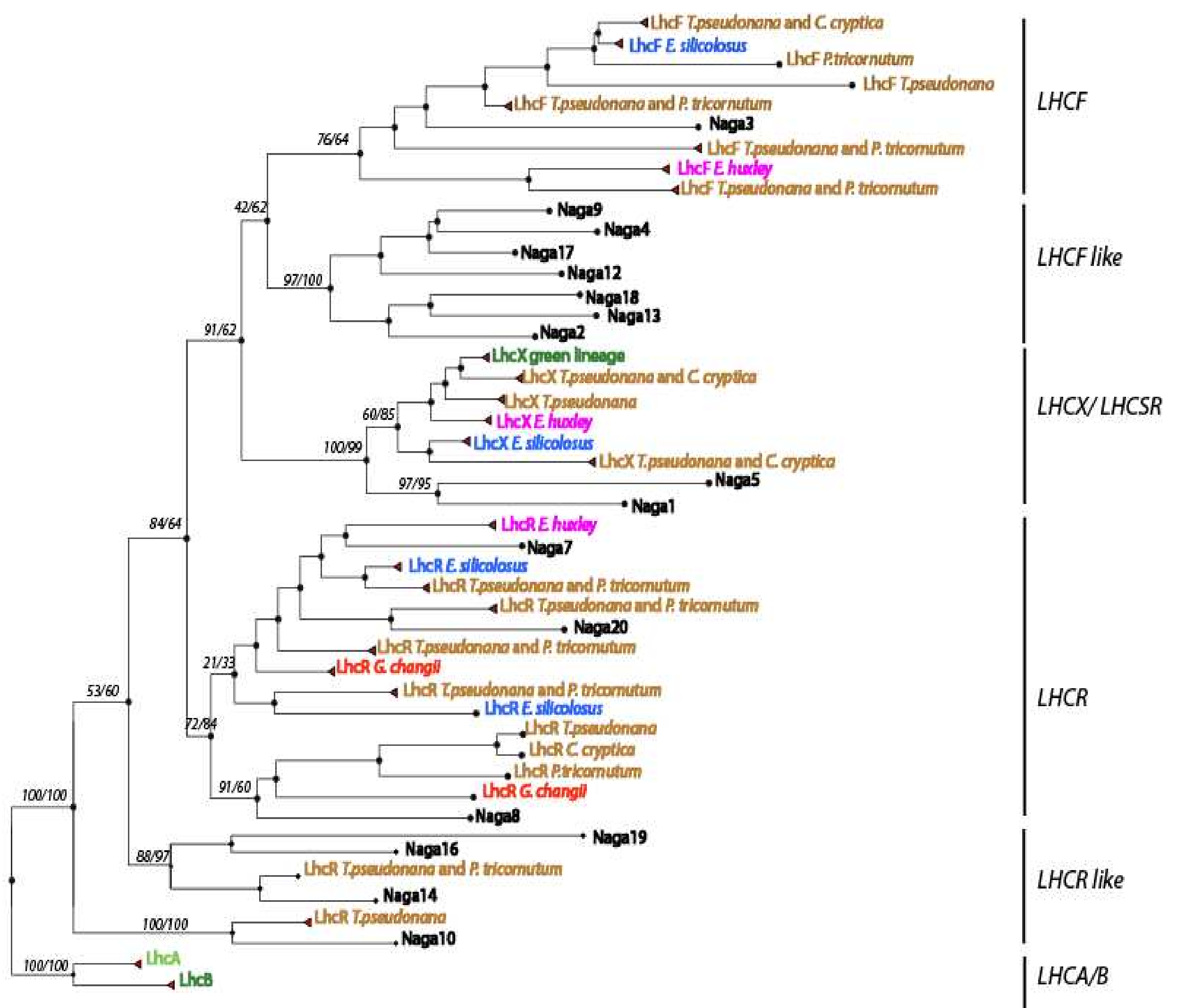


Fig 4.1 Phylogenetic tree of the LHCs identified in Nannochloropsis gaditana. Bootstrap values (100 replicates) for Neighbor-joining and UMPGA are reported in significant nodes. *LHCA/B from C. reinhardtii, P.patens, A.thaliana O. tauris,* were used as outgroup.

Phylogenetic tree presented in Figure 4.1 allows identifying six groups of antenna complexes with a significant statistical support. One is represented by plants and green algae antenna, as expected since they represent an outgroup. Looking to Heterokonta specific sequences, instead, one first observation is that the node including all LHCF sequences from diatoms, *E. silicolosus*, *E. huxley*, also includes Naga3, that corresponds to the VCP, characterized by Sukenik A. et al (1992). Six additional sequences from *N. gaditana* also cluster with this group, but the support is not very high (42/62) suggesting some difference is present. For this reason this group of antennas were tentatively named called LHCF-like. Their difference is supported by the observation of a putative Chl 614 binding site in all LHCF-like but not in LHCF (see below figure 4.2). The following group is instead very well supported and includes LHCX from diatoms and LHCSR from green algae. These are all stress-related LHC which has been shown to play a fundamental

role in NPQ (Peers G. et al. 2009, Alboresi A. et al. 2010). This clustering of sequences from evolutionary distant organisms suggests that these proteins all share a conserved function, which is thus likely for all genes included in this group. The group also includes two *N. gaditana* sequences, Naga1 and Naga5, which thefore likely plays a role in *Nannochloropsis* photoprotection.
The remaining sequences are named LHCR and LHCR-like as named in red algae (Koziol A.G. et al. 2007). Similarly to what was observed in Dittami S. et al. 2010 and Hoffman G. et al. 2011, they are clustered into four different subgroups. The first two groups are named LHCR, and include sequences from *P. tricornutum, T. pseudonana, E. silicolosus, E. huxley, G. changii* as well as three proteins from *N. gaditana,* Naga7, Naga8 and Naga20. The third and fourth group, include proteins from *P. tricornutum, T. pseudonana* and four from *N. gaditana:* Naga10 and Naga14, Naga16, Naga19. These were called LHCR-like because they do not form a unique cluster with LHCSR. Also in previous works LHCR did not form a unique cluster, suggesting that all these genes called LHCR might not have a common evolutionary origin (Dittami S. et al. 2010 and Hoffman G. et al. 2011).
In addition to the five groups previously illustrated, blast search retrieved four additional LHC-like sequences (Naga 6, Naga 15, Naga 21, Naga22), reported in table 4.1, which however did not cluster with Heterokonta LHC and were thus excluded from the tree in figure 4.1.

Name	Gene ID	LHC type	Name	Gene ID	LHC type
Naga1	Naga_100173g12	X-Type	**Naga13**	Naga_100005g99	F-like-type
Naga2	Naga_100027g19	F-like-type	**Naga14**	Naga_100018g45	R-like-type
Naga3	Naga_100012g50	F-type	**Naga15**	Naga_101227g1	LHC-like
Naga4	Naga_100004g86	F-like-type	**Naga16**	Naga_100002g18	R-like-type
Naga5	Naga_100056g42	X-Type	**Naga17**	Naga_100013g28	F-like-type
Naga6	Naga_101036g3	Lhc-like	**Naga18**	Naga_100157g5	F-like-type
Naga7	Naga_100434g4	R-type	**Naga19**	Naga_100168g13	R-like-type
Naga8	Naga_100092g17	R-type	**Naga20**	Naga_100017g83	R-type
Naga9	Naga_100017g59	F-like-type	**Naga21**	Naga_100056g15	Lhc-like
Naga10	Naga_100641g3	R-like-type	**Naga22**	Naga_100030g5	ELIP.
Naga12	Naga_100168g14	F-like-type			

Table 4.1 Summary of the LHC-types found in *Nannochloropsis gaditana* genome.

LHC superfamily comprehends not only three helix Light Harvesting proteins, but also LHC-like (Lil) proteins and the subunit S of photosystem II (PsbS) which is present and expressed only in green lineage (Alboresi A. et al. 2010, Gerotto C. and Morosinotto T. 2013). Among the Lil family six different classes of Lil genes have been identified in *Arabidopsis* (Jansson, 1999; Heddad and Adamska, 2000; Andersson et al., 2003), encoding for six different proteins: the three-helix

early light-inducible proteins (ELIPs), two one-helix proteins (OHP1 and OHP2), and two types of stress-induced proteins SEP1 and SEP2, and the double helix protein LIL3. Naga22 can be identified as an ELIP proteins, while for the other LHC-like proteins it was not possible to derive a specific connection with any other Lil. ELIP proteins are transient inducted and they can be triggered by different physiological conditions, such as light stress, dehydrative processes or morphogenesis (Adamska I. 2001). ELIPs contain two chlorophyll binding motifs located in the first and the third TM helices (Adamska I. 2001).

Conserved site of Chlorophyll bound on Nannochloropsis

Nannochloropsis antennae, as all LHC complexes, show a significant similarity on TM helices. These regions also include the residues coordinating most of the Chl bound to these proteins. Since these proteins function is played thanks to their ability to coordinate pigments the conservation of their binding sites bears a strong functional meaning. Comparing the sequences with the one from LHCII from *Spinacia olearia,* where all Chl binding sites have been identified thanks to the 3D structure (Liu Z. et al. 2004), it was possible to assess the presence of eventual conserved chlorophyll binding site also in *Nannochloropsis* LHCs. As reported in figure 4.2 five conserved residues involved in the binding of five Chl *a* molecules (a602, a603, a610, a612 and a613) are found conserved in all LHC from *Nannochloropsis*, while residue possibly involved in the binding of Chl a614 is found in LHCX and LHCF-like sequences but not in LHCR and LHCF. Residues coordinating Chl *b* in LHCII are instead not as well conserved, with the likely exception of b606. Chl *b* molecules are coordinated especially near the C helix, and this observation is consistent with a lower degree of similarity in this part of the protein between different LHC (Tomitani A. et al. 1999). Bassi R. et al. 1999 and Engelken J. et al. 2010 report that the sequences FDPLGL (or similar), found approximately 15 amino acid positions before the Chlorophyll binding motif in both, A and B TM helices, can be likely involved in carotenoids binding. This sequence is found also in *Nannochloropsis* proteins sequences suggesting the presence of two carotenoids in correspondence with helices A and B, named L1 and L2 in plants LHC (Basso S. et al. 2014).

```
                           α-B
Lhcb_Sp.ol          DPETFAKNRELEVIHCRWAMLGALGCVFPEL-LAR-
Naga1 (LHCX)        SFKELKKWREAELKHGRVAMLAVLGTAVQEN-FHP-
Naga3 (LHCF)        DVEKFNRYRAIEIKHGRVAMLAMLHTLVTGLGVKL-
Naga4 (LHCF-like)   NEGSLRKYRECELKHGRVAMAAMLGIFVQGL-YHL-
Naga20(LHCR)        GLSALKWFREAEIKHGRFAMLAVLGWVAVDMGLRL-
Naga14(LHCR-like)   SGRSLKWYREAEIVHGRVAMLAALGWVFPNIYHLP-
Clustal Consensus   .   .   *  * :* * .  . *:
                             a602a603

                              α-C
Lhcb_Sp.ol          NGVVHAQSILAIWACQVILMG-AVEG-YRI
Naga1 (LHCX)        YPLFWTALLFIIGIIEAR-AG--VK--EDY
Naga3 (LHCF)        PGLGWAQVLLFCSALEVL-AP--QK--EDK
Naga4 (LHCF-like)   PDPAIWQIIVGLGVIEFT-GG-RQK--EDR
Naga20(LHCR)        KSGDMTVGLLAIGFLEVV-MS-KGS--DDR
Naga14(LHCR-like)   PAAGVLQIAATVGAIELF RV RVI  DKEA
Clustal Consensus    .    .        :
                                  b606     b609

                                     α-A                         α-D
Lhcb_Sp.ol          AGGPLPEAFAELKVKEIKNGRLAMFSMFGFFVQAIVTGKGPLENLADHLAD
Naga1 (LHCX)        ICGNLEEAFLSYRNKELNNGRLAMIAAAGITVQEKFVTNGLPEFEFHRFAL
Naga3 (LHCF)        IPGDVEEEALAYQNKEINNGRLAMVAWTGATVGALLTGEDPITTLLAKLG-
Naga4 (LHCF-like)   APGDLEKEFEELQLKELKNGRLAMVASMGALLQEYLTGQGPVEQVLAGHLN
Naga20(LHCR)        AAGDFPAKYARYQVSEIKNGRLAMLAFGGIATQAVLTNGGFPYYQ------
Naga20(LHCR-like)   GFNYSEEEYAEKQLQEIKNGRLAMVGILGLLLQASVTGKGIVQQLGGSFDV
Clustal Consensus     * .       .  *.:***:**..  *  :.. ::   ..
                                   a610a612      a613            a614
```

Fig. 4.2: Polypeptide sequence alignment of the LHC genes from N. gaditana (Naga) with Lhcb from S. olearia (Liu et al. 2004). For Nannochloropsis sequences only one member for each group has been reported (Naga1 is an LhcX, Naga3 is a LHCF, Naga4 is a LHCF-like, Naga14 is an LHCR-like Naga20 is an LHCR) but the residues found conserved are also present in the others. Conserved LHC-residues (bold). Residues are colour-coded: Chl a side chain ligand (green), Chl a backbone ligand via water (blue); Arg in salt bridge to Glu residue in the other helix (red). Residues conserved in the VCP family (*) are partially conserved (.), conservatively substituted (:).

Proteomic analysis

To further investigate *N. gaditana* antenna system with mass spectrometry, thylakoid membranes from *Nannochloropsis gaditana* were solubilized with α or β-DM and separated in six bands by sucrose gradient ultracentrifugation (Fig. 4.3, and Chapter 3). Spectroscopic and Western Blot analysis as described in Basso S. et al. 2014 (Chapter 3) led to identify the presence of free pigments, light harvesting complexes in monomeric form, then in the oligomeric one, PSII core

and PSI-LHC (Fig. 4.3). PSI purified from β-DM gradients showed a reduced number of antenna complexes associated and for this reason named PSI*-LHC.

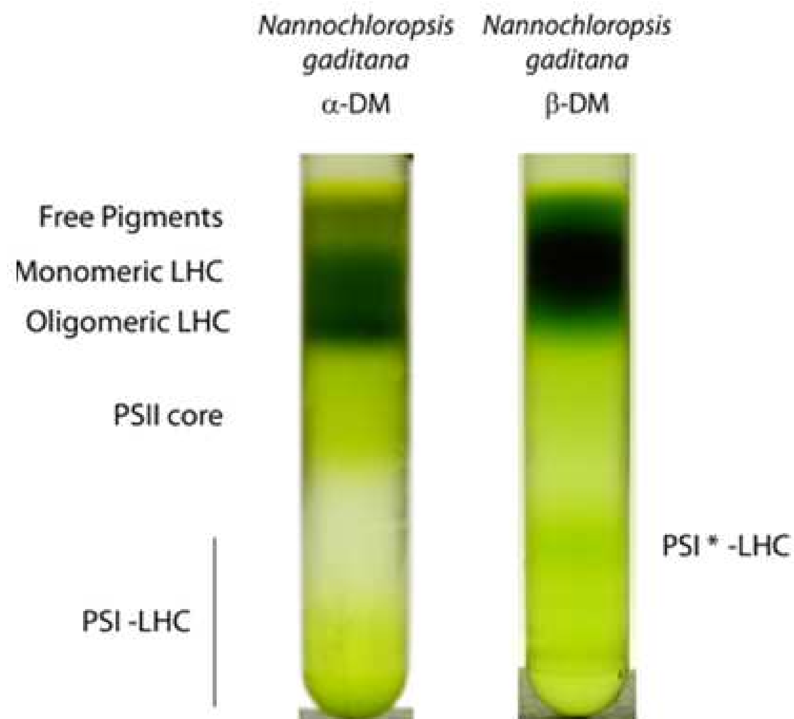


Fig 4.3: Ultracentrifugation sucrose gradient of *Nannochloropsis gaditana* thylakoids after solubilization with 0.4% α-DM on the left, and after solubilization with 1% β-DM on the right.

Proteomic analysis were performed loading on a SDS-PAGE monomeric and oligomeric, PSII core, PSI-LHC solubilized with α-DM and PSI*-LHC solubilized with β-DM. Each sample was loaded twice, and cutted into 30 bands, and performed tryptic digestion.

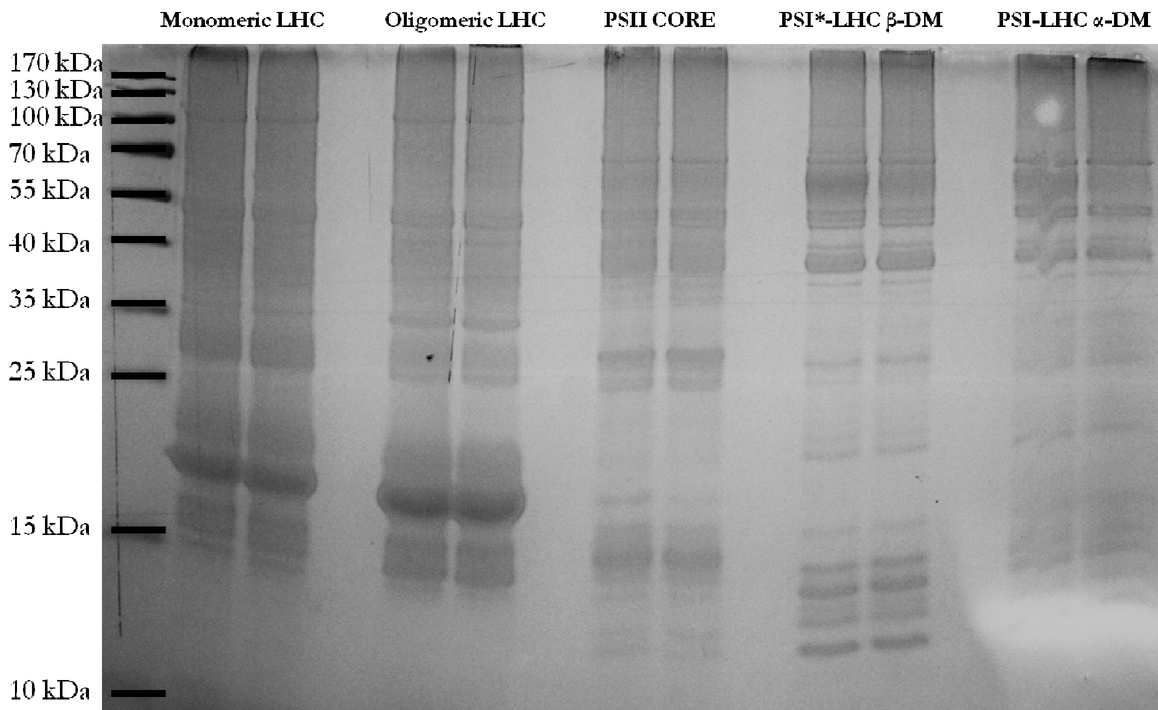


Fig. 4.4 SDS-PAGE F2 and F3 and F4 solubilized with α-DM and F5 solubilized with β-DM from sucrose gradient. Each sample was charged twice, 5 μg per lane. After run gel was stained with Coomassie, than cutted in 30 bands and tryptic digestion was performed.

Presently, proteomic analysis on PSI-LHC solubilized with α-DM fraction are still in progress, so data regards this fraction could not be illustrated here.

It is worth underlining that here 5μg of protein of each fraction were loaded. Starting from a sucrose gradient, however, different fractions are obtained in different yield, as shown in table 4.2. PSII core fraction yield, as example, is more than 10 times less than the one of oligomeric LHC. This means that proteins identified here will be over-represented with respect to their presence in the thylakoids membranes.

Monomeric LHC	Oligomeric LHC	PSII core	PSI*LHC β-DM	PSI-LHC α-DM
22±2.6 μg	52±3.8 μg	3.7±0.9μg	6.5±0.8 μg	4.6±0.85 μg

Table 4.2. Amount of protein extract for each fraction after a centrifugation sucrose gradient with 500 ug Chl *a* as starting material.

MS results showed the presence of several different proteins in the different fraction, as expected since the purification is very simple, and thylakoids isolated from starting material are not strictly isolated from other cellular components. Only proteins identifies as components of the photosynthetic apparatus, however, were analyzed in detail and divided into five different categories: Light Harvesting Protein, Light Harvesting Protein-Like, Photosystem I, Photosystem II, and Cytochrome and ATPase. It is important to underline that samples were not marked before running the analysis and therefore data should only be considered as semi-quantitative.

Monomeric and Oligomeric LHC

These two fractions are enriched in Light-Harvesting protein, but we found also an important amount of proteins belonging to PSII core, like D1 and D2 indicating a partial disassembling of this complex during solubilization. As expected, and found also in other organisms, in these fractions there is also a substantial amount of cytochromes and ATPase subunits which, however, are not pigment binding proteins.

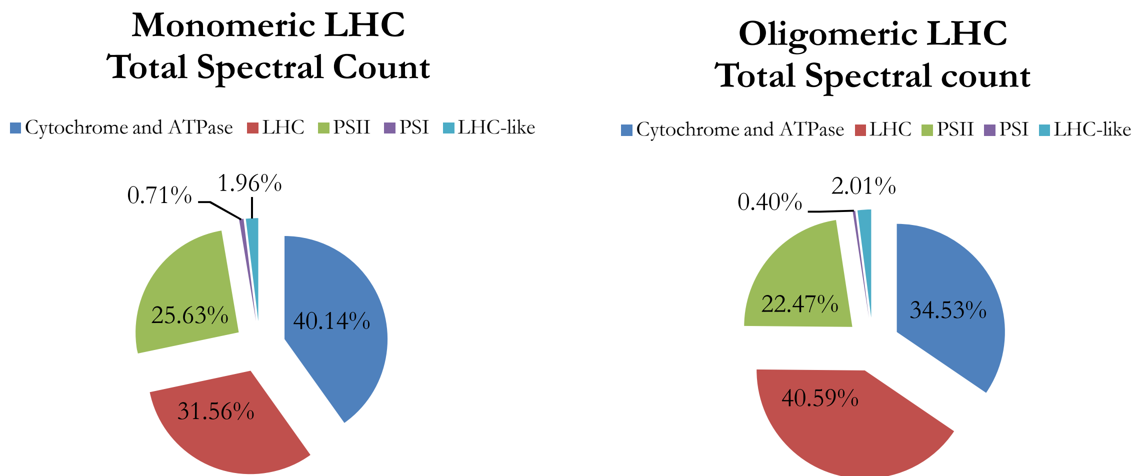


Fig.4.5 Overview of monomeric and oligomeric antenna spectral count from MS-analysis. All spectral counts for proteins belonging to one of the complexes were summed. The spectral count describes the sum of all MS/MS spectra found for peptides identifying the particular protein. The numbers indicate the percentage of the total number of spectra.

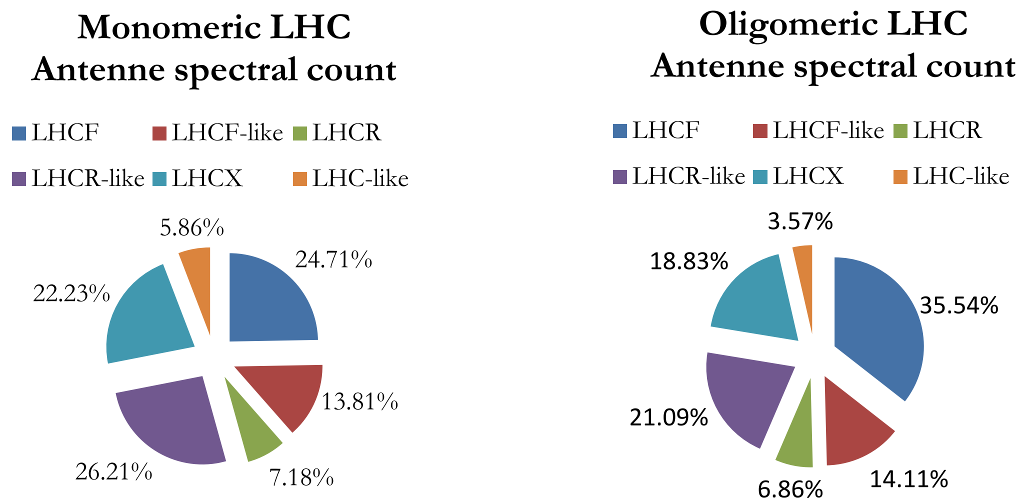


Fig.4.6 Overview of monomeric and oligomeric antenna spectral count from MS-analysis considering only LHC and LHC-like.

LHCF	M-LHC	%	O-LHC	%
Naga3	1679	**24.71**	3043	**35.12**
LHCF-like	**M-LHC**	**%**	**O-LHC**	**%**
Naga 2	73	1.07	88	1.02
Naga 4	184	2.71	215	2.48
Naga9	6	0.09	296	3.42
Naga12	182	2.68	140	1.62
Naga13	71	1.05	61	0.7
Naga17	294	4.33	313	3.61
Naga 18	128	1.88	95	1.1

LHCX	M-LHC	%	O-LHC	%
Naga 1	1455	**21.42**	1559	**17.99**
Naga 5	55	0.81	53	0.61

LHCR	M-LHC	%	O-LHC	%
Naga 7	109	1.6	119	1.37
Naga 8	122	1.8	213	2.46
Naga 20	158	2.33	255	2.94
LHCR-like	**M-LHC**	**%**	**O-LHC**	**%**
Naga 10	99	1.46	102	1.18
Naga 14	**798**	**11.75**	**792**	**9.14**
Naga 16	266	3.92	274	3.16
Naga19	**717**	**10.55**	**638**	**7.36**

LHC-like	M-LHC	%	O-LHC	%
Naga6	97	1.43	163	1.88
Naga15	2	0.03		0.00
Naga21	60	0.88	107	1.23
Naga 22-Elip	239	3.52	138	1.59

TOT	6794	100	8664	100

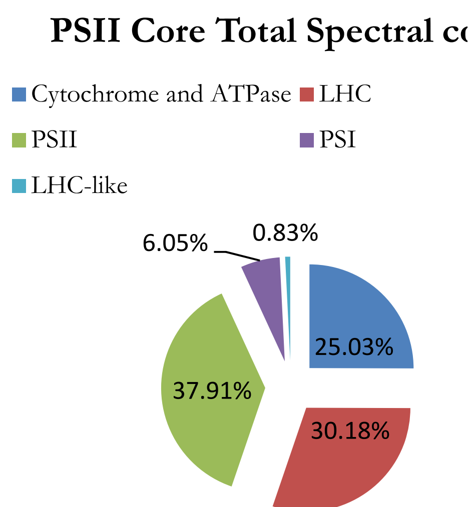
Table 4.3 Table with spectral count from each single LHC in monomeric (M-LHC) and oligomeric (O-LHC) antenna fractions.

Focusing our attention on LHC, in the antenna fractions the major part of the protein is represented by LHCF, with Naga3, the VCP characterized by Sukenik 1992 and 2000, representing the most abundant LHC both in the oligomeric than monomeric form. It is worth

nothing the presence of Naga1, one of the two LHCX isoforms, as the second most abundant protein. Also two LHCRs-like, Naga 14 and Naga19 are present in significant amounts in these fractions. The technique is very sensitive and trace amounts of all LHCs were identified although their content is minor.

Photosystem II

In Photosystem II core fraction almost 38% of identified peptides can be attributed to proteins belonging to PSII, with contaminations from PSI and antennas. Among PSII proteins we are able to recognized the core complex proteins like D1 (PsbA), CP47 (PsbB), CP43 (PsbC), D2 (psbD), PsbE (cytochrome b559 alpha chain) and 10 kDa phosphoprotein PsbH (Naga_1Chloroplast103.1) subunit, PsbF (Naga_1Chloroplast118.1). In this solubilization we isolated PSII with the Oxygen Evolving Complex (OEC), composed by the protein PsbO (Naga_100313g2) PsbQ (Naga_100273), PsbU (Naga_100076g3), and PsbV (Naga_1Chloroplast55.1). The presence of OEC subunits suggests us that PSII core isolated with this protocol is intact. PsbZ (Naga_100005g25) was also identified, this small subunit of PSII has a role in the regulation of electron transfer activity through the two photosystems (Bishop C.L., et al. 2007), and in *C. reinhardtii* and in tobacco PsbZ is implicated in the interaction with the peripheral antenna complexes (Swiatek M. et al. 2001).



PSII core	Gene	Spectral Count
D1(PsbA)	Naga_1Chloroplast27.1	624
CP47 (PsbB)	Naga_1Chloroplast9.1	3318
CP43 (PsbC)	Naga_1Chloroplast17.1	640
D2 (PsbD)	Naga_1Chloroplast28.1	731
PsbE	Naga_1Chloroplast97.1	647
PsbF	Naga_1Chloroplast118.1	65
PsbG	Naga_100003g108	14
PsbH	Naga_1Chloroplast103.1	149
PsbO	Naga_100313g2	11
PsbQ	Naga_100273g6	20
PsbU	Naga_100076g3	9
PsbV	Naga_1Chloroplast55.1	11
PsbZ	Naga_100005g25	13

Fig 4.7 Overview of PSII total spectral count, and Table 4.4 with the spectral count of identified proteins belongs to PSII.

A. thaliana PSII Subunit	*Nannochloropsis gaditana* PSII subunit		
	Present in genome and in proteomic analysis	*Present in genome only*	*Not found in genome*
PsbA	Naga_1Chloroplast27		
PsbB	Naga_1Chloroplast9		
PsbC	Naga_1Chloroplast17		
PsbD	Naga_1Chloroplast28		
PsbE	Naga_1Chloroplast97		
PsbF	Naga_1Chloroplast118.1		
PsbG	Naga_100003g108		
PsbH	Naga_1Chloroplast103		
PsbI		Naga_1Chloroplast125	
PsbJ		Naga_1Chloroplast127	
PsbK		Naga_1Chloroplast117	
PsbL	-		PsbL
PsbM	-		PsbM
PsbN		Naga_1Chloroplast114	
PsbO	Naga_100313g2		
PsbP		Naga_100119g18, Naga_100306g3, Naga_100005g50	
PsbQ	Naga_100273g6		
PsbR	-		PsbR
PsbS	-		PsbS
PsbT		Naga_1Chloroplast123	
PsbU	Naga_100076g3		
PsbV	Naga_1Chloroplast55		
PsbW	-		PsbW
PsbX		Naga_1Chloroplast119	
PsbY		Naga_1Chloroplast126	
PsbZ	Naga_100005g25		

Table 4.5 Comparison between *A. thaliana* PSII subunit and *N.gaditana* PSII subunit, see the text below for further details.

Comparing *N. gaditana* PSII with the *A. thaliana* one (Table 4.5), it is possible to observe that the reaction center, the minimal set of subunits required to oxidize water as experimentally determined (Mulo P. et al. 2009) is detected in both organisms, even if they are evolutionary distant.

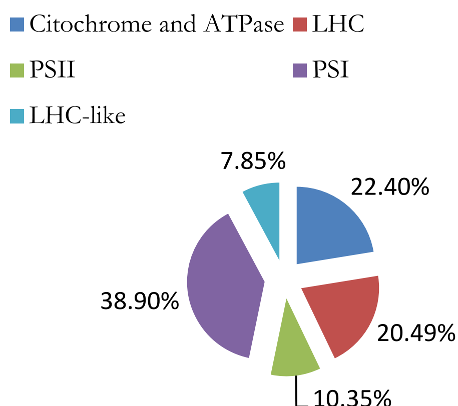
Concerning low molecular subunits, it is interesting to note the presence of three isoforms of PsbP, a nuclear encoded subunit, found in *N. gaditana* genome: this is not surprising because both *A. thaliana* (Yi X et al. 2007) and *Nicotiana benthaminiana* (Perez-Bueno M.L. et al. 2011) present

more than one nuclear encoded isoform of PsbP; all of which are involved in the stability and are fundamental for the optimal activity of the PSII (Ishihara S. et al. 2005 and Perez-Bueno M.L. et al. 2011). PsbP loosely interacts with intrinsic proteins of PSII, and for this reason it is easily lost during solubilization for spectrometry mass analysis within PSII fraction (Suzuki T. et al. 2005, Caffarri S. et al. 2009).

On the contrary there are significant differences between other additional low molecular subunits. In fact *N. gaditana* lacks the following subunits: PsbL, PsbM, PsbR, PsbS and PsbW. The absence of PsbS was expected, due to the fact that this protein, involved in photoprotection mechanisms, evolved during plants land colonization (Alboresi A. et al. 2010, Gerotto C. and Morosinotto T. 2013). PsbR is an important link in the PSII core complex for stable assembly of the oxygen-evolving complex protein PsbP (Suorsa M. et al. 2006). It has been suggested that PsbL (Luo H and Eaton-Rye J.J., 2008), PsbM (Boehm M. et al. 2012) PsbW (Shi et al. 2000) stabilizes the PSII dimer, but all these subunits are typically found in higher plant and in green algae (Shi L.X and Schröder W.P. 2004), so in *N.gaditana* other proteins could be involved in this function.

Photosystem I-LHC solubilized with β-DM

This fraction is well characterized by the major presence of PSI core proteins, represent 39% of the total. There is a trace of PSII, 10%, due to the contamination of D2, D1 CP43 and CP47. The following plastid encoded proteins were identified as *N. gaditana* PSI: PsaA, PsaB, PsaC, and the PSI ferredoxin-binding subunit PsaD, PsaE, PsaF and PsaL. From literature it is known that, in higher plants, the core of PSI is composed of 14 subunits (Jensen, P. E. et al. 2007), among these subunits 8 are conserved and found also in diatoms PSI (Grouneva I. et al. 2011) and in *Nannochloropsis* (see Table 4.7). PsaA and PsaB bind the Chl a responsible for the charge separation while PsaC, D and E are involved in connection with Cyt*c6* in the lumenal side of the membrane or with ferrodoxin in the stromal one.



PSI	Gene	Spectral count
PsaA	Naga_1Chloroplast5.1	437
PsaB	Naga_1Chloroplast6.1	355
PsaC	Naga_1Chloroplast98.1	7
PsaD	Naga_1Chloroplast66.	937
PsaE	Naga_1Chloroplast104.1	135
PsaF	Naga_1Chloroplast48.1	117
PsaL	Naga_1Chloroplast52.1	440

Fig 4.8 Overview of PSI*-LHCβ-DM total spectral count, a table 4.6 with the spectral count of identified proteins belongs to PSI.

A. thaliana **PSI Subunit**	***Nannochloropsis gaditana*** **PSI subunit**		
	Present in genome and in proteomic analysis	*Present in genome only*	*Not found in genome*
PsaA	Naga_1Chloroplast5		
PsaB	Naga_1Chloroplast6		
PsaC	Naga_1Chloroplast98		
PsaD	Naga_1Chloroplast66		
PsaE	Naga_1Chloroplast104		
PsaF	Naga_1Chloroplast48		
PsaG			PsaG
PsaH			PsaH
PsaI			PsaI
PsaJ		Naga_1Chloroplast116	
PsaK			PsaK
PsaL	Naga_1Chloroplast52		
PsaN			PsaN
PsaO			PsaO

Table 4.7 Comparison between *A. thaliana* PSI subunit and *N.gaditana* PSI subunit, see the text below for further details.

Compared to *Arabidopsis* PSI (Jensen et al. 2006) (Table 4.7), *N. gaditana* lack homologues of the subunits PsaG, PsaH, PsaI, PsaK, PsaN and O (Corteggiani Carpinelli E. et al. 2013, Radakovitz

R. et al. 2012, Weil L. 2013), which, with the exception of PsaI and PsaK, are present exclusively in plant and green algae (Vanselow C. et al. 2009).
The subunits F and N represent the likely docking site for plastocyanin, here it is present only PsaF. The lack of PsaN could explain the difference in the soluble electron carrier between *N. gaditana*, which shows the presence of Cyt*c6* instead of plastocyanin (Corteggiani Carpinelli E. 2013, see Chapter 1 for further details), and higher plants, where plastocyanin is found, as explained for *Galdieria sulphuraria* (Vanselow C. et al. 2009) and for diatoms (Grouneva I. et al. 2011).
In contrast to plants and green algae, where several of the small subunits (PsaF, PsaJ, PsaI, PsaK and PsaL) are encoded by the nucleus, the subunits detected in the genome PsaJ and PsaL are encoded by the chloroplasts in *N. gaditana*.

It is interesting to observed also the composition of LHC in this fraction: LHCF and LHCF-like represent 19% of the total, while the remaining is equally divided between LHCR, LHC-like and LHCR and LHCR-like.

PSI*-LHC(β-DM) spectral count

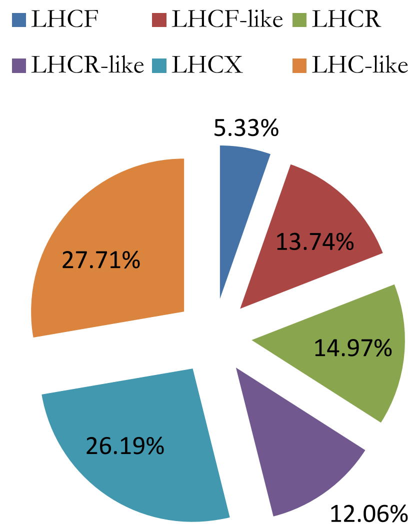


Fig 4.9 Overview of LHC composition in PSI*-LHC-β-DM fraction.

LHCF	PSI*-LHC	%
Naga3	95	5.328099
LHCF Like	**PSI*-LHC**	**%**
Naga 2	18	1.009534
Naga 4	28	1.570387
Naga9	31	1.738643
Naga12	12	0.673023
Naga17	45	2.523836
Naga 18	19	1.06562
Naga 13	92	5.159843

LHCX	PSI*-LHC	%
Naga 1	454	**25.4627**
Naga 5	13	0.729108

LHCR	PSI*-LHC	%
Naga 7	**118**	**6.618059**
Naga 8	**130**	**7.291082**
Naga 20	19	1.06562
LHCR-like	**PSI*-LHC**	**%**
Naga 10	6	0.336511
Naga 14	63	3.533371
Naga 16	16	0.897364
Naga19	**130**	**7.291082**

LHC-like	PSI*-LHC	%
Naga	6	0.336511
Naga 15	-	-
Naga 21	289	**16.20864**
Naga22	199	**11.16096**
TOT	1783	100

Table 4.8 Table with spectral count in PSI-LHCβ-DM fraction.

Analyzing the relative amount of the single protein of PSI*-LHC fraction, as reported in Table 4.8 Naga1, belonging to LHCX, Naga22 and Naga21, belonging to LHC-like, Naga7 and Naga8 belonging to LHCR, and Naga19 are the most abundant proteins detected in this fraction.

Naga7 and Naga8 increase their relative amount in PSI*-LHC respect to monomeric and oligomeric antenna fractions. So they can be the LHC strongly bound with PSI and involved in its supercomplexes, but a confirmation of this hypothesis could arrive only from the proteomic analysis of PSI-LHC solubilized with α-DM.

Moving our attention on Naga1, it also increases its amount in this fraction: also in *P. tricornutum* (Grouneva I. et al. 2011 and Lepetit B. et al. 2010) an LHCX, Lhcx1, was detected within PSI fraction. LHCXs are involved in photoprotective mechanisms and in NPQ and show an homology with Li818 in *Chlamydomonas* (Savard F. et al. 1996 Richard C. et al. 2000).

It is also interesting the increase of the LHC-like, in fact this subgroups reach almost 28% of total antenna spectral count, with Naga 21 and Naga 22 as the most abundant proteins. As previously reported Naga22 is part of ELIPs protein: this 24kDa protein, is similar to ELIP PSI-associated (gi-219110739) found in *P. tricornutum* (Grouneva I. et al. 2011). Studies on *A. thaliana* indicates that OHP2, Lil family's member, is steadily located in PSI, even in low-light conditions and its level increased in response to light stress in a light intensity-dependent manner (Andersson U. et al. 2003). The accumulation of OHP2 in response to light stress in PSI indicates that this photosystem is able to undergo on a dynamic rearrangement of its organization, which possibly takes place in the antenna system. While PSII has a high turnover, especially of D1 protein in case of photodamages, the photoinhibition of PSI is far more dangerous for the

chloroplast because the recovery from photoinhibition, in *A. thaliana*, is not complete even after 1 week (Hihara Y. and Sonoike K., 2001), then the accumulation of OHP2 in PSI exposed to photoinhibitory light might represent one of the strategies to prevent or lower light stress-induced damage(Montané M.H. and Kloppstech K., 2000; Adamska I. 2001). Similarly the presence of an ELIP specific bound to PSI, together with the detection of an LHCX, in *N.gaditana,* could throw a new light on the photoprotection mechanisms of organism in PSI.

Concluding remarks

In *N. gaditana* can be found six different groups of light harvesting complexes: LHCF, LHCF-like, LHCR, LHCR-like and LHCX. The PSI-specific antenna core of *N. gaditana* seems to be composed by two LHCR, Naga7 and Naga8 but further information will be obtained from proteomic analysis of PSI solubilized with α-DM.

It was also found an ELIP specific bound to the PSI. This first analysis raises the possibility to deeper investigate the photoprotection mechanisms involving PSI. It is also known that PSI, respect to PSII is more stable and does not need to a flexible antenna system (Sonoike K. 2011), and its specific antenna play a role on photoprotection (Alboresi A. et al. 2009) so the presence of ELIP and Naga1, an LHCX, could be investigate to detected the mechanisms, against high light, present PSI in *N. gaditana*.

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CHAPTER 5

BIOTECNOLOGICAL OPTIMIZATION OF *NANNOCHLOROPSIS GADITANA*

BIOTECNOLOGICAL OPTIMIZATION OF *NANNOCHLOROPSIS GADITANA*[1]

Abstract

In previous chapters the importance of an optimization of light use efficiency to improve the algae productivity has been underlined. As also discussed earlier, we decided to use *Nannochloropsis* as model organism for the production of biofuels thanks to its capacity to accumulate and high amount of lipids and to maintain a high growth rate under a large range of light intensities. Unfortunately tools for the genetic manipulation of this species are still under development and only recently a few genome sequences become available, making possible to design tools for the biotechnological improvement of this microalga. In this chapter two different methods, homologous recombination transformation and random mutagenesis, were applied on *Nannochloropsis* to generate and isolate possible valuable mutants.

Introduction

The improvement of algae photosynthetic productivity in a large scale cultivation system is an objective requiring a multidisciplinary approach, bringing together a better understanding of the molecular mechanisms influencing light use efficiency in algae with the development of improved growing systems. Several complementary strategies should be investigated to reach a productivity sufficient to make algae large scale cultivation economically and energetically sustainable. As described in Sforza et al. (2012), for instance, an optimized alternation of light/dark cycles has good potential to increase photosynthetic efficiency. In fact, flashes of intense light can be exploited efficiently for photochemistry since they produce reduced electron transporters that can be processed during the following dark period. These data suggest that the optimization of the mixing rates, creating an appropriate alternation of light and dark phases within a photobioreactor, can greatly improve photosynthetic efficiency of the whole culture (Zou N. and Richmond, 2000; Richmond R. et al., 2003; Chen C.Y. et al., 2011). The improvements in photobioreactor design should go in parallel with genetic engineering approaches which can also contribute to increase light use efficiency. This was the scope of this chapter where two methods to obtained optimized strain have been applied to *Nannochloropsis gaditana*.

The first one is the exploitation of homologous recombination (HR) and is based on the work of Kilian O. et al. 2011,that showed good transformation efficiencies in *Nannochloropsis* W2J3B strain. We thus tried to use this approach to generate a *Nannochloropsis* strain Knock-Out (KO) for the VDE. This protein, as previously described (see Chapter 1 and 2 and Chapter 6 for a

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detailed investigation of this enzyme), is a protein involved in the xanthophyll cycle, a photoprotection mechanism active in condition of high light. Xanthophyll cycle however, requires several minutes to be activated and tens of minutes to be de-activated. In the perspective of cultivating algae in mixed photobioreactors where illumination changes have a frequency of seconds or even less, this mechanisms is likely not effective in protecting from excess illumination and could on the contrary be detrimental when cells are in the dark part of the photobioreactor. At least in well mixed photobioreactors thus a VDE KO strain could potentially present an increased productivity.

The second method employed is chemical mutagenesis, aiming to generate *Nannochloropsis gaditana* mutants with altered photosynthetic apparatus regulation through a random mutagenesis with Ethyl methane sulfonate (EMS). EMS is a well-known powerful mutagen, causing a high frequency of nucleotide substitutions, as detected in different plant genomes (Talebi A.B. et al., 2012; Doan T.T.Y and Obbard J.P, 2012). In the past years, EMS has been used to induce the over-production of metabolites in microalgae, including astaxanthin, carotenoids and eicosapentaenoic acid (EPA), an important polyunsaturated fatty acid (PUFA) for the prevention of several human diseases (Doan T.T.Y and Obbard J.P., 2012). In our case one additional advantage is that strains generated by chemical mutagenesis will be testable in outdoor photobioreactors without the necessity of fulfilling the demanding procedures required to cultivate transgenic organisms outdoors.

Materials and Methods

Design of a plasmid for homologous recombination

Four primers were designed to cloned VDE (Naga_100016g50 Corteggiani Carpinelli E. et al. 2013) flanking regions into the pPhaT1-UEP plasmids (Radakovitz R. et al. 2012). Upstream region, which size was 1500 bp each, was inserted into pPhaT1-UEP using the restriction site for BamHI/SpfI present in MCS while downstream region was cloned after the removing of the sequences between HpaI/BspLU11I restriction site. Prior transformation plasmid pPhaT1-UEP-VDE was linearized with NdeI.

VDE UPSTREAM REGION	
Primer For (UPfor)	TCAGGGATCCCGTTCCCTCAGGGATATTG
Primer Rev (UPrev)	ACTATACCTGCAGGTTGAACCAGAAACCCGGC
VDE DOWNSTREAM REGION	
Primer For (DWfor)	TCAGGTTAACATCTCGGAGTTTGAGGGGCG
Primer Rev (DWrev)	ATGACACATGTCCAGAGCCCAAACCCAAGGA

Microalgae growth

Nannochloropsis gaditana from CCAP, strain 849/5, was grown in sterile F/2 medium (Guillard R.R.L. and Ryther J.H., 1962), using sea salts 32 g/L from Sigma Aldrich, 40 mM TRIS/HCl pH

8, Sigma Aldrich Guillard's (F/2) marine water enrichment solution. Cells were grown in Erlenmeyer flasks with 100µmoles photons $m^{-2} s^{-1}$ (µE) of illumination and agitation at 100 rpm. Temperature was set at 22±1°C. Cultures were then treated with an antibiotic cocktail of Ampicillin (100 µg/mL), Streptomycin sulphate (100 µg/mL) and Kanamycin sulfate (100 µg/mL) (Sigma Aldrich) for 48 h to obtain axenic cultures.

Transformation of Nannochloropsis

Transformation protocol described in (Radakovitz R. et al. 2012) with minor modification was used. From a culture in exponential growth phase $5x10^{-8}$cells were harvested for each transformation experiment. Cells were washed four times with 375 mM iced sorbitol before resuspension in 100 µl with 375 mM sorbitol containing 5 µg DNA plasmid linearized with NdeI. Electroporation was done using an ECM630 BTX electroporator (Harvard Apparatus, Inc., Holliston, MA) set at 500 **Ω**, 50 µF and 1200 V using a 1 mm cuvette, resulting in a single 17-20 ms pulse. After electroporation cells were resuspended in F/2 medium and kept overnight on a shaker at room temperature in low light (30 µmol $m^{-2}s^{-1}$). After 24 hours $5x10^{7}$ cells, for each plate, were plated on F/2 selection plates containing 3.5 µg/ml zeocin. Resistant colonies were detected after 4-5 weeks and picked after 7-8 weeks.

Screening of mutants

Picked colonies were mixed in 10 µl of H_2O sterile mQ and incubated for 15 min at 99° then for 15 min on ice to promote the lysis of the cells. 5µl were used as a template for PCR. In the first screening zeocine resistant cassette is amplified and from colonies which present Zeocine resistant cassette DNA was extracted with Chelex 100 (Biorad). The correct integration, for these colonies, was checked with the design of an internal primer and an external one for both flaking region of resistant cassette.

EMS mutagenesis and mortality determination

The microalgae suspension in the late exponential growth phase at 2 $x10^{7}$ cells/mL were mutagenized using 70 mM EMS (Ethyl Methane Sulfonate) for 1 h in darkness at room temperature with mild agitation. Following incubation, treated cells were centrifuged at 5000 g for 8 min to separate cell pellets which were then washed four times with sterile F/2 medium to remove excess EMS. After EMS treatment, cells were then re-suspended in sterile F/2 medium and plated on agar F/2 plates. A number of cells such as to allow a sufficient separation between the growing colonies was plated. Plates were cultured at 22 ± 1 °C, under illumination at 20 µmoles photons m^{-2} s^{-1}, until the algae colonies were emerged. To establish the percentage of mortality induced by the EMS treatment, the number of colonies obtained following EMS treatment was compared to the number of colonies obtained following the treatment with water (control procedure). In the mortality determination procedure the same initial number of cells was plated in F/2 agar plate, both for EMS treated cells and for those water treated.

Mutants selection and screening

Twenty days after EMS treatment, the obtained colonies were visually selected for altered coloration, an indication of altered photosynthetic apparatus. After this first retention, colonies were analysed by in vivo fluorescence based screening which allowed to identify the ones affected in regulation of photosynthetic apparatus. Fluorescence kinetic curves were recorded with a video-imaging apparatus: FluorCam FC 800 (Photon Systems Instruments) (saturating light was set at 400 μmoles photons m^{-2} s^{-1}). Colonies showing significant differences with respect to the wild type (WT) were retained for further analysis.

Results and Discussion

Homologous recombination on Nannochloropsis gaditana

Plasmid for HR in Nannochloropsis was developed using the pPhaT1-UEP developed by Radakovitz and coworkers (2012). As reported in Fig. 5.1, 1500bp upstream and downstream of VDE sequence were amplified from Nannochloropsis genome and cloned into this plasmid. The correct insertions were confirmed by DNA sequencing.

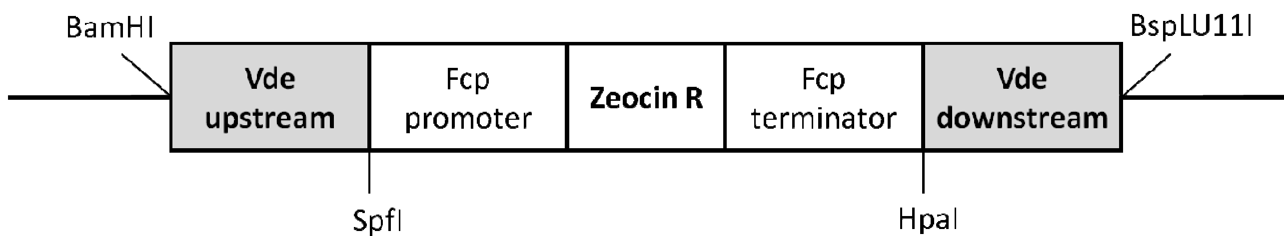

Fig 5.1 Schematic representation of the developed plasmid linearized with NdeI. BamHI/SpfI lead the insertion of upstream region, while HpaI/BspLU11I permit the insertion of the downstream region.

Transformation was conducted as in Radakovits R. et al 2012. From a *Nannochloropsis gaditana* culture in exponential growth phase $5x10^8$ cell were pelletted and washed four times with 375mM iced sorbitol. with 5 μg of linearized plasmid. After electroporation cells were resuspended in F/2 and kept overnight in low light (30 μmol $m^{-2}s^{-1}$), the day after cells were plated on F/2 selection plates. Resistant colonies were detected after 5-6 weeks and picked after 7-8 weeks. The presence of the zeocin resistance cassette was verified with a colony PCR. The PCR product was of 371 bp. More than 95 % of the resistant colonies presents the predicted band suggesting that the transformation was successful in inserting the heterologous DNA in *Nannochloropsis* cells.

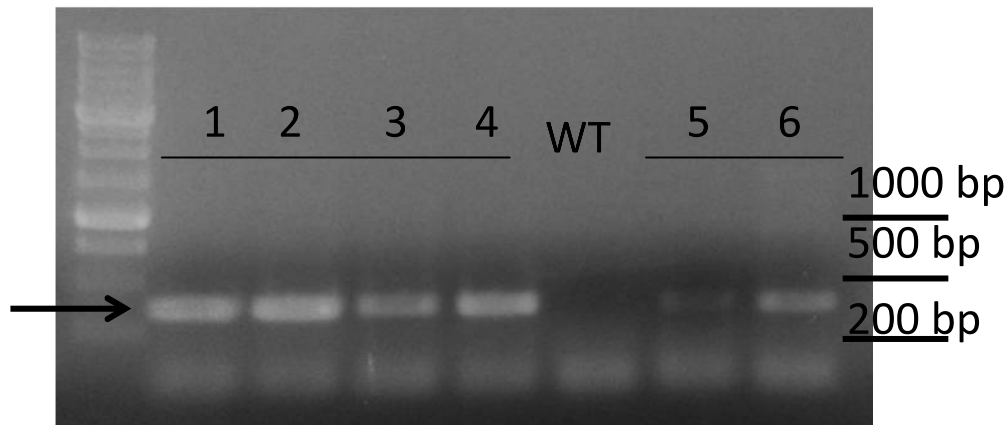

Fig 5.2 PCR colony. PCR confirms the presence of the resistance cassette in some mutants.

These positive colonies were duplicated in plates without the selective agent and after 2 weeks these colonies were again transferred to a solid media with zeocin, in order to select only the colonies with a stable DNA integration. Survival rate of this process was over 90%, suggesting that in *N. gaditana* integration of external DNA occurred with high efficiency.

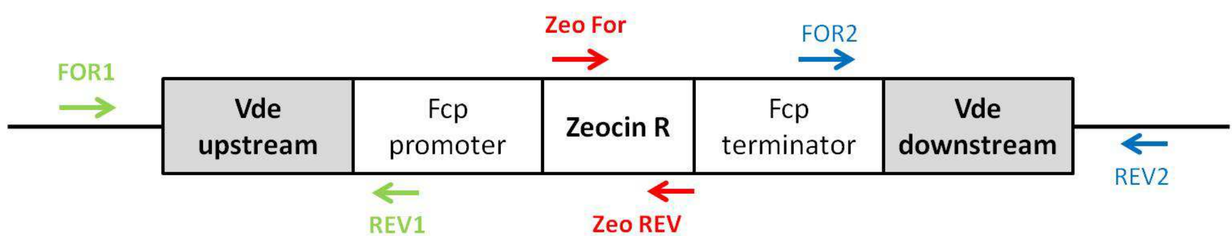

Fig 5.3 Schematic representation of the screening methods to verify the correct integration of resistance cassette.

To control if the resistance cassette was correctly introduced in *N. gaditana* genome, we design four plasmids FOR1/REV1 and FOR2/REV2 to verify the correct integration on the genome of the resistance cassette (Fig. 5.2). Despite several tests in different conditions up to now we did not observed any positive result. This does not exclude the possibility that *Nannochloropsis gaditana* is capable of performing homologous recombination but it shows that this species, unlike *Nannochloropsis sp. W2J3B*, is not able to perform homologous recombination with high efficiency.

Chemical mutagenesis on Nannochloropsis gaditana

A *Nannochloropsis gaditana* culture in the late exponential growth phase was the starting material to perform EMS treatment. Conditions were set to induce a 90% cells mortality and thus ensure a high mutation rate. Following exposure to EMS, surviving cells were cultured in F/2 medium for 20 days prior to isolation of target mutants. $7x10^3$ EMS treated colonies were visually analysed to select around 150 strains (2 %) with an apparent altered pigmentation (Figure 5.3).

Selected strains were thus re-cultured in F/2 agar plates and subjected to a further screening by measuring *in vivo* chlorophyll (Chl) fluorescence, to select those mutants with altered photosynthetic apparatus composition and regulation. Fluorescence measurements are an useful tool to gain indirect information on photosynthetic efficiency and in fact these analysis have become one of the most powerful and widely used techniques to investigate photosynthetic apparatus and its regulatory mechanisms.

We here exploited *in vivo* fluorescence to test photosynthetic functionality of the selected mutants to identify mutants affected in photosynthetic apparatus. We employed a PAM-imaging (PSI instruments Czech Republic) apparatus to measure simultaneously multiple colonies on agar plates. One parameter monitored was the fluorescence of dark-adapted cells (Fo) which, normalized for the area of the colony yields of Fo/Area parameter and thus provides an estimation of the Chl content of the colony.

Differences in this parameter are present when a colony has a reduced Chl content per cell, as expected for a mutant strain with altered photosynthetic apparatus. For this reason, all colonies with a significant alteration in this parameter were retained as potentially interesting mutants.

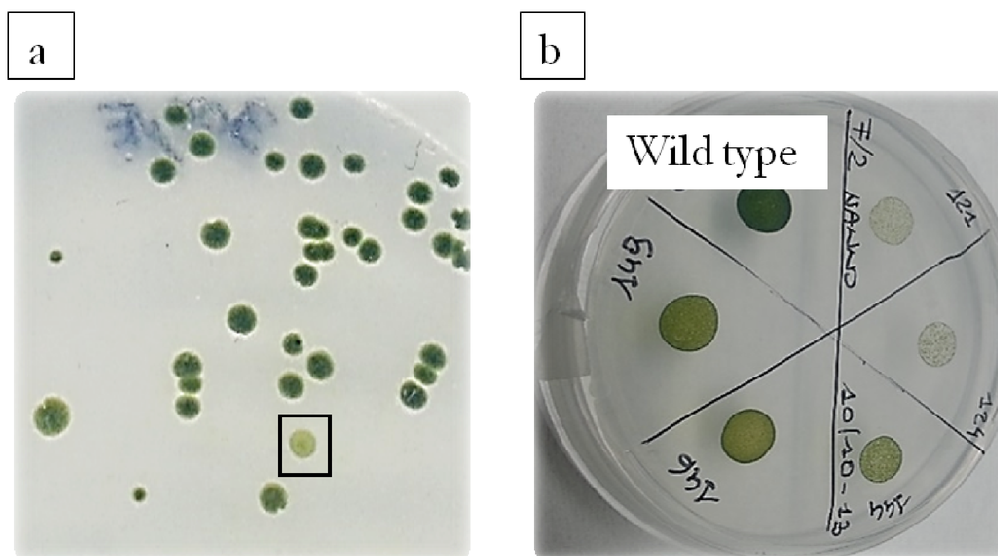

Fig. 5.3: In (a) is presented a F/2 agar plate surface, showing the *N. gaditana* colonies, grown following EMS exposure. In the black square is shown a selected mutant colony, thanks to its pale color gradation, with respect to the other ones grown on the same agar plate. In (b) is displayed a F/2 agar plate, showing five mutants; it is clearly evident the difference in the color gradation of the selected mutants, in relation to the WT. Note that all spots were inoculated with similar cellular concentration (quantified by OD_{750}).

It is important to underline, however, that differences in these parameters can emerge also if the colony contains less cells and thus also if the mutation affects growth rate for a number of reasons independent from the photosynthetic apparatus. To reduce the number of false positives, it was important to inoculate different strains as homogenously as possible (Figure 5.3b).

Additional video-imaging measurements were further used to select *bona fide* mutants. For instance mutants affected in the antenna system are expected to have a higher saturation point of photosynthesis because of their reduced light harvesting ability. Saturation of photosynthesis can be monitored by measuring the quantum yield of Photosystem II (ΦPSII), that estimates the proportion of the light absorbed, exploited for photochemistry (Maxwell K. and Johnson G.N., 2000). When cells are exposed to strong illumination, this value decreases because photosystems are oxidized and thus in a "closed" state, not available for photochemistry. The decrease in this parameter can be exploited as an indication of the photosynthesis saturation in light adapted cells and we expect mutants impaired in light harvesting to have higher values of this parameter with respect to the WT. We found that some mutants with a reduced Fo/Area also showed a decreased saturation of photosynthesis, thus suggesting that these are genuine antenna mutants. Twelve mutants, showing these characteristics, were identified and are currently under further investigation to verify their effective reduction of Chl content per cell, as well the detailed alteration in photosynthetic apparatus.

Concluding remarks

N. gaditana has emerged as model organisms for biofuels production, but genomic information about its capacity to accumulate high amount of lipids has just started to be available and represents just a starting point for further investigations.

The aim of this work is to develop different tools for *Nannochloropsis*'s manipulation with the goal to find mutants which are not impaired in the basic reactions of photosynthesis but where photosynthetic apparatus composition and regulation are altered and allow a better exploitation of light in all the photobioreactor volume.

Today for *Nannochloropsis* the highest-transformation efficiency is reached with the electroporation at elevated electric field strengths, with endogenous promoter driving the selection-marker expression and linearized plasmids (Radakovits R. et al. 2012, Vieler A. et al. 2012, Kilian O. et al. 2011). Our data on homologous recombination seems to be in contrast with the results obtained by Kilian O. et al. 2011, where the HR occurs with a frequencies of 94% (Kilian O. et al. 2011).

It is worth to underline that HR is not common in algae and indeed many eukaryotes, prefer the non-homologous end-joining (NHEJ) to repair the double strand break (Apt KE et al. 1996). Despite the fact that HR is a more accurate method of repair, however it requires the presence of an intact sister chromatid, a requite not always available in case of strand break. During NHEJ, the two broken ends of DNA are simply pieced together, sometimes after limited processing of the DNA ends, resulting in quick, but error-prone, repair. The NHEJ process is driven by two proteins Ku70/Ku80, two proteins evolutionarily conserved from bacteria to human, that form an heterodimer binding to the end of double strand break and promoting the repair process (Lodish K. et al. 2000). In *Nannochloropsis* is present only Ku70 protein (Jinkerson R.E. et al.

2012), so lacking one of the proteins involved in NHEJ, it is possible to suppose that *Nannochloropsis* is more prone to HR process than the NHEJ one; maybe a difference in the gene regulation of Ku70 in *Nannochloropsis* W2J3B strain, respect to *N. gaditana* could explain the high rates obtained in HR to one strain respect to the other. In *Neurospora crassa* it was demonstrated that the elimination of Ku70 or Ku80 improve greatly the frequency of HR (Nynomiya Y. et al. 1999), so in the future a deletion in Ku70 *N. gaditana* gene could be taking in account to develop a particularly efficient strain in HR.

But presently HR in *N. gaditana* is not a valuable tool to manipulate and improve this algae, so for us it was important to investigate a method completely different: the chemical mutagenesis approach. This technique shows some advantages respect to the HR: it could be easily applied to different organisms modifying the concentration of the chemical agent and it is easily to obtain a relatively high density of random mutations (Heinikoff S. and Comai L., 2003) that are heritable in the next generation, and moreover the mutant obtained with this protocol can be easily tested on outdoor photobioreactor. The main disadvantage of this kind of mutation is the detection-approach: looking for phenotypes which may have a positive effect on photosynthetic performances of this alga growing in a large scale system help us to improve a simple methods to isolate the mutants. In this work we screened a *Nannochloropsis gaditana* random mutants collection, obtained following treatment with Ethyl methane sulfonate (EMS). The rationale of this effort is that WT algae isolated in nature have regulatory mechanisms of photosynthesis which are optimized to survive and thrive in their natural environment. Instead, in order to achieve an optimal productivity in the new artificial environment of photobioreactors there is thus the need to select improved strains.

For instance, the mutants with a reduced chlorophyll content per cell should reduce self-shading and then increase homogeneity in light distribution with a beneficial effect on productivity. In this work we managed to select mutants presenting potentially interesting photosynthetic properties which can indeed provide an higher productivity on a large scale.

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CHAPTER 6

PROTEIN REDOX REGULATION IN THE LUMEN: THE CASE OF VIOLAXANTHIN DE-EPOXIDASE

PROTEIN REDOX REGULATION IN THE LUMEN: THE CASE OF VIOLAXANTHIN DE-EPOXIDASE[1]

Abstract

Light influences the regulation of many chloroplast metabolic processes in photosynthetic organisms. One mechanism to achieve this regulation is the light dependent modulation of cysteines redox state. Several chloroplast stroma enzymes are in fact well known to be oxidized in the dark while upon illumination disulphide bridges are reduced and thus broken. This change in oxidation state is correlated with the light availability and results in the modulation of their activity.. The presence of such a redox regulation in thylakoidal lumen proteins have been recently suggested but never demonstrated up to now. If this regulation is present, however, the redox regulation state in the lumen would be the opposite with respect to the stroma and thus oxidizing under illumination and reducing in the dark.

In this work the role of cysteines residues was investigated in *Arabidopsis thaliana* Violaxanthin De-Epoxidase (VDE), a lumenal enzyme involved in the xanthophyll cycle, able to convert the carotenoid violaxanthin into zeaxanthin in condition of high light. VDE presents 13 cysteins with 11 of them concentrated in the N-terminal domain which thus represents a possible site for redox regulation. In this work, 13 mutants each missing one of the cysteines were produced with site directed mutagenesis. In all cases, but one, the mutation led to a drastic reduction of enzymatic activity, demonstrating the importance of these residues for protein activity. These 12 cysteines are also shown to be likely involved in the formation of six disulphide bridges in the active form of the protein.

Introduction

As reported in the previous chapters, photosynthesis is a complex phenomenon finely tuned according to environmental conditions which is far from being completely understood. As discussed earlier, this understanding is also seminal in the perspective of algae biofuels production in order to make genetic manipulation efforts more effective.

One of the main target in this field is the xanthophyll cycle, a photoprotection mechanism which is activated under strong irradiation (Bugos R. C. et al. 1999), present in most photosynthetic

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eukaryotes including *Nannochloropsis* (Gentile M.P. and Blanch H.W. 1992). As discussed in Chapters 1 and 2, a better understanding of its regulation is fundamental for genetic improvement of algae photosynthetic efficiency . Zeaxanthin synthesis is activated under strong illumination and the enzyme responsible of this reaction is called violaxanthin de-epoxidase (VDE). VDE activity is well known to depend from a decrease in lumenal pH occurring under strong illumination. In this chapter a possible further level of regulation, depending from the redox state, was investigated. The study was performed on *A. thaliana* VDE because this protein was already available in our laboratory together with reliable protocols for protein expression and activity tests. As showed below, however, cysteines residues analyzed are well conserved in many other species of plants and algae, thus all conclusions are most likely valid for *Nannochloropsis* protein as well.

As mentioned above VDE is a key enzyme in regulation of photosynthesis. VDE is well known to have a pH dependent regulation and, when photosynthesis is saturated, a high concentration of H^+ in the lumen leads to its activation and zeaxanthin synthesis (Pfündel E.E. and Dilley R.A. 1993). This enzyme undergoes to a conformational change (Arnoux P. et al. 2009), and associates with thylakoid membranes, where violaxanthin is found (Hager A. and Holochener K., 1994, Morosinotto T. et al. 2002). It belongs to the lipocalins family protein, whose members shared a conserved structural organization with an 8 strands β-barrel and often bind hydrophobic molecules (Hieber A. D. et al. 2002). It is formed by three domains.

Fig. 6.1 Schematic domain organization of VDE (Arnoux P. et al. 2009)

The structure of the lipocalin domain has been recently resolved from crystals grown at acidic and neutral pH (Arnoux et al. 2009), showing the pH dependent conformation changes related to VDE activation. The analysis on the structure at pH 5 suggests that active VDE is a dimer, where both violaxanthin epoxide rings can accommodate and react at once. The reducing power necessary for the reaction is provided by ascorbate whose binding site was identified by Saga G. et al. (2010). A recent work (Fufezan C. et al. 2012) demonstrated that four protonable residues play a fundamental role in inducing the first step of activation and starting the pH dependent conformational change.

The other two domains were named after their peculiar amino acid composition as Glutamate-rich and Cys-rich domains (Hieber A. D. et al. 2002, Bugos R. C. et al. 1996).

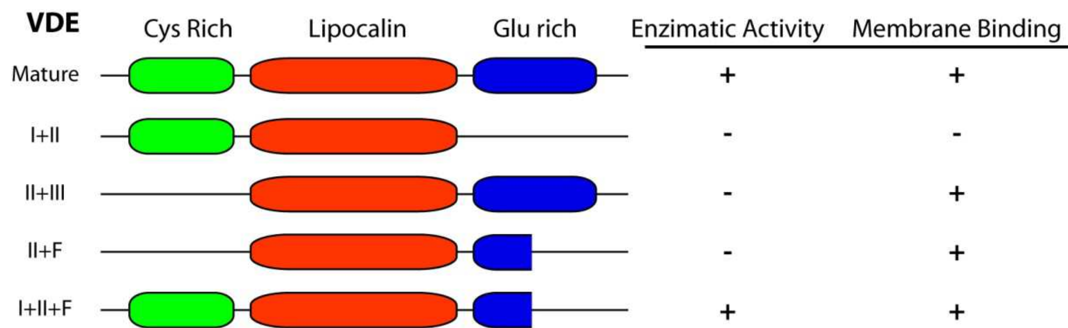

Fig. 6.2 VDE activity in different combinations of domains. N-terminal domain and part of C-terminal domain, called F, are seminal for activity and membrane binding. For further details see Hieber et al. 2002

As reported in figure 6.2, even if the lipocalin domain includes the active site, the other two domains are nevertheless fundamental for enzymatic activity and the protein binding to thylakoids.

Chloroplast is the cellular organelle where photosynthesis takes place. Light, however, not only provides the energy to support photosynthesis but also plays a seminal influence on the regulation of chloroplast metabolisms. One key regulation involves the light-dependent modulation of chloroplast enzymes redox state which leads to different activation states (Buchanan B. 1980). As shown in figure 6.3, light drives to the reduction of ferredoxin (Fd), which is responsible of electron transport between PSI and NADPH (Figure 1.5). Ferredoxin, however, can also reduce thioredoxin (Trx), thanks to the activity of ferredoxin-thioredoxin reductase (FTR) (Buchanan B. 2005a).

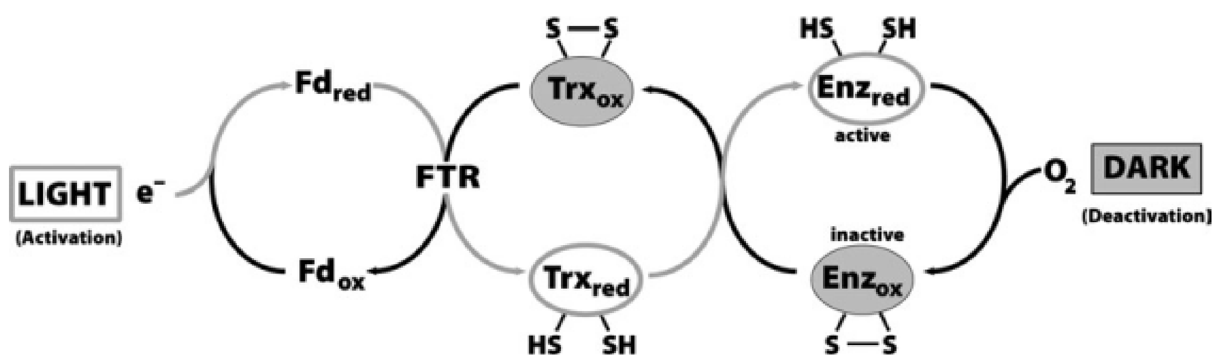

Fig 6.3. Ferrodoxin-Thioredoxin system in the chloroplast. In the light condition Fd is reduced and using FTR, reduced Fd can reduce Trx. Thioredoxin, through thiol/disulfide exchange, then activates carbohydrate synthesis enzymes. As a result, light uses FTR to activate carbohydrate biosynthesis. From Buchanan et al. 2005a

Thioredoxin is a disulphide protein, capable to interact with several chloroplast enzymes and reduce their disulphide bonds. Depending on their redox state these target enzymes assume a different active/inactive state which thus ultimately depends on the available light. Buchanan B. B.(1991) demonstrated that this system controls enzymes involved in the dark phase of photosynthesis allowing plants to balance carbohydrate synthesis and degradation according to the light availability and photosynthesis rate: in fact in dark condition Fd remains oxidized as Trx

and the enzymes of the Calvin-Benson cycle. These enzymes, in the oxidized form, are inactive and as a consequence CO_2 fixation is inhibited, allowing for carbohydrate breakdown. When cells are illuminated, instead, Fd is actively reduced and it drives to the reduction of Trx and Calvin-Benson enzymes, leading to their activation and triggering of CO_2 fixation.

This redox regulation has been well established for several enzymes in the chloroplast stroma, where Calvin cycle takes place. On the contrary it is not clear if a light dependent redox regulation also affects thylakoid lumen proteins. Historically considered only a compartment where oxygen evolution is performed, proteomic analysis throw a new light on thylakoidal lumen, discovering that it contains numerous enzymes associated with the light reactions (Gupta R. et al., 2002a and b; Peltier J. B. et al., 2002; Schubert M. et al., 2002; Spetea C. et al., 2004). The function of most of them is still unknown but studies like Karamoko M. et al. (2011), Hall M. et al. (2010), Gopalan G. et al. (2004) and Kieselbach T. (2013) evidenced how these proteins play a fundamental role in the assembly, function and maintenance of photosynthetic apparatus.

While disulfide bridges reduction in stromal targets activates several enzymes (Buchanan B.B. et al. 2005 and Balmer Y., Meyer Y. et al., 2009, fig 6.3), it seems that several lumenal proteins are active in their oxidized state, presenting disulfide bridges (Gopalan G. et al. 2004 Buchanan B.B. and Luan S. 2005). In order to verify if a redox regulation indeed is affecting lumenal proteins one fundamental piece of information missing is the understanding of how the flow of reducing equivalents are able to move from the stromal compartment to lumen and which enzymes are involved. Recently Motohashi K. and Hisabori T. (2010) have postulated the existence of a special Trx, called Trx-*m*, which could play function in the transfer of reducing equivalent across the thylakoid membrane. However, this protein has never been identified and at present it is not clear how it could receive electrons from Fd which is found on the stromal side of PSI (Karamoko M. et al. 2013). Also since Fd is reduced in illuminated leaves it is difficult to understand how reducing equivalents could be transferred to the lumen in the dark. Karamoko and coworkers (2010) identified in *A. thaliana* Lumen Thiol Oxidoreductase1 (LTO1) an enzyme able to form disulfide bonds in the thylakoid lumen. This protein presents a thioredoxin-like domain which interacts with PsbO, a lumenal PSII subunit known to be disulfide bonded, and a recombinant form of the molecule can introduce a disulfide bond in PsbO in vitro. In 2013 Lu Y. demonstrated that LTO1 have a broad range of substrates in the lumen. Among the various lumenal Trx target, it is important to underline the presence of the two D1-processing proteases, and the VDE indicating that Trx interactions are involved in the assembly of PSII and photoprotection.

In this study we investigate the possible influence of redox state on VDE, analyzing the role of the 13 Cysteines found in *A. thaliana* VDE. Some of these residues most likely influence VDE activity as shown by the fact that DTT is a strong inhibitor (Yamamoto H. Y. and Kamite L. 1972). Their role and the role of redox potential in VDE regulation, however, have never been investigated in detail. We show here that all these cysteines but one have a fundamental role in VDE activity and are involved in multiple disulphur bridges in the active protein.

Materials and Methods

Vde expression and purification

The construct expressing mature WT *A. thaliana* VDE cloned in pQE60 was kindly provided by Prof. Yamamoto (Hieber A.D. et al. 2002). Site-specific mutants of cysteines were produced using the QuickChange site-directed mutagenesis kit (Stratagene) with cysteine substituted by alanine. WT and mutants of VDE were expressed in Origami B strain (Prinz W. A. et al. 1997) of *Escherichia coli* coltured in LuriaBertani (LB) medium (Tryptone 10g (Sigma Aldrich) Yeast Extract 5g (Sigma Aldrich) NaCl10g (Sigma Aldrich)) with a 600-nm absorbance of 0.6 were induced with 1 mM isopropyl-D-1-thiogalactopyr-anoside (IPTG) for 3 h at 37 °C in continuous mixing. Cells were there then centrifuged at 4000 g and 4 °C for 10 min, then resuspended in 25mM Tris-HCl pH 8, 250 mM NaCl, and lysed by sonication.
VDE was then purified on a nickel affinity column (Sigma-Aldrich). Fraction eluted with 100 mM imidazole was stored at -80 °C.

Vde activity test, HPLC

The activity of purified VDE was determined by monitoring the change in the absorbance at 502 nm as previously described (Yamamoto H. Y. 1985; Bugos R.C. et al., 1999). The enzyme assay contained 1% BSA, 1.5 μM Violaxanthin (purified from spinach by HPLC), 9μM MGDG (Lipid Products), 60 mM Ascorbate (Sigma-Aldrich), and 60mM Citrate (Sigma-Aldrich), pH 5. In the case of mutants, the protein amount required for the activity test comparable to the wild type was quantified by SDS-page (Laemmli U. K., 1970) and Western blotting. The presence or the absence of Zeaxanthin production in the mutants were confirmed by HPLC (Havaux M. et al., 2007). Carotenoids were extracted with 100 % diethyl-ether and 125mM NaCl. The organic phase containing the carotenoids was collected, dried 2 hours in a SpeedVac, and resuspended in 80% acetone for HPLC analysis (Farber A. et al., 1997).

Determination of the redox potential of VDE

Redox titration was performed monitoring the change in absorbance at 502 nm as in *Vde activity test*. VDE was incubate 2 hours at 25° C with 20mM at various dithiol (DL-Dithiothreitol)-disulfide(*trans*-4,5-Dihydroxy-1,2-dithiane) ratios of DTT (Sigma-Aldrich), in 50mM Tris pH 8, 1mM EDTA. Control experiments were performed under the same conditions but in the absence of dithiol-disulfide ratios of DTT. Redox titration results were fit with Boltzmann equation (OriginPro8).

Non reducing (redox) SDS-page and Western Blot

Purified VDE was treated with 100mM TCEP (Tris-(2-Carboxyethyl)phosphine-Hydrochloride Invitrogen) or 100 mM H_20_2 (Sigma-Aldrich), to fully reduce or oxidize the protein, in 100mM Hepes pH 7 and incubated for 90 min at room temperature. Samples were then alkylated with 20

mM AMS (4-Acetamido-4'-Maleimidylstilbene-2,2'-Disulfonic Acid, Disodium Salt, Invitrogen) and incubated for 90 min at room temperature. Samples were run on 10% acrylammide non reducing SDS-PAGE, and then transferred to nitrocellulose membranes (Pall Corporation). VDE was detected with home-made antibody raised against *A. thaliana* protein.

Results and Discussion

Cysteines are strongly conserved in VDE

N-terminal region of VDE protein sequences from different species was compared to identify conserved features in the Cys-rich domain. As shown in the figure 6.4, 10 out of the 11 cysteines present in this domain (two additional cysteines are found in the lipocalin domain, Fig. 6.5) are conserved in all organisms presenting a xanthophyll cycle (Fig. 6.4). Cys07 is the only exception, being conserved only in higher plants sequences.

It is worth underlining that this domain does not show a high degree of overall conservation: out of a total of 80 residues, only 23 (29%) are identical. On the contrary over 90% of cysteines are conserved , suggesting the presence of a strong selective pressure for the conservation of these residues and therefore a strong relevance for function of this part of the protein.

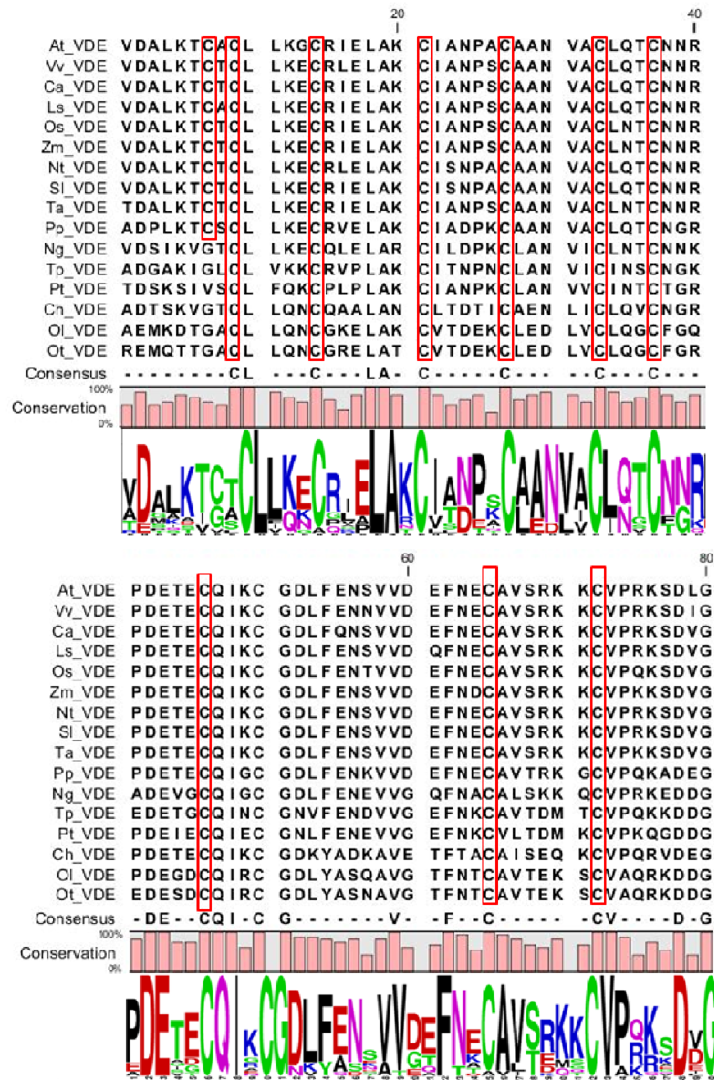

Fig. 6.4 VDE N-terminal domain alignment. Plants: At(Arabidopsis thaliana), Vv(Vitis vinifera), Ca(Coffea arabica), Ls(Lactuca sativa), Os(Oryza sativa), Zm(Zea mais), Nt(Nicotiana tabacum), Sl(Solanum lycopersicum), Ta(Triticum aestivum), Pp(Physcomitrella patens); and algae: Ng(Nannochloropsis gaditana), Tp(Thalassiosira pseudonana), Pt(Phaeodactylum tricornutum), Ch(Chlorella sp), Ol(Ostreococcus lucimarinus), Ot(Ostreococcus tauri). Conserved cysteines (C118 and C249 are highlighted with red rectangles)

In the lipocalin domain are found the two additional cysteines, Cys118 and Cys249, as shown in fig. 6.5 they are also conserved both in higher plant and algae.

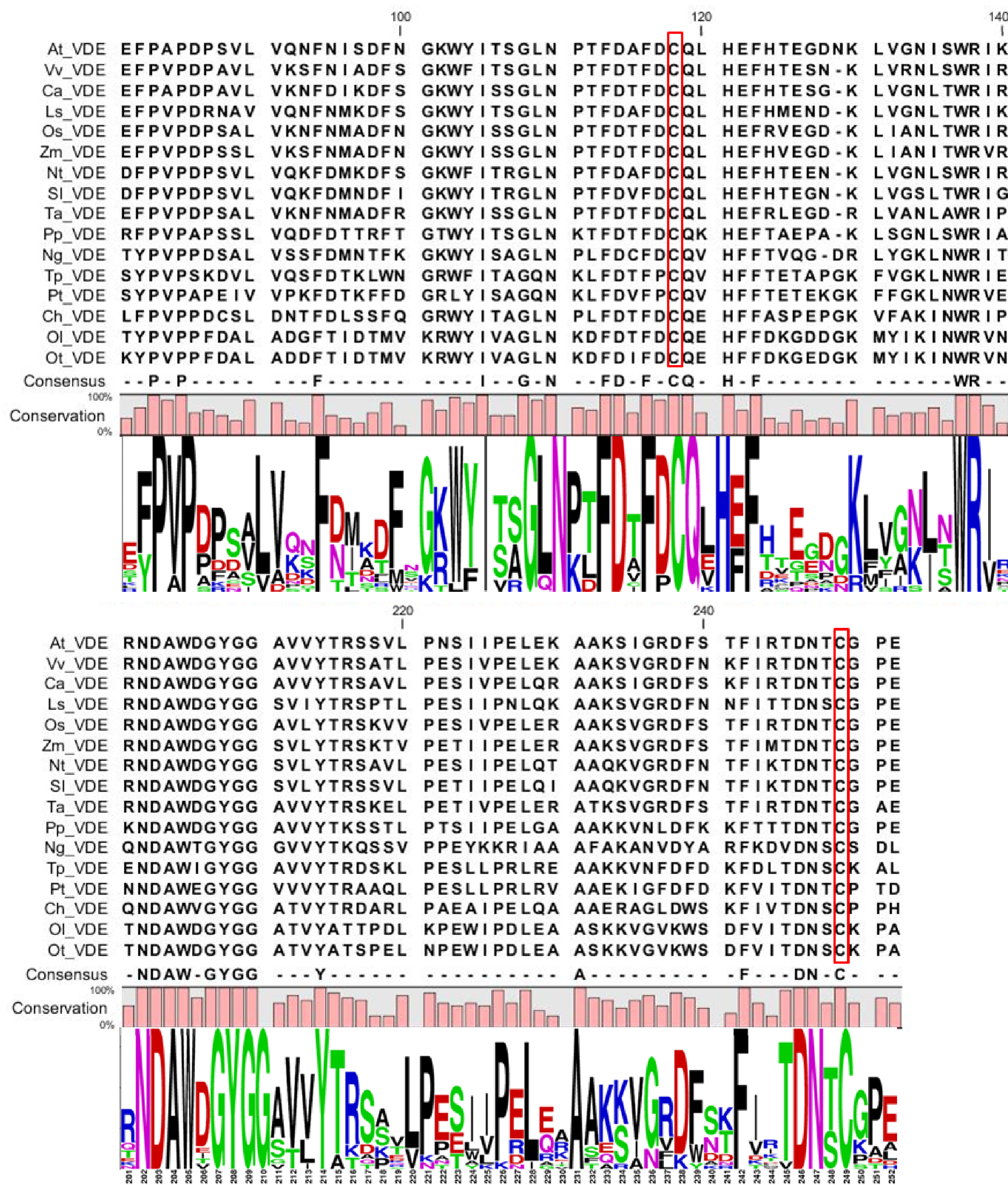

Fig. 6.5 VDE partial lipocalin domain alignment (from amino-acid 81 to 140, and from amino-acid 200 to 252). Plants: At(*Arabidopsis* thaliana), Vv(*Vitis vinifera*), Ca(*Coffea arabica*), Ls(*Lactuca sativa*), Os(*Oryza sativa*), Zm(*Zea mais*), Nt(*Nicotiana tabacum*), Sl(Solanum *lycopersicum*), Ta(*Triticum aestivum*), Pp(*Physcomitrella patens*); and algae: Ng(*Nannochloropsis gaditana*), Tp(*Thalassiosira pseudonana*), Pt(*Phaeodactylum tricornutum*), Ch(*Chlorella sp*), Ol(*Ostreococcus lucimarinus*), Ot(*Ostreococcus tauri*). Conserved cysteines (C118 and C249) are highlighted with red rectangles.

The crystal structures of the lipocalin domain clearly showed that C118 is forming a disulfide bridge with C249 in both structures at pH 5 and 7 (Fig. 6.6).

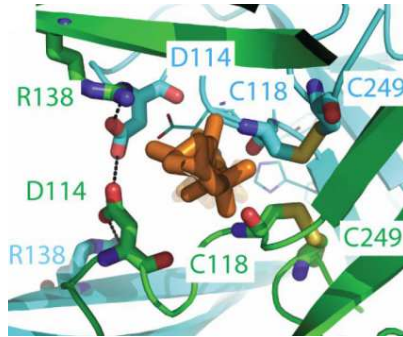

Fig.6.6 C118 and C249 are involved in a disulfide bridge. Here two monomers of VDE are indicated in cyan and green.

Cysteines are fundamental for VDE activity

In order to verify the importance of these residues for VDE activity, all Cysteines were mutated with an Alanine, a non polar amminoacid with a *steric* hindrance *similar to Cystein.*

Residue	Mutation	Residue	Mutation
Cys07	Ala	Cys46	Ala
Cys09	Ala	Cys50	Ala
Cys14	Ala	Cys65	Ala
Cys21	Ala	Cys72	Ala
Cys27	Ala	Cys118	Ala
Cys33	Ala	Cys249	Ala
Cys37	Ala		

Table 6.1 Cysteines belonging to N-terminal domain mutated in Alanine.

All mutant proteins were expressed in E. coli Origami cells and purified by affinity chromatography. Western blot technique was used to quantify purified enzymes and the expression levels was found to be similar for WT and all mutants. Since a strong alteration in protein folding would cause a reduction in purification yield, this suggest that mutations did not alter significantly protein folding.

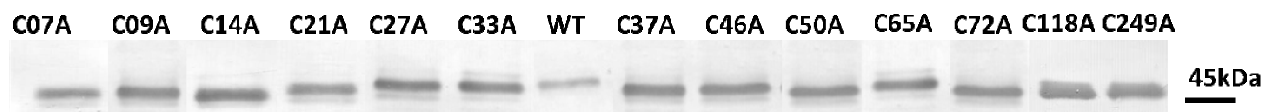

Fig. 6.7 Western blot quantification for VDE WT and VDE Mutants for Cysteines. In the sample a similar amount of protein was charged.

Enzymatic activity of all mutants were afterwards determined. Mutant C07A was the only one showing an activity close to the WT. All other mutations instead caused a drastic reduction of

VDE activity. C50A and C65A showed a strong reduction with an activity between 1 and 3% of the WT.

With the spectrophotometric assay we were not able to detect significant conversion of violaxanthin into zeaxanthin. Since sensitivity of the test is limited (≈1 % of WT) in order to be able to detect smaller activities, we decided to prolonged the reaction and tested the eventual zeaxanthin production by HPLC, a more sensitive method. This alternative approach confirmed previous results for C07A, C50A and C65A but also showed the presence of small but detectable zeaxanthin formation in C09A, C21A, C37A and C72A. In the case of C14A, C27A C33A C46A, C118A and C249A instead there was no zeaxanthin formation also increasing the time for the reaction, suggesting these mutants were completely inactive (Table 6.2).

Residue	**Cys07**	**Cys09**	**Cys14**	**Cys21**	**Cys27**	**Cys33**	**Cys37**
Mutation	A	A	A	A	A	A	A
Activity % WT	85.2 ± 16	< 1 %	nd	< 1 %	Nd	Nd	< 1 %
Residue	**Cys46**	**Cys50**	**Cys65**	**Cys72**	**Cys118**	**Cys249**	
Mutation	A	A	A	A	A	A	
Activity % WT	Nd	3.1 ± 2.5	1.15 ± 1	< 1 %	Nd	Nd	

Table. 6.2 VDE mutants activity shown as a percentage respect to WT, after HPLC analysis

These results support the idea that Cys residues have a seminal function for VDE enzymatic activity, an observation consistent with the high sensitivity to DTT showed by this enzyme (Yamamoto H. Y. and Kamite L. 1972). The only exception is *C07 which has a limited influence, consistently with its presence only in higher plants sequences.*

All conserved cysteines are involved in disulphide bridges

Mutational analysis revealed the importance of the cysteine residues for VDE activity. In order to better understand their role it is fundamental to clarify their redox state in the active protein. This was addressed using a method proposed by Wakabayashi K.I. and King S.M. (2006) in which different oxidation states of the protein were detected using labeling with AMS. This molecule is able to bind covalently to free thiols, increasing the protein weight by 440Da for each free thiol (Fig. 6.8). The number of free cysteines can thus be estimated from the increase in molecular weight. For this purpose, the purified protein was incubated with AMS in the purification buffer or in the presence of either a reducing (TCEP) or oxidant (H_2O_2) agent. After incubation the samples were analyzed by non reducing SDS-PAGE and Western Blot (Fig. 6.9) to detect eventual changes in VDE molecular weight. TCEP was used instead of DTT as reducing agent because the former does not react with maleimide group of AMS.

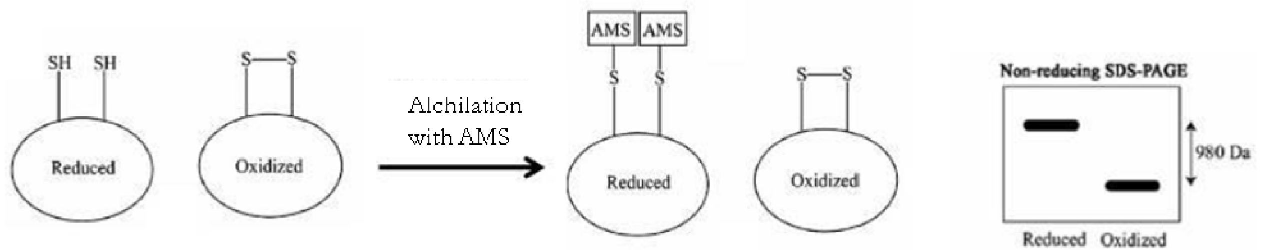

Fig. 6.8 Scheme detailing the AMS method to distinguish oxidized and reduced dithiols. The maleimide group of AMS reacts only with reduced thiols and, thus, a protein with a reduced vicinal dithiol will incorporate two AMS moieties (980 D). Adapted from Wakabayashi K.I. 2006.

As shown in Fig. 6.9, the samples treated with TCEP and AMS migrate slower compared to the untreated samples or the oxidized ones. This could be explained by the fact that in these samples there are several free thiols that can react with AMS leading to an increase in the molecular weight. It is interesting to note that on the contrary control and the oxidized samples do not show any detectable differences in the molecular weight, suggesting that there are no additional disulfide bridges formed due to the oxidation. The number of free cysteines binding AMS in the control and oxidized samples thus remains the same. Finally it is worth underline that both bands have similar weight as the control without AMS labelling suggesting that the number of free cysteines in control is very low, likely one. All these results are well consistent with the hypothesis that all cysteines except one are involved in disulphide bridges in the native protein.

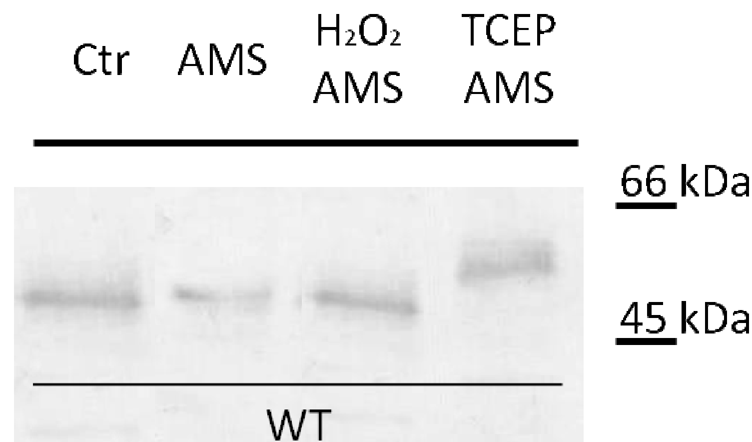

Fig. 6.9. Non reducing SDS-PAGE and Western blot of VDE treated with TCEP or H202 and then with AMS. Ctr indicates the control (WT untreated), AMS indicates WT incubated with AMS, while H_20_2/AMS and TCEP/AMS represent the samples mixed first with the oxidant and reducing agent respectively and then treated with AMS.

A rapid change in redox potential led to a rapid inactivation of VDE activity

Analysis with TCEP and AMS revealed that VDE is completely oxidized in its native form. In order to better investigate this characteristic we made a redox titration of VDE, testing its activity

after incubation at different redox potentials. As shown in figure 6.10 VDE displays a sharp dependence from redox potential showing inactivation at lower potentials, with an inflection point at -320 ± 3 mV.

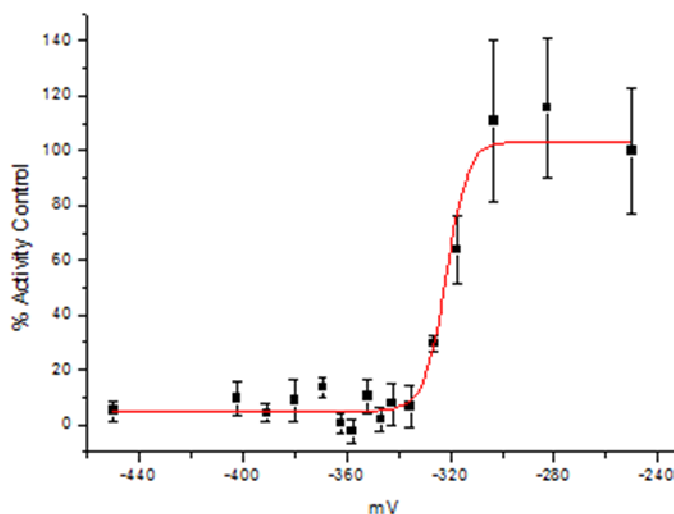

Fig. 6.10. Redox titration of VDE. **VDE was incubated for 2 hours at 25° with 20mM at various dithiol-disulfid ratios of DTT. Completely oxidized enzyme shares the same activity of the control; the activity of the protein mixed with different dithiol/disulphide ratios was calculated respect to the completely oxidized enzyme (100% of activity respect to control).**

Concluding remarks

This study involved the enzyme responsible for the zeaxanthin synthesis in the xanthophyll cycle: the Violaxanthin De-Epoxidase. Zeaxanthin plays a role of crucial importance protecting the photosynthetic apparatus from damages caused by high light, acting as a scavenger of ROS and promoting the dissipation of the light as heat (Demming Adams B. et al. 1996, Havaux M. et al. 2007). Zeaxanthin production must be finely regulated to allow a correct balance between photoprotection at high irradiation and the necessity of photosynthetic organisms to exploit solar energy, with the maximum efficiency, when light conditions are limiting. While the pH dependent regulation of the VDE, involving an important conformational change, was largely investigated (Arnoux P. et al. 2009, Saga G. et al. 2010, Fufezan C. et al. 2012), other possible mechanisms have been less studied.

In this work we focused our attention on the N-terminal domain, enriched in cysteines to assess if a second regulatory mechanisms based on the change in the redox state is possible for this enzyme. The works by Karamoko M. et al. (2010), Gopalan G. et al. (2004) and Buchanan B.B. (2005) suggested a possible redox regulation in thylakoidal lumen differing from the one active in the stroma. While in the stroma light causes a cysteines reduction and led to more active proteins, in the lumen (Fig 6.11) illumination could cause the oxidation of thiol groups. This kind of

control could also be light dependent because combines the light absorbed by the photosystems, which allows the electron transport chain flow and the reduction of the thioredoxins, and the CO_2 fixation into the organic compound such as lipids, proteins, carbohydrates which depends on the reducing power and free energy coming from light phase of photosynthesis.

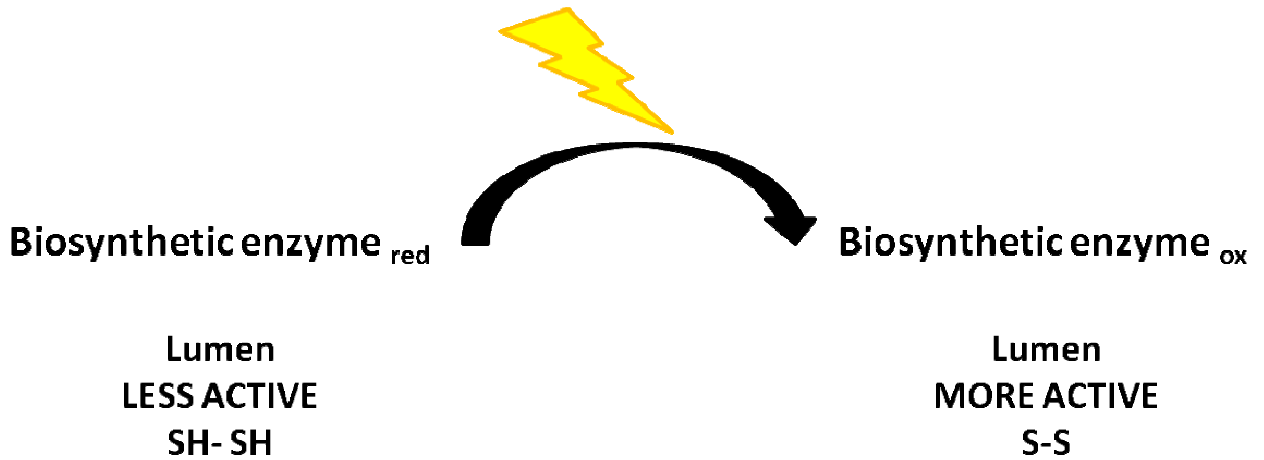

Fig. 6.11 schematic mechanism of redox regulation in thylakoidal lumen. Adapted from Gopalan 2004

If this hypothesis is correct in thylakoid lumen must be present a system able to oxidize and reduce proteins in a light-dependent manner. Thylakoidal lumen is the place where molecular oxygen is produced and in illuminated leaves, thanks to the photosynthesis, there is a high O_2 concentration which should be sufficient to oxidize proteins. Another explanation, exposed first by Gopalan G. et al., (2004) postulates the presence of an intermediate lumenal protein oxidized from O_2 that is responsible of oxidizing target proteins, as VDE. The presence of this protein however is controversial: it was first described in 2008 by Hall and coworkers, with the name of peroxiredoxin Q-like protein, but experimental evidences that definitely prove the presence of this particular thioredoxin in the thylakoidal lumen are still missing. In this case, even less clear would be how lumenal proteins are reduced in the dark when photosynthesis process, and consequently the reducing equivalents flow, is blocked. Concerning the eventual existence of this protein a more recent work (Motohashi K. and Hisabori T. 2010) supposed a different function of this enzyme with its involvement in the transfer of reducing equivalent across the thylakoid membrane.
Concerning VDE, it is evident from our results, that this enzyme is extremely sensitive to reduction state and only in a condition of completely oxidation VDE is active: this protein likely presents 6 disulfide bridges and the disruption of only one of them seems to be sufficient to inactivate the protein, as demonstrated from test activity (Table 6.2). These data, together with the steep dependence from redox potential of protein activity, outlines a picture where redox regulation is not suitable for a fine tuning of VDE activity. On the contrary a modulation of redox potential can completely activate/inactivate the protein. It is possible to hypothesize a scenario where a redox regulation might be important: in fact, in the morning plants coming from a dark night adaptation are gradually exposed to illumination and they start to perform

photosynthesis. In this condition, proton concentration in the lumen gradually increases and as a consequence it is possible to activate VDE. If light is not in excess this is not beneficial for plants because solar energy, which should be use to perform photosynthesis, could be dissipated as heat by the activation of xantophylls cycle and not exploited to produce chemical energy. During the night, photosynthesis is not performed, and VDE is found in a condition of inactivation due to reduction state of the lumen. If VDE could be activated by oxidation due to molecular oxygen produced by photosynthesis, this means that VDE could be activated only when light intensities are higher thus when a sufficient concentration of molecular oxygen has been produced, avoiding a premature activation of energy dissipation and ensuring better regulation of photoprotection mechanisms and a better exploitation of solar energy.

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APPENDIX 1

CHLOROPHYLL TRIPLET QUENCHING IN THE VIOLAXANTHIN-CHLOROPHYLL-LIGHT HARVESTING PROTEIN FROM *NANNOCHLOROPSIS GADITANA*, AS REVEALED BY ODMR AND EPR SPECTROSCOPIES.

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Abstract

Violaxanthin-Chlorophyll-Protein (VCP) is the major Light-Harvesting Complex (LHC) of the Heterokonta *Nannochloropsis gaditana* binding only Chlorophyll *a*, violaxanthin and vaucheriaxanthin, in the form of 19' deca/octanoate esters. Photosynthetic apparatus of algae belonging to this group, have been poorly characterized in the past, but there is now an increasing interest also because of their possible biotechnological application in biofuel production.

In this work, isolated VCP proteins have been studied by means of advanced EPR techniques in order to investigate the presence of the photoprotective mechanism based on triplet-triplet energy transfer (TTET). Optically Detected Magnetic Resonance (ODMR) has been used to identify the triplet states populated by photoexcitation, and describe the optical properties of the chromophores carrying the triplet states. Time-resolved EPR (TR-EPR) has been employed to get insight into the TTET mechanism and reveal the structural features of the pigment sites involved in photoprotection. The analysis of the data shows a strong similarity in terms of triplet state populations between VCP, FCP from diatoms and LHC-II from higher plants. Even if these antenna proteins have differentiated sequences and binds different pigments, the results suggest that in all members of the LHC superfamily there is a core represented by two central carotenoids surrounded by five Chlorophyll *a* molecules with a conserved structural organization and a fundamental photo-protective function in Chl triplets quenching.

Abbreviations

$^{1}Chl^{*}$: singlet Chl excited molecules

$^{1}O_2$: singlet oxygen

^{3}Chl: Chl triplets

Car, Carotenoid;

Chl, Chlorophyll;

Cyt, Cytochrome;

DTT, dithiotreitol;

Fdx, ferredoxin;

FNR, Ferredoxin NADP + reductase;

FTR: ferredoxin-thioredoxin reductase

HR, homologous recombination;

KO, knock-out;

LHC, Light harvesting complex;

LHCA, antenna polypeptides of Photosystem I;

LHCB antenna polypeptides of Photosystem II;

LHCF, FCP fucoxanthin chlorophyll *a*/*c*-binding protein

LHCR: LHC in red algae/diatoms,

LHCSR,LHCX Lhc-like protein Stress Related (previously called Li818)

Lil: LHC-like proteins,

NHEJ, Non-Homologous End Joining;

NPQ, Non Photochemical Quenching;

PC, plastocyanin;

PCR, polymerase chain reaction;

PQ/PQH 2 , plastoquinone/plastoquinol;

PSBS photosystem II subunit S;

PSII (PSI): photosystem II (I);

ROS, Reactive oxygen species;

RT, room temperature;

RuBisCO, ribulosio 1,5biphosphate carbossilase-oxygenase; SDS, sodium-dodecyl-sulphate;

Trx: Thioredoxin reductase

VDE, Violaxanthin de-epoxidase;

WT, wild type.

ZE, Zeaxanthin epoxidase