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# 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.



## 1. Introduction

Organisms that perform oxygenic photosynthesis are capable of converting light into chemical energy due to the activity of photosystem (PS[2]) I and PSII, 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

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[2] 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.

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exploitation for biodiesel production due to their rapid growth rate and ability 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.

## 2. Materials and methods

### 2.1. Cell growth

*N. 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 μmol of photons $m^{-2}$ $s^{-1}$ (μE) of illumination and air enriched with 5% $CO_2$. The temperature was set at 22 ± 1 °C.

### 2.2. 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 min of centrifugation at 4 °C with a Beckman Allegra 250 at 4000 ×*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 s 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 ×*g*, 15 min, 4 °C), and the supernatant was collected. The supernatant containing the broken cells and thus the thylakoids was centrifuged at 15,000 ×*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 resuspended 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.

### 2.3. Thylakoid solubilization and sucrose gradients

Thylakoid membranes corresponding to 500 μg of Chl were washed with 50 mM EDTA and then solubilized for 20 min 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 15,000 ×*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) (280,000 ×*g*, 18 h, 4 °C). The green fractions of the sucrose gradient were then harvested with a syringe.

### 2.4. 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).

### 2.5. 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.

### 2.6. Electrophoresis and western blotting

A 12% SDS-PAGE analysis for both the sucrose gradient fractions and thylakoid extracts was performed using a TRIS–glycine buffer system, as described [42]. The samples were solubilized for 20 min 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 min at 15,000 ×*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*.

### 2.7. 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.

## 3. Results

### 3.1. Isolation of pigment-binding complexes from N. gaditana

The first requirement for characterizing pigment-binding protein complexes from *N. 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 thylakoid 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 Fig. 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, *P. tricornutum*, and one plant, *P. patens* (Fig. 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). 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 (Fig. S1). This finding suggests that the number of antenna subunits associated with PSI in *Nannochloropsis* is closer to the nine found in *Chlamydomonas* than to the four present in plants [47,48].

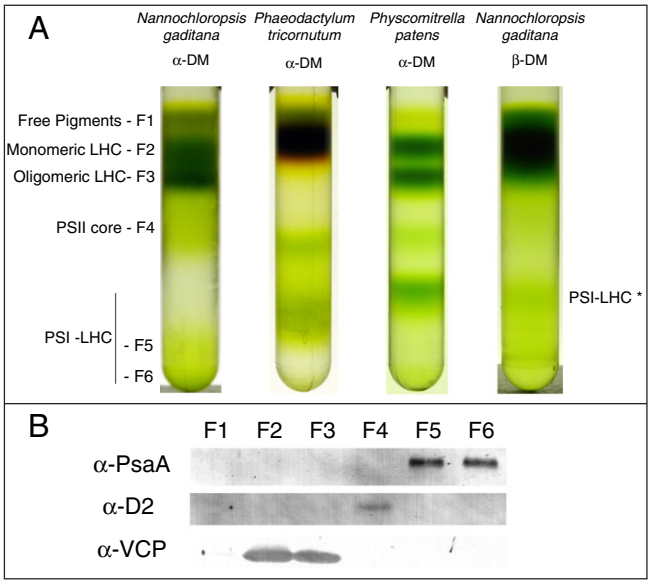


**Fig. 1.** Isolation of pigment binding proteins from *Nannochloropsis gaditana* by sucrose gradient ultracentrifugation. A) *Nannochloropsis* sucrose gradient after mild solubilization with 0.4% α-DM. Five distinct bands are distinguishable (F1–F5) while a sixth is present at the bottom of the tube (F6). Mobility of sucrose gradients after solubilization is compared with other species, a diatom (*Phaeodactylum tricornutum*) and a plant (*Physcomitrella patens*). Band 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 band 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.

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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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] (Fig. 2B).

### 3.2. 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 Fig. 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 (Fig. 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 (Fig. 3B); the spectra of these two bands are very similar, supporting the identification of F6 as PSI-LHC aggregates or PSI-enriched membrane particles.

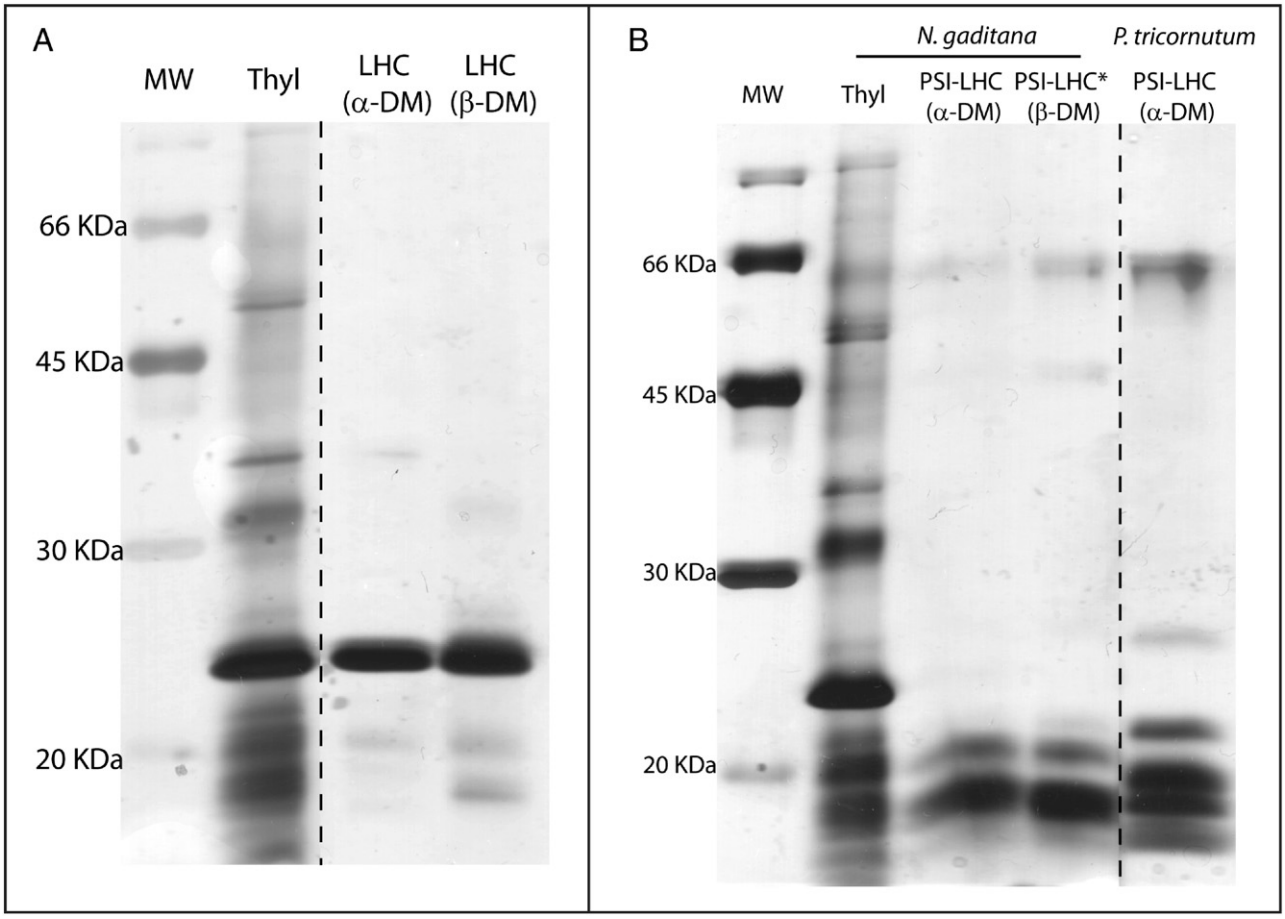


**Fig. 2.** Analysis of sucrose band polypeptide composition. A) SDS-PAGE of *Nannochloropsis* thylakoids and antenna bands from α and β-DM gradients (F3 and F2 respectively). 4 and 2 Chl μg were loaded for thylakoids and LHC bands respectively. B) SDS-PAGE of PSI bands, comparing PSI from *Nannochloropsis* 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.

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 (Fig. 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 (Fig. 3A and B), in agreement with its identification. In Fig. 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.

*Nannochloropsis* has a peculiar carotenoid composition, with violaxanthin, β-carotene, and vaucheriaxanthin as the major pigments (Table 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.

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 spectrum 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 (Fig. 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, Fig. 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*.

## 4. Discussion

Algae are a very diverse group of organisms, and their photosynthetic apparatus shows variable protein and pigment compositions.

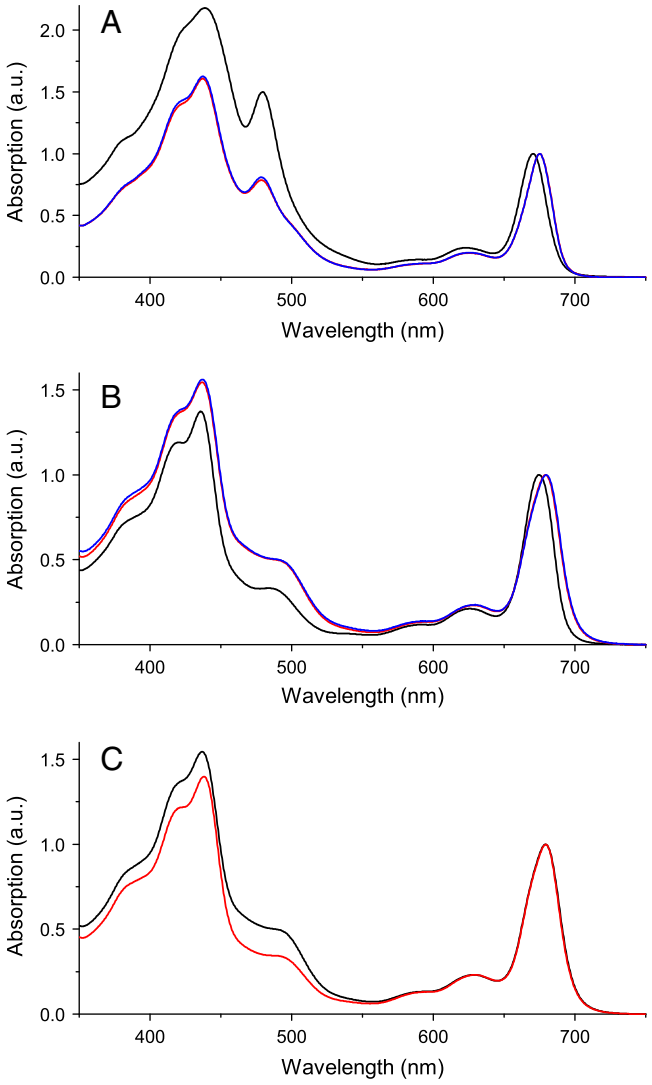


**Fig. 3.** Absorption spectra of sucrose gradient bands. A) Free pigments (F1, black), monomeric and oligomeric antennas (F2, red and F3, blue). F1 has a maximum in the Qy region at 670, typical for free Chl *a* in a detergent solution, Chls maximum in monomeric and oligomeric antennas is at 675 nm. B) PSII core complex (F4, black), PSI-LHC (F5, red and F6, blue). PSI-LHC presents a typical absorption over 700 nm. 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.

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

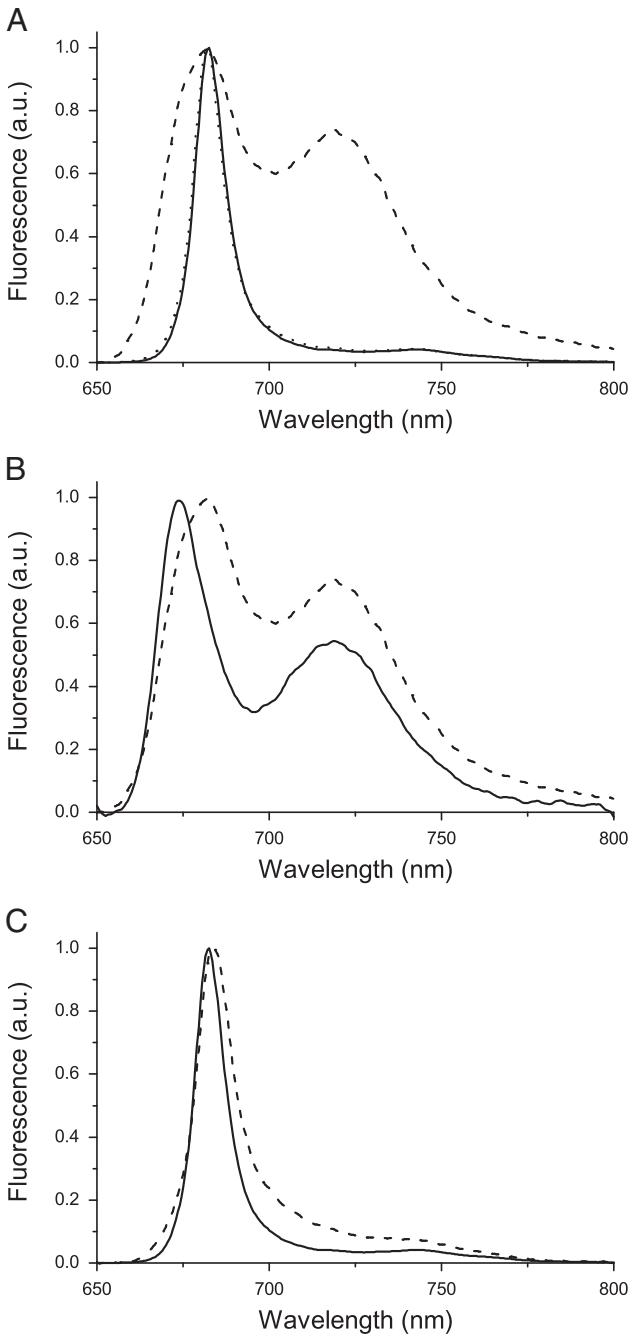


**Fig. 4.** Fluorescence LT spectra. A) Comparison of 77 K fluorescence spectra of monomeric and trimeric antennas (F2, solid, F3, dotted) and PSI-LHC (F5, dashed). Fluorescence at 720 nm, due to red shifted Chl is detected only in PSI-LHC. B) Spectra of PSI-LHC solubilized with α-DM (dashed) and β-DM (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).

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

| Fractions | Name | Viola-xanthin | Vaucheria-xanthin | Antera-xanthin | Zea-xanthin | 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 |

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].

### 4.1. 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], Fig. S3). A notable difference with diatoms and any other known heterokontan, however, is that the *Nannochloropsis* antenna has the unusual property of binding only Chl *a*, with no accessory Chls.

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 (Fig. 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.

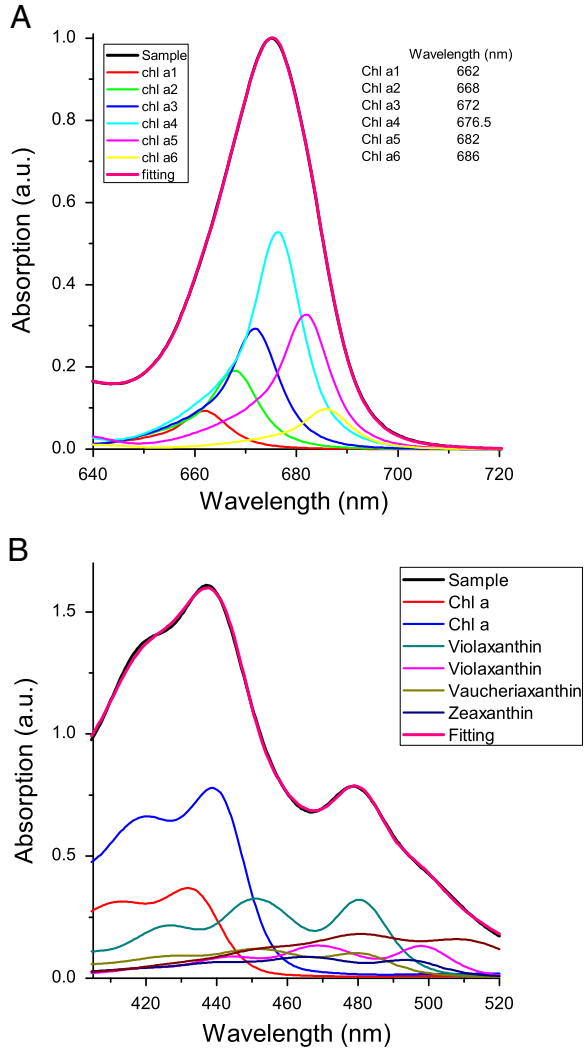


**Fig. 5.** Analysis of LHC complexes absorption spectra from *N. gaditana*. A) Distribution of Chl spectral forms in Qy region of the spectrum. In order to describe the absorption spectrum four major Chl forms absorbing at 668, 672, 676.5 and 682 nm were employed together with two minor forms at 662 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].

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 (Fig. 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 plant 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.

### 4.2. 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 (Figs. 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 (Fig. 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 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 (Fig. S1) [47,48].

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 (Fig. 2B), distinct from the PSII antenna complexes in which the major band is larger (Fig. 2A). This difference clearly suggests that a distinct set of LHC proteins is preferentially associated with PSI and PSII in *N. gaditana*.

### 4.3. 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 photosystems 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 weights identified in the PSII and PSI fractions (Fig. 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 Fig. 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, *C. 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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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbabio.2013.11.019.

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