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Molecular basis of chromatic adaptation in pennate diatom *Phaeodactylum tricornutum*

Miroslava Herbstová [a,b], David Bína [a,b], Peter Koník [b], Zdenko Gardian [a,b], František Vácha [a,b], Radek Litvín [a,b,*]

[a] Institute of Plant Molecular Biology, Biology Centre CAS, Branisovska 31, 37005 Ceske Budejovice, Czech Republic
[b] Faculty of Science, University of South Bohemia, Branisovska 1760, 37005 Ceske Budejovice, Czech Republic

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ABSTRACT

The remarkable adaptability of diatoms living in a highly variable environment assures their prominence among marine primary producers. The present study integrates biochemical, biophysical and genomic data to bring new insights into the molecular mechanism of chromatic adaptation of pennate diatoms in model species *Phaeodactylum tricornutum*, a marine eukaryote alga possessing the capability to shift its absorption up to ~700 nm as a consequence of incident light enhanced in the red component. Presence of these low energy spectral forms of Chl *a* is manifested by room temperature fluorescence emission maximum at 710 nm (F710). Here we report a successful isolation of the supramolecular protein complex emitting F710 and identify a member of the Fucoxanthin Chlorophyll *a/c* binding Protein family, Lhcf15, as its key building block. This red-shifted antenna complex of *P. tricornutum* appears to be functionally connected to photosystem II. Phylogenetic analyses do not support relation of Lhcf15 of *P. tricornutum* to other known red-shifted antenna proteins thus indicating a case of convergent evolutionary adaptation towards survival in shaded environments.

1. Introduction

Marine photosynthesis powers a substantial part of the biosphere and forms a major portion of global carbon cycle. Diatoms (Bacillariophyceae), a group of eukaryote algae, play a considerable role in oceanic carbon fixation [1]. Diatoms belong to the group of Heterokonta (Stramenopila), a group of protists originating around the beginning of the Palaeozoic [2]. However, the line leading to them is much older and is based on a common eukaryote ancestor which engulfed a red alga, creating a secondary plastid [3,4]. The unifying characteristic of this diverse group of photosynthetic protists is the cell wall made of silicon dioxide.

The symmetry of diatom silica cell walls has been used to establish a simple classification into pennate (bilaterally symmetric) and centric (radially symmetric) diatoms. This split is partially reflected in their lifestyles — centric diatoms are often planktonic whereas the pennates are mostly benthic [5,6]. More recent phylogenetic studies have complicated this traditional distinction by identifying radial centric diatoms as the basal group with respect to a branch containing both multipolar centric and pennate diatom clades [7,8]. Due to their importance, diatoms were the first heterokont algae to have their genomes sequenced, first the centric *Thalassiosira pseudonana* [9] followed by pennate *Phaeodactylum tricornutum* [10].

Although diatom ancestry includes red algae their light harvesting machinery does not rely on phycobilisomes but is based on intrinsic membrane light harvesting complexes known as fucoxanthin-chlorophyll *a/c* binding proteins (FCPs). FCPs of diatoms contain three transmembrane α-helices and belong to the LHC superfamily [11]. Diatom nuclear genomes contain several tens of FCP genes [9,12].

Based on function and sequence homologies, FCPs are classified into three major clades encoded by: (i) *lhcf* genes representing major antenna proteins of diatoms, (ii) *lhcr* genes homologous to PSI antennas of red algae [13–15] and (iii) *lhcx* genes related to stress-related (LhcSR) proteins (formerly called LI818) of green alga *Chlamydomonas* [16–18]. The major Lhcf type of FCPs is known to form trimers and higher oligomeric states [19–21]. As to the specific proteins forming these trimers and oligomers, the available information is still limited. Although it has been found that Lhcf1 and 5 proteins of *P. tricornutum* form a stable trimer [22], analyses of wild type samples always show a mixture of many isoforms [14,15,23,24].

It has been known for a long time that some pennate diatom species exhibit a shift of their emission spectra to the far-red region, a phenomenon accompanied by a change in absorption spectrum, in response to changes of the quality of incident light [25–31]. A first successful step towards characterization of the protein species involved in this process

Abbreviations: CLH, *Chromera* light harvesting; DL, day light; DM, n-dodecyl β-D-maltoside; F, fluorescence; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; FCP, fucoxanthin-chlorophyll protein; Chl(s), chlorophyll(s); LL, low light; MS, mass spectrometry; PCP, peridinin-chlorophyll protein PS, photosystem; Q, plastoquinon; RC, reaction centre; RL, red light; RT, room temperature SDS-PAGE, sodium dodecyl-sulphate-polyacrylamide gel electrophoresis; SG, sucrose gradient; VCP, violaxanthin-chlorophyll *a* binding protein

* Corresponding author at: Institute of Plant Molecular Biology, Biology Centre CAS, Branisovska 31, 37005 Ceske Budejovice, Czech Republic. Tel.: +420 38 777 5523.
E-mail address: litvin@umbr.cas.cz (R. Litvín).

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was made by Fujita and Ohki in *P. tricornutum* [32]. These results suggested that the presence of a specific antenna protein of the FCP family, rather than the modulation of PSII:PSI ratio implicated earlier [33], was responsible for the spectral shift.

Such enhancement of the absorption in the near-infrared region is reminiscent of several cyanobacterial species, *Acaryochloris* and *Halomicronema hongdechloris*, where it is achieved by using unique pigmentation [34,35] and was interpreted as an adaptation to existence in shaded niches that are enriched in the far-red light [36].

It is well known that even organisms with Chl *a* can achieve a similar feat resulting in a significant change in their emission and absorption spectra, the best understood example being the red-shifted antenna of PSI, LHCI, of green plants [37]. However, red shifted Chl *a*-based antenna systems associated with photosystem II have also been observed. A coral-associated prasinophyte green alga *Ostreobium* sp. contains an antenna complex with absorption and fluorescence shifted towards and beyond 700 nm [38–40]. The red-shifted absorption and fluorescence have also been recently described in *Chromera velia* [41,42], a relative of apicomplexan parasites which nevertheless has an antenna system which is undoubtedly heterokont in origin [43,44]. The chromatic adaptation of *C. velia* is based on synthesis of specific antenna proteins of the LHC group and the process apparently shares many similarities with spectral changes described in diatoms.

The present study brings additional insight into the functioning of chromatic adaptation of a marine pennate diatom *P. tricornutum* on the molecular level. We have successfully isolated a thylakoid membrane fraction preserving the 710 nm fluorescence emission at room temperature, which allowed the identification of the protein required for the red-shifted antenna complex formation and development of F710 emission. Present results also suggest that the red-light induced F710 antenna complex is functionally connected, that is capable of transferring excitation, to photosystem II (PSII), similarly to red-shifted antenna complexes of *C. velia* and *Ostreobium*. In accordance with the original study of Fujita and Ohki, we have observed a fast disappearance of F710 emission in vitro indicating that the shift of Chl *a* transition to lower energies occurs due to intermolecular interactions within a fragile supramolecular protein assembly.

2. Material and methods

2.1. Growth conditions

Diatom, *P. tricornutum* (SAG culture collection, strain 1090-1a) was grown in a modified artificial sea water f/2+Si medium [45]. Cells were cultivated in 5 l Erlenmeyer flasks under two different spectral qualities hereby denoted as red light (RL) and day light (DL). A halogen lamp OSRAM HALOPAR38 and a metal halide lamp OSRAM POWERSTAR HQI 250 W/D PRO were used as a source of RL and DL, respectively. For each spectral quality, cultures were maintained at 22 °C under constant aeration achieved by bubbling with sterile air, and at light intensity of ~20 μmol photons m^{-2} s^{-1} in a 16/8 h light/dark regime.

2.2. Fractionation of pigment–protein complexes

Cells in the late logarithmic phase were collected by centrifugation (7000 ×*g*, 5 min). Isolation of pigment–protein complexes was performed as described in [32]. In our experiments, freshly harvested cells were washed in Tricine buffer (pH 7.5) and resuspended in the same buffer containing EDTA-free Protease Inhibitor Cocktail (Roche). Subsequently, cells were broken by two passages through an EmulsiFlex-C5 high pressure cell disrupter (Avestin Inc., Canada) at 10,000 psi. Chlorophyll concentration of the disrupted cells was adjusted to 1 mg ml^{-1} and the detergent n-dodecyl β-D-maltoside (DM) was added to final concentration of 0.5% (w/v). The solubilization step was performed for 15 min with continuous mixing in the dark on ice. After removal of unsolubilized material (30,000 ×*g*, 20 min, 4 °C), supernatant was loaded onto a fresh 0.1–1.0 M linear sucrose density gradient prepared in Tricine buffer (pH 7.5) with 0.01% DM (w/v). Ultracentrifugation was carried out with using SW 40 Ti swing-out rotor (Beckman Coulter) for 17 h at 100,000 ×*g*, 4 °C. The fractions containing the different isolated pigment–protein complexes were collected with a syringe and immediately used for the experimental measurements.

2.3. Gel filtration

Sucrose density gradient zones of interest were concentrated on Amicon Ultra centrifugal filter devices (Merck Millipore) with assigned molecular weight cut-off 10 kDa and applied to a pre-packed gel filtration column Superdex 200 10/300 GL (GE Healthcare). Gel filtration was carried out in Tricine buffer (pH 7.5) containing 0.01% DM (w/v) at a flow rate of 0.5 ml min^{-1} using Econ 4020 FPLC system (Econ, Czech Republic). Chromatograms were recorded at 435 nm. The collected fractions were directly used for the spectroscopic measurements.

2.4. Spectroscopy

Chlorophyll concentration was determined spectroscopically using UV300 spectrophotometer (Spectronic Unicam, UK) in 80% (v/v) acetone according to [46].

Room temperature absorption spectra were recorded using Shimadzu UV-2600 spectrometer. For measurements of whole cells, the instrument was equipped with ISR-2600 Plus integrating sphere.

Room temperature and low temperature (77 K) fluorescence emission spectra were recorded using Spex Fluorolog-2 spectrofluorometer (Jobin Yvon, Edison, NJ, USA) in the spectral range of 640–760 nm (slit width 2 nm) with an excitation wavelength of 435 nm (slit width 3.2 nm). The instrument was fitted with a locally built light source equipped with an incandescent lamp. The irradiance provided by the measuring beam was $\ll 1$ μmol(photons)m^{-2} s^{-1} (as measured using Hansatech Quantitherm light meter, Hansatech, UK). DCMU was added to the final concentration of 250 μM. The samples with DCMU were incubated for 5 min in the dark prior to measurement.

The room-temperature emission was recorded in direction perpendicular to excitation beam, using 1 cm × 1 cm fluorescence cuvettes. The 77 K spectra were recorded using front-face illumination on samples immersed in liquid nitrogen inside a Dewar vessel using locally made holders. Chlorophyll concentration of the samples used for spectroscopic measurements corresponded to ~10 μg $ml^{-1}$of Chl *a*.

2.5. Protein composition

Protein composition was determined by SDS-PAGE using precast 14% polyacrylamide SDS gels (Serva, Germany). The protein pattern in the gels was visualized with Coomassie Brilliant Blue G250 staining. Apparent molecular weights of the proteins were estimated by co-electrophoresis of a low molecular weight protein standard (Fermentas or Applichem). Gels were loaded on an equal chlorophyll basis.

2.6. Pigment composition

All steps of pigment extraction were performed on ice in the dark. An aliquot of 50 μl of cell suspension was resuspended in 1500 μl of 100% methanol by vortexing for 10–15 s. Samples were sonicated using an ultrasonic homogenizer 3000MP (Biologics, Inc., USA) at 20% power for 6 × 15 s waiting 5 s between pulses, then centrifuged at 13,000 ×*g* for 1 min at 4 °C. Supernatant was collected and pellet was subjected to another run of extraction repeating the same procedure until the pellet was colourless. All supernatants were pooled, and placed in an exsiccator connected to vacuum rotary pump to evaporate solvent for storage.

Pigment analysis was performed by high-performance liquid chromatography (HPLC), using a system consisting of a Pump Controller

Delta 600, Autosampler 2707 injection system and a PDA 2996 detector (Waters, USA). Pigments were separated on a reverse phase Zorbax SB-C18 column (4.6 × 150 mm, 5 μm, silica-based, non-endcapped; Agilent, USA) using a linear gradient elution. A tertiary solvent system used was as follows: solvent A (80:20 methanol:0.5 M ammonium acetate (aq., pH 7.2 v/v)), solvent B (90:10 acetonitrile:water), solvent C (100% ethyl acetate) [47]. The flow rate was 1 ml min^{-1}.

The pigment molar ratios were estimated from areas under the chromatograph peaks displayed at wavelengths corresponding to the respective extinction coefficients. Following molar extinction coefficients (in $dm^3 mmol^{-1}$ cm^{-1}) were used: Chl a: 78.3 at 662 nm; Chl c: 211 at 443 nm; fucoxanthin 109.4 at 450 nm, diadinoxanthin 130 at 448 nm; β-carotene: 134 at 453 nm [47].

2.7. Mass spectrometry

The bands of interest were cut out from a Coomassie-stained gel. Gel slices were prepared for tryptic digestion and subjected to in-gel digestion using sequencing grade trypsin as described previously [44]. Tryptic digests/peptides were analyzed on a nano-scale UPLC on-line coupled to ESI-Q TOF premier mass spectrometer (Waters). MS/MS spectra were searched against the public protein sequence databases.

2.8. Phylogenetic methods

Publicly available LHC sequences of *P. tricornutum* and *T. pseudonana* were used as the basis for phylogenetic analysis which was further enhanced by adding select sequences of *C. velia*, *Nannochloropsis gaditana*, *Emiliania huxleyi* and diverse sequences found during GenBank searches for homologies. Emphasis was placed on obtaining close homologs of both Lhcf15 and *C. velia* red-CLH. Three green lineage Lhcs were used as outgroup. In total, 132 LHC sequences were used for the analysis. Amino acid sequences were aligned by MAFFT (mafft.cbrc.jp/alignment/software/), using E-INS-i settings [48,49] with default parameters and the phylogeny was constructed by MrBayes 3.2.2 [50] using GTR+Γ+I model with default settings. The analysis was run well beyond convergence (5,000,000 generations, final standard deviation of split frequencies was 0.015).

3. Results

3.1. Chromatic adaptation in pennate diatoms revisited

As a first step, we sought to replicate the results of Fujita & Ohki [32] on red-shifted absorption and fluorescence in *P. tricornutum*. Cells were grown under red-enhanced light (RL, provided by an incandescent light bulb) and day light (DL, approximated by a metal halide lamp) at low irradiance. These broadband light sources were chosen instead of monochromatic LED blue and red illumination to ensure the availability of weak full spectrum light to the cells in both treatments.

Cell culture parameters and pigment contents under both growth conditions are summarized in Table 1. The F_v/F_M parameter was essentially identical in both cultures but much higher than the values reported previously [32] and similar to values reported for healthy *P. tricornutum* cells [51]. Thus, the RL growth condition did not significantly affect the efficiency of *P. tricornutum* photosystem II. The growth rate was slower under RL condition in agreement with the fact that a significant part of the absorption spectrum of *P. tricornutum* is not fully utilized on RL (for growth curves of the cultures refer to Suppl. Fig. 1A, in 1B see the spectra of cultivation lamps compared to the solar spectrum). The pigment composition of the cells differed considerably. Diadinoxanthin remained in constant proportion to Chl *a* but the RL-grown cells contained about 10% less fucoxanthin and 25% less Chl *c* when compared to DL-grown cells.

3.1.1. F710 as a spectroscopic signature of chromatic adaptation

In agreement with earlier report [32], prolonged exposure (8 days) to RL brought about considerable changes of the room temperature fluorescence spectrum of *P. tricornutum* cells (Fig. 1A) whereby the emission maximum was red-shifted by more than 30 nm, to 712 nm (further denoted as F710). We have also observed F710 emission after transferring the cells from DL to RL conditions. Upon full acclimation to RL, the ~681 nm emission band (emission maximum of DL-grown cells) only remained as a shoulder on the blue edge of the cell emission spectrum. Approximately a week under RL conditions was required for full development of the F710 signal, when the cultivation was initiated from the DL-adapted culture. This was in agreement with observations of Fujita and Ohki [32] who characterized the development of the F710 signal under monochromatic light in cultures adjusted to equal growth rates. In line with these previous results, these changes likely indicated a slow acclimation process rather than mere rearrangement of existing thylakoid membrane components (cf. [32,41]). A signal similar to F710 was also observed in dense DL cultures (OD > 0.3) in the form of a small shoulder to the main (~681 nm) emission band (Suppl. Fig. 2).

As expected, the F710 fluorescence of whole cells was more pronounced in low temperature fluorescence spectra where it became prominent even in DL cultures, indicating that the responsible pigment acted as an excellent exciton trap (Suppl. Fig. 2). The development of the F710 species was accompanied by changes of absorption spectra (Fig. 1B). The region of the Q_Y band of Chl *a* showed broadening of the main peak and an enhancement of absorption towards the far-red part. RL cell absorbance was ~45% higher above 690 nm when compared to DL cells (when the spectra were normalized to equal areas in the 600–750 nm region).

3.1.2. Association of the F710 emitter with photosystems

We have compared room temperature fluorescence spectra of whole RL cells in presence and in absence of a PSII inhibitor DCMU (Fig. 2) and also emission spectra of fluorescence induction (Kautsky curve) of RL-grown cells (Suppl. Fig. 3). Upon blocking of PSII the fluorescence yield grew approximately 2× across the emission spectrum but the spectrum shape remained essentially identical and was still dominated by F710. Thus, if excitation prevented from being trapped in PSII was emitted from the red-shifted Chl *a*, part of the pool of the complexes emitting F710 fluorescence had to be functionally connected to PSII. The extent of the relative DCMU-induced change of fluorescence ($1 - Fo/F_{DCMU} \approx 0.55$, Fig. 2B) indicated that the PSII-associated red antenna forms a major component of the red-shifted Chl *a* pool. However, it is evident (Fig. 2B), that the spectral range <700 nm displayed a slightly larger yield enhancement by DCMU than >700 nm. This can be interpreted so that the antenna system of PSII also contained complexes emitting (e.g. regular FCP) ~680 nm and that a small part of the red antenna did not respond to DCMU. Note that the cell samples for fluorescence were diluted so as to avoid reabsorption. The assignment of the

Table 1
Culture parameters and pigment content of *P. tricornutum* cells grown under RL and DL. Values are mean ± standard deviation of at least six pigment and F_v/F_M determinations from independent cell harvests. Growth rates were determined in three replicates.

Culture	F_v/F_M	μ^* [d^{-1}]	Chl c^* [mol/mol Chl *a*]	Fucoxanthin* [mol/mol Chl *a*]	Diadinoxanthin [mol/mol Chl *a*]	β-carotene [mol/mol Chl *a*]
RL	0.68 ± 0.01	0.19 ± 0.03	11.1 ± 1.4	60.2 ± 2.6	13.1 ± 2.2	6.1 ± 0.4
DL	0.69 ± 0.02	0.52 ± 0.07	14.7 ± 0.6	67.5 ± 4.2	13.4 ± 3.2	6.2 ± 0.3

* Statistically significant difference ($p < 0.01$, *t*-test) between RL and DL cultures.

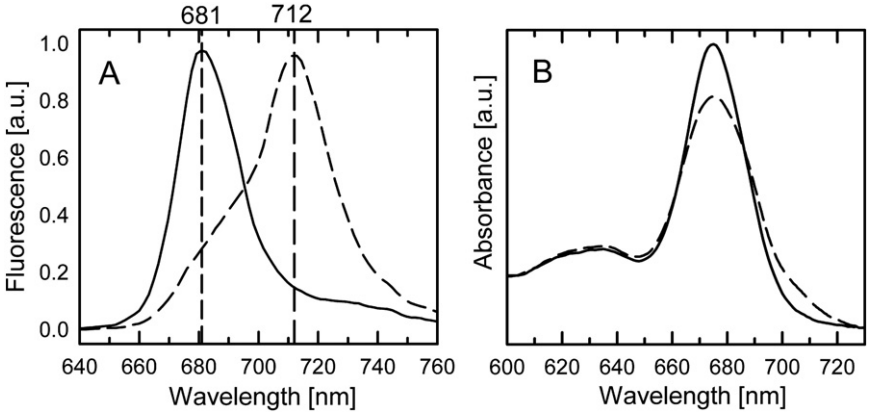

Fig. 1. Representative room temperature fluorescence emission spectra of DL- (solid) and RL-grown cells (dashed) of pennate diatom *Phaeodactylum tricornutum*. (A); excitation wavelength 435 nm. Room temperature absorption spectra of whole cells of *P. tricornutum* grown in DL (solid) and RL (dashed) conditions (B). The absorption spectra were normalized to equal area in 600–750 nm regions.

red antenna to the light-harvesting machinery of PSII was also corroborated by the spectra of Kautsky induction curve (Suppl. Fig. 3) where the shape of the spectra did not change during the course of actinic illumination (Suppl. Fig. 3A) and, consequently, the time course of the F710 peak was identical to that of the "standard" emission maximum around 680 nm (Suppl. Fig. 3C). To make this point clearer, we have used transitional cells for this experiment which had the standard ~681 nm emission still visible as a well-defined peak. For comparison, results of the same experiment performed on plant (*Hibiscus rosa-sinensis*) leaf are shown (Suppl. Fig. 3B, D) to demonstrate how the fluorescence signal would have been affected by the presence of a significant PSI contribution [52].

Due to the striking difference of emission spectra of DL and RL cells, we have used the room temperature F710 feature in the following work as a spectral signature of the presence of red-shifted pigments in the search for the molecular basis of this phenomenon.

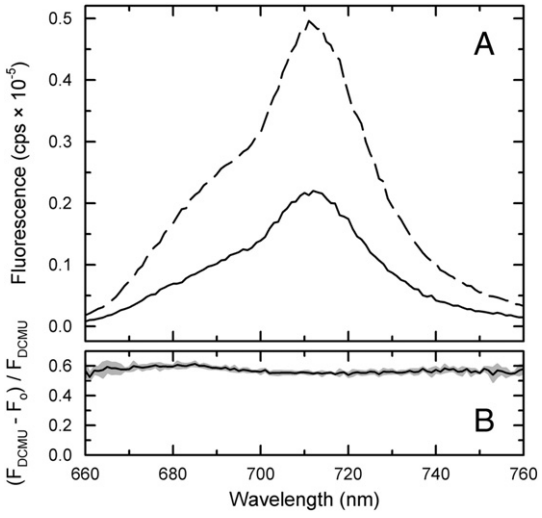

Fig. 2. Effect of PSII inhibitor DCMU on the fluorescence of whole cells of the red-light culture of *P. tricornuntum*. (A) Raw (in cps) emission spectra measured before (solid line) and after addition of 250 μM DCMU (dashed line) on the same sample, representative example. (B) Relative fluorescence change due to DCMU calculated as $F_v/F_M = 1 - Fo/F_{DCMU}$; mean (solid line) ± SD (grey area) obtained from measurements on four different cultures. The cells were diluted to OD at 674 nm = 0.1 to eliminate reabsorption.

3.2. Identification of the F710 emitter

3.2.1. Membrane fractionation and preservation of F710

Solubilized thylakoid membranes from RL- and DL-grown cells were first subfractionated by sucrose density gradient ultracentrifugation. The gradient separation pattern was identical for both cultivation conditions. Five zones (Fig. 3A, Suppl. Fig. 4A) differing in colour could be reproducibly distinguished: zones 2 and 5 were brown, suggesting presence of FCP antennas with fucoxanthin, while the remaining zones 1, 3 and 4 were green. To assess the presence of F710 signal, fluorescence spectra of each zone were measured at both room temperature and 77 K (Suppl. Fig. 4B and C, respectively). The red-shifted fluorescence was only observed in zone 5 of RL-culture gradient (Fig. 3B, C).

It should be noted here that the F710 signal was highly unstable and all the experimental work had to be done on ice and as fast as possible to preserve the maximal 712/681 ratio. The signal was lost when previously frozen cells were used, thus only freshly harvested cells were used for preparations. After cell breakage, the F710 signal disappeared completely within 1 h when exposed to room temperature (see Suppl. Fig. 6A). Gentle sample handling without undue shaking was required to preserve maximum of the F710 signal. Even with these precautions, the detergent extract of thylakoids retained only about 30% of the initial F710 (Suppl. Fig. 6B).

Protein composition of the sucrose gradient zones was determined by SDS-PAGE (Suppl. Fig. 5). The upper green zone 1 consisted of a mixture of presumably monomeric proteins and free pigment (data not shown), zone 2 (brown) contained most of the light harvesting antennas (the bands of molecular weights below 20 kDa) while the main subunits of both photosystems dominated in lower SG zones 3 and 4 (green). The position of zone 5 (brown) on the bottom of the gradient suggested that it contained supramolecular protein complexes as well as membrane rafts (see below). It thus appears that the very mild solubilization was the condition necessary for preserving the F710 signal.

The major difference between RL- and DL-grown cells was in the antenna protein composition (Suppl. Fig. 5). FCP band of ~16 kDa was present in both growth conditions whereas an extra protein band with apparent molecular mass of ~18 kDa could be distinguished in RL-grown cells. The RL-specific band was present mainly in the upper zones 1 and 2, and in significant amount in zone 5 of RL-grown cells (Suppl. Fig. 5A, red arrow). Zones 1 and 2 of DL-grown cells contained bands of similar mass, but in much lower amounts than in RL cells, and, more importantly, these proteins were not present in zone 5. Hence, our hypothesis was that this protein band represents a light harvesting antenna protein responsible for the F710 fluorescence in analogy to the red-induced antenna in *C. velia* [41,42]. The abundance of this

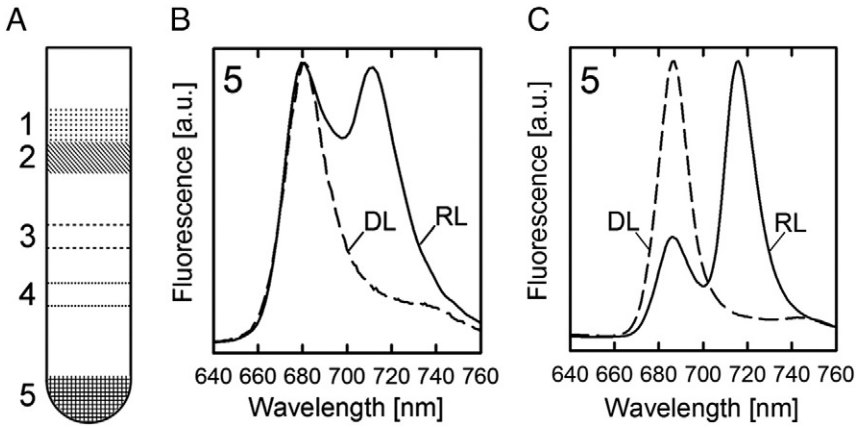

Fig. 3. (A) A typical separation pattern of RL- and/or DL-grown *P. tricornutum* on a linear sucrose density gradient; the original images of both cultures are available in Suppl. Fig. 4. Fluorescence emission spectra of zone 5 of DL (dashed) and RL (solid line) culture recorded at RT (B) and at 77 K (C). Excitation wavelength was 435 nm; spectra were normalized to their maxima. Data is representative of seven and three replicates for RL and DL sample, respectively.

protein in the upper zones of the sucrose gradient from RL cells that lacked the F710 fluorescence could then be explained straightforwardly by fragility of the complex in which the red-shifted spectral feature appears only as the result of inter-complex interaction and is not the property of individual proteins, in clear parallel to the case of *C. velia*.

3.2.2. Purification of the complex harbouring the F710 emitter

In order to further characterize the RL-specific pigment–protein complex and to identify the F710 emitter, zones 5 from both RL- and DL-grown *P. tricornutum* were analyzed by gel filtration chromatography. Based on the elution profiles (Fig. 4A, D) and absorption spectra (data not shown), four and three fractions were resolved in RL- and DL-grown *P. tricornutum*, respectively. SDS-PAGE protein profiles of fractions 1 and 2 of RL cells and 1 of DL cells (Fig. 4B, E) showed the presence of components of both photosystems and antenna proteins. A strong band corresponding to the major proteins of PsaA/B heterodimer of PSI reaction centre was obvious in both fractions 1 and 2 (RL) and fraction 1 (DL). In fraction 1 of DL culture, bands

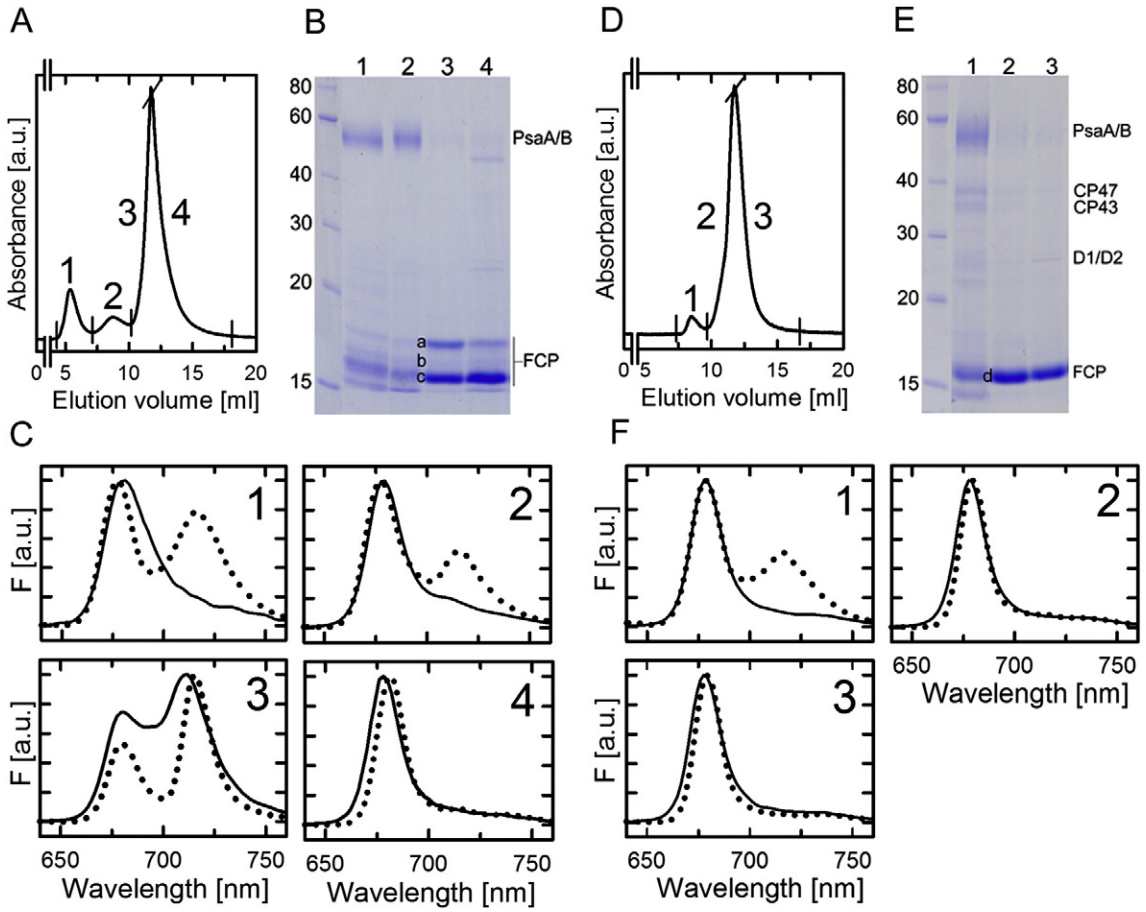

Fig. 4. Biochemical–biophysical identification and characterization of RL-induced antenna of *P. tricornutum*. Gel filtration chromatograms of sucrose gradient zone 5 from RL- (A) and DL-grown (D) *P. tricornutum*; Coomassie stained SDS-PAGE gels (B, E) and fluorescence emission spectra measured at RT (solid line) and at 77 K (dotted line) (C, F) of the gel filtration fractions. Main photosystem subunits are marked on SDS gels. Data is representative of seven and three replicates for RL and DL sample, respectively.

corresponding to core subunits of PSII reaction centre were visible on the gel. Similarly, PSII proteins were also detectable in fractions 1 and 2 of RL culture after silver staining or higher sample loading (data not shown). Identity of the above mentioned PSI and PSII protein subunits was confirmed by MS analysis of the respective SDS-PAGE bands (data not shown).

The nature of the first fractions of gel filtration was also tested by observing negatively stained samples by TEM. Membrane rafts showing protein components were visible. As these did not contain the red-shifted antenna they will not be further discussed here and the analysis of these rafts will be published elsewhere (manuscript in preparation).

Fractions 3 and 4 of RL cells and 2 and 3 of DL cells consisted of FCP antenna proteins (Fig. 4B, E) presumably in trimeric and/or oligomeric form [21,23]. In accordance to data obtained on sucrose gradient zones (see above; Suppl. Fig. 5), the additional ~18 kDa band was present in RL-grown cells only. We have attempted to image these fractions with TEM as well but apart from the presence of FCP trimers the results were inconclusive (data not shown).

3.2.3. Spectroscopic characterization of purified complex containing F710

Immediately as the fractions were eluted from the column, fluorescence emission spectra at RT and 77 K were measured (Fig. 4C, F). At room temperature (solid lines in Fig. 4C, F), only fraction 3 of RL-grown *P. tricornutum* had the long wavelength emission at 712 nm, even more pronounced than the 681 nm fluorescence. As expected, in DL-grown *P. tricornutum* no fraction from gel filtration chromatography exhibited the red shifted emission.

The low temperature fluorescence spectra (dotted lines in Fig. 4C, F) demonstrated several phenomena. The fractions known from SDS-PAGE to contain photosystems (fractions 1 and 2 in RL and fraction 1 in DL) had two distinct emission peaks at 677 and 715 nm. The 677 nm peak can be attributed to light harvesting antennas, either co-purified or partially detached from photosystems and the peak at 715 nm can be attributed to low-energy chlorophylls of PSI [53]. The FCP antenna fractions (fractions 3, 4 in RL and 3 in DL) had a single emission peak at ~680 nm [20,21,54]. The only fraction that possessed the F710 emission at room temperature, RL fraction 3, had also two sharp emission peaks at 77 K, centred at ~680 and 715 nm. In Suppl. Fig. 8, we present the absorption spectra of fraction 3 of the RL sample. Comparison to the antenna fraction of DL sample (fraction 2, Fig. 4D, F) shows enhanced absorption in the >700 nm region.

It should be emphasized that there were several properties distinguishing the far-red emission of RL fraction 3 (F710 complex) from fractions 1 and 2 (PSI): (i) negligible far-red emission was recorded from photosystem fractions at room temperature, in agreement with literature on PSI [55]; (ii) at 77 K, the width of the 715 nm emission band of fraction 3 (F710) was about 60% of the PSI emission band (Suppl. Fig. 7A); (iii) at 77 K, with equal chlorophyll content, the fluorescence intensity of the F710 emission was several times higher compared to PSI (Suppl. Fig. 7B).

3.2.4. Lhcf15 protein required for F710

To identify the specific polypeptides forming the antenna fractions 3, the bands marked in Fig. 4B and E were cut out and analyzed by mass spectrometry (Table 2, Suppl. Table 1). As expected, due to a large number of genes coding for light-harvesting proteins of similar sizes in *P. tricornutum*, none of the antenna protein bands on the gel consisted of a single protein. Five Lhcf-, five Lhcr-family proteins and two low molecular mass PSI subunits were recovered from analyzed protein bands of SDS-PAGE in RL culture (Table 2). Major light-harvesting proteins of diatoms encoded by *lhcf* genes were predominantly found in the 16 kDa band present in both cultures (DL and RL). A faint 17 kDa band, exclusively containing Lhcr-type proteins, was visible only in RL culture. However, it was more evident in PS fractions 1 and 2 where no F710 signal was recorded.

Table 2
Proteins identified in three biological replicates by MS analyses of 18 (a), 17 (b) and 16 (c, d) kDa SDS-PAGE band of gel filtration fractions isolated from RL- and DL-grown *P. tricornutum*. The protein bands subjected to MS are depicted in Fig. 4B, E. Proteins marked with an asterisk were found in both cultures.

Band	Red light	# peptides[a]	Band	Day light	# peptides[a]
	Protein identified			Protein identified	
a	Lhcf15	4/(8)			
a	Lhcr4	4/(16)			
b	Lhcr13	4/(13)			
b	Lhcr2	1/(10)	d	Lhcf10	7/(11)
c	Lhcf8*	5/(17)	d	Lhcf3/4*	7/(14)
c	Lhcf2*	3/(10)	d	Lhcf8*	5/(17)
c	Lhcr12	3/(12)	d	Lhcf2*	4/(10)
c	Lhcf3/4*	3/(14)	d	Lhcf6/7	4/(10)
c	Lhcf11*	3/(11)	d	Lhcf11*	4/(11)
c	PsaL	2/(10)	d	Lhcf1	4/(10)
c	PsaF*	2/(12)	d	Lhcf5	3/(10)
c	Lhcr14	2/(14)	d	PsaF*	3/(12)

[a] Number of matched/theoretical peptides.

In Table 2, the proteins marked with an asterisk, i.e. Lhcf8, Lhcf2, Lhcf3/4 and Lhcf11, were present in both the RL and DL cultures, while proteins Lhcf10, Lhcf6/7, Lhcf1 and Lhcf5 were only found in the DL culture. Therefore, all Lhcf proteins except Lhcf15 can be excluded as candidate F710 emitters. Several Lhcr-type proteins were also found in the RL culture fraction, all of them (Lhcr2, Lhcr4, Lhcr12, Lhcr13, Lhcr14) were previously found attached to PSI [15].

Our data confirmed the findings presented in [15]. For convenience, we present the comparison of Lhcr's found in our fractions 1 and 3 from RL cells (Fig. 4A–C) with the proteins identified in the PSI–antenna complex by [15] in Suppl. Table 2. Also of note is the similarity of the SDS-PAGE analysis of fraction 1 and PSI–antenna complex given in [15]. These data enabled us to exclude Lhcr4, Lhcr13, Lhcr2, Lhcr12, and Lhcr14 (Table 2) from the list of candidate F710 emitters. The source of Lhcr in fraction 3 might be the small amount of contaminating PSI that can be seen in fraction 3 upon closer inspection of Fig. 4B (upper part of the 3rd lane).

Upon comparison of results from DL and RL cultures, we propose Lhcf15, which has been previously found to be regulated by quality of light [56], to be the crucial component required for F710 emission and absorption spectrum changes in RL-grown *P. tricornutum* (see below for detailed discussion).

It should be noticed here that the Lhcf15 proteins were detected by MS analysis in zones 1 and 2 of sucrose density gradient of the RL sample (data not shown) although these zones emit no F710 signal (see Suppl. Fig. 4B). This suggests that preservation of the F710 signal requires an oligomeric assembly of Lhcf15 proteins which most likely exists in zone 5 of sucrose gradient but not in upper zones 1 and 2.

3.2.5. Phylogenetic analysis of P. tricornutum antenna proteins

To provide further context and to assess how these proteins are related to the Red-CLH antenna of *Chromera* a phylogenetic tree of light harvesting proteins from *P. tricornutum* and related organisms was constructed (Fig. 5, Suppl. Fig. 9). The full tree recovered all major properties of the LHC protein family of heterokont algae and related proteins [57]. Three major types of the Chl *a*/*c* LHC proteins (Lhcr, Lhcf and Lhcx) were well resolved. The Lhcr clades included sequences of diatoms, red algae, brown algae, eustigmatophytes and *Chromera* [58]. The major light harvesting proteins related to diatom FCP (Lhcf) formed alga-class-specific clusters of pennate and centric diatom FCP, brown algae FCP, raphidophyte FCP, dinoflagellate acpPC, haptophyte (*Emiliania*) FCP, eustigmatophyte (*Nannochloropsis*) VCP and *Chromera* CLH. Finally, the stress-related Lhcx/LHCSR/LI818 proteins [59] of *Chlamydomonas*, diatoms, brown algae and others also formed a separate well supported cluster. Between these previously described proteins two additional LHC protein clusters are highlighted. One is

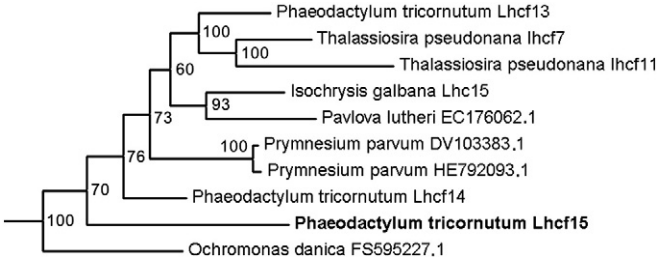

Fig. 5. Details from a phylogenetic analysis of Lhc sequences (Suppl. Fig. 9) showing the sequences closely related to Lhcf15, the protein required for the far-red emission in *P. tricornutum*. Amino acid sequences were aligned by MAFFT using E-INS-i settings with default parameters and the phylogeny was constructed by MrBayes 3.2.2 using GTR+Γ+I model. Node supports (in percents) are indicated.

related to the *Chromera* Red-CLH proteins as described recently [42] and the other one is centred around the aforementioned Lhcf15 protein of *P. tricornutum*.

The LHC phylogenetic tree showed that the Lhcf15 protein is divergent from major diatom Lhcf sequences and embedded in an interesting assortment of LHCs with good support. There were two more sequences of *P. tricornutum* (Lhcf13 and Lhcf14), two from a centric diatom *Thalassiosira* (Lhcf7 and Lhcf11) and one additional heterokont sequence of chrysophycean alga *Ochromonas*. Curiously, several haptophyte LHC sequences also showed high similarity to *P. tricornutum* Lhcf15. The same clade of antenna proteins containing haptophyte sequences and Lhcf15 of *P. tricornutum* has been previously recovered in a broad LHC analysis (labelled as cluster VIIb) [57] or in a survey of *P. tricornutum* LHCs [23,60].

4. Discussion

The absorption spectra presented in Fig. 1B showed that the thylakoid membrane of *P. tricornutum* responds to changed spectrum of incident radiation by chromatic adaptation involving synthesis of a novel component of the light harvesting machinery containing a distinct spectral form of Chl *a* absorbing above 690 nm. The presence of such low-energy pigment in RL-grown cells was strikingly demonstrated by the significant change in room temperature fluorescence emission spectrum (Fig. 1A) which helped us track the F710 presence during biochemical analyses. As suggested before [41], this change of room temperature fluorescence spectrum might be used in searches for further candidate species possessing red-shifted light harvesting antennas. Comparison of absorption capabilities of the RL and DL adapted cultures above 690 nm indicated that the RL-grown cells could absorb as much as 45% more far-red light. This might prove to be a significant advantage in an environment where most of the photosynthetically active radiation is absorbed by other photosynthetic organisms (see [61] for discussion of light-harvesting function of red-shifted pigments).

We find it important to emphasize that the pronounced changes in light harvesting apparatus described here were provoked by broadband light sources. These were used intentionally to ensure that weak excitation was available across the spectrum so that all potential light sensors, such as phytochromes [62], present in the cells were covered. This was done in order to maintain ecologically relevant cultivation conditions using light quality closer to natural environment where monochromatic illumination cannot be assumed.

One of the issues pertaining to the spectroscopic properties of the complexes containing red-shifted Chl *a* that warrants further discussion is the contribution of PSI to the far-red emission. This issue breaks down to two questions: (i) the contribution of PSI itself; and (ii) the association of the red-shifted antenna with photosystems. As for the first question, it has been shown before that diatom PSI does not emit strongly above 700 nm at room temperature [55] in agreement with our results (Fig. 4C, F). This is the direct consequence of the fact that only a small number (~3) of red-shifted Chl *a* is present in PSI in diatoms [63]. Considering that the relative intensity of emission is proportional to the probability of an exciton being found in respective pigment pool, one can assume that the statistics of the energy equilibration in such complex containing hundred(s) of Chl *a* molecules in total [55] is in favour of the more abundant, if higher, energy levels. Thus the far red emission becomes visible only at cryogenic temperatures where the system is devoid of thermal energy needed to populate the higher (~680 nm) energy levels of the system. But even at 77 K, in case a larger number of FCP antenna is present in addition to the PSI core, the relative contribution of PSI emission in the 715–720 nm region can be diminished [13,63,64]. In this regard, it is illustrative to refer to Suppl. Fig. 4 showing the sucrose gradient zone 5 of the DL culture apparently lacking the far-red emission. Only after the zone was subjected to further purification to separate the PSI–antenna fraction from other complexes, the red-shifted PSI emission became manifested.

In addition to that, even though peripheral antenna system associated specifically with PSI exists, these complexes lack the far-red emission [55,64,65]. Thus, if there is a complex present that exhibits the red-shifted emission at room temperature (by virtue of its larger number of red-shifted Chl *a* states, in contrast to PSI), it would dominate the emission spectrum at low temperature as well, likely obscuring any PSI core contribution. We thus assume that the most likely source of red emission in dense DL cultures as seen in Suppl. Fig. 2 is the F710 emitting antenna induced by self-shading of the dense culture.

At closer inspection, our low temperature spectra from gel filtration fractions provided another helpful tool for disentangling antenna and PSI emission in the case of isolated supercomplexes (Suppl. Fig. 7). Although the red PSI emission signal is positioned at the same wavelength (~715 nm at 77 K) as the F710 antenna signal, it is clearly much broader (30 nm full width at half maximum vs. 19 nm for F710) [65]. The F710 signal is of equal width as the corresponding emission of major FCP antenna at 680 nm upon cooling to 77 K. On the other hand, even if the red emitter is unambiguously assigned to the antenna, the issue of its connection to photosystems remains. While the far red forms of Chl *a* are typically associated with (plant) PSI, it has been shown before that red-shifted antenna complexes are capable of driving PSII photochemistry [40,41], despite the difference in the respective energies (corresponding to ~680 nm for PSII and ~700 nm for the low antenna states).

While a more detailed investigation of the larger-scale organization of the membrane complexes and the physiological aspects of the far-red antenna were beyond the scope of the present paper, our results suggest that the far-red antenna in *P. tricornutum* is also associated with PSII (as suggested already by Fujita and Ohki [32]), as its emission responded strongly to changes of the PSII RC redox state, as seen from the fluorescence induction curves (Supplementary Fig. 3, cf. [41]), and the reaction to DCMU, an inhibitor of PSII electron transfer (Fig. 2). On the other hand, the association to PSI cannot be presently excluded based on the fluorescence data alone. Since the diatom thylakoid membrane is not differentiated into specific domains corresponding to grana and stromal lamellae of plant thylakoids, with either PSII or PSI dominating the protein complement [66], it is likely that the F710 complex serves as an external antenna of both photosystems, mirroring the situation in *C. velia* [41].

As for the identity of the complex carrying the far-red forms of Chl *a*, we identified the protein marked Lhcf15 as the most likely candidate. This is in agreement with the previous results [56,60] that the Lhcf15 protein expression is light quality-dependent and its elevated level has been observed in response to the exposure to the red light or to prolonged dark treatment, respectively. In the dataset of [56], Lhcf15 was by far the most upregulated antenna protein (about 10×), and also Lhcf13, Lhcf2 and Lhcf5 showed upregulation on RL by about 20%. It can be concluded that Lhcf15 production is induced by red light exposure and it is a key component of a newly formed red antenna complex emitting F710 signal. Most likely, this antenna complex is formed by aggregation of Lhcf15 alone and/or possibly other Lhcf-family proteins of

existing or only slightly modified FCP trimers or higher oligomers participate in the oligomerization process. Determining other key proteins in this process seems challenging. Although some differences in the presence of the major FCP components between RL and DL culture were observed in our MS/MS data (Table 2), e.g. Lhcf10, Lhcf6/7, Lhcf1 and Lhcf5 besides some Lhcr proteins, it appears premature to make a conclusion based on these data. As seen in Table 2, Lhcf15 was accompanied in the ~18 kDa band (a) (Fig. 4B) in SDS-PAGE from RL culture by another protein, Lhcr4. However, previous proteomic studies [56,60] which also identified this protein in *P. tricornutum* cells did not indicate its light-dependent regulation, as opposed to Lhcf15. Moreover, as our later investigation indicated, the Lhcr4 is indeed, as its label suggests, a part of the antenna associated with PSI (manuscript in preparation) known to lack the far-red emission, we excluded this protein from further consideration in regard to the origin of the F710 emission.

The pigment composition of RL versus DL cells shows an interesting analogy with the situation in *Chromera* [42] where the Red–CLH complex is enriched in violaxanthin, a xanthophyll cycle pigment, at the expense of isofucoxanthin, the major light harvesting carotenoid in that species [44,67]. Diatoms use fucoxanthin in major light harvesting role [68] and diadinoxanthin in the xanthophyll cycle. The RL cells contained significantly lower amounts of Chl *c* and fucoxanthin (relatively to Chl *a*) when compared to DL cells. This is surprising because if the RL culture was light-limited, as the growth rates suggested, there should have been more light harvesting antennas present to compensate for the low irradiation. FCP antennas contain most of cellular fucoxanthin and Chl *c*. Hence, if the content of these pigments decreased in RL cells it indicated that the F710 related proteins are depleted in these pigments and probably contain mostly Chl *a* and diadinoxanthin. Since Red-CLH and F710 proteins are not closely phylogenetically related (see below) this similarity of carotenoid content appears to be a peculiar convergent feature. Unfortunately, the extreme fragility of the far-red complex of *P. tricornutum* seems to preclude any detailed investigation of excitation energy flows within the complex and their comparison to "canonical" fucoxanthin containing complexes.

The phylogenetic analysis of *P. tricornutum* antenna proteins (Fig. 5, Suppl. Fig. 9) showed that Lhcf15 protein is a member of a well supported protein clade formed by complexes of varied phylogenetic origin, comprising both diatom and haptophyte sequences. It would be probably premature to suggest that all these sequences are related to the chromatic adaptation — we have not found Lhcf13 and Lhcf14 in any relation to the F710 antenna and we have not found the F710 signal in centric *Cyclotella* (not shown, cf. [32]), suggesting that *Thalassiosira*, another centric diatom, will also lack it. However, it can be assumed that there has to be some functional aspect shared by these proteins, perhaps associated with the assembly of specific oligomeric complexes.

The placement of haptophyte sequences within the Lhcf15 clade is particularly surprising. Haptophytes are an ancient evolutionary lineage sharing common ancestor with heterokonts (stramenopiles), dinoflagellates, apicomplexans and ciliates, together forming the chromalveolate supergroup [69]. Chromalveolates have common plastid origin in secondary endosymbiosis of a red alga [3]. Although haptophytes have a long evolutionary history independent of heterokont lineage [69], their LHCs align firmly along the other predominantly Chl *a*/*c* containing antenna proteins [57].

It has been proposed recently [51] that the F710 fluorescence (detected in low temperature spectra) is related to NPQ capability of diatom species, particularly *P. tricornutum*. As NPQ capacity was not a focus of our work we cannot comment upon this phenomenon as the growth conditions used differ considerably from the cited work. The F710 protein complex is an excellent exciton trap and its involvement in NPQ currently cannot be ruled out. Also the hypothesized higher content of diadinoxanthin in F710 complex points to its possible NPQ involvement. However, the conditions which enhanced NPQ in *P. tricornutum* in the work of Lavaud and Lepetit were either stationary phase of growth ('medium' NPQ condition) or intermittent growth light ('high' NPQ condition) resulting in very low growth rates [51]. Both of these conditions are bound to enhance the F710 signal. The 77 K fluorescence spectra presented are reminiscent of our spectra of slightly self-shaded cells (Suppl. Fig. 2), also noted previously elsewhere [70].

Of the organisms known to utilize red-shifted light harvesting antennas, *Acaryochloris* has been found epiphytic on red algae [71] and in an ascidian, shaded by another cyanobacterium *Prochloron* [36]. *H. hongdechloris* comes from dense biofilms of stromatolites [72]. Red-shifted *Ostreobium* was found inside a coral, shaded by dinoflagellates [38]. *Chromera* is known to reside in or around corals as well [73]. Therefore the capability to shift at least part of the chlorophyll Q_Y absorption band to or even beyond 700 nm has appeared several times in marine organisms dwelling in the shaded environment of both warm and cold (*P. tricornutum*) seas [74].

Due to the fact that water preferentially absorbs red light, the environmental niche for red-shifted light harvesting antennas is limited to at most the first few metres below surface. In the case of *P. tricornutum*, the F710 antenna is probably an adaptation to a benthic lifestyle in shallow coastal waters [75]. *P. tricornutum* is capable of inducing some F710 signal by self-shading (Suppl. Fig. 2) but the full development of F710 and enhanced absorption above 700 nm is only possible under significantly red-shifted growth light. Therefore such environmental shading cannot be provided just by high density population of diatoms or other algae with similar absorption properties. Solar spectrum screening needed for creating 'red' light in situ can be provided by e.g. the light harvesting proteins of cyanobacteria (phycobilins) or dinoflagellates (PCP) living 'above' *P. tricornutum* [42] whether in water column or in a biofilm. For illustration of the above stated points we present in Fig. 6 the simulation of the light conditions at one metre depth in presence of shading by dinoflagellate or cyanobacterial cells. The spectra were modelled using solar spectrum at the ground level (AM1.5 global-ASTM G173, [76]) modified by water absorption [78], absorption spectrum of PCP from *Symbiodinium* sp. [77] or absorption spectrum of phycobilin-containing cyanobacterium (*Synechococcus* PCC 7002), both spectra were adjusted to OD = 1 at 670 nm.

Based on these considerations we can predict that members of one or both of these groups should be present in the *P. tricornutum* habitat at least for part of the annual cycle.

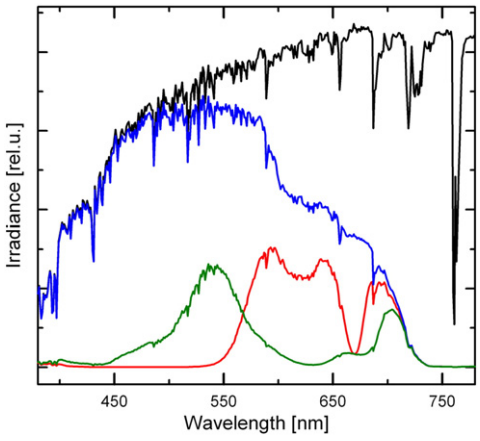

Fig. 6. Illustration of utility of red-shifted antenna systems in conditions where water absorption is combined with screening of photosynthetically active radiation by Chl *a*-containing phototrophs in the water column. Simulated light conditions under one metre thick water layer (blue) and in additional presence of dinoflagellates (red) or cyanobacteria (green), both adjusted $OD_{670} = 1$. Incident solar spectrum is shown in black. For details see text.

4.1. Conclusions

We have described the molecular basis of chromatic adaptation of pennate diatoms in model species *P. tricornutum*. Based on phylogenetic analyses, Lhcf15, the key protein likely required for the red-shifted oligomeric FCP complex formation, is not related to previously described red-shifted light harvesting proteins of *C. velia* and *Ostreobium* sp. Therefore, these adaptations most probably evolved independently and represent a case of convergent evolution. *P. tricornutum* is a third well described case of marine eukaryote alga possessing the capability to shift its absorption towards 700 nm. It can be predicted that the number of algae which independently adapted to their environment by developing red-shifted light harvesting antennas based on Chl *a* is even higher. We are currently broadening our search for red-shifted light harvesting antennas of algae, with a particular focus on heterokonts. The haptophyte algae also seem likely candidates possessing Chl *a* based red-shifted antennas.

6. Transparency Document

The Transparency Document associated with this article can be found, in the online version.

Acknowledgements

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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.2015.02.016.

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