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Identification of several sub-populations in the pool of light harvesting proteins in the pennate diatom *Phaeodactylum tricornutum*

Kathi Gundermann [a], Matthias Schmidt [a], Wolfram Weisheit [b], Maria Mittag [b], Claudia Büchel [a,*]

[a] Institute of Molecular Biosciences, University of Frankfurt, Max-von-Laue-Str. 9, 60438 Frankfurt, Germany
[b] Institute of General Botany and Plant Physiology, Friedrich Schiller University Jena, Am Planetarium 1, 07743 Jena, Germany

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ABSTRACT

Diatoms are major contributors to the photosynthetic productivity of marine phytoplankton. In these organisms, fucoxanthin-chlorophyll proteins (FCPs) serve as light-harvesting proteins. We have explored the FCP complexes in *Phaeodactylum tricornutum* under low light (LL) and high light (HL) conditions. Sub-fractionating the pool of major FCPs yielded different populations of trimeric complexes. Only Lhcf and Lhc-like proteins were found in the trimers. Under LL, the first polypeptide fraction contained six different Lhcfs and was mainly composed of Lhcf10. It was characterised by the highest amount of fucoxanthin (Fx). The second was dominated by Lhcf10, Lhcf5 and Lhcf2, and had a lower Fx/Chl c ratio. Little Fx/Chl c also characterised the most abundant FCP complexes, found in fraction 3, composed mainly of Lhcf5. These FCPs bound Fx molecules with the strongest bathochromic shift. The last two fractions contained FCP complexes that were built mainly of Lhcf4, harbouring more Fx molecules that absorbed at shorter wavelengths. Under HL, the same main polypeptides were retrieved in the different fractions and spectroscopic features were almost identical except for a higher diadinoxanthin content. The total amount of Lhcf5 was reduced under HL, whereas the amount of the last two fractions and thereby Lhcf4 was increased. Lhcf11 was identified in different LL fractions, but not detected in any HL fraction, while two new Lhc-like proteins were only found under HL. This is the first report on different trimeric FCP complexes in pennate diatoms, which differ in polypeptide composition and pigmentation, and are differentially expressed by light.

1. Introduction

Diatoms are unicellular, eukaryotic organisms that perform oxygenic photosynthesis and are of high ecological importance since they contribute to approx. 25% of the yearly biomass production on earth [1,2]. Their plastids derived from a secondary endosymbiotic event, whereby ancient rhodophytes were identified as endosymbionts [3]. Diatom plastids are, therefore, surrounded by four membranes, in contrast to e.g. higher plants, and the differentiation into grana and stroma thylakoids is missing. Despite the relationship to red algae, diatom chloroplasts do not contain phycobilisomes, but membrane intrinsic light harvesting complexes, which are called fucoxanthin chlorophyll proteins (FCPs) because of the major pigments bound.

In general, FCPs bind chlorophyll (Chl) a, Chl c and fucoxanthin (Fx), whereby the carotenoid to Chl ratio is extremely high with four Fx per five Chls [4] compared to higher plant light harvesting proteins. In addition, substoichiometric amounts of diadinoxanthin (Dd) and diatoxanthin (Dt) are present, whereby the amount of these xanthophyll cycle pigments in cells depends strongly on the light conditions during growth [5], and is significantly influenced by the method chosen for protein isolation [6,7].

Despite the marked ecological importance of diatoms and the decisive role of FCPs in the energetics of these organisms, there is a lack of knowledge about the precise built of these light harvesting systems compared to higher plants. Interest in FCPs started early, and the first reports on isolated FCPs date back to the 80s [8–11]. One major pool of FCPs was usually purified, but for long no idea about the molecular built of FCP complexes or about a special attribution of certain FCP polypeptides to photosystem (PS) I or II existed. Only some reports were available about different pools of FCP complexes [12,7]. With the sequencing of the whole genomes from a centric (*Thalassiosira pseudonana*) and a pennate (*Phaeodactylum tricornutum*) diatom [13,14], information about FCPs increased significantly. Nowadays, three major groups are distinguished by their sequence, but also by their function. One group of FCPs, encoded by *Lhcr* genes, was hypothesised to function as antenna for PSI, due to the similarity with rhodophyte intrinsic PSI antennas, and this could later be proven biochemically [15–18]. Another group of FCP polypeptides, Lhcx, showed high similarity to LI818 or LhcSR proteins of *Chlamydomonas reinhardtii*, which were later identified as

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; Chl, chlorophyll; Fx, fucoxanthin; Dd, diadinoxanthin; Dt, diatoxanthin; WT, wild type; FCP, fucoxanthin chlorophyll protein; LL, low light; HL, high light; LC-ESI tandem MS, liquid chromatography electrospray ionisation tandem mass spectrometry; SDS-PAGE, SDS polyacrylamide gel electrophoresis; IEX, ion exchange chromatography

* Corresponding author. Tel.: +49 69 798 29602; fax: +49 69 798 29600.
E-mail address: c.buechel@bio.uni-frankfurt.de (C. Büchel).

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functional homologues to psbS of higher plants [19], thus needed for protection against a surplus of light. The involvement of such a Lhcx protein in photoprotection, Fcp6 of the diatom *Cyclotella meneghiniana*, had already earlier been proposed due to its ability to modulate the fluorescence yield of the FCPs [6,20], and was later proven for *T. pseudonana* and *P. tricornutum* [21,22]. The last group, Lhcf, constitutes the major part of the genes, and proteins function in light harvesting.

Membrane intrinsic, pigmented proteins are not easily prepared in a fully functional state. Another difficulty concerns the variety of FCPs encoded by the many Lhc genes present in diatoms. 17 Lhcf genes, 14 Lhcr and 4 Lhcx are annotated for the pennate diatom *P. tricornutum* and for the centric diatom *T. pseudonana* 11 Lhcf, 14 Lhcr and 7 Lhcx genes are described. All are expressed according to EST data, or their expression was proven otherwise [23].

Previous studies on FCPs focused often on the centric diatom *C. meneghiniana*, a close relative to *T. pseudonana*, due to its better biochemical accessibility. For *C. meneghiniana*, two major FCP complexes could be purified and biochemically characterised, FCPa being a trimer built of Fcp2 and Fcp6, members of the Lhcf and Lhcx family, respectively, and FCPb being a higher oligomer out of Fcp5, again a Lhcf protein [7,6]. A comparable built of antenna complexes was later shown for *T. pseudonana* [18]. The composition of the trimeric complexes in *C. meneghiniana* is dependent on light intensity and iron supplement during growth [6,24]. In addition, a functional heterogeneity could be detected as well, since the fluorescence yield of FCPa was shown to depend on the interaction of these trimers, their diatoxanthin content and the pH. All these features pointed to an involvement of FCPa in the protection mechanism called non-photochemical quenching, as already suggested by its Fcp6 content [20,25], whereas FCPb lacks these features and seems to be a pure light-harvesting complex.

P. tricornutum was early on taken as a model species for a pennate diatom, also concerning investigations of the photosynthetic apparatus [8–11]. Lepetit et al. [26] were the first to analyse a FCP pool fraction isolated via sucrose density centrifugation concerning oligomeric states; they found mainly trimers. As polypeptides Lhcf3/4 and Lhcf5 were reported. Using less detergent they also isolated FCPs in higher oligomeric states. Later, Lepetit et al. [17] did a more extensive analysis and recovered all Lhcf proteins except Lhcf12, as well as almost all Lhcr proteins and two of the Lhcx family, Lhcx1 and Lhcx2, in the same FCP pool preparations used before. Grouneva et al. [18] chose the more stringent blue native polyacrylamide gel electrophoresis (BN-PAGE) for separation. Thus, this group was able to analyse the trimer composition separately. In the trimeric FCPs, only Lhcfs, the main antenna proteins, were found, but none of the members of the other groups of FCPs. Lhcf1 to Lhcf12 except Lhcf6 and Lhcf7 were detected, and are thus constituents of trimeric FCPs.

Since different trimers might serve distinguished functions and might thus also be regulated in a different way, we wanted to determine whether different trimeric FCP complexes containing different Lhcf members exist, and whether their amount depends on one of the most prominent factors for photosynthetic organisms, the light intensity during growth. Thus, we sub-fractionated the pool of FCP proteins from *P. tricornutum* obtained by sucrose density centrifugation further by ion exchange chromatography, in order to get more insight into the biochemical and functional heterogeneity. Different trimeric complexes with changing members of Lhcfs were found, as well as differences under high light (HL) and low light (LL) conditions.

2. Material and methods

2.1. Cell culture

P. tricornutum Böhlin WT (UTEX culture collection, strain 646) was grown in ASP medium for 7 days at 18 °C in batch cultures at a light intensity of 40 μmol photons m^{-2} s^{-1} (LL) or 140 μmol photons m^{-2} s^{-1} (HL) and a 16/8 h light/dark cycle.

2.2. Purification of FCPs

1 l of cells was harvested in the third till fourth hour of light by a centrifugation at 4500 *g* for 3–5 min at 4 °C, and cells were resuspended in ~10 ml homogenisation buffer (1 M sorbitol, 10 mM HEPES, 2 mM KCl, 5 mM EDTA at pH 7.4). Thylakoid preparation was done as described by Veith and Büchel [15] with slight modifications. Cells were broken by four or five frenchpress cycles (French-Press-Cell-Disrupter, Polytec/Thermo) at 20,000 psi cell pressure. Unbroken cells were pelleted by centrifugation for 2 min at 3000 *g* at 4 °C. To recover all broken cells, the pellet was re-suspended in homogenisation buffer and spun again. Supernatants were pooled and remaining unbroken cells removed by centrifugation. To obtain good yields, membranes were then pelleted in an ultracentrifuge at 300,000 *g* for 1 h at 4 °C, and resuspended in washing buffer (=homogenisation buffer without sorbitol). After a spin at 200,000 *g* for 20 min at 4 °C, the thylakoids were resuspended with a little amount of buffer B1 (2 mM KCl, 25 mM Tris at pH 7.4). Chlorophyll concentration of the thylakoid suspension was determined spectrometrically according to Jeffrey and Humphrey [27].

Thylakoids of *P. tricornutum* with a concentration of 0.25 mg Chl a/ml were solubilised with 20 mM ß-dodecyl maltoside (Applichem) for 20 min on ice by gentle shaking. Unsolubilised material was removed by a short spin at 10,000 *g* at 4 °C for 5 min. The supernatant was loaded immediately onto discontinuous sucrose gradients, which contained 25, 15, 12.5, 10, 7.5 and 5% sucrose in buffer B1 supplemented with 0.03% ß-dodecyl maltoside (buffer B1a). Runs were carried out as described in Joshi-Deo et al. [28] and the only dark brown band containing the FCP pool was harvested. Lowering the detergent concentration to 10 mM led to poorer yields, but still only one band could be observed. In addition, no sufficient separation between the photosystems and FCPs was obtained (data not shown).

Pre-purified FCPs (100–400 μg Chl a) were loaded onto an ion exchange chromatography (IEX) column (DEAE-650S Toyopearl, Tosho Bioscience) according to Beer et al. [6]. A linear gradient of 0–200 mM NaCl in 10 column volumes was used for elution. Fractions were identified via their absorption and samples containing identical FCPs were pooled, washed with Buffer B1 and concentrated using concentration devices (Centricon, cut-off 30 kDa). Subsequently the samples were measured spectroscopically, aliquoted for further analysis, frozen in liquid nitrogen, and stored at −80 °C until further use. For comparison, FCPa and FCPb from *C. meneghiniana* were isolated exactly as described in Gundermann and Büchel [20].

2.3. Analytical gel filtration

Samples were applied to a self packed gel filtration column (high precision bore column, 400×6.6 mm, BioChem Fluidics) connected to an ÄKTA purifier P-900 (Amersham Biosciences). Flow-rate of B1a buffer was maintained at 0.3 ml min^{-1} during the run and absorbance of the eluates were analysed at 437 nm. The column was calibrated with FCPa and FCPb complexes from *C. meneghiniana* since their oligomeric status and molecular weights were previously established [6].

2.4. Spectroscopy

Absorption spectra were recorded with a Jasco V-550 spectrophotometer from 370 to 750 nm using 1 nm bandpass. All FCP fractions were checked for intactness by fluorescence excitation spectra in a Jasco F-6500 fluorospectrometer as described in Beer et al. [6]. In brief, samples were excited from 400 to 600 nm and fluorescence emission was detected in the Chl a emission maximum (675 nm), whereby a rhodamin spectrum was used for correction.

2.5. SDS-PAGE and liquid chromatography-electrospray ionisation tandem mass spectrometry (LC-ESI tandem MS)

Proteins were separated by high-percentage (10% and 12.5%, respectively) SDS-PAGE that contained 0.3% piperazine diacrylamide as a cross-linker [29]. Subsequent to the gel electrophoresis, the gel was silver stained [30]. For calculation of the relative abundance of bands on the gels ImageJ was used. This method is only semi-quantitative but allows the calculation of relative abundance of identical polypeptides from HL and LL samples on the same gel. Data were expressed as relative abundance of one band in relation to all proteins of the same fraction. For MS analysis, bands from HL and LL FCPs from two to three gels were excised, slices destained with freshly prepared destaining solution (15 mM $K_3[Fe(CN)_6]$ and 50 mM NaS_2O_3) for 8 min at room temperature and washed four times with MS-grade water. Proteins in the gel slices were in-gel tryptic digested, and peptides were subjected to nano LC-ESI-MS/MS using an UltiMate 3000 nano HPLC apparatus (Dionex) coupled online with a linear ion trap nano ESI mass spectrometer (Finnigian LTQ; Thermo Electron) as described previously [15]. In some cases, data-dependent MS was done with low abundant proteins. Therefore, a mass list of potential precursor ions specific for the protein of interest was created and the MS was set to trigger these precursor ion masses. Results from such runs are specially labelled within the tables. Data were analysed using the Thermo Electron Corp. Proteome Discoverer software (version 1.0) that includes Sequest algorithm [31]. As database the public available protein sequences of JGI Phatr2 (http://genome.jgi-psf.org/Phatr2/Phatr2.download.ftp.html) combined with the 132 plastid encoded proteins listed on NCBI (GenBank reference NC_008588.1) were used along with a false discovery rate (FDR) of equal or less than 1%. The minimum Xcorr for the three charge states was set to 2.0 for +1, 2.5 for +2 and 3.0 for +3. Only proteins that were identified with at least three different peptides (labelled unique peptides) have been considered further and are listed in the tables.

2.6. Sequence analysis

ClustalW was used to create a phylogenetic tree of all annotated Lhcf sequences from the genome of *T. pseudonana* (version 3) and *P. tricornutum* (version 2) retrieved from http://genome.jgi-psf.org/.

3. Results

In this study, we wanted to determine whether different FCP complexes exist in the pennate diatom *P. tricornutum*, and whether their amount is regulated depending on the light conditions during growth. Thus, we grew the cells under low light (LL) or high light (HL) conditions (see Material and methods). We used sucrose gradient centrifugation to separate the pool fraction of FCP complexes at first from other proteins of the *P. tricornutum* thylakoid membranes. In contrast to purifications from *C. meneghiniana*, where the same conditions had been used [7], only one band of FCP complexes was obtained, even when lowering the detergent concentration (data not shown). This whole pool was then harvested and further sub-fractionated by ion exchange chromatography, a method adopted from the protocols for *C. meneghiniana* [6] by which FCPs differing in polypeptide composition can be separated, while their oligomeric state is retained. We observed several fractions upon elution with a salt gradient (Fig. 1). Elution profiles from HL and LL cells were similar with few exceptions. One main difference regarded the second of the first two elution peaks, both labelled 0, which were more prominent in HL complexes. In the combined fraction 0, no discrete FCP bands could be detected on SDS-PAGE (data not shown). Thus, these fractions resembled mainly free pigments as demonstrated by their absorption spectra, which were dominated by carotenoids (Fig. 2a). This was confirmed by HPLC analyses (data not shown). No excitation energy transfer to Chl a could be measured (inset Fig. 2a), since Chl a emission was only measured upon Chl a excitation (~440 nm), but not upon carotenoid (~450–530 nm) excitation. In summary, fraction 0 contained no FCPs, but only free pigments. As already described earlier for *C. meneghiniana* FCPs [6,32], the usage of IEX for preparation of sub-populations of FCPs removed the more loosely bound Dd, Dt and Fx molecules, which are still present if preparing the whole pool of FCP by sucrose density centrifugation [28,26,24].

Six further fractions were reproducibly observed after salt elution of the ion exchange chromatography column (labelled 1–6; Fig. 1) and characterised. Fraction 1 eluted a bit later in FCPs from HL thylakoids, but only small differences between HL and LL FCPs except for pigmentation could be detected (see below). Fraction 2 was minor and followed by the most prominent fraction of all, fraction 3 that revealed in addition a separate shoulder (named fraction 4). Then, two small fractions (5 and 6) followed, whereby fraction 6 was almost missing in LL FCPs.

The absorption and excitation spectra of different fractions (1, 3 and 5) from LL cells are shown in Fig. 2b. Fractions 2 and 4 are omitted due to almost identical features compared to fraction 3, and the same holds for fraction 6, which was almost indistinguishable from fraction 5 (Fig. S1). All typical features of FCPs were present [26,7]: Chl c was visible by its absorption at around 625 nm and 465 nm, and Fx showed the typical broad absorption in the range from 490 to 565 nm. All these FCPs showed similar excitation energy transfer from Fx to Chl a as demonstrated by the excitation spectra (insets Fig. 2b). However, despite all similarities, differences were also obvious. FCPs eluting very early and those with the most acidic apparent pI showed almost identical absorption. In contrast, FCPs in the main fraction 3 exhibited the most red shifted Fx absorption and little absorption around 490 nm, an extinction previously attributed to the presence of Dd or Dt. This absorption at around 490 nm was generally more pronounced in FCPs from HL cells (Fig. S1). Besides this difference in absorption at 490 nm, HL and LL samples showed no spectroscopic differences.

The variations in absorption were mirrored by differences in pigment composition (Fig. 3). We compared the Chl c/Chl a, Fx/Chl a and Dd/Chl a ratios in the main fractions. As already judged from the higher absorption at around 490 nm, all HL FCPs had a trend to harbour more Dd than the corresponding LL FCPs. Fraction 1 had the highest amount of Dd, but we cannot rule out that this was due to its elution close to free pigments enriched in Dd. The Dd values for the other fractions are probably too low. It was already described earlier that preparation of FCPs by ion exchange methods leads to

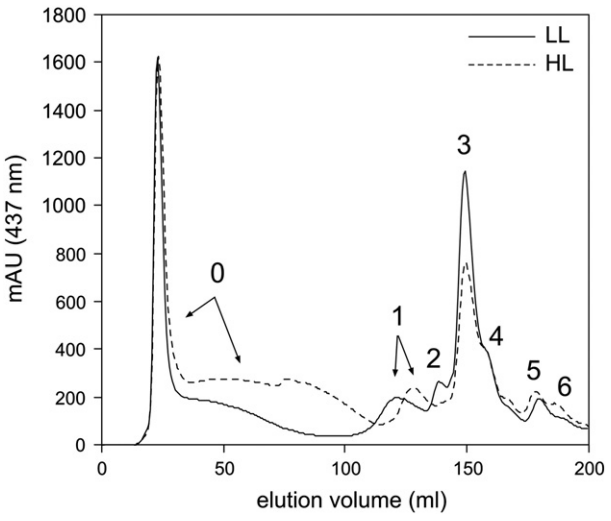

Fig. 1. Elution profiles of LL FCPs (solid line) and HL FCPs (dashed line) from *P. tricornutum*, separated on an IEX column.

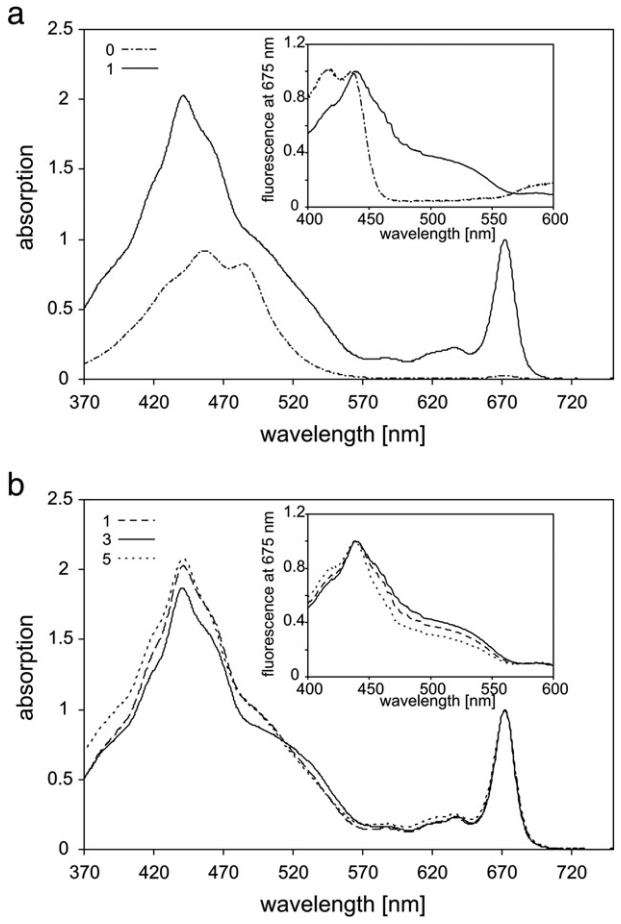

Fig. 2. Absorption and fluorescence excitation spectra recorded at 675 nm (inset) of representative fractions from the IEX runs of LL FCPs. In (a), fraction (0) (see Fig. 1) is represented by the dot-dashed line and fraction 1 by the solid line. In (b), fraction 1 (dashed line), fraction 3 (solid line) and fraction 5 (dotted line) are depicted. For better comparison, absorption spectra of fractions 1, 3 and 5 were normalised in the Q_Y absorption, whereas the excitation spectra and the absorption spectrum of fraction (0) were normalised in the maximum.

losses of Dd but also Dt. Indeed, in none of the FCPs a significant amount of Dt was present. Fx binding also differed between the complexes. In fraction 1, the Fx content was high, whereas the other FCPs

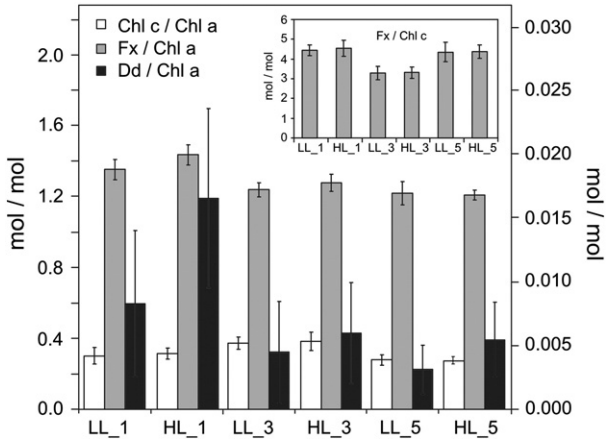

Fig. 3. Pigment stoichiometries in mol/mol of some representative fractions from the IEX runs. Chl c/Chl a (white bars) and Fx/Chl a (grey bars) are plotted on the left y-axis, whereas Dd/Chl a (black bars) is plotted on the right y-axis. In the inset the Fx/Chl c values are depicted. Data represent means and standard deviations of 3–4 measurements of samples from independent preparations.

contained a similar but lower amount of Fx. Chl c content differed in the various pigment–protein complexes derived from the IEX as well. This becomes extremely obvious when comparing Fx/Chl c ratios (Fig. 3, inset). The main fraction 3, whether coming from HL or LL, was characterised by the lowest Fx/Chl c ratio. Since the values of HL and LL complexes were almost identical in contrast to what is reported from FCPs isolated by sucrose gradients [17], these pigments should represent the core pigments bound to the FCP complexes, despite the losses in peripheral carotenoids.

The proteins from the different IEX fractions from HL and LL FCPs were separated on SDS-PAGE (Fig. 4), and the relative abundance of single bands inside a fraction was calculated (Table 1). All major bands had a molecular weight of approx. 18 kDa or slightly below, as demonstrated by a comparison with FCPa and FCPb from *C. meneghiniana*, where proteins of 19 kDa and 18 kDa are present [6]. In addition, some polypeptides in the 19 kDa range became visible as well. All sub-populations of FCPs showed a distinctive composition, except for the main fraction 3 and its shoulder, where no differences could be found. The relative contribution of polypeptides changed slightly between HL and LL samples in all fractions (Table 1). For fraction 2 it should be noted that the distribution was not reproducibly consistent under HL, most probably due to the small size of this fraction. Thus, these values were omitted from Tables 1 and 2.

To identify the different FCPs, bands were excised, proteins in gel trypic digested and peptides were used for mass spectrometric analysis by LC-ESI tandem MS (Table 2). Strict criteria were chosen for identification of proteins with a FDR rate of equal or less than 1% and at least three unique peptides per protein (see Material and methods). Supplemental Table 1 lists all identified proteins along with the details about evaluated peptides. *P. tricornutum* contains 17 *Lhcf* genes, coding for proteins of very similar sizes. Thus, heterogeneity had to be expected. Indeed none of the protein bands on the gels consisted of a single polypeptide. We did not apply a quantitative labelling approach for the MS analysis, but the number of total peptides found in a sample still gives an estimate of the protein abundance, especially when proteins of similar sizes are compared [33]. Indeed, peptide numbers of the main polypeptides inside a silver-stained band from the same light condition roughly correlated with the values for protein abundance as calculated from the density of bands in the gel, although the latter provides no unambiguous quantification as well (Table 1). Taking together the quantification from the gels and from the mass spectrometric analyses for the

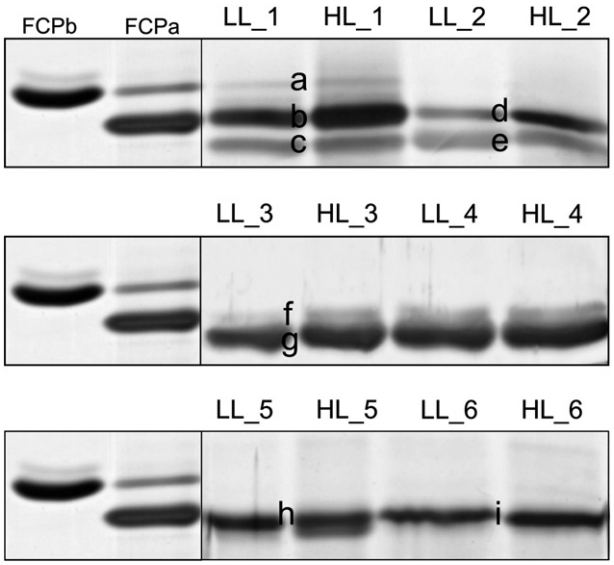

Fig. 4. SDS-PAGE of proteins from fractions after the IEX runs. For each lane, 5 μg of Chl a was loaded. FCPa and FCPb complexes from *C. meneghiniana* were separated for comparison. Small letters refer to bands that were quantified (Table 1) and excised for LC-ESI-tandem MS analysis (Table 2).

Table 1
Relative distribution of protein bands from LL and HL in a given fraction as calculated from SDS-PAGE.

Fraction	Band on gel (Fig. 4)	Abundance on gel[+]	
		LL	HL
1	a	0.06	0.05
	b	0.67	0.72
	c	0.27	0.23
2	d	0.45	n.c.
	e	0.55	n.c.
3, 4	f	0.08	0.15
	g	0.92	0.85
5	h	1.00	1.00
6	i	1.00	1.00

[+]Relative proportion of a single band of a fraction relative to the sum of all bands of the same fraction.
n.c.: data were non-consistent.

LL samples, Lhcf10 is the main protein of fraction 1, since it is most abundant in the band which accounts for 67% of all proteins in fraction 1 (Table 1). But this fraction also consists of Lhcf1, Lhcf2, Lhcf8, and tiny amounts of Lhcf9 and Lhcf4. LL fraction 2 is characterised by Lhcf 10, Lhcf2 and Lhcf5, but also includes Lhcf1, 8 and 11 in small amounts. In LL fraction 3/4 Lhcf5 is dominating, since it is the major polypeptide in the band which accounts for 92% of total proteins of fraction 3 (Table 1). It is accompanied by tiny amounts of Lhcf1, 2, 4, 10, and Lhcf11. Lhcf4 is the major constituent of the later fractions, but fraction 6 also includes Lhcf10, Lhcf5 and Lhcf2 as well as Lhcf11.

The data obtained from the LL FCPs were then compared to FCPs isolated from cells grown under HL (Table 2). Fraction 2 data were omitted from the results due to the reasons mentioned above. In the other fractions, the major polypeptides identified remained the same, but some differences were obvious. First, the relative amounts of the fractions from the IEX columns varied, with fraction 3 being more prominent under LL, whilst fraction 6 is only pronounced under HL (Fig. 1). Second and interestingly, Lhcf11 seems to be typical for LL FCPs. On the other hand, HL samples contained proteins that had not been identified in any LL fraction. These are two Chl-binding proteins not annotated as Lhcs, whereby in the JGI data base LHL1 is discussed as an early light induced protein and jgi11006 is only described as antenna protein, but is related to Lhcrs.

As mentioned above, we harvested the complete pool of FCPs from sucrose gradients. To examine if the FCPs in this pool and in the IEX fractions are present as monomers or in trimeric or oligomeric complexes, we performed gel filtration chromatography (Fig. 5). Mainly trimers were found when analysing the whole FCP pool harvested from sucrose gradients directly. A tiny shoulder might represent a very minor amount of oligomers as well. However, these oligomers were not stable enough to be present after IEX, in contrast to what is known from *C. meneghiniana* FCPb [6]. Thus, in the IEX fractions FCPs were also mostly trimeric, especially in the main fraction 3, but monomers were found

Table 2
Identified Lhcf proteins isolated from *P. tricornutum*, sub-fractionated by IEX and separated by SDS-PAGE by LC-ESI tandem MS analyses.

Fraction	Band on gel	LL	# peptides		HL	# Peptides		#AAs (including presequences)	Description
		Lhc identified	Unique	Total	Lhc identified	Unique	Total		
1	a	Lhcf9	5	22				206	jgi|Phatr2|30031|estExt_Genewise1.C_chr_200072
		Lhcf4	3	6				199	jgi|Phatr2|25168|estExt_Genewise1.C_chr_20040
	b	Lhcf10	4	138	Lhcf10	3	7	200	jgi|Phatr2|22006|estExt_gwp_gw1.C_chr_150122
		Lhcf2	5	94	Lhcf2	3	5	199	jgi|Phatr2|25172|estExt_Genewise1.C_chr_20044
					LHL1	3	3		jgi|Phatr2|17326|estExt_gwp_gw1.C_chr_10213
	c	Lhcf1	4	58				197	jgi|Phatr2|18049|estExt_gwp_gw1.C_chr_20047
		Lhcf8	4	37				201	jgi|Phatr2|22395|estExt_gwp_gw1.C_chr_170070
		Lhcf10	4	31	Lhcf10	3[a]	4	200	jgi|Phatr2|22006|estExt_gwp_gw1.C_chr_150122
					Jgi 11006	3	17	168	jgi|Phatr2|11006|e_gw1.4.352.1
2	d	Lhcf10	4	133	n.c.			200	jgi|Phatr2|22006|estExt_gwp_gw1.C_chr_150122
		Lhcf2	5	60				199	jgi|Phatr2|25172|estExt_Genewise1.C_chr_20044
		Lhcf5	3	27				198	jgi|Phatr2|30648|estExt_Genewise1.C_chr_240105
	e	Lhcf5	4	125	n.c.			198	jgi|Phatr2|30648|estExt_Genewise1.C_chr_240105
		Lhcf1	3	22				197	jgi|Phatr2|18049|estExt_gwp_gw1.C_chr_20047
		Lhcf8	3	9				201	jgi|Phatr2|22395|estExt_gwp_gw1.C_chr_170070
		Lhcf11	3	7				198	jgi|Phatr2|51230|estExt_fgenesh1_pm.C_chr_240004
3, 4	f	Lhcf4	4	11				199	jgi|Phatr2|25168|estExt_Genewise1.C_chr_20040
		Lhcf10	3	10	Lhcf10	4	30	200	jgi|Phatr2|22006|estExt_gwp_gw1.C_chr_150122
					Lhcf5	3	26	198	jgi|Phatr2|30648|estExt_Genewise1.C_chr_240105
					Lhcf2	4	24	199	jgi|Phatr2|25172|estExt_Genewise1.C_chr_20044
	g	Lhcf5	5	56	Lhcf5	4	142	198	jgi|Phatr2|30648|estExt_Genewise1.C_chr_240105
		Lhcf1[b]	3	26	Lhcf1	3	17	197	jgi|Phatr2|18049|estExt_gwp_gw1.C_chr_20047
		Lhcf2[b]	3	26				199	jgi|Phatr2|25172|estExt_Genewise1.C_chr_2004
		Lhcf11	4	10				198	jgi|Phatr2|51230|estExt_fgenesh1_pm.C_chr_240004
		Lhcf10[b]	4	9				200	jgi|Phatr2|22006|estExt_gwp_gw1.C_chr_150122
		Lhcf8[b]	3	8				201	jgi|Phatr2|22395|estExt_gwp_gw1.C_chr_17007
5	h	Lhcf4	4	24	Lhcf4	5	11	199	jgi|Phatr2|25168|estExt_Genewise1.C_chr_20040
6	i	Lhcf4	11	357	Lhcf4	7	278	199	jgi|Phatr2|25168|estExt_Genewise1.C_chr_20040
		Lhcf5	5	14	Lhcf5	4	21	198	jgi|Phatr2|30648|estExt_Genewise1.C_chr_240105
		Lhcf11	5	11				198	jgi|Phatr2|51230|estExt_fgenesh1_pm.C_chr_240004
		Lhcf10	6	8	Lhcf10	4	25	200	jgi|Phatr2|22006|estExt_gwp_gw1.C_chr_150122
		Lhcf2	4	8	Lhcf2	5	11	199	jgi|Phatr2|25172|estExt_Genewise1.C_chr_20044
		Lhcf1[b]	3	7				197	jgi|Phatr2|18049|estExt_gwp_gw1.C_chr_20047
		Lhcf8[b]	3	6				201	jgi|Phatr2|22395|estExt_gwp_gw1.C_chr_17007

[a] The usual MS analysis resulted in the identification of two unique peptides. A special MS run that triggered Lhcf10 specific peptides resulted in the identification of the third peptide (NDAIDFGWDTFDEETK). Note that the analyses of LL and HL samples derived from different gels. n.c.: data were non-consistent (see Table 1).
[b] In some cases the identified peptides fit also to other Lhcfs due to extended homologous regions within these Lhcfs. If a Lhcf protein cannot be exclusively identified by at least one peptide not overlapping with another Lhcf.

as well (fractions 1 and 5). An increase in the content of monomers was the only difference found between the main fraction and its shoulder, fraction 4 (Fig. S2), which might explain the different elution behaviour. In addition, monomers were more prominent in fraction 6 than in fraction 5 (Fig. S2). Our data suggest that stable FCPs exist mainly as trimeric complexes in *P. tricornutum*. Moreover, different trimeric complexes are present that are composed of different Lhcfs. Thereby, Lhcf5 dominates in the main fraction 3, while others such as Lhcf10, Lhcf2 and Lhcf4, respectively, are major components of other fractions (see Table 2, total peptides). In some cases, also light quantity affects the abundance of the Lhcfs (see Table 1, abundance on gels).

4. Discussion

This is the first report about sub-fractionating the trimeric FCP pool of the pennate diatom *P. tricornutum*, thus being able to depict Lhc members from different trimeric sub-pools. So far, only data about the composition of the whole pool existed. No Lhcr or Lhcx proteins were detected as before [18], and the same Lhcf polypeptides were identified, except for Lhcf12. Lhcf12 was not detected at all in LC-ESI-MS/MS analysis even when lowering the strict identification criteria and considering only two unique peptides for the identification of a protein. Application of such less stringent criteria would have additionally revealed Lhcf6 in tiny amounts (2 total peptides). All other Lhcfs, which were not found by us or in the analysis by Grouneva et al. [18], but by Lepetit et al. [17], might thus be expressed only in minute amounts. The lack of Lhcf12 in the FCP pool might be due to technical reasons of the different preparations. But it might be also related to the different strains of *P. tricornutum* used in the two studies.

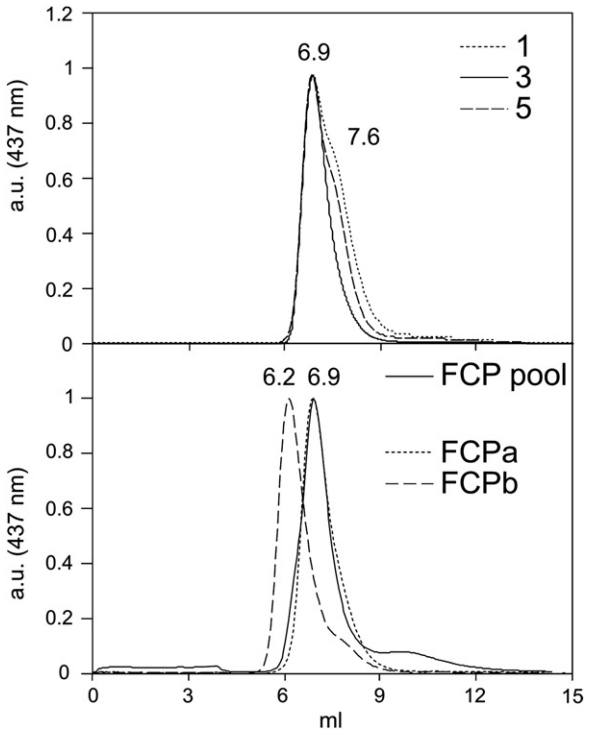

Fig. 5. Gel filtration of the FCP pool and IEX fractions. Fractions 1 (dotted line), 3 (solid line) and 5 (dashed line) (upper panel) as well as the whole pool of FCP from *P. tricornutum* as harvested from the sucrose gradients (lower panel, FCP pool, solid line) were separated on a gel filtration column and analysed at 437 nm. The column was calibrated with FCPa (dotted line) and FCPb (dashed line) complexes from *C. meneghiniana* (lower panel), since their oligomeric status and molecular weights were previously established [6]. For better comparison, all curves were normalised in their maxima. At 6.9 ml trimers eluted and the shoulders at 7.6 ml represent monomers. No higher oligomers eluting at 6.2 ml could be detected in any of the FCPs from *P. tricornutum*.

From all sub-pools of *P. tricornutum* FCP complexes derived from IEX, fraction 3 was the most prominent and it was dominated by Lhcf5. This polypeptide seems to be the most abundant Lhcf protein; it was additionally found in nearly every fraction from the IEX runs except for number 1. It was also found as a prominent protein in an earlier analysis from Lepetit et al. [26]. Another very abundant protein is Lhcf10, the major constituent of fraction 1 (together with Lhcf2) as well as of fraction 2 (closely followed by Lhcf5). The other polypeptide already identified in the early work by Lepetit et al. [26] was Lhcf4, which eluted only in a small fraction from the IEX column and thus is present in smaller amounts in thylakoids compared to Lhcf5.

Unfortunately, little is known about specific protein interactions of FCPs in *P. tricornutum* or other pennates. Joshi-Deo et al. [28] could identify Lhcf5 as the partner of Lhcf1 in trimers. Those Lhcf5/1 trimers are most probably found in fractions 2 and 3/4, albeit Lhcf1 is of lower overall abundance in these fractions compared to Lhcf5. According to the Lhcf distributions within the different fractions together with the abundance as estimated by total peptides (Tables 1 and 2), Lhcf10 might be also an interaction partner of Lhcf5 in fraction 2. Lhcf5 seems to form homotrimers as well, especially in fraction 3, where it is almost the sole constituent. The same holds true for Lhcf4 in the later fractions. On the other hand, Lhcf10 (as well as Lhcf2 and Lhcf5) most likely form trimers with several interaction partners as well (fraction 1).

In several fractions, changes in abundance could be observed between HL and LL FCP complexes (Table 1). For example, in fraction 3 the amount of the polypeptides in the upper band on the gel (band f) was almost doubled under HL, and contained Lhcf10, Lhcf5 and Lhcf2 instead of Lhcf4 and Lhcf10 under LL. Another important difference is the appearance of Lhcf11 under LL in fractions 2, 3 and 6, but never in HL FCPs. This result is not obvious from the mRNA data supplied by Nymark et al. [23], where no HL regulation was observed for Lhcf11 and may suggest some posttranscriptional control mechanism. On the other hand we could detect two further antenna proteins under HL conditions. One of them, Lhl1, was already reported by Lepetit et al. [17] in the whole pool of FCP complexes from *P. tricornutum*, whereas jgi11006 was not reported before.

Not only the composition of the trimers changed slightly when comparing LL and HL conditions, but also the abundance of the different trimers changed as demonstrated by the elution profiles of the IEX columns (Fig. 1). Fraction 3, consisting mainly of Lhcf5, was significantly reduced under HL, which corresponds to the mRNA levels after HL shift as published by Nymark et al. [23]. Fraction 6 appeared under HL in addition to fraction 5. Since both fractions contained mainly Lhcf4, the overall amount of this polypeptide is increased under HL conditions.

Trimers containing mainly Lhcf4 were characterised by a high Fx/Chl c ratio, which was due to low Chl c content, and also Dd was present in the smallest amount of all other FCP complexes. In contrast, the most abundant FCP complexes, i.e. fraction 3, had a low Fx to Chl c ratio with around 3.5. Since in fraction 3 Lhcf5 is most abundant, this polypeptide is very likely the one binding less Fx/Chl c. The Fx molecules bound were the ones absorbing at the longest wavelengths. Fx was already shown to be bound to FCPs in different ways, leading to variations in the bathochromic shift and thus creating 'blue', 'green' and 'red' absorbing Fx [34,32] in the FCPs of the centric diatom *C. meneghiniana*. Fractions 1, 5 and 6 were characterised by more 'blue' absorbing Fx. Since both fraction 1 and 2 contained Lhcf10 in prominent amounts, the significant difference in pigment binding between these two fractions has to be due to the other FCPs present. Lhcf2 might thus be responsible for the binding of higher amounts of ('blue') Fx, as well as Lhcf4, the dominant component of fractions 5 and 6. Szabó et al. [35] could also detect functionally different Fx in whole cells of *C. meneghiniana* and *P. tricornutum*, and the red forms were hypothesised to transfer more excitation energy

into photosystem II than to photosystem I. Taking these findings together one could propose that Lhcf2 and Lhcf4, in addition to the Lhcr polypeptides, might be more closely associated with PSI than with PSII *in vivo*, whereas Lhcf5 might be the dominant harvester for PSII. In addition, the 'red' Fx molecules were more abundant in LL cells [35]. This is in accordance with the higher amount of fraction 3 under LL conditions seen here. This shift from FCPs binding more 'red' Fx (Lhcf5) under LL to FCPs enriched in 'blue' Fx (Lhcf2, Lhcf4) in HL will then also influence the overall absorbance features of the cells. Such differences in pigmentation of trimers were never reported before for pennate diatoms. In centrics, both trimers and oligomers were found to contain the various Fx forms, whereby in FCPb the 'red' forms absorbed at even longer wavelengths [32].

In this study, as well as in the work by Grouneva et al. [18] using BN-PAGE, all complexes were trimeric except for some monomerisation, whereas in the centric diatoms *T. pseudonana* as well as *C. meneghiniana* the same methods resulted in separation of trimers and stable higher oligomers [18,7,6]. When comparing *Lhcf* genes of *P. tricornutum*, *Lhcf1–11* cluster in one group, and are well separated from *Lhcf12–17* (Fig. 6). The relationships between the *Lhcfs* of pennate diatoms like *P. tricornutum* and centric diatoms like *T. pseudonana* are relatively weak, especially for the *Lhcf1–11* genes of *P. tricornutum* on one hand and the *Lhcf1–6, 8, 9* genes of *T. pseudonana* on the other. In *C. meneghiniana*, the trimers are composed mainly of Fcp1–3 and Fcp6, and indeed the homologues Lhcf1/2 and Lhcx1 were found in *T. pseudonana* trimers as well. The oligomers contain Fcp5, and the homologue Lhcf8/9 appeared in the MS analysis of oligomers of *T. pseudonana* with the highest abundance, followed by Lhcf7. Only the latter protein is closely related to Lhcfs in *P. tricornutum*, i.e. to Lhcf12–14, but those were not found in our analysis. Only Lhcf12 was detected by Grouneva et al. [18] in the trimeric FCP fraction of *P. tricornutum*, but with the lowest abundance of all polypeptides. Thus, the polypeptides of highest abundance in the pennate *P. tricornutum* have no direct homologues in centrics and *vice versa*. These variations are likely to be the reasons for the differences seen in oligomeric state, the presence of Lhcx proteins in trimers, but also in pigmentation. All reports on *P. tricornutum* FCPs describe higher Fx/Chl a ratios compared to e.g. data from *C. meneghiniana* [6]. These data underline the importance to study the light harvesting complexes in both pennate and centric diatoms in depth.

Despite contradictory reports, the most prominent differences in the FCP complex organisation between centric and pennate algae, i.e. the lack of Lhcx in stable FCP trimers of pennates and the lack of specific polypeptides forming stable higher oligomers shown in the work by Grouneva et al. [18] have now been confirmed using a different biochemical approach. This is the first report about specific sub-fractions and their abundance in the FCP pool in pennate diatoms, whereby we could show that not only polypeptide composition, but also pigmentation differs between the subtypes. Despite the high similarity of proteins which make up the different trimers, the specific composition allows for adaptation of the absorption abilities due to the change from more 'red' to 'blue' Fx containing FCPs (Lhcf5 and Lhcf4/Lhcf2, respectively) during HL acclimation.

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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.2012.10.017.

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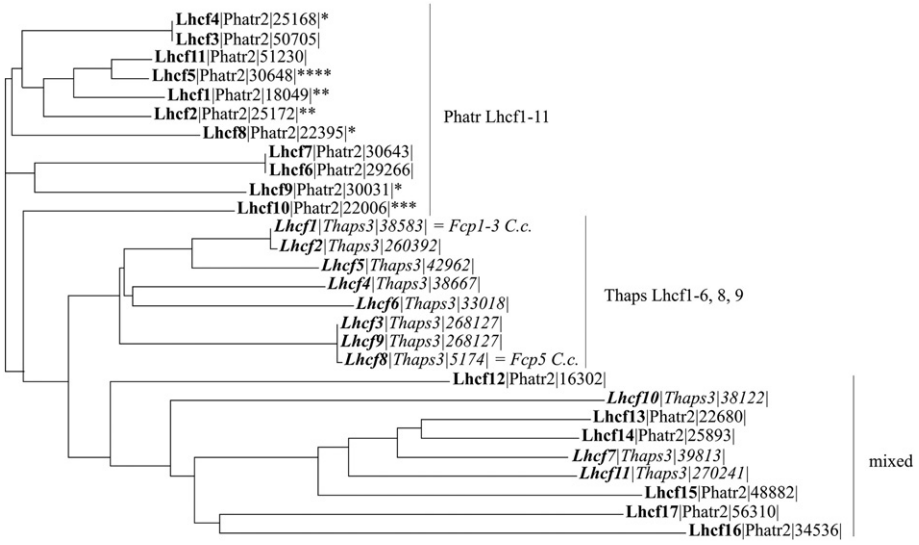

Fig. 6. Phylogenetic tree of Lhcfs from *P. tricornutum* (Phatr2) and *T. pseudonana* (Thaps3). Protein identification according to the jgi data base is given as well. In case of *T. pseudonana*, gene names are given in italics for better visualization of the clusters. *Cyclotella cryptica (=meneghiniana)* sequences are marked as well (C.c.). Numbers of stars refer to the overall amount of the proteins found in the FCP pool in our analysis.

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