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Supramolecular organization of fucoxanthin–chlorophyll proteins in centric and pennate diatoms

Zdenko Gardian · Radek Litvín · David Bína · František Vácha

Abstract Fucoxanthin–chlorophyll proteins (FCP) are the major light-harvesting proteins of diatom algae, a major contributor to marine carbon fixation. FCP complexes from representatives of centric (*Cyclotella meneghiniana*) and pennate (*Phaeodactylum tricornutum*) diatoms were prepared by sucrose gradient centrifugation and studied by means of electron microscopy followed by single particle analysis. The oligomeric FCP from a centric diatom were observed to take the form of unusual chain-like or circular shapes, a very unique supramolecular assembly for such antennas. The existence of the often disputed oligomeric form of FCP in pennate diatoms has been confirmed. Contrary to the centric diatom FCP, pennate diatom FCP oligomers are very similar to oligomeric antennas from related heterokont (Stramenopila) algae. Evolutionary aspects of the presence of novel light-harvesting protein arrangement in centric diatoms are discussed.

Keywords Photosynthesis · Transmission electron microscopy · Fucoxanthin–chlorophyll proteins · Diatoms · Light harvesting · Thylakoid membrane

Abbreviations
Chl   Chlorophyll

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MES	2-(*N*-morpholino)ethanesulfonic acid
DM	*n*-Dodecyl-β-D-maltoside
TEM	Transmission electron microscopy
FCP	Fucoxanthin–chlorophyll proteins
LHC	Light-harvesting complex
PSI	Photosystem I
PSII	Photosystem II
SDS-PAGE	Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate
CD	Circular dichroism

Introduction

Diatoms (Bacillariophyceae) are a ubiquitous group of eukaryotic algae that contribute significantly to marine biomass production (Field et al. 1998; Falkowski et al. 2004). Phylogenetically, diatoms belong to a group of algae with secondary plastids of red algal origin—Stramenopila. They are classified into two groups distinguished by the symmetry of their silica cell walls—radially symmetric centric diatoms and bilaterally symmetric pennate diatoms. This division is generally reflected in their lifestyles where centric diatoms are mostly planktonic whereas pennate diatoms are benthic (Falciatore and Bowler 2002).

While the overall features of the photosynthetic membranes of diatoms are similar to those of higher plants, there are important differences. First of all, the diatom photosynthetic membranes do not form grana and stroma lamellae but rather assemble into stacks of three thylakoids. Second, the photosystems are not segregated but only randomly distributed across the membrane (Pyszniak and Gibbs 1992).

The major peripheral light-harvesting complexes of diatoms are fucoxanthin–chlorophyll proteins (FCPs). Besides fucoxanthin, the main light-harvesting carotenoid, they contain diadinoxanthin and chlorophylls (Chls) *a* and *c*. Diatom FCPs are encoded by a family of nuclear genes phylogenetically related to the Lhcr genes of the red algal ancestor of diatom chloroplasts (Hoffman et al. 2011). Both FCPs and Lhcr are members of a huge gene family of chlorophyll binding proteins (*cab* family, Dittami et al. 2010). The FCP proteins are structurally similar to other members of *cab* family (Eppard and Rhiel 1998). They contain three transmembrane helices binding a number of cofactor molecules though the carotenoid to chlorophyll ratio is higher in FCP than in LHC I and II proteins—four carotenoids per five Chls (Papagiannakis et al. 2005; Premvardhan et al. 2010).

In higher plants, light-harvesting proteins of the *cab* family are functionally specialized into PSI-specific (Wientjes et al. 2009), PSII-specific (Boekema et al. 1999) and mobile antenna complexes (Wientjes et al. 2013). Higher plant LHCIs assemble in a belt on one side of photosystem I (Ben-Shem et al. 2003) whereas LHCIIs form trimeric and higher oligomeric complexes (Dekker et al. 1999; Barros and Kühlbrandt 2009). In red algae, Lhcr proteins assemble a crescent on one side of PSI, in analogy to the situation in higher plants (Gardian et al. 2007; Busch et al. 2010).

So far, no PSII-specific peripheral antenna proteins have been described in Stramenopile algae (Lepetit et al. 2012) and it is assumed that the main antenna pool is shared between the two photosystems (Lepetit et al. 2012). In addition, PSI light harvesting is supplemented by complexes arranged in a manner similar to LHCI/LHCr complexes of higher plants and red algae (Ikeda et al. 2013). In Stramenopile algae, trimeric complexes of antenna proteins appear to be the main building block as have been described in phaeophytes (Katoh et al. 1989; Fujii et al. 2012), xanthophytes (Gardian et al. 2011) and diatoms (Joshi-Deo et al. 2010; Grouneva et al. 2011). These trimers have been described to assemble into higher oligomers in all mentioned groups of algae (Katoh and Ehara 1990; Büchel 2003) as well as in *Chromera velia*, which has closely related antenna proteins (Tichy et al. 2013).

The diatom genes encoding the main FCP antenna proteins form two clusters split along the phylogenetic line between centric and pennate diatoms (Gundermann et al. 2013). In centric diatoms, the oligomeric complexes of FCPs are well known (Büchel 2003) whereas in pennate diatoms no conclusion has been reached so far. Lepetit et al. (2007) reported on weakly bound oligomeric FCP complex in *Phaeodactylum* but many others found little evidence of such complex (Guglielmi et al. 2005; Joshi-Deo et al. 2010; Grouneva et al. 2011; Gundermann et al. 2013; Nagao et al. 2013).

In this work, we report projection structural maps of oligomeric FCP complexes isolated from representatives of centric (*Cyclotella meneghiniana*) and pennate (*Phaeodactylum tricornutum*) diatoms. We have found that the arrangements of these complexes are markedly different. Circular dichroism spectroscopy also supported the structural difference between centric and pennate diatom FCPs.

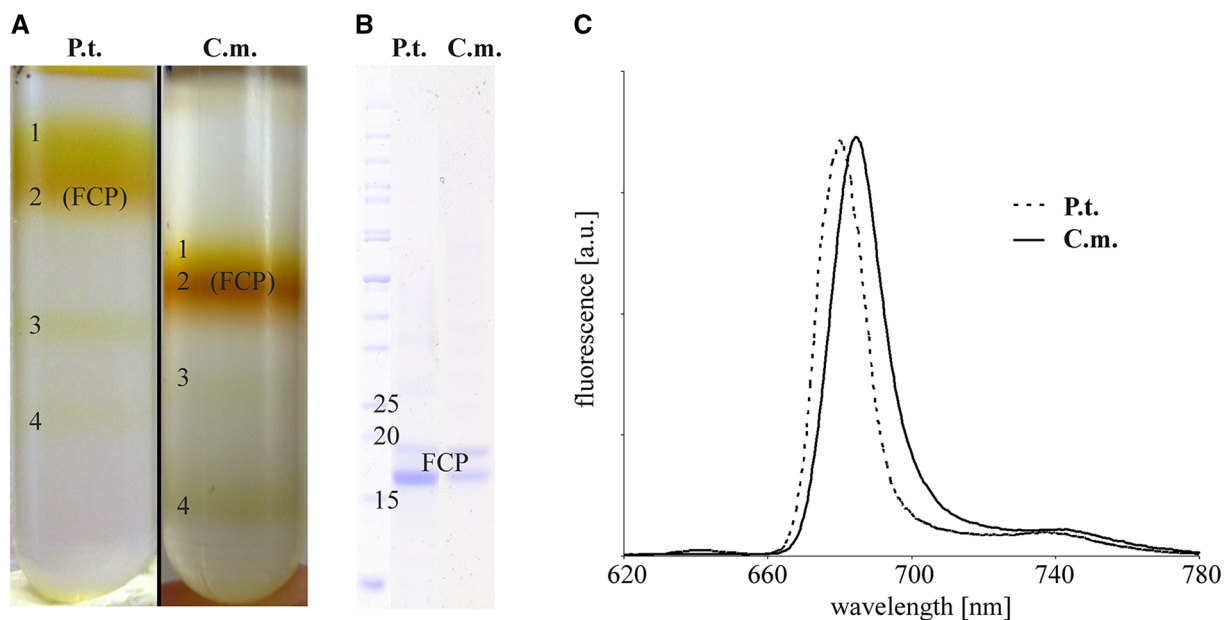## Materials and methods

Culture and growth conditions

Cyclotella meneghiniana (SAG 1020-1a) and *P. tricornutum* (SAG 1090-1a) were batch cultivated in 5 l flasks at room temperature in ASW f/2 medium. The light intensity was ~ 50 μmol photons m^{-2} s^{-1}. Cells were harvested by centrifugation at $5,000 \times g$ for 5 min, washed with distilled water, and resuspended in a buffer containing 10 mM MES (pH 6.5), 2 mM KCl, 5 mM EDTA, 1 M Sorbitol.

Thylakoid membrane isolation

Cells were broken by several passages through an EmulsiFlex-C5 High Pressure cell disrupter (Avestin Inc., Canada) at a pressure of 100–120 MPa, while keeping the apparatus refrigerated on ice in the dark. All following isolations steps were carried out at 4 °C and dim light conditions. Unbroken cells were removed by centrifugation for 10 min at $1,000 \times g$. The supernatant was then centrifuged for 30 min at $60,000 \times g$ to pellet thylakoid membranes. Membranes were resuspended in MES buffer (MES (pH 6.5), 2 mM KCl, 5 mM EDTA) and solubilised with 0,5 % *n*-dodecyl-β-D-maltoside at chlorophyll concentration of 1 mg (Chl) ml^{-1} for 20 min (detergent:Chl 10:1). The unsolubilized material was removed by centrifugation for 20 min at $40,000 \times g$, and the supernatant was loaded onto a fresh 0–1.2 M continuous sucrose density gradient prepared by freezing and thawing the centrifuge tubes filled with a buffer containing MES (pH 6.5), 2 mM KCl, 5 mM EDTA, 0.01 % DM, 0.6 M sucrose. The following centrifugation was carried out using a SW-40 swing-out rotor (Beckman Coulter) at $150,000 \times g$ for 16 h, the zone with light-harvesting antennae was collected. The antenna zone was further exposed to a gel filtration using Sephadex G-75 (Amersham Biosciences, Sweden), to reduce the amount of sucrose and improve contrast in TEM.

Protein composition and spectroscopic analysis

The protein composition was determined by SDS-PAGE using a precast 12 % polyacrylamide SDS gel (C.B.S.

Fig. 1 a Sucrose density gradient of solubilized thylakoids of *P. tricornutum* (*left*) and *C. meneghiniana* (*right*). The second (brown) zone of both gradients contains the most of FCP. **b** SDS-PAGE analysis of FCP zones from *P. tricornutum* (*left*) and *C. meneghiniana* (*right*) shows prominent bands at about 17–19 kDa, which are characteristic for polypeptides of FCP. **c** 77 K fluorescence emission spectra of FCP zones from *P. tricornutum* (*dashed line*) and *C. meneghiniana* (*solid line*). The excitation wavelength was 435 nm. Spectra are normalized to their maxima

Scientific) and visualized with Coomassie Brilliant Blue or by silver staining. Apparent molecular weights were estimated by co-electrophoresis of a low molecular weight protein standard (Fermentas).

Chlorophyll concentration was determined according to Ogawa and Vernon (1971). Low temperature fluorescence emission spectra were measured at liquid nitrogen temperature using a Fluorolog-2 spectrofluorometer (Jobin–Yvon, Edison, NJ, USA) with an excitation wavelength of 435 nm and a chlorophyll concentration of 10 µg (Chl a ml^{-1}). Circular dichroism (CD) spectra were recorded at 4 °C with a Jasco J-715 spectropolarimeter with a slit width of 2 nm and optical path length of 1 cm.

Transmission electron microscopy (TEM) and image analysis

Freshly prepared FCP complexes and photosynthetic membrane of *C. meneghiniana* were immediately used for TEM. The specimen was placed on glow discharged carbon-coated copper grids and negatively stained with 2 % uranyl acetate. TEM was performed with a JEOL JEM–2100F transmission electron microscope (JEOL, Japan) using 200 kV at 20,000× magnification. TEM images were recorded by a bottom-mount Gatan CCD Orius SC1000 camera, corresponding to a pixel size of 3.4 Å.

Image analyses were carried out using Spider and Web software package (Frank et al. 1996). Manually selected

projections were rotationally and translationally aligned, and treated by multivariate statistical analysis in combination with classification procedure (van Heel and Frank 1981; Harauz et al. 1988). Classes from each of the subsets were used for refinement of alignments and subsequent classifications. For the final sum, the best of the class members were summed using a cross-correlation coefficient of the alignment procedure as a quality parameter (van Heel and Frank 1981).
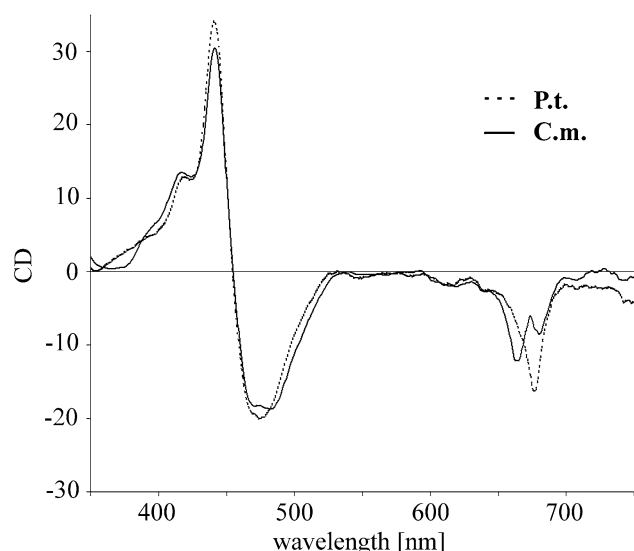
Results and discussion

In this work, we obtained purified FCP complexes from *C. meneghiniana* and *P. tricornutum* for electron microscopy analysis. Solubilised thylakoid membranes of both organisms were separated into 4 zones by sucrose density gradient centrifugation (Fig. 1a).

For studies of arrangement of FCPs, we were interested in the second (brown) zones of both gradients as they contain the most of FCPs as indicated by SDS-PAGE. The SDS-PAGE gels (Fig. 1b) show prominent bands at about 17–19 kDa, corresponding to polypeptides of FCP antennas (Büchel 2003; Gundermann and Büchel 2008). In *C. meneghiniana*, the protein composition of trimers and oligomers is known to differ (Büchel 2003)—the trimers are primarily assembled from the lighter (∼18 kDa) proteins whereas the higher oligomers are primarily made up
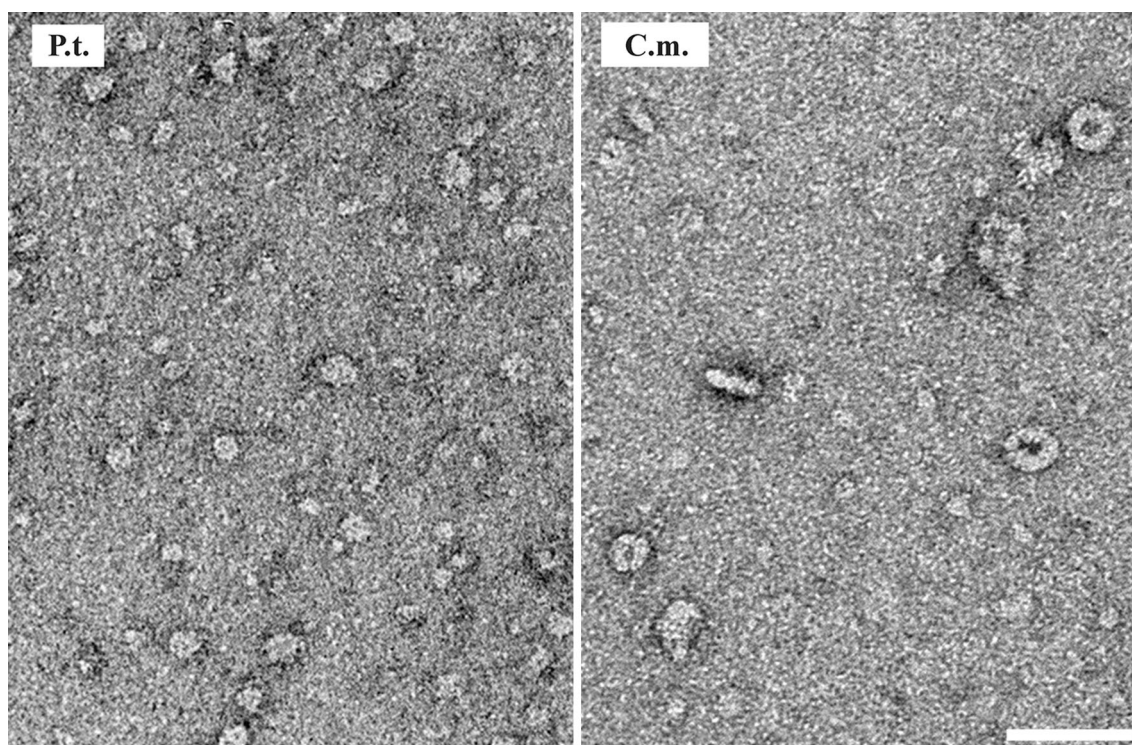
of the heavier ($\sim$ 19 kDa) proteins. The intensity of the two bands in our preparation (Fig. 1b) was about equal so the zone was most probably a mixture of both trimers and oligomers as was the case in Büchel (2003)

Fig. 2 Circular dichroism spectra of the isolated antenna complexes of *P. tricornutum* (*dashed line*) and *C. meneghiniana* (*solid line*)

The protein composition of oligomeric FCP from *P. tricornutum* described by Lepetit et al. (2007) did not differ from the FCP trimers. However, given the position of the FCP gradient band in our experiment compare to that of Lepetit et al. (2007) and also to our *C. meneghiniana* gradient, we expect it probably consisted mostly of trimeric FCP. It should be noted here that our prime concern was minimizing the number of purification steps in order to increase the probability of observing near-native oligomeric complexes. The heterogeneity of FCP oligomerization in collected fractions was of secondary importance because in the analysis of TEM images only the oligomeric particles were readily picked and processed.

The 77 K fluorescence emission spectra of FCP zones (Fig. 1c) of the two diatoms clearly differ. In *P. tricornutum*, the Chl *a* emission peaks at 680 nm in good correspondence with spectra reported by Lepetit et al. (2007) while in *C. meneghiniana* the emission peaks at 685 nm. Given that both of the samples are supposedly very closely related FCP isolations, this is a significant difference. The bathochromic shift of emission in *C. meneghiniana* might be indicative of higher aggregation or oligomerization status of its LHC proteins (Pascal et al. 2005; Miloslavina et al. 2008) which is also suggested by slightly higher width of the emission peak and different positions of the respective bands in sucrose density gradients. A

Fig. 3 TEM images of FCP zone from *C. meneghiniana* (*right*) and *P. tricornutum* (*left*). Samples were negatively stained with 2 % uranyl acetate. The *scale bar* represents 50 nm

comparison of 77 K emission spectra of *C. meneghiniana* FCP zone with those of Gundermann and Büchel (2012) shows good correspondence with their FCPb (oligomeric) complexes contrary to the FCPa (trimeric) complexes. However, our SDS-PAGE and CD spectroscopy data (see below) indicate a mixture of oligomeric states in this zone.
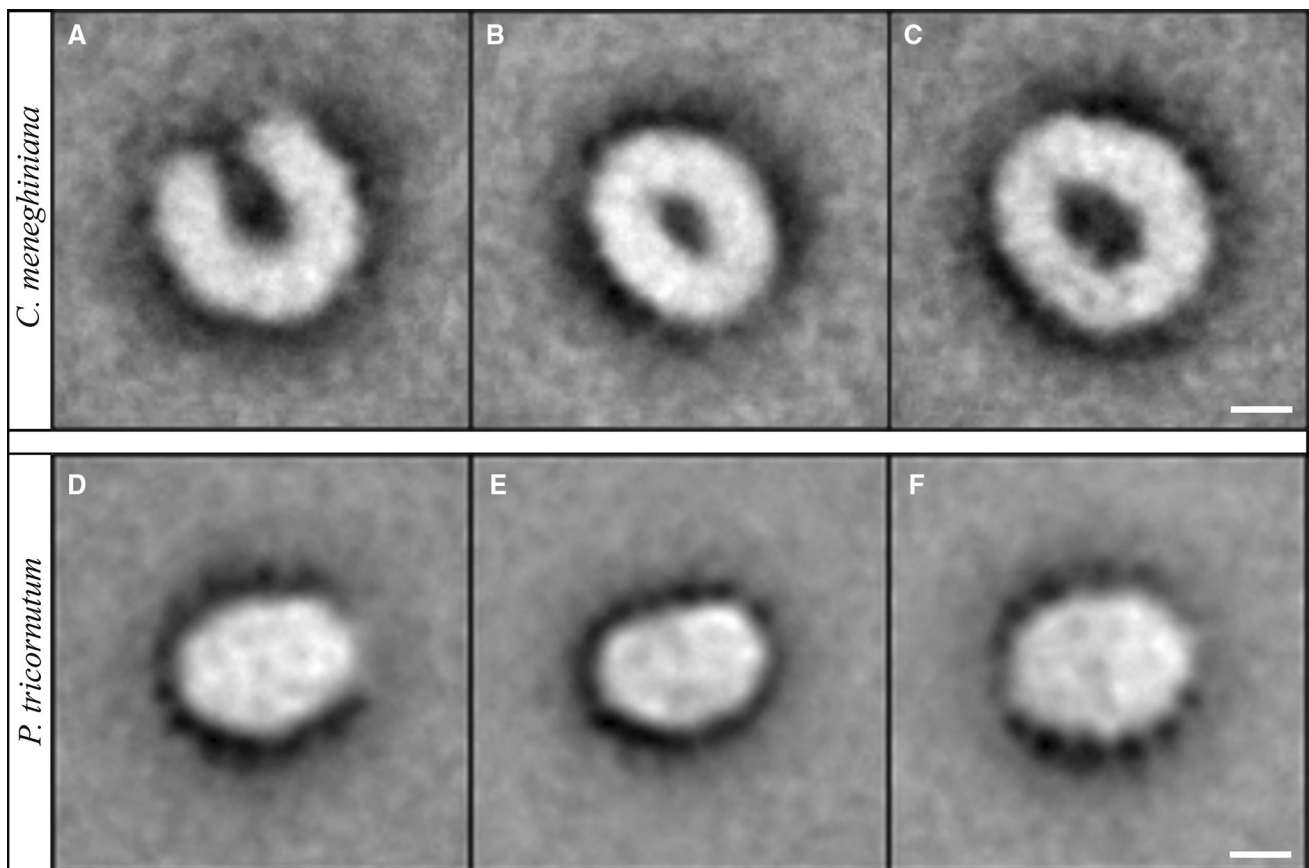
The circular dichroism (CD) spectra of the two antenna isolations (Fig. 2) differed markedly mainly in the Qy region of chlorophyll *a*, in agreement with published data (Büchel 2003; Lepetit et al. 2007). The two negative features of the CD of *C. meneghiniana*, at 663 and 680 nm, are characteristic for FCPs isolated from this diatom. The CD of trimers shown in Büchel (2003) has equal intensity of these two peaks whereas the CD of oligomers has only the higher energy peak. Our CD spectrum has about 1:2 ratio of the 680:663 peaks which indicates that the FCP band of the sucrose density gradient is a mixture of FCP trimers and oligomers, in line with the results of SDS-PAGE (Fig. 1 b). The CD spectrum of the FCP band of *P. tricornutum* does not allow distinguishing between trimeric or oligomeric complexes (Lepetit et al. 2007). While subtle, the differences in CD spectra of FCPs of closely related

organisms provide an independent line of evidence of structural differences of these proteins.

Purified FCP complexes of both organisms were negatively stained with 2 % uranyl acetate, visualized by TEM and processed by image analysis. Typical TEM images of FCP zone from *C. meneghiniana* and *P. tricornutum* are shown in Fig. 3. It is apparent that both FCP sucrose density gradient zones contained at least two types of particles, presumably small FCP complexes and bigger oligomeric forms of FCPs.

For single particle analysis, top-view projections of the oligomeric particles (in diameter bigger that 10 nm) were manually selected. 8,000 particles were selected from the *P. tricornutum* FCP zone and 5,200 particles from *C. meneghiniana* FCP zone. After the classification steps, the particles were separated into 18 and 28 classes for *P. tricornutum* and *C. meneghiniana*, respectively. The most representative classes of particles are shown in Fig. 4.

There were striking differences in the shapes and sizes of the FCP oligomers from the two organisms. The FCP oligomers isolated from *P. tricornutum* form approximately elliptical structure with major and minor axis of

Fig. 4 The most representative class averages obtained by classification of 5,200 particles from *C. meneghiniana* (*up*) and 8,000 particles from *P. tricornutum* (*down*). The numbers of averaged particles are 280 (**a**), 312 (**b**), 277 (**c**) for *C. meneghiniana* and 582 (**d**), 528 (**e**), 478 (**f**) for *P. tricornutum*. The *scale bar* represents 5 nm
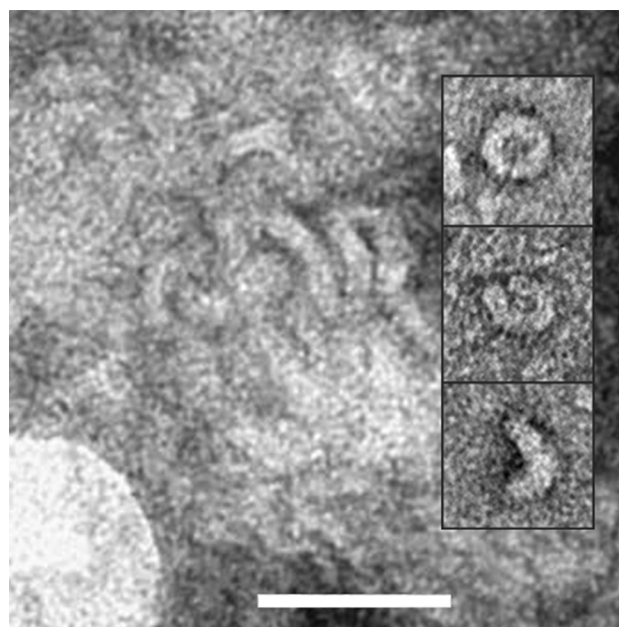
about 15 and 11 nm, respectively. This structure is very similar to the phylogenetically related oligomeric antennas of *Xanthonema* XLH (Gardian et al. 2011) and *Chromera* CLH (Tichy et al. 2013). Extrapolating from these results and also the particles of a brown alga FCP (Katoh and Ehara 1990), one might be tempted to conclude that all Stramenopile algae share the same peripheral antenna quaternary structure. Yet, the *C. meneghiniana* FCPs are very different from all these despite the fact that they are, in terms of their amino acid sequences, the most similar to *P. tricornutum* FCPs.

The FCP oligomers from *C. meneghiniana* assemble flexible chain-like structures, often enclosed to form a ring of varied shape. The size of these complexes (about 17 nm in diameter) is bigger than in *P. tricornutum*. The apparent flexibility of these structures caused an increase in the number of particle classes revealed during the single particle analysis. The higher noise in the averaged projections is also stemming from the lower number of particles per class of *C. meneghiniana* FCP oligomers.

The high variability of the shape of observed FCP oligomers from *C. meneghiniana* raises the question of what is the native shape of these complexes. One attractive possibility is that the antenna could encircle a photosystem in analogy to bacterial LH1 antennas (Walz et al. 1998) but there are no data supporting such speculation. Direct observation of the diatom photosynthetic membrane would be very informative in this matter (Kouril et al. 2013). The localization of FCP proteins in *Cyclotella* was studied by immunogold electron microscopy of freeze-fractured thylakoid membranes (Westermann and Rhiel 2005), but the method does not provide enough resolution to visualize shapes of the complexes.

To resolve this issue, we tried to visualize *C. meneghiniana* photosynthetic membrane by the TEM negative staining method. It was possible to observe many particles with similar thickness as our FCP oligomeric complexes but essentially linear arrangement (Fig. 5, Supplementary Fig. 1). Due to the abundance of such particles in the photosynthetic membrane and their shape, we interpret these particles as the native FCP antenna oligomers. The ring arrangement displayed in Fig. 4b, c is then most probably an artifact associated with the high affinity of FCPs to themselves. Unfortunately, no photosystem particles could be unambiguously assigned by this method.

The presence and abundance of oligomeric forms in our *P. tricornutum* FCP isolation is perhaps surprising given the literature consensus (reviewed by Gundermann et al. 2013) and also our own data presented in Fig. 1. A likely explanation is that the oligomeric form of pennate FCP is very fragile, and we used a very crude isolation method. It is possible that the oligomeric FCP will not survive multistep purification where the sucrose gradient is followed by

Fig. 5 Electron microscopy image of negatively stained photosynthetic membrane of *C. meneghiniana*. The *insets* (*right*) show three raw particles from the single particle analysis of oligomeric FCP antennas of *C. meneghiniana*. The *scale bar* represents 50 nm. Additional images are presented in supplementary Fig. 1

other procedures, such as ion exchange chromatography or gel filtration.

The presence of unique light-harvesting protein arrangement in *C. meneghiniana* is puzzling. Recent diatom evolution studies using molecular data (Sims et al. 2006; Kooistra et al. 2007) split diatoms into three subgroups—radial centric diatoms, multipolar centric diatoms, and pennate diatoms—where the latter two groups form together a single clade that includes both *C. meneghiniana* (multipolar centric) and *P. tricornutum* (pennate). To our knowledge, no radial centric diatom FCPs have been studied in detail, the best known member is probably *Coscinodiscus* (Lang and Kroth 2001). In light of the results presented here, it will be interesting to know if the arrangement of antenna observed in *C. meneghiniana* FCP is unique to this species or whether it is characteristic of a larger phylogenetic group. Also, it remains to be seen if this particular geometry of supramolecular aggregates represents any advantage in terms of regulation of light-harvesting efficiency, and might thus be an adaptation to the living condition of the organism. This is an important question given the significance of diatoms as marine primary producers.

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