Subunit Composition and Pigmentation of Fucoxanthin−Chlorophyll Proteins in Diatoms: Evidence for a Subunit Involved in Diadinoxanthin and Diatoxanthin Binding†

Anja Beer, Kathi Gundermann, Janet Beckmann, and Claudia Büchel*

Institute of Molecular Biosciences, Department of Biosciences, University of Frankfurt, Siesmayerstrasse 70, 60323 Frankfurt, Germany

Received June 23, 2006; Revised Manuscript Received August 28, 2006

ABSTRACT: Two different fucoxanthin−chlorophyll protein complexes (FCP) were purified from the centric diatom *Cyclotella meneghiniana* and characterized with regard to their polypeptide and pigment composition. Whereas the oligomeric FCPb complex is most probably composed of *fcp5* gene products, the trimeric FCPa has subunits encoded by *fcp1-3* and *fcp6/7*. The amount of the latter polypeptide is enhanced when FCPa is isolated from algae grown under HL conditions. This increase in Fcp6/7 polypeptides is accompanied by an increase in the pool of xanthophyll cycle pigments, diadinoxanthin and diatoxanthin, and a concomitant decrease in fucoxanthin content. In addition, the de-epoxidation ratio, i.e., the amount of diatoxanthin in relation to the pool of xanthophyll cycle pigments, is increased by a factor of 2. With regard to fluorescence yield, HL FCPa was quenched in comparison to LL FCPa. This is in accordance with the larger amount of diatoxanthin that is bound, which is supposed to act as a quencher like zeaxanthin in higher plants. Thus, we conclude that the enhanced content of diatoxanthin in FCPa plays a protective role, which is paralleled by a weakened light harvesting function due to a smaller amount of fucoxanthin.

Diatoms (Bacillariophyceae) are one of the major players in the biochemical cycles of carbon, nitrogen, phosphorus, and silica with a strong impact on global climate not only in the ocean but also in the freshwater environment (for a recent review, see ref *1*). They are eukaryotic, unicellular organisms capable of oxygenic photosynthesis. Their chloroplast was acquired by a secondary endosymbiosis event, i.e., the engulfment of a eukaryotic, photosynthetic cell by a eukaryotic host. Thus, the chloroplast of recent diatoms is related to red algae chloroplasts (*2*−*4*), despite differences in the thylakoid arrangement and light harvesting.

Photosynthesis relies on the absorption of light energy, which is carried out by pigment−protein complexes. Like in higher plants, light-harvesting systems in diatoms are membrane intrinsic proteins. However, pigmentation differs considerably and thus the absorption capabilities, which have a major influence on the photosynthetic performance in different environments. Whereas higher-plant LHCs[1] bind chlorophyll (Chl) *b*, the antenna proteins of diatoms bind Chl *c*. Additionally, the major carotenoid in diatoms is fucoxanthin which has spectral characteristics resembling only those of peridinin (*5*). When fucoxanthin binds to the protein, an extreme bathochromic shift of absorbance occurs, enabling absorption between 460 and 570 nm, a range of wavelength not used by higher plants and green algae. Another, yet unexplained difference between the antenna proteins of diatoms, called fucoxanthin−chlorophyll proteins (FCP), and LHCs concerns the stoichiometry of the pigments. Whereas LHCs bind far more Chls than carotenoids (Car), in FCP the Chl:Car ratio is ∼1 (*6*−*8*). In addition, Chl *c* is present in FCPs in much smaller amounts than is Chl *b* in LHCs.

Early sequencing of cDNA clones of the major genes for these antennae in *Cyclotella cryptica* and *Phaeodactylum tricornutum* found some homology with LHCII of higher plants, placing these proteins in the same family (*9*, *10*). Three membrane-spanning helices were predicted from sequence analysis, whereby homology to LHC is mainly found in helices 1 and 3 (*11*). Generally, FCPs are smaller in size (18−22 kDa) than higher-plant LHCs, mainly due to shorter loops and termini. Three groups of FCP proteins can be distinguished on the basis of sequence analyses. One group represents the major FCP in diatoms and is most closely related to FCP proteins in other heterokont algae, e.g., brown alga (*12*−*14*). *fcp1*−*5* in *C. cryptica* and *fcpA-F* in *P. tricornutum* belong to these genes. The second group of *fcp* genes is closely related to the one intrinsic light-harvesting protein in red algae, *lhca*, and to the membrane intrinsic antenna in cryptophytes. A member of this group was first detected (*10*) in *C. cryptica* (*fcp4*) and later also found in the *Thalassiosira pseudonana* genome (*15*). The last major group is related to a protein found in the green

† This work was kindly supported by the DFG (SFB 472 and BU 812/2-2) and the EU (MRTN-CT-2003-505069, Intro2).

* To whom correspondence should be addressed. Phone: +49-69-798-24768. Fax: +49-69-798-24822. E-mail: C.Buechel@bio.uni-frankfurt.de.

[1] Abbreviations: Car, carotenoid; Chl, chlorophyll; FCP, fucoxanthin−chlorophyll protein complex; Fcp, fucoxanthin−chlorophyll protein subunit; HL, high light; LHC, light-harvesting complex; LL, low light.

10.1021/bi061249h CCC: $33.50
Published on Web 10/05/2006

alga *Chlamydomonas reinhardtii* but not in higher plants: a light inducible member of the LHC family, LI818. In *C. cryptica*, *fcp6*, *-7*, and *-12* belong to this group (*14*). In the *P. tricornutum* EST database, sequences representing this type of Fcp protein can also be found, and they are also present in the genome of *T. pseudonana* (*15*).

Despite this detailed knowledge of genes and their relation to other genes from the LHC family, biochemical information about FCPs is scarce. A major obstacle might be the instability against most detergents. In addition, different FCPs show a high degree of similarity with regard to size, sequence, and pigmentation. This makes the separation of the different proteins extremely difficult. Usually, one major FCP has been purified from diatoms (*6*, *16*) and the related brown algae (*17*−*19*) and was interpreted as an antenna for both photosystems. Recently, two distinguishable FCP complexes intact in excitation energy transfer were purified from *Cyclotella meneghiniana* (*20*). Interestingly, the oligomeric state differed. The 18 kDa proteins assembled into trimers, which contained small amounts of 19 kDa subunits as well. The other FCP complex consisted exclusively of 19 kDa subunits arranged in stable higher oligomers. However, considering the genes annotated so far, one might expect other FCPs, albeit in smaller amounts. Using antibodies directed against the minor LHC in green plants, some of the diatom Fcp proteins were tentatively assigned to minor antenna proteins (*21*), but this has still to be confirmed by more thorough biochemical studies.

No molecular structure for any of the FCP complexes is available yet. Even precise data about pigment:protein ratios are missing. Still, on the basis of fluorescence emission spectra, ultrafast kinetic data about excitation energy transfer, and sequence analyses of conserved pigment binding sites, a preliminary structural model was developed (*8*). This model depicts four Chl *a* molecules, one Chl *c* molecule, and four fucoxanthin molecules per Fcp monomer (*1*). Diadinoxanthin and diatoxanthin were found in only substoichiometric amounts in biochemical preparations of FCPs and thus omitted from the model.

Antenna proteins primarily serve the function of light harvesting; however, protection against a surplus of light is another role carried out by those proteins. FCPs additionally bind the xanthophyll cycle pigments diadinoxanthin and diatoxanthin. The de-epoxidation of diadinoxanthin to diatoxanthin was shown to correlate with the non-photochemical quenching (NPQ) of chlorophyll fluorescence in vivo like the conversion of violaxanthin to zeaxanthin in higher plants (*22*−*25*). Thus, diatoxanthin is supposed to be the carotenoid species responsible for NPQ in diatoms. The precise localization of the quenching species is still not known (*7*). The amount of these pigments in purified FCP complexes intact in excitation energy transfer varies in the different reports and is dependent on species and growth conditions, but it is always below $^1/_{10}$ of the Chl *a* content, i.e., most probably substoichiometric (*7*, *8*, *26*, *27*). Under some light regimes, even the xanthophyll cycle pigments usually found in higher plants, zeaxanthin and violaxanthin, are synthesized in *P. tricornutum* as well; however, nothing is known about their location (*28*, *29*).

Here we present a new method for purifying two different FCP complexes from the centric diatom *C. meneghiniana*. Using specific antibodies, the polypeptide composition was probed, and for the first time, subunits could be identified. In addition, differences in polypeptide composition of one of the FCPs in cells grown under high and low light intensities could be detected. These changes can be related to adjustments in pigment content, the de-epoxidation ratio of xanthophyll cycle pigments, and fluorescence quenching properties.

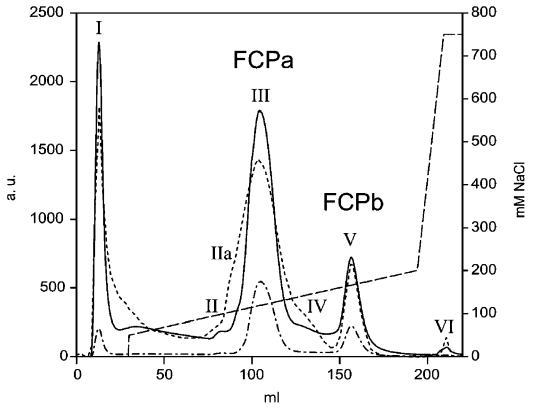

FIGURE 1: Elution profile of the ion exchange chromatography of thylakoid protein complexes prepared from LL and HL cells. For LL samples, the solid line shows the absorption at 437 nm whereas the dotted−dashed line represents the absorption at 530 nm. The dotted line depicts the elution profile of HL samples at 437 nm. The NaCl gradient used for elution is presented as well (dashed line). The peaks in the elution profiles were attributed to the following fractions (for an explanation, see the text): I, free pigment; II, IIa, IV, and VI, photosystems; III, FCPa; and V, FCPb.

MATERIALS AND METHODS

Growth Conditions. The diatom *C. meneghiniana* (Culture Collection Göttingen, strain 1020-1a) was grown for 2 weeks in batch cultures in ASP-2 medium (*30*) supplemented with 1 mM silica at 18 °C under a 16 h light−8 h dark cycle. Low-light (LL) cells were illuminated by 40 μmol of white light m^{-2} s^{-1}, whereas for high-light (HL) cultures, 145 μmol m^{-2} s^{-1} was used.

Preparation of FCP. Cells were harvested in the early light phase by centrifugation. Thylakoid membranes were isolated according to the method described in ref *20* except that they were finally resuspended in a buffer containing EDTA to further reduce chlorophyllase activity {buffer A [10 mM MES, 2 mM KCl, and 5 mM EDTA (pH 6.5)]}.

Thylakoids were then solubilized at 0.25 mg of Chl *a*/mL with 15 mM β-1,4-dodecyl maltoside [β-DDM, 31:1 (w/w) β-DDM:Chl *a* ratio] for 20 min on ice. Separation of solubilized proteins was carried out on an ion exchange column [DEAE Toyopearls 650S (Tosoh)] in 25 mM Tris, 2 mM KCl, and 0.03% DDM at pH 7.4 (buffer B). The column was attached to a FPLC system (ÄKTA Purifier P-900, Amersham Biosciences) with three-wavelength detection. The sample was loaded on top of the column, and fractions were eluted using a gradient from 0 to 750 mM NaCl in buffer B at 2 mL/min (for details, see Figure 1). Fractions were pooled and concentrated using Amicon filtration devices with a cutoff of 30 kDa and characterized spectroscopically and biochemically.

Spectral Characterization. Determination of the amount of chlorophyll was carried out according to the method of ref *31*. For absorption spectra, a Jasco spectrophotometer (V-550) was used with a 1 nm bandpass and an optical path

length of 1 cm at a Chl *a* concentration of 5 μg/mL. Fluorescence spectra were measured at room temperature in a Jasco FP-6500 fluorometer with an optical path length of 5 mm for emission. Correction for the excitation side was carried out using a rhodamine B spectrum as a reference, and the photomultiplier was corrected using a calibrated lamp spectrum. Excitation spectra at an emission wavelength of 675 nm were recorded at a Chl *a* concentration of 1 μg/mL and excitation and emission bandwidths of 3 and 10 nm, respectively. When testing for Chl *c* to Chl *a* excitation transfer, we recorded fluorescence emission spectra using an excitation wavelength of 465 nm, an excitation bandwidth of 10 nm, and an emission bandwidth of 3 nm. The Chl concentration was adjusted to 1 μg/mL. In the case of probing for the relative fluorescence yield, an excitation wavelength of 437 nm was used and emission and excitation bandwidths were set to 3 nm. To make the samples virtually free of reabsorption, the Chl *a* concentration was lowered to 0.3 μg/mL. When different FCPs were compared, the emission spectra were corrected to account for their different absorbance.

Characterization of the Oligomeric State, Polypeptide Composition, and Western Blots. To analyze the oligomeric state of the FCPs, analytical gel filtration was carried out using a Superose6 column (Pharmacia) attached to a SMART system (Pharmacia). Run conditions were identical to the ones given in ref *20*.

To confirm the purity of the preparations and for Western blotting, Tris/Tricine gels, described in ref *32*, consisting of a 4% (w/v) stacking gel together with a 12% (w/v) separating gel were used. Samples were delipidated by suspending them in a 10-fold volume of acetone and incubating them at −22 °C for 20 min, followed by a short centrifugation. Gels were stained with Sigma Brilliant Blue G or silver. For Western blotting, proteins separated by gel electrophoresis were electrotransferred to PVDF membranes and decorated with antibodies against all Fcp proteins (α-ccFcp) and against specific Fcp polypeptides (α-Fcp2 and α-Fcp6). Detection was achieved using the ECL method (Amersham). Stripping of the primary and secondary antibody to probe the same blot with different antibodies was carried out as recommended in the ECL leaflet. All antibodies are directed against polypeptides of the related species *C. cryptica* and were kind gifts from E. Rhiel (University of Oldenburg, Oldenburg, Germany).

Pigment Analysis. Pigment stoichiometries of isolated FCPs were determined by analytical HPLC (Merck Elite LaChrom, L-2130/L-2450), after precipitation of the proteins and extraction of the pigments in 90% methanol (final concentration). Pigments were separated and quantified using an RP18 column and a photodiode array detector as described in ref *8*.

RESULTS

Purification of FCP Complexes. To improve the quantity and quality of the isolated FCP complexes, a new purification procedure was developed using ion exchange chromatography instead of sucrose density centrifugation. Figure 1 shows the elution profile of solubilized thylakoid proteins from a LL culture of *C. meneghiniana*. The first peak eluting at 0 mM NaCl (fraction I) could be attributed to free pigments,

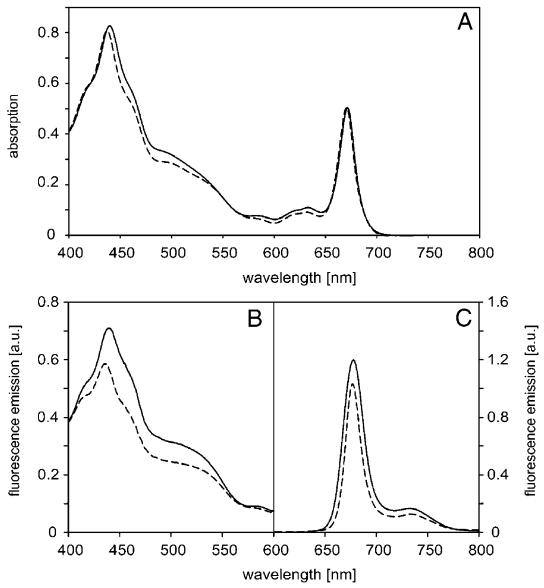

FIGURE 2: Spectral analyses of FCPa (—) and FCPb (- - -) prepared from LL cells. Panel A shows the absorption spectra at a Chl concentration of 5 μg/mL, panel B the fluorescence excitation spectra recorded at 675 nm, and panel C the fluorescence emission spectra upon excitation at 465 nm. The fluorescence spectra were measured at a Chl concentration of 1 μg/mL each.

exhibiting comparable spectra and polypeptide pattern as described for the free pigment fractions from sucrose density centrifugation (*20*). Some fractions, i.e., fractions II, IIa, IV, and VI, were characterized by a smaller A_{530}/A_{437} ratio of 0.14 compared to that of fractions III and V (0.30), indicative of a smaller amount of fucoxanthin in relation to Chl *a*. The decrease in the amount of carotenoid could indeed be confirmed by absorption spectroscopy (data not shown), where these fractions were also characterized by a Q_y band of Chl *a* above 673 nm. Thus, these fractions most probably represent photosystems and were not considered further. Here we focus on the two major fractions, fraction III and fraction V, which were named FCPa and FCPb, respectively. Whereas in the case of FCPb almost all peak fractions could be harvested, for FCPa only the middle fractions were collected to prevent contamination by photosystems.

Figure 2A demonstrates that both fractions exhibited the typical absorption spectra of FCP. The Q_y band of Chl *a* in these complexes absorbs at 671.5 nm in the case of FCPa, whereas FCPb shows a slightly shorter Q_y absorption at 670.5 nm. In both cases, the Q_y band of Chl *c* is only visible as a small peak at 636 nm, whereas the Soret band is clearly distinguishable in the form of a shoulder at 465 nm. As described earlier, there is a strong absorption between 475 and 565 nm due to the carotenoids bound to the FCPs, mainly fucoxanthin (*20*). Both FCPs were intact with regard to excitation energy transfer to Chl *a* as proven by their fluorescence excitation and emission spectra (Figure 2B). The fluorescence excitation spectra show excellent energy transfer when fucoxanthin (from 475 to 565 nm) or Chl *c* (around 465 nm) was excited. Further proof of energetic coupling of Chl *c* comes from the emission spectra, where only Chl *a* emission is visible, although Chl *c* was preferentially excited. Both fluorescence excitation and fluorescence emission spectra exhibited a weaker maximal signal in the case of FCPb. Since the spectra were recorded at the same Chl *a* concentration but not calibrated for their differences in

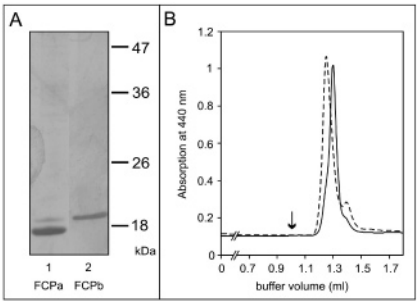

FIGURE 3: Biochemical characterization of FCPa and FCPb prepared from LL cells. Panel A shows results of silver-stained SDS−PAGE, whereby FCPa was loaded in lane 1 and FCPb in lane 2. In panel B, gel filtration runs of FCPa (—) and FCPb (- - -) are depicted. The arrow shows the void volume of the column.

pigment composition, this is partly due to differences in absorbance around 465 nm (Figure 2A). The remaining difference has to be due to a smaller fluorescence yield of FCPb compared to that of FCPa (see below).

Figure 3A shows denaturing SDS−PAGE of the two FCP fractions, which were of high purity as only bands which can be attributed to Fcp polypeptides are visible. FCPa (Figure 3A, lane 1) contained two polypeptides of around 18 and 19 kDa, and the 18 kDa proteins were more abundant. In *C. cryptica* (*10*, *12*), the predicted molecular mass of Fcp1−3 is 18.4 kDa and that of Fcp4 is supposed to be 18.1 kDa. Fcp5, Fcp6, and Fcp7 polypeptides are larger with predicted masses of 19.8 (Fcp5) and 19.2 kDa (Fcp6 and -7). FCPb contained exclusively the 19 kDa polypeptides, i.e., the *fcp5−7* gene products. The polypeptide composition of FCPa and FCPb and their spectra resembled those of the trimeric and oligomeric FCPs prepared by the method described in ref *20* using the combination of sucrose gradients followed by gel filtration. Thus, the samples were also checked for their oligomeric state by gel filtration (Figure 3B), especially since here high-salt conditions were applied in contrast to the low salt concentrations on sucrose gradients. For both complexes, two peaks were obtained, whereby the fraction eluting at 1.4 mL was known to represent monomers (*20*). The elution times of the main peaks on the gel filtration system, 1.25 mL in the case of FCPb and 1.3 mL in the case of FCPa, were identical to those reported in ref *20*, indeed confirming that FCPa is trimeric while FCPb is the fraction of the higher oligomeric state. In addition, this shows again that the oligomeric state of the FCPs did not depend on the salt conditions, as already shown in ref *20*.

Comparison of HL and LL Cultures. mRNA levels of Fcp polypeptides are known to depend on the light intensity during growth (*33*). However, nothing is known about the steady-state protein levels, the pigmentation, or the polypeptide composition of the different FCP complexes in HL or LL. Thus, we compared different *C. meneghiniana* cultures with respect to these parameters and with regard to their spectral properties.

Figure 4 shows the overall polypeptide composition of thylakoids in HL and LL cultures. It has long been known that the ratio of photosystem to antenna proteins changes during these adaptation processes (*34*, *35*). For the gels, an equal amount of chlorophyll was loaded, and thus, it is easily seen that in HL cultures the amount of other polypeptides relative to FCPs is increased in comparison to that in LL

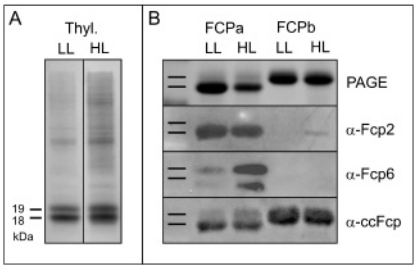

FIGURE 4: Characterization of HL and LL FCPs by SDS−PAGE and Western blotting. Black lines mark the bands with molecular masses of 18 and 19 kDa. Panel A compares HL and LL thylakoids on a Coomassie-stained SDS−PAGE gel. In panel B, FCPa and FCPb from HL and LL cells are compared using SDS−PAGE and immunolabeling with three different antibodies. Antibodies were made against proteins from *C. cryptica*. α-ccFcp detects all Fcp polypeptides, whereas α-Fcp2 and α-Fcp6 are specific for the Fcp2 and Fcp6 polypeptides, respectively.

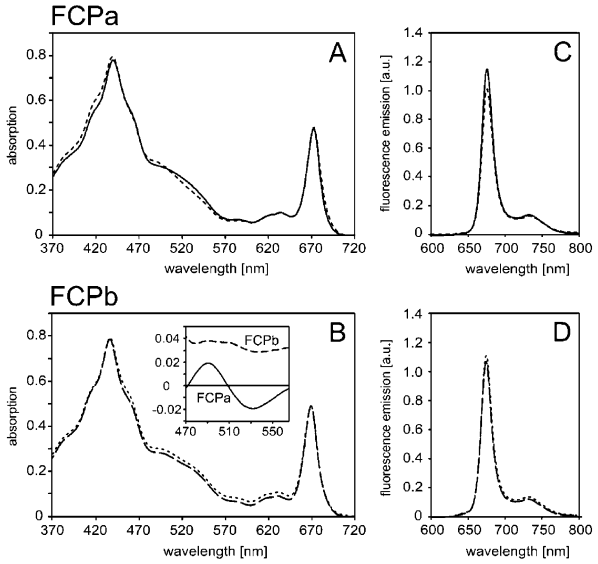

FIGURE 5: Comparison of the spectral properties of FCPs prepared from HL and LL cells. In panels A and B, the absorption spectra of FCPa [LL (—) and HL (- - -)] and FCPb [LL (- - -) and HL (···)] are given. The inset of panel B shows an enlargement of the difference absorption spectrum of HL minus LL FCPa (—) and FCPb (- - -) in the range of 470−570 nm. Panels C and D demonstrate the fluorescence emission spectra corrected for absorbance upon excitation at 437 nm of FCPa (C) and FCPb (D) accordingly. Absorption spectra were recorded at a concentration of 5 μg/mL Chl *a* and fluorescence spectra at 0.3 μg/mL Chl *a*.

cells. In addition, under HL, there is a slight shift in the ratio of 18−19 kDa polypeptides compared to that under LL, where the 18 kDa polypeptides are more clearly dominant.

Characterization of HL and LL FCPs and Identification of Subunits. Using these thylakoids, the different FCPs were purified as described above. The elution profiles were similar, except that the amount of FCPs compared to the other bands was slightly decreased in HL (Figure 1) as expected from the SDS−PAGE of thylakoids. Panels A and B of Figure 5 show the absorption spectra of the antenna complexes. In the case of FCPb (Figure 5B), a slight increase between 455 and 640 nm occurred without a change in spectral shape. In contrast, the shape of the spectrum between 470 and 565 nm of FCPa (Figure 5A) changed under HL conditions. This is further demonstrated by the difference spectra shown as an inset: the differences for FCPa are mainly due to an

increase in absorption around 490 nm and a concomitant decrease around 530 nm in HL FCPa compared to LL. The fluorescence excitation spectra have similar features for both FCPs under all conditions, but the signal height was different when using the same chlorophyll concentration (data not shown). This difference becomes obvious in the fluorescence emission spectra shown in panels C and D of Figure 5. While almost no change can be seen for FCPb, the relative fluorescence yield for FCPa under HL conditions is reduced by ~10% compared to that in LL. As stated above, in LL cells the fluorescence yield of FCPa was higher than the yield of FCPb. This difference is abolished in HL cells due to the decreased fluorescence yield of HL FCPa.

To examine the polypeptide composition, SDS−PAGE and Western blots using antibodies for specific Fcp polypeptides were carried out. Figure 4B compares the different FCPs from HL and LL cultures. It must be emphasized that identical amounts of complexes based on Chl content were loaded in each case. Thus, only relative changes in polypeptide composition inside the FCP complexes can be detected, but not the absolute expression of Fcps under the different light regimes. FCPa is composed of 18 and 19 kDa polypeptides as mentioned above. Under HL conditions, the amount of 19 kDa compared to the amount of 18 kDa subunits is increased, whereas no change in the composition of FCPb is visible from Coomassie-stained gels.

To gain more detailed information about the subunit composition, the same Western blot of HL and LL FCPs was probed with antibodies specific against different Fcp proteins of the related species *C. cryptica* (Figure 4B). As a control, α-ccFcp was used, an antibody reacting indeed with all polypeptides of the different FCP complexes. *fcp2* encodes a polypeptide predicted to have a molecular mass of 18.4 kDa, as is the case for the very similar gene products of *fcp1* and *fcp3* [98−99% similar on the protein level (*10*)]. As expected, the 18 kDa subunits of FCPa reacted with an antibody specific for Fcp2, α-Fcp2. *fcp6* and *fcp7* are predicted to encode proteins of 19.2 kDa, which are 98% homologous (*10*). Thus, the α-Fcp6 antibody is most likely specific for Fcp6 as well as Fcp7. It reacted with two bands in the case of FCPa. However, one labeling was found at around 16 kDa, which most probably resembles a breakdown product as no genes which encode Fcp proteins of this size are known. The antibody further recognized the 19 kDa band of FCPa, and the reaction was much stronger when HL FCPa was probed. Thus, the 19 kDa polypeptide in FCPa that becomes more abundant under HL conditions can be identified as Fcp6/7. In contrast, no reaction could be found with the 19 kDa polypeptides of FCPb, in neither HL nor LL.

To further characterize the different complexes, HPLC analysis was carried out. Figure 6 gives the molar stoichiometries per Chl *a* and the de-epoxidation ratio, i.e., the amount of the de-epoxidised xanthophyll diatoxanthin in relation to the total pool of diadinoxanthin and diatoxanthin. No traces of β-carotene could be found in any of the fractions, further demonstrating that no contamination by photosystem complexes was present. When FCPa is compared with FCPb, FCPa is enriched in fucoxanthin compared to FCPb under both light conditions. The relative amounts of Chl *c* differ only slightly; a decrease can be observed when FCPa is compared to FCPb in LL. In contrast to the pigments having light harvesting function, the differences for the

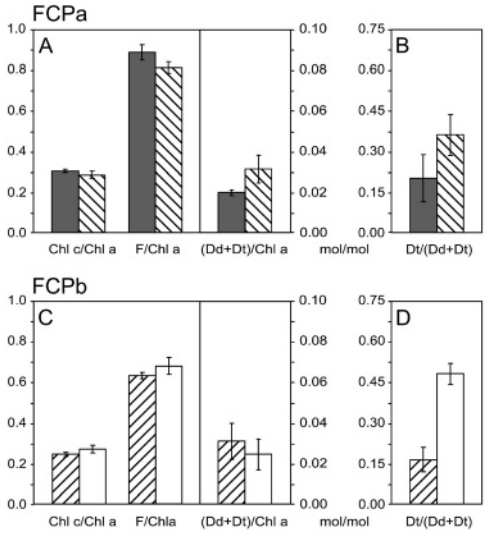

FIGURE 6: Comparison of the pigment stoichiometries in FCPs isolated from HL and LL cultures. Values for pigments are given in moles per mole, as means ± standard deviation of three independent FCP preparations and three measurements each. F is fucoxanthin, Dd diadinoxanthin, and Dt diatoxanthin. In panels A and B, the values for FCPa from LL (black bars) and HL (hatched bars) cultures are shown. Panels C and D show values for FCPb prepared from LL (hatched bars) and HL (white bars) cells. In both cases, the ratios of pigment to Chl *a* (A and C) and the de-epoxidation ratios (B and D) are compared.

protective xanthophylls are not uniform. In LL cultures, diadinoxanthin with diatoxanthin is more frequently bound to FCPb than to FCPa, whereas under HL conditions, the xanthophyll cycle pigment content is increased in FCPa.

When the same complexes are compared under HL and LL conditions, the most prominent change occurs in the de-epoxidation ratio, which is increased in HL by a factor of more than 2 in both FCPa and FCPb. In HL FCPa, fucoxanthin and Chl *c* are present in smaller amounts than they are in LL FCPa, whereas the pool of xanthophyll cycle pigments grows. The reverse is true for FCPb, but to a smaller extent concerning the changes in the diadinoxanthin and diatoxanthin pool. Thus, the changes become only visible in the absorption difference spectra of FCPa via an increase in absorption at 490 nm and a concomitant decrease at 530 nm in HL FCPa (inset of Figure 5B).

In summary, the changes in the pool of xanthophyll cycle pigments are accompanied by an opposite change in the fucoxanthin and Chl *c*:Chl *a* ratio. The most prominent changes are the increase in the de-epoxidation ratio of the xanthophyll cycle pigments under HL conditions and the increase in their pool size in HL FCPa. As diatoxanthin is thought to promote chlorophyll fluorescence quenching, this difference might explain the changes seen in fluorescence yield.

DISCUSSION

Purification of FCPa and FCPb. The aim of this study was to identify some of the subunits which constitute the different FCP complexes found in the diatom *C. meneghiniana*. To this end, a new purification protocol was used which had the advantage of allowing for the purification of both FCPa and FCPb complexes in one step from solubilized thylakoids by ion exchange chromatography. The intactness

of the complexes as proven by their fluorescence spectra and the purity as shown by SDS−PAGE were identical to those of the complexes described before (*20*), yet the yield per preparation was significantly improved. In addition, the complexes were of the same oligomeric state, i.e., similar to those purified using sucrose density centrifugation followed by gel filtration. As the lateral distribution of the different FCP complexes in the thylakoid membrane of diatoms is still not known and to avoid confusion with antenna proteins specific for photosystem I or II, we here name the trimeric complex FCPa and the oligomeric complex FCPb.

Polypeptide Composition of FCPa and FCPb. We checked FCPa as well as FCPb from HL and LL cultures for the appearance of some of the subunits. Using specific antibodies, we could show that Fcp1−3 and Fcp6/7 are inserted into FCPa complexes and that the relative amount of the latter is higher in cells grown under high-light conditions. The increase in the amount of *fcp6/7* gene products at the expense of *fcp1−3* under HL conditions confirms what was seen for the mRNA levels (*33*). However, it must be noted that even under HL FCPa is a mixed complex with regard to polypeptide composition since Fcp6/7 is apparent only in substoichiometric amounts in the trimeric complex. This resembles the situation from higher-plant LHCII, where Lhc3 can be found in only some of the trimers (*36*). In the green alga *Ch. reinhardtii*, LI818 was described as a protein not very much related to LHC proteins, which appears in the early phase of greening and might protect photosystems, especially photosystem II cores (*37*). The diurnal regulation in the diatom *C. cryptica*, where the related Fcp genes, *fcp6/7*, are transcribed early in the morning, argues for a similar role for the Fcp6/7 polypeptides (*38*). However, here we could show that Fcp6/7 is a constituent of the trimeric FCPa complexes and thus more closely associated with the antenna system than with the reaction centers.

Against expectations, no fcp6/7 could be found in FCPb, there being only 19 kDa subunits in these complexes. From *C. cryptica*, several genes encoding proteins of >18 kDa are known besides *fcp6/7* (*10*, *12*): *fcp5* encoding a polypeptide of 19.8 kDa and *fcp12* encoding a protein of 21.7 kDa. The estimation of molecular masses from SDS−PAGE yields only apparent masses, which can differ significantly from the actual mass especially in the case of membrane proteins. In addition, predicted molecular masses have to be treated with caution as little is known about how the transit peptide of the nuclear-encoded Fcps is cleaved. Thus, we cannot rule out the possibility that *fcp12* is encoding polypeptides which appear to have masses of 19 kDa. However, since in our gel system the apparent masses of Fcp2 as well as Fcp6 are almost identical to the theoretical values, we consider this highly unlikely. In addition, more *fcp12* transcripts are found under HL conditions than under LL conditions (*33*), whereas FCPb appeared to be reduced under stronger illumination. The lack of a 21.7 kDa polypeptide might be due to low expression levels under our culture conditions or the fact that a similar polypeptide is missing in *C. meneghiniana*, as we were never able to detect a band of that size, despite using polyacrylamide gels with high resolution in this range. We thus conclude that the oligomeric FCPb consists of Fcp5 and that FCPa is composed of at least Fcp1−3 and Fcp6/7, the latter polypeptides being upregulated under HL conditions.

Pigmentation of the FCP Complexes. The increase in the amount of Fcp6/7 of FCPa under HL was accompanied by an enhanced pool size of diadinoxanthin and diatoxanthin and an increase in the de-epoxidation ratio. Thus, we hypothesize that Fcp6/7 is responsible for binding the additional diadinoxanthin and diatoxanthin molecules. As already pointed out, Fcp6/7 is only present in some of the FCPa trimers. The same holds for the xanthophyll cycle pigments.

However, in FCP fractions prepared using sucrose density centrifugation (*8*), the size of the pool of xanthophyll cycle pigments was significantly larger, albeit still substoichiometric. The pool of light-harvesting pigments exhibited only very minor differences. From the pronounced variations concerning diadinoxanthin, we have to conclude that using ion exchange chromatography the xanthophyll cycle pigments are more easily lost than by using sucrose gradients, but complexes are purer with regard to polypeptides and contain less lipids (data not shown). Thus, pigments might have been lost with the lipids or with contaminating polypeptides. More probably, the reduction is due to a more peripheral binding site for diadinoxanthin and diatoxanthin compared to fucoxanthin, Chl *c*, and Chl *a*. The complexes purified here thus retain only a very tightly bound fraction of the diadinoxanthin and diatoxanthin pool, and thus, our conclusions concern only this part of the pool. Further studies will have to clarify whether the additional diadinoxanthin and diatoxanthin is more closely associated with either FCPa or FCPb in vivo.

In *P. tricornutum*, two antenna protein fractions, F and D, could be separated using sucrose density centrifugation, which differed in the amount of diadinoxanthin, and similar fractions were obtained by gel filtration (*27*). Whereas the F fraction contained almost exclusively polypeptides in the range of Fcps, the D fraction and comparable fractions were composed of several subunits, some of them of a molecular mass like Fcps. F exhibited excitation energy transfer from the bound carotenoids to Chl *a*, but this was not the case for fraction D. The level of accumulation of diadinoxanthin in fraction D was much greater than the increase we see here for FCPa under HL conditions. However, it must be stressed that FCPa is an antenna complex functional in light harvesting and consisting of only Fcp subunits, while fraction D is not. Although the analogue to fcp6/7 in *P. tricornutum* might be one of the polypeptides seen in fraction D, bands with lower molecular masses seem to be the more likely candidates in *P. tricornutum* (*27*).

Protection by the Xanthophyll Pigment Diatoxanthin. The establishment of NPQ, i.e., the dissipation of excess energy as heat instead of fluorescence, is accompanied by a de-epoxidation mainly of diadinoxanthin to diatoxanthin in diatoms (*7*, *23*, *28*). Thus, diatoxanthin is involved in the decrease in the chlorophyll fluorescence yield and might quench the fluorescence in isolated complexes as well. Indeed, the higher de-epoxidation ratio and the enhanced pool size in HL FCPa in comparison to those of LL FCPa resulted in a lower yield, in contrast to that of FCPb. Thus, very likely, the increased amount of diatoxanthin in the HL FCPa is responsible for the quenching seen here. However, as we are examining purified protein complexes, this diatoxanthin has to belong to a pool which is only slowly converting in

vivo. The time courses shown for de-epoxidation (*23*, *26*, *39*), and epoxidation (*28*), which is more relevant, depict times for conversion much faster than the times needed for harvesting and breaking of the cells. Whether the variable pool which is converted rapidly is also bound to FCP complexes or belongs to other proteins has still to be clarified. However, even the tightly bound pigments are converted under HL conditions.

Interestingly, in FCPb, the higher de-epoxidation ratio was not accompanied by a higher fluorescence quenching. Thus, it seems that the sole content of diatoxanthin is not sufficient to decrease the fluorescence yield of a FCP complex. In FCPb, the binding site for diatoxanthin might have become changed during preparation of the complexes or the diatoxanthin bound to FCPb is only quenching when FCPb is in its natural environment, e.g., quenching chlorophylls not bound to FCPb but to neighboring complexes. This will have to be clarified by further experimental work.

The increase in the rate of heat dissipation might help to protect the cells against a surplus of energy, but it is not only the increase in diatoxanthin which helps. As described in refs *28* and *29*, an increase in the level of diatoxanthin is accompanied by a decrease in fucoxanthin content in whole cells. From these data, the authors conclude that fucoxanthin as well as diatoxanthin can be derived from diadinoxanthin. In addition, the (de-)epoxidases fine-tune the diadinoxanthin:diatoxanthin ratio. Thus, accumulation of xanthophyll cycle pigments, especially diatoxanthin, disfavors the amount of fucoxanthin. This increase in diatoxanthin content at the expense of fucoxanthin or vice versa is also true for purified complexes, FCPa as well as FCPb. Increasing diatoxanthin content while reducing fucoxanthin content has an advantage in that not only can protection by diatoxanthin occur, but the light harvesting function is also suppressed, making the mechanism more effective in shielding the photosynthetic apparatus, especially the photosystems, from too much light energy.

In summary, we could show that FCPb is most probably built from Fcp5 subunits, whereas FCPa contains Fcp1−3 and variable amounts of Fcp6/7, the latter being increased under HL conditions. The larger amount of Fcp6/7 is accompanied by a larger diadinoxanthin and diatoxanthin pool in FCPa, which is much more de-epoxidized under HL conditions. The larger amount of diatoxanthin present most probably accounts for the higher fluorescence quenching at room temperature found in FCPa with large amounts of Fcp6/7 polypeptides. This part of the pool of xanthophyll cycle pigments is strongly bound to the FCPa and de-epoxidized, probably helping the HL cells to cope with the high fluxes of illumination.

ACKNOWLEDGMENT

We gratefully acknowledge the kind gift of antibodies by E. Rhiel. K. Pieper is thanked for her perpetual technical assistance.

REFERENCES

1. Wilhelm, C., Büchel, C., Fisahn, J., Goss, R., Jakob, T., LaRoche, J., Lavaud, J., Lohr, M., Riebesell, U., Stehfest, K., Valentin, K., and Kroth, P. G. (2006) The Regulation of Carbon and Nutrient Assimilation in Diatoms is significantly different from green algae, a putative consequence of secondary endosymbiosis, *Protist 157*, 91−124.
2. Bhattacharya, D., and Medlin, L. (1995) The phylogeny of plastids: A review based on comparisons of small-subunit ribosomal RNA coding regions, *J. Phycol. 31*, 489−498.
3. Yoon, H., Hackett, J. D., Pinto, G., and Bhattacharya, D. (2002) The single, ancient origin of chromist plastids, *Proc. Natl. Acad. Sci. U.S.A. 99*, 15507−15512.
4. Wolfe, G. R., Cunningham, F. X., Durnford, D., Green, B. R., and Gantt, E. (1994) Evidence for a common origin of chloroplasts with light-harvesting complexes of different pigmentation, *Nature 367*, 566−568.
5. Mimuro, M., and Akimoto, S. (2003) Carotenoids of light harvesting systems: Energy transfer processes from fucoxanthin and peridinin to chlorophyll, in *Photosynthesis in Algae* (Larkum, A. W., Douglas, S. E., and Raven, A., Eds.) pp 335−349, Kluwer Academic Publisher, Dordrecht, The Netherlands.
6. Berkaloff, C., Caron, L., and Rousseau, B. (1990) Subunit organization of PS I particles from brown algae and diatoms: Polypeptide and pigment analysis, *Photosynth. Res. 23*, 181−193.
7. Lavaud, J., Rousseau, B., and Etienne, A. L. (2003) Enrichment of the light-harvesting complex in diadinoxanthin and implications for the nonphotochemical fluorescence quenching in diatoms, *Biochemistry 42*, 5802−5808.
8. Papagiannakis, E., van Stokkum, I., Fey, H., Büchel, C., and van Grondelle, R. (2005) Spectroscopic characterisation of the excitation energy transfer in the fucoxanthin-chlorophyll protein of diatoms, *Photosynth. Res. 86*, 241−250.
9. Bhaya, D., and Grossman, A. R. (1993) Characterization of gene clusters encoding the fucoxanthin chlorophyll proteins of the diatom *Phaeodactylum tricornutum*, *Nucleic Acids Res. 21*, 4458−4446.
10. Eppard, M., and Rhiel, E. (1998) The genes encoding light-harvesting subunits of *Cyclotella cryptica* (Bacillariophyceae) constitute a complex and heterogeneous family, *Mol. Gen. Genet. 260*, 335−345.
11. Green, B., and Pichersky, E. (1994) Hypothesis for the evolution of three-helix Chl a/b and Chl a/c light-harvesting antenna proteins from two-helix and four-helix ancestors, *Photosynth. Res. 39*, 149−162.
12. Eppard, M., Krumbein, W. E., von Haesler, A., and Rhiel, E. (2000) Characterization of fcp4 and fcp12, two additional genes encoding light harvesting proteins of *Cylotella cryptica* (Bacillariophyceae) and phylogenetic analysis of this complex gene family, *Plant Biol. 2*, 283−289.
13. Durnford, D. G., Aebersold, R., and Green, B. R. (1996) The fucoxanthin-chlorophyll proteins from a chromophyte alga are part of a large multigene family: Structural and evolutionary relationships to other light-harvesting antennae, *Mol. Gen. Genet. 253*, 377−386.
14. Green, B. R. (2003) The evolution of light-harvesting antennas, in *Light-Harvesting Antennas in Photosynthesis* (Green, B. R., and Parson, W. W., Eds.) pp 129−168, Kluwer Academic Publishers, Dordrecht, The Netherlands.
15. Armbrust, E. V., Berges, J. A., Bowler, C., Green, B. R., Martinez, D., Putnam, N. H., Zhou, S., Allen, A. E., Apt, K. E., Bechner, M., Brzezinski, M. A., Chaal, B. K., Chiovitti, A., Davis, A. K., Demarest, M. S., Detter, J. C., Glavina, T., Goodstein, D., Hadi, M. Z., Hellsten, U., Hildebrand, M., Jenkins, B. D., Jurka, J., Kapitonov, V. V., Kröger, N., Lau, W. W. Y., Lane, T. W., Larimer, F. W., Lippmeier, J. C., Lucas, S., Medina, M., Montsant, A., Obornik, M., Schnitzler Parker, M., Palenik, B., Pazour, G. J., Richardso, P. M., Rynearson, T. A., Saito, M. A., Schwartz, D. C., Thamatrakoln, K., Valentin, K., Vardi, A., Wilkerson, F. P., and Rokhsar, D. S. (2004) The genome of the diatom *Thalassiosira pseudonana*: Ecology, evolution, and metabolism, *Science 306*, 79−86.
16. Owens, T. G., and Wold, E. R. (1986) Light-harvesting function in the diatom *Phaeodactylum tricornutum*. I. Isolation and characterization of pigment-protein complexes, *Plant Physiol. 80*, 732−738.
17. Katoh, H., Mimuro, M., and Takaichi, S. (1989) Light-harvesting particles isolated from a brown alga, *Dictyota dichotoma*. A supramolecular assembly of fucoxanthin-chlorophyll-protein complexes, *Biochim. Biophys. Acta 976*, 233−240.
18. Passaquet, C., Thomas, J. C., Caron, L., Hauswirth, N., Puel, F., and Berkaloff, C. (1991) Light-harvesting complexes in brown algae. Biochemical characterization and immunological relationships, *FEBS Lett. 280*, 21−26.
19. De Martino, A., Douady, D., Rousseau, B., Duval, J. C., and Caron, L. (1997) Characterization of two light-harvesting subunits isolated

from the brown alga *Pelvetia canaliculata*: Heterogeneity of xanthophyll distribution, *Photochem. Photobiol. 66*, 190−197.
20. Büchel, C. (2003) Fucoxanthin-Chlorophyll Proteins in Diatoms: The 18 kDa and 19 kDa subunits assemble into different oligomeric states, *Biochemistry 42*, 13027−13034.
21. Rhiel, E., Marquardt, J., Eppard, M., Mörschel, E., and Krumbein, W. E. (1997) The light-harvesting system of the diatom *Cyclotella cryptica*. Isolation and characterisation of the main light harvesting complex and evidence for the existence of minor pigment proteins, *Bot. Acta 110*, 109−117.
22. Ting, C. S., and Owens, T. G. (1993) Photochemical and nonphotochemical fluorescence quenching processes in the diatom *Phaeodactylum tricornutum*, *Plant Physiol. 101*, 1323−1330.
23. Lavaud, J., Rousseau, B., van Gorkum, H. J., and Etienne, A. L. (2002) Influence of the diadinoxanthin pool size on photoprotection in the marine planctonic diatom *Phaeodactylum tricornutum*, *Plant Physiol. 129*, 1398−1406.
24. Arsalane, W., Rousseau, B., and Duval, J. C. (1994) Influence of the pool size of the xanthophyll cycle on the effects of light stress in a diatom: Competition between photoprotection and photoinhibition, *Photochem. Photobiol. 60*, 237−243.
25. Demmig-Adams, B., and Adams, W. W. (1996) The role of xanthophyll cycle carotenoids in the protection of photosynthesis, *Trends Plant Sci. 1*, 21−26.
26. Lavaud, J., Rousseau, B., and Etienne, A.-L. (2004) General features of photoprotection by energy dissipation in planktonic diatoms (Bacillariophyceae), *J. Phycol. 40*, 130−137.
27. Guglielmi, G., Lavaud, J., Rousseau, B., Etienne, A.-L., Houmard, J., and Ruban, A. V. (2005) The light-harvesting antenna of the diatom *Phaeodactylum tricornutum*. Evidence for a diadinoxanthin-binding subcomplex, *FEBS Lett. 272*, 4339−4348.
28. Lohr, M., and Wilhelm, C. (1999) Algae displaying the diadinoxanthin cycle also possess the violaxanthin cycle, *Proc. Natl. Acad. Sci. U.S.A. 96*, 8784−8789.
29. Lohr, M., and Wilhelm, C. (2001) Xanthophyll synthesis in diatoms: Quantification of putative intermediates and comparison of pigment conversion kinetics with rate constants derived from a model, *Planta 212*, 382−391.
30. Provasoli, L., Mc.Laughlin, J. J. A., and Droop, M. R. (1957) The development of artificial media for marine algae, *Arch. Mikrobiol. 25*, 392−428.
31. Jeffrey, S. W., and Humphrey, G. F. (1975) New spectrometric equations for determing chlorophyll a, b, c_1 and c_2 in higher plants, algae and natural phytoplankton, *Biochem. Physiol. Pflanz. 167*, 191−194.
32. Schägger, H., and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate−polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal. Biochem. 166*, 368−379.
33. Oeltjen, A., Krumbein, W. E., and Rhiel, E. (2002) Investigations on transcript sizes, steady state mRNA concentrations and diurnal expression of genes encoding fucoxanthin chlorophyll a/c light harvesting polypeptides in the centric diatom *Cyclotella cryptica*, *Plant Biol. 4*, 250−257.
34. Falkowski, P. G. (1980) Shade adaptation in marine phytoplankton, in *Primary productivity in the sea* (Falkowski, P. G., Ed.) pp 99−189, Plenum Press, New York.
35. Janssen, N., Bathke, L., Marquardt, J., Krumbein, W. E., and Rhiel, E. (2001) Changes in the photosynthetic apparatus of diatoms in response to low and high light intensities, *Int. Microbiol. 4*, 27−33.
36. Standfuss, J., and Kühlbrandt, W. (2004) The three isoforms of the light-harvesting complex II, *J. Biol. Chem. 279*, 36884−36891.
37. Richard, C., Ouellet, H., and Guertin, M. (2000) Characterization of the L1818 polypeptide from the green unicellular alga *Chlamydomonas reinhardtii*, *Plant Mol. Biol. 42*, 303−316.
38. Oeltjen, A., Marquardt, J., and Rhiel, E. (2004) Differential circadian expression of genes *fcp2* and *fcp6* in *Cyclotella cryptica*, *Int. Microbiol. 7*, 127−131.
39. Jakob, T., Goss, R., and Wilhelm, C. (1999) Activation of diadinxanthin de-epoxidase due to a chlororespiratory proton gradient in the dark in the diatom *Phaeodactylum tricornutum*, *Plant Biol. 1*, 76−82.

BI061249H