Evidence for the Existence of One Antenna-Associated, Lipid-Dissolved and Two Protein-Bound Pools of Diadinoxanthin Cycle Pigments in Diatoms[C][W]

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We studied the localization of diadinoxanthin cycle pigments in the diatoms *Cyclotella meneghiniana* and *Phaeodactylum tricornutum*. Isolation of pigment protein complexes revealed that the majority of high-light-synthesized diadinoxanthin and diatoxanthin is associated with the fucoxanthin chlorophyll protein (FCP) complexes. The characterization of intact cells, thylakoid membranes, and pigment protein complexes by absorption and low-temperature fluorescence spectroscopy showed that the FCPs contain certain amounts of protein-bound diadinoxanthin cycle pigments, which are not significantly different in high-light and low-light cultures. The largest part of high-light-formed diadinoxanthin cycle pigments, however, is not bound to antenna apoproteins but located in a lipid shield around the FCPs, which is copurified with the complexes. This lipid shield is primarily composed of the thylakoid membrane lipid monogalactosyldiacylglycerol. We also show that the photosystem I (PSI) fraction contains a tightly connected FCP complex that is enriched in protein-bound diadinoxanthin cycle pigments. The peripheral FCP and the FCP associated with PSI are composed of different apoproteins. Tandem mass spectrometry analysis revealed that the peripheral FCP is composed mainly of the light-harvesting complex protein Lhcf and also significant amounts of Lhcr. The PSI fraction, on the other hand, shows an enrichment of Lhcr proteins, which are thus responsible for the diadinoxanthin cycle pigment binding. The existence of lipid-dissolved and protein-bound diadinoxanthin cycle pigments in the peripheral antenna and in PSI is discussed with respect to different specific functions of the xanthophylls.

In their natural environment, diatoms are exposed to rapidly changing light regimes when they are transported from deep water layers below the euphotic zone to full sunlight at the water's surface. These drastic changes in light intensities must be faced by means of efficient photoprotection mechanisms, thus minimizing the risk of damage to the photosynthetic apparatus. The most important photoprotective mechanism in diatoms is the so-called diadinoxanthin (Ddx) cycle (Lavaud et al., 2002; Goss et al., 2006; Lavaud, 2007; Goss and Jakob, 2010). It is located in the chloroplast and consists of a one-step deepoxidation from the monoepoxy xanthophyll Ddx to the epoxy-free diatoxanthin (Dtx), which is catalyzed by the enzyme Ddx deepoxidase and takes place when diatoms are illuminated with high light (HL) intensities (Stransky and Hager, 1970). Low light (LL) stimulates the back reaction of the cycle, in which Dtx is epoxidized to Ddx (for a recent review, see Goss and Jakob, 2010).

The majority of the Ddx cycle pigments are located within the peripheral antenna complexes of the diatoms, the so-called fucoxanthin chlorophyll protein (FCP) complexes (Lavaud et al., 2003; Lepetit et al., 2007). Basically, three different protein families constitute the diatom antenna: (1) the "classical" light-harvesting complex proteins, called Lhcf; (2) the Lhcr proteins, related to the red algal LHCI proteins; and (3) the ancient Li818 proteins, called Lhcx (Eppard et al., 2000; Green, 2007; Koziol et al., 2007). Recently, the latter have been demonstrated to be involved in a photoprotective mechanism (Zhu and Green, 2010). In *Cyclotella meneghiniana*, two peripheral antenna complexes, the trimeric FCPa and the hexameric FCPb, can be isolated (Büchel, 2003). Using western-blot analysis, Fcp6, which belongs to the Lhcx protein family, and Fcp2, belonging to the Lhcf family, were identified in FCPa. It was shown that FCPb is mainly composed of Fcp5, an Lhcf protein (Beer et al., 2006). Furthermore, a protein belonging to the Lhcr family was detected within PSI (Veith et al., 2009). In contrast to these results, Brakemann et al. (2006) identified a protein of the Lhcr (Fcp4) and Lhcf (Fcp2) families in the peripheral antenna of the related diatom, *Cyclotella cryptica*. The pennate diatom *Phaeodactylum tricornutum* contains only one antenna fraction, which, however, can be isolated in different oligomeric states, with the trimer as the basic unit (Lepetit et al., 2007; Joshi-Deo

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et al., 2010). Using mass spectrometry, Lepetit et al. (2007) demonstrated the presence of the FcpA to FcpF proteins, all belonging to the Lhcf family, in the antenna complexes of *P. tricornutum*. Despite our increasing knowledge about the composition of the diatom antenna complexes, it is not exactly clear at present which Lhc proteins bind the pigments of the Ddx cycle. Evidence for the existence of a special Ddx-binding protein in diatoms was found for *C. meneghiniana*, where the FCPa complex is enriched in these pigments, whereas the FCPb complex seems to bind only very small amounts of Ddx (Gundermann and Büchel, 2008). In addition, Guglielmi et al. (2005) reported the existence of special Ddx-binding FCPs, whose identity remained unclear. Furthermore, Lepetit et al. (2008) showed that a small amount of Ddx is connected to FCPs, which are tightly associated with the PSI core complex.

The deepoxidation of Ddx is strongly enhanced in the presence of the nonbilayer lipid monogalactosyldiacylglycerol (MGDG; Goss et al., 2005, 2007). MGDG is the main thylakoid membrane lipid of vascular plants, followed by the bilayer-forming digalactosyldiacylglycerol (DGDG) as the second most abundant lipid (Joyard et al., 1998; Siegenthaler, 1998). The lipid classes of vascular plants are comparable to those in isolated diatom thylakoids (Goss et al., 2009; Goss and Wilhelm, 2009). With regard to the concentration of charged lipids, however, significant differences exist. Diatom thylakoids can contain more than 40% of their total membrane lipids in the form of the negatively charged lipids sulfoquinovosyldiacylgylcerol (SQDG) and phosphatidylglycerol (PG; Goss et al., 2009), whereas in plant thylakoid membranes, these two lipid species amount to only 20% or less (Murata and Siegenthaler, 1998; Siegenthaler, 1998). Thereby, SQDG surpasses DGDG as the second most abundant lipid (Vieler et al., 2007).

Two aspects are responsible for the enhancement of deepoxidation by MGDG. First, MGDG has the highest capacity of the thylakoid lipids to solubilize the hydrophobic xanthophyll cycle pigment Ddx. Second, MGDG forms inverted hexagonal structures (H_{II} phases) in an aqueous medium (Shipley et al., 1973; Israelachvili and Mitchell, 1975), which promote efficient Ddx deepoxidation in artificial lipid systems (Goss et al., 2005, 2007). In the natural thylakoid membrane, the H_{II} phases most likely enable the binding of the xanthophyll cycle deepoxidases to regions of the thylakoid membrane, which are enriched in antenna proteins and MGDG (Schaller et al., 2010).

The deepoxidation of Ddx to Dtx is an essential prerequisite of the thermal dissipation of excess excitation energy, visible as strong nonphotochemical quenching (NPQ) of the chlorophyll (Chl) *a* fluorescence (Lavaud et al., 2002; Goss et al., 2006; for review, see Goss and Jakob, 2010). Interestingly, drastic increases of the total amount of Ddx cycle pigments, which can be observed in HL-grown diatom cultures (Lavaud et al., 2002; Schumann et al., 2007), do not necessarily lead to a stronger NPQ. In a detailed study, Schumann et al. (2007) were able to show that the linear correlation between NPQ and the concentration of the deepoxidized Ddx cycle pigment Dtx is only valid until a certain concentration of Dtx has been reached. This means that in algae with a high amount of Dtx, a portion of the Dtx molecules loses the ability to quench the fluorescence nonphotochemically, indicating that these pigments fulfill other functions and might not be protein bound.

In vascular plants, two specific sites exist for the binding of the violaxanthin (Vx) cycle pigment Vx within the Lhc proteins: these are the V1 site in the trimeric LHCIIb, and possibly also in Lhca3, and the L2 site in all other Lhc proteins (Morosinotto et al., 2003; Jahns et al., 2009). However, several measurements have indicated that a part of the Vx cycle pigment pool may be located in the lipid phase of the membrane. Zeaxanthin (Zx), which normally acts as an enhancer of NPQ (Niyogi, 2000; Dall'Osto et al., 2005; Horton et al., 2005), may therefore have another significant photoprotective role as an antioxidant, detoxifying reactive oxygen species (ROS) produced during photosynthesis under HL conditions (Havaux and Niyogi, 1999; Triantaphylidès and Havaux, 2009; Dall'Osto et al., 2010).

Because the exact localization of the Ddx cycle pigments within the different photosynthetic pigment protein complexes is not known, one objective of this study was to carefully analyze the Ddx cycle pigment composition of isolated pigment protein complexes of the diatoms *C. meneghiniana* and *P. tricornutum* grown under LL and HL conditions. Special attention was paid to the additional Ddx cycle pigments that are synthesized under HL illumination and that do not participate in the mechanism of NPQ. After the analysis of the pigment distribution, the protein composition of the Ddx-binding proteins was analyzed by mass spectroscopy. As we assumed that a large part of the HL-synthesized Ddx cycle pigments are located within special lipids, the lipid composition of the individual pigment protein complexes was carefully investigated and related to the lipid composition of the thylakoid membrane.

RESULTS

Pigment Composition of LL- and HL-Cultivated *C. meneghiniana* Cells, Thylakoids, and Pigment Protein Complexes

The cultivation of *C. meneghiniana* cells at HL illumination with a light intensity of 160 to 180 μmol m^{-2} s^{-1} induced a 3- to 4-fold increase of the Ddx cycle pigment pool of cells and thylakoids compared with cultivation under LL illumination with an intensity of 10 to 15 μmol m^{-2} s^{-1} (Table I). Despite the drastic changes in the concentration of the Ddx cycle pig-

Table I. *Pigment content of C. meneghiniana cells, thylakoid membranes, and isolated pigment protein complexes*

C. meneghiniana cultures were grown at LL intensities of 10 to 15 μmol m^{-2} s^{-1} PAR or at HL intensities of 160 to 180 μmol m^{-2} s^{-1} PAR. Pigment concentrations are depicted as mM pigment M^{-1} Chl *a*. The table shows the mean values of at least four pigment determinations from independent preparations with the respective SDs.

Sample	Light	Pigment Content			
		Chl *c*	Fx	β-Carotene	Ddx + Dtx
		mM M⁻¹ Chl a			
Cells	LL	88 ± 32	565 ± 9	49 ± 3	237 ± 89
	HL	75 ± 11	518 ± 40	86 ± 29	738 ± 169
Thylakoid	LL	131 ± 27	592 ± 78	38 ± 18	205 ± 88
	HL	105 ± 12	586 ± 103	26 ± 8	809 ± 261
FCP[a]	LL	215 ± 14	853 ± 67	3 ± 2	200 ± 56
	HL	185 ± 12	855 ± 98	15 ± 8	1,001 ± 223
FCPa[b]	LL	226 ± 19	885 ± 97	2 ± 2	208 ± 64
	HL	230 ± 43	933 ± 82	15 ± 7	1,070 ± 212
FCPb[b]	LL	270 ± 24	971 ± 87	5 ± 4	76 ± 25
	HL	273 ± 25	1,028 ± 76	6 ± 5	270 ± 44
PSII	LL	9 ± 5	63 ± 22	92 ± 22	55 ± 10
	HL	6 ± 4	57 ± 22	101 ± 36	73 ± 9
PSI	LL	18 ± 2	140 ± 11	63 ± 9	158 ± 9
	HL	17 ± 2	131 ± 9	56 ± 15	173 ± 10

[a]The highly oligomeric FCP was obtained after solubilization of thylakoids with a DM to Chl ratio of 10. [b]FCPa and FCPb were obtained after solubilization with a DM to Chl ratio of 40.

ments, the content of the other pigments remained largely unchanged. This indicated that the ratio of antenna complexes to the photosystem core complexes was relatively stable under the different light regimes. Comparable results were obtained for the other diatom used in these experiments, *P. tricornutum*. Due to the stronger pigment degradation in isolated thylakoids and pigment protein complexes of *P. tricornutum*, which was caused in part by high activity of the chlorophyllase, we chose to present the pigment and lipid data and the absorption and fluorescence spectra from *C. meneghiniana*.

To investigate the exact localization of the newly synthesized Ddx cycle pigments, thylakoids were solubilized with a dodecyl β-D-maltoside (DM)-to-Chl ratio of 10 and separated by Suc gradient centrifugation (Fig. 1). Four different fractions were obtained: a free pigment, an FCP, a monomeric PSII, and a PSI fraction. In contrast to *P. tricornutum*, both photosystem fractions from *C. meneghiniana* existed as discrete separate bands, so that a pure PSI fraction could be obtained. Another fraction, which was located at a higher density than the PSI complex, exhibited identical absorption and fluorescence emission and excitation spectra as PSI and therefore most likely represented oligomeric PSI complexes. The FCP fraction was oligomeric, similar to the FCPo of *P. tricornutum* described by Lepetit et al. (2007). Due to the low detergent concentration used in our experiments, the free pigment fraction contained only very small amounts of pigments and was not analyzed further.

When a higher detergent per Chl ratio was applied (Fig. 1, B and D), the FCPo disaggregated into the trimeric FCPa and the hexameric FCPb (Büchel, 2003). In *P. tricornutum*, the FCP fraction was isolated as trimeric FCP (Lepetit et al., 2007), which represents the smallest basic unit of the peripheral antenna of diatoms (Lepetit et al., 2007; Joshi-Deo et al., 2010). The oligomeric PSI fraction disappeared under the harsher detergent treatment. Additionally, a decreased concentration of the monomeric PSII band was observed, accompanied by a concomitant increase of the dimeric PSII fraction, which was located slightly above the PSI fraction in the Suc gradient. This is in line with recent results from Takahashi et al. (2009), who showed that

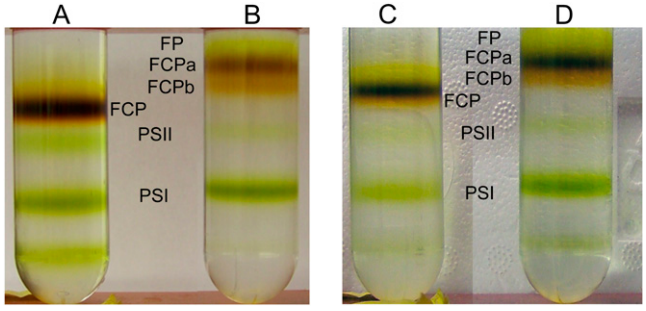

Figure 1. Separation of the pigment protein complexes of *C. meneghiniana* by Suc density gradient centrifugation. A and B, LL pigment protein complexes. C and D, HL pigment protein complexes. A and C show the typical separation pattern when a low DM per Chl ratio of 10 was used. Higher detergent per Chl ratios (DM to Chl ratio of 20 or above) led to a dissociation of the oligomeric FCP complex into two distinct bands, FCPa and FCPb (B and D). [See online article for color version of this figure.]

an increase in the detergent concentration during solubilization leads to a dimerization of the native monomeric PSII complex in non-grana-containing photosynthetic organisms. It also has to be noted that no significant difference in the fractionation pattern of LL and HL thylakoids under both solubilization conditions was detected.

When the Ddx cycle pigment content of the different density gradient fractions was analyzed, it was obvious that the PSII band was almost free of Ddx and Dtx (Table I). The PSI fraction, however, contained a significant amount of Ddx cycle pigments. Interestingly, this amount was similar in PSI fractions isolated from HL- or LL-cultivated *C. meneghiniana* cells. The largest part of the Ddx cycle pigments was localized within the peripheral antenna (i.e. the FCP fraction). Moreover, the additional Ddx and Dtx molecules, which were synthesized during the HL cultivation, were exclusively bound to the FCP. The use of higher detergent concentrations revealed that, within the FCP, the FCPa was the major site of Ddx cycle pigment binding. Calculations showed that the LL FCP fractions contained approximately 85% of the total Ddx cycle pigments, while the FCP isolated from HL-cultivated cells contained more than 95% of the total Ddx and Dtx. With respect to the light-harvesting pigments fucoxanthin (Fx) and Chl *c*, no significant changes were observed in the FCP or the PSI and PSII fractions isolated from LL- and HL-grown *C. meneghiniana* cultures.

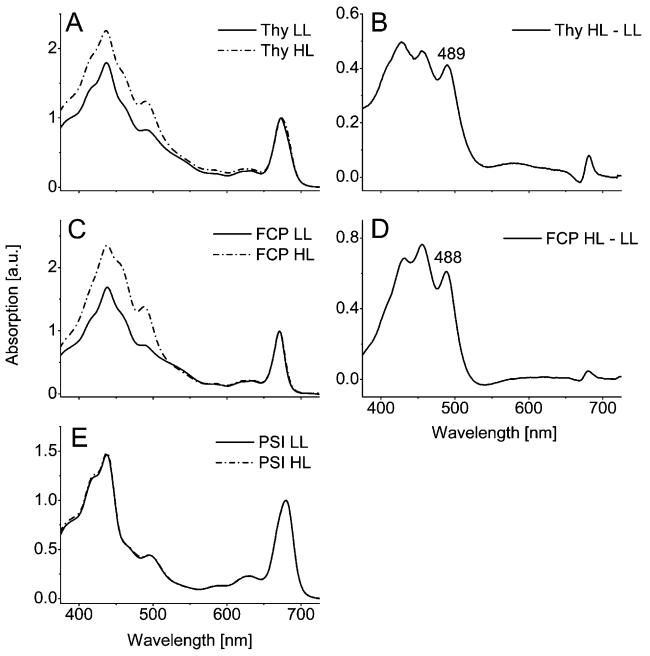

Figure 2. A, C, and E, Absorption spectra of thylakoid membranes (A), FCP (C), and PSI (E) complexes isolated from LL- and HL-grown *C. meneghiniana*. The FCP fractions were isolated after solubilization of thylakoids with a DM to Chl ratio of 10, and the PSI fractions were isolated after solubilization with a DM to Chl ratio of 10 and 40. Spectra are averages of at least four different samples. Note that the spectra were normalized to the Q_Y band of Chl *a*. B and D, Difference absorption spectra of HL minus LL thylakoids (B) and HL minus LL FCPs (D). The wavelength of the third maximum is indicated.

Spectroscopy of Cells, Thylakoids, and Pigment Protein Complexes of LL- and HL-Grown *C. meneghiniana* Cultures

The huge increase of the Ddx cycle pigments in the HL-cultivated algae caused significantly more pronounced absorption in the blue and blue-green range of the absorption spectrum of the respective thylakoids and FCP complexes compared with thylakoid membranes and FCPs derived from LL-grown *C. meneghiniana* cells (Fig. 2). In agreement with the pigment analysis data, the difference spectra of the HL minus LL thylakoids and FCPs exhibited major changes only in the carotenoid region. The difference spectrum of the FCP fractions closely resembled the absorption spectrum of purified carotenoid molecules with respect to the shape of the spectrum and the localization of the absorption maxima. The wavelength of the third absorption maximum was located at 489 and 488 nm in the thylakoids and FCPs, respectively.

Interestingly, the massive increase in the absorption spectrum of HL thylakoids and FCP complexes caused by the increased Ddx and Dtx content was not accompanied by a concomitant increase in the blue/blue-green region of the fluorescence excitation spectrum (Fig. 3, A and B). On the contrary, the fluorescence excitation spectra of LL and HL FCPs were similar both in shape and the extent of fluorescence. Thus, most of the additional Ddx and Dtx synthesized under HL illumination was unable to transfer excitation energy to Chl *a*. This indicated that the largest part of these pigments was either not bound to the antenna apoproteins or existed in a protein-binding site where no functional energy transfer between Ddx and Chl *a* could be realized, as was described for Vx in the V1 site of the LHCIIb from vascular plants (Caffarri et al., 2001). However, in HL FCPs, a slight increase of the fluorescence excitation at 497 nm was observed compared with LL FCPs (Fig. 3, inset). As there was no difference in the pigment content of LL and HL FCPs, with the exception of Ddx, this maximum at 497 nm most likely represented a small pool of protein-bound Ddx. This protein-bound Ddx was also identified within the FCPa subfraction at higher detergent concentrations, while the FCPb was largely devoid of it (Supplemental Fig. S1).

The absence of changes in the content of light-harvesting pigments (the Fx-to-Chl *a* as well as the Chl *c*-to-Chl *a* ratios remained constant) indicated that no further pigment-binding sites were available in HL-grown algae. Together with the lack of excitation energy transfer from the majority of the HL-induced Ddx and Dtx molecules, this led to the assumption that the additional Ddx cycle pigments might be located

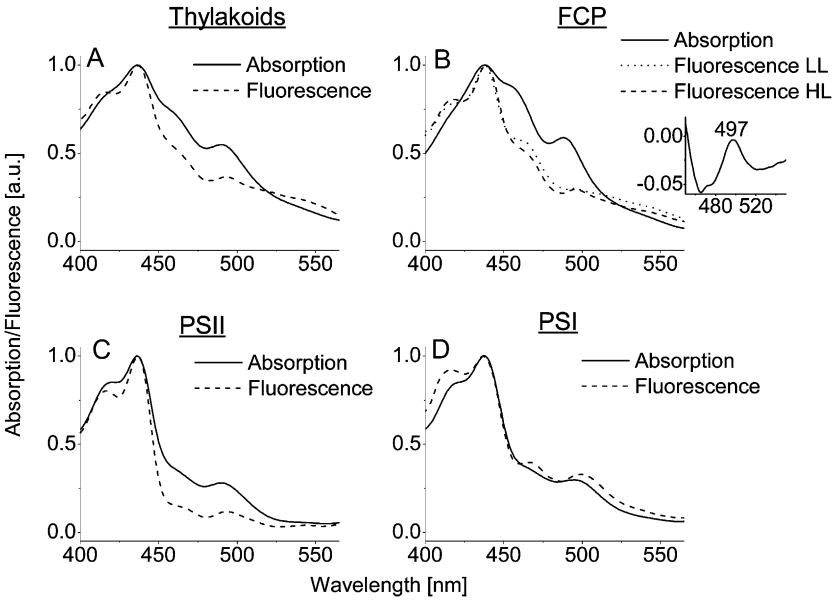

Figure 3. Comparison of the absorption and the 77 K fluorescence excitation spectra of thylakoids (A), FCP (B), PSII (C), and PSI (D) from HL-cultivated *C. meneghiniana*. In B, the 77 K fluorescence excitation spectrum of the LL FCP is additionally presented. The inset shows the difference fluorescence excitation spectrum of the HL minus LL FCP in the range of 450 to 550 nm. For the fluorescence excitation spectrum, the fluorescence emission was set to the emission maximum of the Chl *a* fluorescence, which was located at 686 nm in the thylakoids, at 683 nm in the FCP, at 687 nm in PSII, and at 715 nm in PSI. Spectra are averages of at least four different samples.

within a shield of membrane lipid molecules associated with the respective protein complexes (see below). To test this hypothesis, the difference absorption spectra of HL minus LL FCPs were compared with an absorption spectrum of isolated Ddx, which was dissolved in the purified membrane lipid MGDG at a molar ratio of MGDG to Ddx of 7.25:1 (Fig. 4). Note that this lipid-to-pigment ratio has been shown to ensure the complete solubilization of Ddx (Goss et al., 2005). The absorption spectrum of Ddx dissolved in MGDG closely resembled the difference absorption spectra of the FCP complexes with respect to the overall shape of the spectrum and the location of maxima and minima. The shape of the difference spectrum of HL minus LL thylakoids differed slightly from the absorption spectrum of Ddx in MGDG (compare Figs. 2B and 4). However, the characteristic Ddx maximum was located at almost identical wavelengths (489 nm for thylakoids and 490 nm for Ddx in MGDG).

To check whether the difference absorption spectrum of the isolated FCPs was influenced by the detergent DM, which was used during the isolation of the pigment protein complexes, Ddx was additionally dissolved in DM (data not shown). Very high concentrations of DM were needed to solubilize the same amount of purified Ddx as in the solubilization experiments with MGDG (the ratio of DM to Ddx was 780 compared with the ratio of MGDG to Ddx of 7.25). These concentrations were more than 10-fold higher than those employed during the solubilization of the thylakoids. Furthermore, the absorption maxima of the Ddx spectrum in DM were significantly shifted toward shorter wavelengths. The characteristic third maximum already peaked at a wavelength of 484 nm, compared with the absorption maxima at 488 nm in the FCP and 490 nm in pure MGDG. Both experimental results argued against an influence of the detergent treatment on the native difference absorption spectrum. Most importantly, thylakoids, not being influenced by the detergent, exhibited an almost identical Ddx absorption maximum as the isolated FCP complexes.

To obtain further evidence that the majority of the HL-synthesized Ddx and Dtx were indeed placed within a lipid shield surrounding the FCP complexes, it was necessary to determine the absorption maximum of Ddx within its binding site of the photosynthetic apoproteins. Although the fluorescence excitation spectra of HL FCPs already indicated a localization of the third absorption peak of protein-bound Ddx at 497 nm (Fig. 3), additional independent results were needed, as diatoms contain the so-called "Fx green" molecules, which absorb at approximately 500 nm (Premvardhan et al., 2009; Szábo et al., 2010). Very minor amounts of Ddx were detected within PSII.

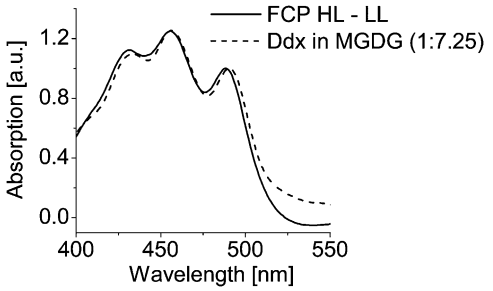

Figure 4. Comparison between the difference absorption spectrum of HL FCP complexes minus LL FCP complexes and the absorption spectrum of purified Ddx dissolved in MGDG. For the MGDG experiments, a ratio of MGDG to Ddx of 7.25 was chosen. The Ddx concentration in the MGDG experiment was 0.4 μM. Spectra were normalized to the third absorption peak.

However, these pigments were not involved in energy transfer, as the fluorescence excitation in the carotenoid region was much lower than the respective absorption (Fig. 3C). The weak fluorescence excitation maximum at 492 to 493 nm was most probably caused by β-carotene, which has been shown to transfer the absorbed energy to Chl *a* in PSII (Siefermann-Harms, 1987) and, when protein bound, exhibits an absorption maximum at the respective wavelength in vascular plants (Croce et al., 2007) and diatoms (Nagao et al., 2010). Thus, it is likely that Ddx within the PSII band represented a slight contamination by the FCP and PSI fractions of the Suc gradient. In contrast, the PSI fraction contained significant amounts of Ddx cycle pigments. The similarity between the fluorescence excitation and absorption spectra of the PSI band (Fig. 3D) indicated that Ddx associated with PSI was transferring the excitation energy to Chl *a*, which could only be realized in the case of a tight binding of pigment and protein. Because the PSI isolated from HL cultures showed a higher deepoxidation state (DES) than the LL PSI (DES HL PSI 0.3, DES LL PSI 0; i.e. a higher concentration of Dtx and a lower concentration of Ddx), the estimation of the absorption maxima of protein-bound Ddx was possible by calculating the difference spectrum of LL minus HL PSI. This difference spectrum was compared with a difference absorption spectrum of native lipid-dissolved Ddx minus Ddx/Dtx in intact cells from HL-grown *C. meneghiniana* cultures (recall that HL cells contained a large part of the total Ddx cycle pigment pool in a lipid-dissolved form). To achieve a comparable DES of 0.3 in the cells (i.e. a lower concentration of Ddx), absorption spectra were immediately recorded after removing the cells from the HL cultivation chamber. Another sample of the HL cells was LL adapted for 45 min to allow the complete epoxidation of Dtx to Ddx before the absorption spectrum was recorded. In addition, the absorption spectrum of pure Ddx in MGDG was compared with a MGDG solution, which contained a lower concentration of Ddx but additional Dtx, to achieve a simulated DES of the Ddx cycle pigment pool of 0.3.

Notably, a significant difference in the location of the Ddx absorption maxima in PSI and the HL cells was observed. The characteristic maximum of lipid-dissolved Ddx in the cells was located at 488 nm (Fig. 5). This was almost identical to the maximum found in the difference absorption spectrum of MGDG enriched in dissolved Ddx minus MGDG containing lower concentrations of Ddx and additional Dtx (489 nm). In PSI, the Ddx absorption maximum was significantly shifted toward the longer wavelength of 497 nm. This was directly comparable to the location of the fluorescence excitation maximum derived for Ddx, which was bound to the FCP (Fig. 3). These findings provide strong evidence that the wavelength at 497 nm represented the absorption of protein-bound Ddx.

Since the samples of the intact cells, the PSI fractions, and the MGDG solutions with a DES of 0.3 contained significant amounts of Dtx, the absorption maxima of protein-bound and lipid-dissolved Dtx were also accessible in the difference spectra. Due to the fact that the difference spectra depicted in Figure 5 were calculated from a sample without Dtx minus a sample containing Dtx, these absorption wavelengths became visible as minima in the difference absorption spectra. For protein-bound Dtx, the wavelength of the third absorption peak could be determined at 513 nm, whereas the wavelength for lipid-dissolved Dtx was located at 508 nm.

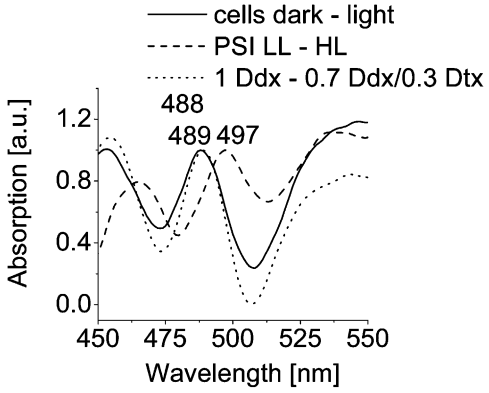

Figure 5. Comparison of the difference absorption spectrum of LL-adapted HL cells (DES 0) minus cells that were directly taken from the growth light of 160 to 180 μmol m^{-2} s^{-1} (DES 0.3) with the difference absorption spectrum of LL PSI (DES 0) minus HL PSI complexes (DES 0.3). Additionally, the difference absorption spectrum of Ddx dissolved in MGDG (DES 0) minus Ddx and Dtx dissolved in MGDG (DES 0.3) is depicted. For the MGDG experiments, Ddx or Ddx/Dtx concentrations of 0.4 μM were used. The MGDG per Ddx (Ddx/Dtx) ratio was 7.25. The wavelengths of the respective characteristic Ddx absorption maxima are depicted.

Lipid Composition of Thylakoids and Pigment Protein Complexes Isolated from LL- and HL-Cultivated *C. meneghiniana*

To analyze which lipids of the native thylakoid membrane incorporate Ddx, the lipids of thylakoids and isolated FCP complexes were separated by thin-layer chromatography. In accordance with recent results (Goss et al., 2009), diatom thylakoids derived from a LL-grown culture contained a high amount of the negatively charged lipid SQDG (Fig. 6A). The concentration of SQDG was further increased in thylakoid membranes isolated from HL-cultivated *C. meneghiniana* cells, where SQDG even surpassed MGDG as the main thylakoid membrane lipid. To investigate the specific lipid composition of the FCPs, increasing concentrations of DM were used to remove nonspecifically bound lipids. However, a higher ratio of DM per Chl *a* caused a dissociation of the higher oligomeric FCP complex into the two antenna complexes FCPa and FCPb (Fig. 1). As FCPa contained the largest part of the newly synthesized Ddx cycle pig-

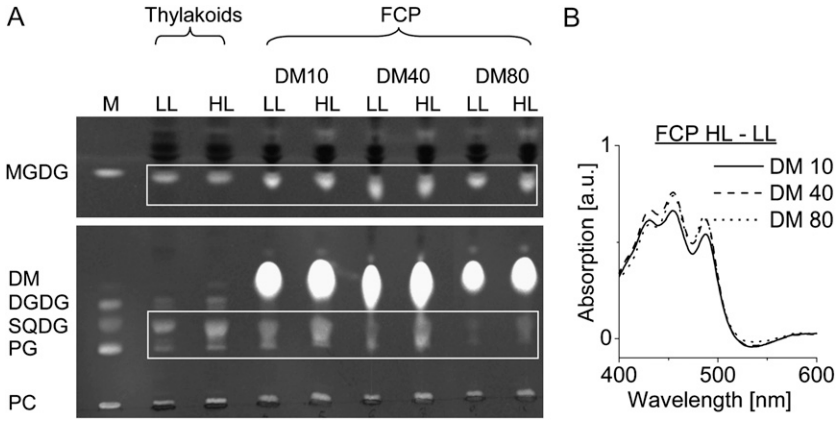

Figure 6. A, Lipid composition of thylakoids and FCP complexes isolated from LL- or HL-grown *C. meneghiniana* cultures. FCP complexes were isolated with the different DM per Chl ratios of 10, 40, and 80. For separation by high-performance thin-layer chromatography, samples corresponding to a Chl *a* content of 2 μg were subjected to the plates. MGDG was separated with an eluent according to Ventrella et al. (2007), while the other lipids were separated with the eluent reported by Mock and Kroon (2002). B, Difference absorption spectra of HL FCP complexes minus LL FCP complexes. The FCP complexes were isolated with the different DM per Chl ratios mentioned above.

ments (Table I), the lipid composition of the oligomeric FCP was compared with that of the FCPa complex. Note that, due to the detergent, precise quantification of the lipids DGDG, SQDG, and PG was impossible, but qualitative information was obtained. The FCP complex isolated with a DM per Chl ratio of 10 exhibited a lipid composition comparable to the isolated thylakoid membranes. However, the amount of MGDG was increased (Table II). Higher detergent concentrations caused a dramatic loss of DGDG, SQDG, and PG in the FCP, while the MGDG content remained virtually unchanged, as well as a minor amount of phosphatidylcholine (PC). Based on a rough estimation, MGDG represented 80% of total lipids of the FCP fraction isolated with a ratio of DM to Chl of 80. Importantly, increasing concentrations of DM were unable to remove the Ddx cycle pigments from the FCP fractions, as indicated by the similarity of the difference absorption spectra of the different FCP complexes (Fig. 6B). Combining the information that MGDG is the only lipid that remains in higher concentrations in the FCPs treated with a high amount of DM, and that the Ddx content of the FCPs is stable, the HL-synthesized Ddx cycle pigments must be dissolved in an MGDG shield surrounding the antenna complexes.

Lipid analyses of the PSI fraction were also conducted during these experiments. However, the lipid concentration of the isolated PSI was so low that even a 10-fold concentration of the lipid samples subjected to the thin-layer plates did not yield significant amounts of the different lipid classes (data not shown). From this result, it is concluded that a lipid shield, as described above for the FCP, does not exist around the PSI core complex. This is in line with the spectroscopic data, which showed that the Ddx cycle pigments of PSI are protein bound.

Another interesting observation was that the different cultivation light intensities did not have a strong impact on the concentration of MGDG in either the isolated thylakoids or the FCPs (Fig. 6A; Table II), indicating that the amounts of MGDG and Chl *a* are correlated. The calculation of the ratio of MGDG per Ddx and Dtx revealed that, under LL conditions, six to seven molecules of MGDG per molecule of Ddx and Dtx were available, while this ratio dropped to only around 1.5 in FCPs isolated from HL cultures. In HL thylakoids, comparable ratios to those in the isolated FCP complexes were observed, whereas in thylakoid membranes isolated from LL cultures, the ratio of MGDG per Ddx and Dtx was slightly lower than in the isolated FCP.

Thermoluminescence Measurements of HL Thylakoids

To gain insight into a possible antioxidant function of the newly synthesized, lipid-dissolved Dtx molecules, thermoluminescence (TL) measurements of HL thylakoids were performed. It is important to note that the thylakoid preparation used in these experiments allowed us to determine the direct antioxidant properties of Dtx. The isolated thylakoids were not able to generate a significant proton gradient (data not shown), which would have been required for the buildup of NPQ (Lavaud et al., 2002; Goss et al., 2006). This means that photoprotection in the isolated thylakoids through enhanced thermal dissipation,

Table II. *MGDG content of LL and HL C. meneghiniana thylakoid membranes and isolated FCP complexes*

The DM to Chl ratio used for the solubilization is indicated for the FCP fractions. The MGDG concentrations are depicted as μg MGDG μg^{-1} Chl *a* and are the average values of two to three independent samples. The SD was in the range between 10% and 20%. The table also shows the ratio of MGDG to Ddx cycle pigments.

Sample	Thylakoid LL	Thylakoid HL	FCP10 LL	FCP10 HL	FCPa40 LL	FCPa40 HL	FCPa80 LL	FCPa80 HL
MGDG (μg μg^{-1} Chl *a*)	0.81	0.86	1.32	1.41	1.17	1.25	1.16	1.41
MGDG/(Ddx + Dtx) (mol mol^{-1})	4.6	1.2	7.5	1.6	6.4	1.3	6.2	1.8

which might have masked photoprotection by the antioxidant properties of Dtx, was impossible. Furthermore, Dtx synthesis in the isolated thylakoid membranes was triggered by a dark incubation in a pH 5.2 medium in the presence of ascorbate. Dark induction of Zx synthesis in vascular plants has been shown to generate deepoxidized xanthophyll cycle pigments, which are unable to induce NPQ (Goss et al., 2008). In our experiments here, this treatment caused a very high DES of 0.8 in *P. tricornutum* thylakoids, but a fast relaxing NPQ could not be observed after an illumination of the thylakoids with a light intensity of 5,000 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR) for 10 min, as it was used directly before the TL measurements (data not shown). Moreover, the Dtx-enriched thylakoids did not show a significant difference in NPQ compared with Ddx-containing membranes, where Dtx synthesis at pH 5.2 was blocked through the omission of the cosubstrate of the deepoxidase, ascorbate. TL glow curves of Ddx- and Dtx-enriched thylakoids were recorded to detect lipid peroxidation caused by an illumination with very HL intensities. The absence of the B-band in the TL curves, which originates from charge recombination of the $S_2Q_B^-$ radical pair state of PSII, indicates a severe photoinhibition of the isolated thylakoids after the 10-min HL illumination with 5,000 μmol m^{-2} s^{-1} PAR (Fig. 7). However, the HTL-2 band, which is caused by excited triplet carbonyls and singlet oxygen formed by thermoinduced decomposition of lipid peroxidation products (Vavilin and Ducruet, 1998; Havaux, 2003), was much more pronounced in the Ddx-enriched thylakoids compared with the thylakoids that contained high amounts of Dtx (Fig. 7). This indicates that lipid-dissolved Dtx might indeed fulfill an antioxidant function in the native diatom thylakoid membrane.

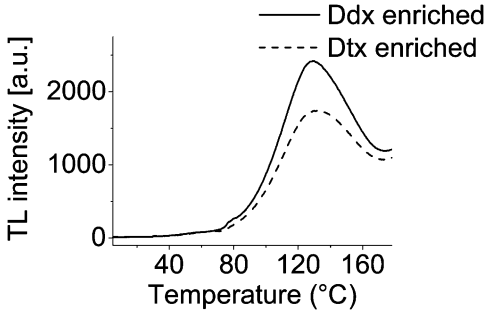

Figure 7. TL glow curves of HL thylakoids from *P. tricornutum* that contained low concentrations (Ddx enriched, 130 mmol Dtx mol^{-1} Chl *a*) or high concentrations (Dtx enriched, 710 mmol Dtx mol^{-1} Chl *a*) of Dtx. The thylakoids were illuminated with 5,000 μmol m^{-2} s^{-1} PAR before the TL measurements were started. The TL glow curves represent average values of four independent measurements with three repetitions each. A Student's *t* test calculation for the difference between means based on the maxima at 130°C revealed a statistical significance of $P < 0.001$. For further details, see "Materials and Methods" and "Results."

Lhc Proteins within the FCP and the PSI

Because the results from the pigment analysis showed that the PSI fraction and the peripheral FCP contained protein-bound Ddx and Dtx, we were interested in the analysis of the specific antenna proteins binding these pigments. As complete genomic data are not available for *C. meneghiniana*, the PSI and FCP fractions of *P. tricornutum* were analyzed. To exclude artifacts that might have been caused by slight contaminations with other Suc gradient fractions, only those identified proteins were considered as relevant for which at least three different peptides were identified by tandem mass spectrometry (Table III). In addition, only those proteins were listed that were detected in 50% or more of the examined samples. (A table containing all identified antenna proteins, protein identifiers, single amino acid sequences, and their respective masses as well as the sequence coverage is presented as Supplemental Table S1.) In the PSI fraction, eight of the 14 known Lhcr proteins were identified, whereas only one out of the 15 different Lhcf proteins was detected. In contrast, the protein composition of the peripheral antenna was remarkably different. Here, most of the Lhcf proteins were identified, together with a significant number of Lhcr proteins. However, there were fewer Lhcr proteins present within the FCP compared with the PSI. Additionally, one member of the Lhcx family, Lhcx1, was detected. Three Fcp-related proteins, whose exact classification awaits further research, were also significantly expressed within the FCP.

This tandem mass spectrometry analysis did not allow us to quantify the protein expression in HL and LL fractions. However, it is noteworthy that in HL FCPs, the Lhcr4, Lhcr6, Lhcr8, and Lhcr10 proteins could be detected with a few peptides, while they were completely missing in the LL FCP.

DISCUSSION

Protein-Bound Ddx Cycle Pigments in PSI

Based on our results here, one pool of Ddx and Dtx is associated with the PSI fraction. The ability of Ddx to transfer excitation energy to Chl *a*, thus acting as a typical accessory pigment, indicates that the Ddx cycle pigments exist in a protein-bound form. The existence of Ddx cycle pigments in purified PSI complexes has been reported for the diatom *P. tricornutum* (Berkaloff et al., 1990; Lepetit et al., 2007, 2008). Recently, Veith et al. (2009) presented evidence that the PSI of *C. meneghiniana* also contains significant concentrations of Ddx and Dtx. Moreover, by using specific antibodies and applying mass spectrometry, they concluded that a protein belonging to the Lhcr family is bound to the PSI core complex, which is in line with earlier assumptions based on sequence homologies (Eppard et al., 2000). These results are confirmed and extended

Table III. *Tandem mass spectrometry identification of antenna proteins in the FCP and the PSI fraction of P. tricornutum*

The protein sequences were taken from UniProt and the *P. tricornutum* database (Joint Genome Institute *P. tricornutum* version 2.0; http://genome.jgi-psf.org/Phatr2/Phatr2.home.html). Three independent LL and HL FCP samples and two independent LL and HL PSI samples were analyzed. The amount of identified peptides is depicted in parentheses, as well as the old names of the Lhcf proteins identified by Lepetit et al. (2007). Only those proteins that occurred with at least three different peptides and that were found in a minimum of 50% of the examined samples are depicted. The exact peptide sequences are listed in Supplemental Table S1.

Protein	FCP	PSI
Lhcf1 (FcpA)	X (5)	
Lhcf2 (FcpB)	X (6)	
Lhcf3/4 (FcpC/D)	X (8)	X (3)
Lhcf5 (FcpE)	X (6)	
Lhcf6/Lhcf7	X (4)	
Lhcf8	X (9)	
Lhcf9	X (6)	
Lhcf10	X (5)	
Lhcf11 (FcpF)	X (7)	
Lhcf14	X (8)	
Lhcf16	X (4)	
Lhcf17	X (3)	
Lhcr1	X (4)	X (8)
Lhcr2	X (4)	X (5)
Lhcr3	X (4)	X (6)
Lhcr4		X (4)
Lhcr11	X (5)	X (5)
Lhcr12	X (6)	X (3)
Lhcr13		X (3)
Lhcr14	X (5)	X (3)
Fcp47485	X (3)	
Fcp17531	X (3)	X (3)
Fcp48798	X (3)	
Fcp24119	X (3)	
Lhcx1	X (9)	

in our experiments here with *P. tricornutum*, which, like *C. meneghiniana*, exhibits a significant enrichment of Ddx cycle pigments in PSI. Several polypeptides belonging to the Lhcr family are shown to be associated with the PSI core complex. As the PSI core is generally highly conserved (Green and Durnford, 1996; Amunts and Nelson, 2008) and does not contain binding sites for Ddx cycle pigments, Ddx and Dtx must be bound to these Lhcr proteins. This reasoning is in line with the notion that the Lhcr proteins of red algae also serve as PSI antennae (Wolfe et al., 1994; Marquardt and Rhiel, 1997) and that the Lhcr of red algae is able to bind high amounts of the Vx cycle pigments Zx and antheraxanthin (Grabowski et al., 2000). In addition, Lhcr is able to attach Ddx and Fx in a functional manner (Grabowski et al., 2001).

Protein-Bound Ddx Cycle Pigments in the FCP

The largest part of the Ddx cycle pigments in both LL- and HL-cultivated *C. meneghiniana* cells was found in the FCP complexes. More specifically, the FCPa complex was associated with the majority of the Ddx cycle pigments, whereas the FCPb fraction was almost completely devoid of Ddx and Dtx but enriched in the light-harvesting pigments Fx and Chl *c*. The heterogeneous distribution of Ddx cycle pigments within the peripheral antenna in *C. meneghiniana* is comparable to the situation in vascular plants, where numerous studies have shown that the Vx cycle pigments are bound in different stoichiometries to the major LHCIIb and the minor Chl *a*/*b*-binding proteins of PSII (Bassi and Büchel, 1993; Ruban et al., 1994; Goss et al., 1997; Morosinotto et al., 2003). Vx cycle pigments are also found in the PSI holocomplex (i.e. the PSI core complex with its attached PSI antenna; Thayer and Björkman, 1992; Morosinotto et al., 2003). Our experiments here show that a small fraction of the total Ddx cycle pigments associated with the FCPa are protein bound. These pigments are visible in the fluorescence excitation spectra, which means that they act as accessory pigments in the peripheral FCP. FCPb complexes also contain protein-bound Ddx pigments, but in a much lower amount compared with FCPa (Gundermann and Büchel, 2008; Gildenhoff et al., 2010). Importantly, in *P. tricornutum*, protein-bound Ddx cycle pigments are also present in the FCP (data not shown).

In this study, the protein-bound Ddx cycle pigments, which transfer excitation energy to Chl *a*, were masked by the large amount of Ddx cycle pigments, which did not interact excitonically with Chl *a*. Thus, a quantification of the protein-bound Ddx cycle pigment pool was not possible. However, several pigment determinations were performed before with FCPs, which were isolated with anion-exchange chromatography or a two-step purification protocol using Suc gradient centrifugation in combination with anion-exchange chromatography (Beer et al., 2006; Gundermann and Büchel, 2008; Gildenhoff et al., 2010; Premvardhan et al., 2010). All these purifications have in common that the Ddx cycle pigment content of the isolated complexes was strongly reduced in comparison with the content of the intact cell. It can be assumed that in these preparations, the remaining Ddx represents the protein-bound Ddx molecules, while the lipid-dissolved Ddx cycle pigments are lost during the purification. Accordingly, Premvardhan et al. (2010) were able to assign eight Chl *a*, eight Fx, and two Chl *c* molecules to the FCP monomer by combining information about the LHCIIb of vascular plants, results from resonance Raman spectroscopy of purified FCPa and FCPb complexes of *C. meneghiniana*, and previously obtained pigment data. However, a Ddx-binding site within the FCP could not be found and remains an open question. Therefore, the excitation fingerprint of the protein-bound Ddx obtained in this study represents, to our knowledge, the first experimental evidence that a small portion of the Ddx pool is protein bound to the peripheral FCP. Since, in vascular plants, Vx bound to the L2 site transfers its excitation energy to Chl *a* (Gradinaru et al., 2000), it can be speculated that a similar pigment-binding site in the

FCP harbors the Ddx molecules active in light harvesting.

Our results here do not allow for a specific assignment of the proteins that bind the Ddx cycle pigments in the peripheral FCP. However, mass spectrometry analysis of the basic unit of the *P. tricornutum* antenna, the trimeric FCP, significantly increased the amount of identified antenna proteins compared with our previous analysis (Lepetit et al., 2007). In this study, the existence of many members of both the Lhcf and Lhcr families was revealed. In addition, Lhcx1 and Lhcx2 sequences were identified. In line with these results, Beer et al. (2006) and Veith et al. (2009), using antibodies, demonstrated the presence of Lhcf and Lhcx proteins within the peripheral antenna of *C. meneghiniana*, while Brakemann et al. (2006) identified Lhcf and Lhcr proteins within the antenna of *C. cryptica*. Taking into account that Lhcr proteins in diatoms are expressed to approximately the same extent as Lhcf proteins (Westermann and Rhiel, 2005), Lhcr proteins, which were shown to bind Ddx cycle pigments within PSI, might also carry these pigments within the peripheral antenna. Ddx binding by Lhcr proteins is in agreement with recent results demonstrating that, in *P. tricornutum*, the transcript levels of Lhcr6, Lhcr8, and Lhcr10 are increased after prolonged exposure to HL (Nymark et al., 2009). In our experiments here, we also identified these proteins in HL FCPs (Supplemental Table S1). Importantly, the presence of Lhcr6, Lhcr8, and Lhcr10 seems to be correlated with the slight increase of protein-bound Ddx in the HL FCP. Therefore, the basic unit of the peripheral antenna, the trimeric FCP, can most likely be divided into smaller units, some of them binding Ddx, while others cannot. Accordingly, it was recently shown that the trimeric FCP contains a distinct composition of apoproteins and does not randomly combine the different antenna proteins that are expressed (Joshi-Deo et al., 2010).

Lipid-Dissolved Ddx Cycle Pigments Associated with the FCP

The HL cultivation of *C. meneghiniana* caused a 5-fold increase of Ddx cycle pigments in the isolated FCP fractions. Based on the following experimental results, we concluded that the largest part of these newly synthesized pigments is dissolved in a lipid shield surrounding the FCP. (1) The HL-synthesized Ddx exhibited the same absorption properties as Ddx dissolved in the purified, isolated thylakoid membrane lipid MGDG. The absorption spectra of HL-synthesized and lipid-dissolved Ddx were significantly different from the spectra of protein-bound Ddx cycle pigments within the FCP and PSI. (2) No decrease of other pigments (i.e. Fx, Chl *c*, and Chl *a*) was observed within the HL FCP, which could have led to the existence of unoccupied pigment-binding sites for the high amounts of HL-synthesized Ddx cycle pigments. (3) The newly formed Ddx cycle pigments neither excitonically interacted with Chl *a* nor were involved in the process of NPQ (Schumann et al., 2007).

Our experiments suggest that the majority of these pigments are dissolved in MGDG molecules, which tightly surround the FCPs as a lipid shield. SQDG, which is highly abundant in the thylakoid membrane of diatoms, does not show a strong association with the antenna complexes and is most likely present in other areas of the thylakoid membrane, in line with its strong inhibitory effect on Ddx deepoxidation (Goss et al., 2009).

However, it has to be mentioned that for the HL-synthesized Ddx cycle pigments, a protein binding to a hypothetical V1 site within the FCPs must also be taken into account. Vx cycle pigments bound to the V1 site of the LHCIIb of vascular plants do not interact excitonically with Chl *a* (Caffarri et al., 2001), similar to what was observed for the HL-synthesized Ddx in this study. In addition, Vx bound to the V1 site of the LHCII showed a significantly shorter absorption wavelength of the third peak compared with Vx located in the L2 site (i.e. 485 nm compared with 492 nm; Caffarri et al., 2001). The shift of the Vx absorption maximum toward shorter wavelengths is in line with the absorption maximum observed for the additionally HL-synthesized Ddx molecules found in our experiments. On the other hand, Zx, which binds to the V1 site after Vx deepoxidation, acts as an allosteric modulator of the conformational changes of the antenna, which lead to enhanced heat dissipation (Johnson et al., 2009). This is different from the observation that the additionally synthesized Dtx does not participate in NPQ (Schumann et al., 2007). Moreover, recent determinations of the pigment-binding sites of the FCP monomers (Premvardhan et al., 2010) are in favor of our hypothesis that the HL-synthesized Ddx cycle pigments are rather dissolved within an MGDG shield than bound to so far unidentified V1-like sites. As already mentioned, each individual FCP monomer contains eight Chl *a*, eight Fx, and two Chl *c* molecules, summing up for a total of 18 protein-bound pigments (Premvardhan et al., 2010). This is directly comparable to the 18 pigment molecules bound by the LHCIIb monomer (Liu et al., 2004; Standfuss et al., 2005), which is not surprising, as the FCP and LHC proteins both belong to the LHC protein family and are highly related (Green and Durnford, 1996; Koziol et al., 2007). With regard to the amount of HL-synthesized Ddx, the HL FCP monomers would have needed to incorporate eight additional Ddx pigment molecules, which would have yielded a total of 26 pigments per FCP monomer. Moreover, while in LHCIIb one V1 site per monomer is present (Liu et al., 2004), in the FCP monomers approximately seven V1-like sites would be needed to bind the excitonically inactive Ddx, assuming that one L2-like site incorporates the small pool of Ddx that transfers energy to Chl *a*. Such an assumption, a total pigment-binding capacity of 26 pigments and seven V1-like sites per monomer, is highly unlikely, especially when taking into account that so far no evidence

for further pigment-binding sites apart from the 18 known sites (Premvardhan et al., 2010) has occurred. Nevertheless, regarding the relatedness of LHC and FCP (Green and Durnford, 1996; Koziol et al., 2007), a V1-like site might exist in the FCP that binds a minor amount of Ddx cycle pigments, in addition to the Ddx molecules bound to an L2-like site and those dissolved in the MGDG shield.

In vascular plants, an increase of the xanthophyll cycle pigment pool can also be observed in HL-grown plants, albeit to a lesser extent (Demmig-Adams et al., 1996; Verhoeven et al., 1999; Demmig-Adams and Adams, 2006). In the Arabidopsis (*Arabidopsis thaliana*) *sChyb* mutant, β-carotene carboxylase is overexpressed, which results in a 3-fold increase of the Vx cycle pigment pool (Johnson et al., 2007). The authors showed that all of these additional pigments were found in an oligomeric LHCII fraction, which contained 90% of the total Vx cycle pigment pool. Although this situation strongly resembles our results obtained for the two diatoms, Johnson et al. (2007) suggested a protein binding of the additional Vx cycle pigments to the trimeric LHCIIb. This is clearly different from our observation that the majority of the HL-synthesized Ddx is likely dissolved in an MGDG shield surrounding the FCP. A possible explanation for this discrepancy is provided by the fact that the Ddx cycle pigment pool in the diatoms was five times larger than the Vx cycle pigment pool in the stressed Arabidopsis *sChyb* mutant calculated on a Chl *a* basis. An important finding of this work is that a difference exists between "free" xanthophyll cycle pigments in the free pigment fraction of a Suc gradient or anion-exchange chromatography separation and those free xanthophyll cycle pigments that are purified with the antenna protein fractions but are lipid dissolved within a lipid shield surrounding the respective protein complexes. Such a detailed differentiation has not been done for vascular plants, but a recent study, using spinach (*Spinacia oleracea*) thylakoids, demonstrated that the amount of LHCIIb-associated Vx decreases with a decreasing concentration of MGDG. This indicates that at least a minor part of the Vx cycle pigments is dissolved in an MGDG shield surrounding the PSII antenna (Schaller et al., 2010). Generally, in vascular plants, the differentiation of Vx cycle pigments into lipid-dissolved or protein-bound pigments is hampered by two main considerations. First, the carotenoids of the LHCIIb, lutein, neoxanthin, Vx, antheraxanthin, and Zx, all exhibit comparable absorption spectra. In diatoms, Ddx and Dtx show clearly distinct absorption properties compared with the only other carotenoid in the FCP, Fx. Second, the amount of Vx cycle pigments does not reach the values of the Ddx cycle pigments. Future measurements have to clarify if the Vx cycle pigment content in vascular plants is limited by the availability of the Vx cycle pigment-binding sites V1 and L2 or if, as in diatoms, under certain stress conditions the Vx cycle pigments might be dissolved in larger amounts within the lipid shield of the antenna complexes.

Thus far, most studies on the lipids of antenna complexes have focused on the intrinsic lipids of the LHCII. Although MGDG does not seem to be a structural component of the LHCII, some experimental results point to a specific role of MGDG in the formation and stabilization of higher order structures of LHCII (Reinsberg et al., 2000; Simidjiev et al., 2000; Yang et al., 2006). Recently, Schaller et al. (2010) demonstrated that MGDG is needed to trigger the deepoxidation of Vx in the isolated LHCII. These authors concluded that, in the native membrane, MGDG realizes the solubilization of Vx and targets the Vx deepoxidase to the LHCII, where the majority of the xanthophyll cycle pigments are located. The localization of Ddx cycle pigments within an antenna-associated MGDG phase is in line with this study. Accordingly, in HL-cultivated diatoms, the majority of Ddx cycle pigments are localized in the exact lipid environment that allows very efficient and fast operation of the Ddx cycle.

In in vitro experiments using isolated MGDG and Ddx, a lipid-to-pigment ratio of 6 to 7 was necessary to completely solubilize the pigment (Goss et al., 2005, 2007). However, in our experiments here, we determined a ratio of MGDG per Ddx of only 1.5 in the FCPs isolated from HL-cultivated algae. Although this MGDG-to-Ddx ratio is significantly lower than the optimum ratio, it seems to be sufficient to enable efficient xanthophyll cycling for several different reasons. First, a part of the Ddx cycle pigment pool might be dissolved within the lipid PC, which is also tightly bound to the FCP. Second, it has been shown that efficient Ddx deepoxidation can already be observed at MGDG-to-Ddx ratios of 2 and lower in in vitro enzyme assays (Goss et al., 2005). Third, Schaller et al. (2010) were able to show that xanthophyll solubilization in the natural membrane occurs at much lower MGDG concentrations than in in vitro experiments.

The significant amount of PC in the diatom FCPs is noteworthy, as in vascular plants PC does not even represent a thylakoid lipid (Moreau et al., 1998). However, diatoms generally contain a high PC content (Kates, 1987; Vieler et al., 2007) as well as significant concentrations of the lipid phosphatidylsulfocholine (Bisseret et al., 1984), which is chromatographically indistinguishable from PC (Anderson et al., 1978). Our data here suggest that PC/phosphatidylsulfocholine are tightly associated with the FCP and may be important for its function and structure.

Function of the Lipid-Dissolved Ddx Cycle Pigments

The majority of Ddx and Dtx, which are synthesized during HL cultivation of diatom cells, are likely localized within a special MGDG shield surrounding the FCP complexes. Numerous studies on vascular plants have shown that different pools of the deepoxidized Vx cycle pigment Zx exist in the thylakoid membrane (Havaux et al., 1991; Eskling et al., 1997; Havaux and Niyogi, 1999; Kalituho et al., 2007). While a part of Zx

replaces Vx in its binding to the LHCII, another part remains in the lipid phase of the membrane, where it acts as an important antioxidant (i.e. prevents lipid peroxidation), which may be triggered by the presence of ROS (Böhm et al., 1997; Edge et al., 1997; Havaux et al., 2007). Interestingly, it was shown recently in the LHCII antenna-free *ch1* mutant of Arabidopsis that the antioxidant potential is strongly reduced, when Zx is randomly distributed within the lipids, without being in close association with the PSII core complex and the PSII antenna (Dall'Osto et al., 2010). The authors suggested that light-harvesting complexes with their associated Zx molecules might form an antioxidant protective shield around the photosystem, thus preventing the PSII core from being attacked by peroxylipids during photooxidative stress. We assume a similar function for the additional Ddx and especially Dtx molecules synthesized in HL-cultivated *C. meneghiniana* and *P. tricornutum* cells. Their localization in the lipid interface between the peripheral antenna and the core complexes may facilitate the detoxification of singlet oxygen ($^1O_2$), which is formed through the interaction with antenna-based triplet Chl *a* (^{3}Chl *a*) molecules (Siefermann-Harms, 1987; Mozzo et al., 2008). This is especially important for diatoms, as these algae contain high concentrations of polyunsaturated fatty acids (Berge et al., 1995; Guschina and Harwood, 2006; Vieler et al., 2007), which are a primary target for $^1O_2$. In addition, the high concentration of Ddx and Dtx within the antenna-based MGDG shield might be able to prevent an attack of peroxylipids on the PSII core, as suggested by Dall'Osto et al. (2010) for vascular plants. The reduction of the HTL-2 band in TL measurements of Dtx-enriched thylakoids versus Ddx-enriched thylakoids depicted in this study provides, to our knowledge, the first indication that Dtx, like Zx in vascular plants, is a better antioxidant than Ddx (Vx in vascular plants). Future measurements will be needed to verify this hypothesis.

In addition to the hypothetical function in ROS detoxification, the Ddx/Dtx MGDG domain may serve another important function. According to the results of Lohr and Wilhelm (1999), photoprotective Ddx cycle pigments, which are synthesized during HL illumination of diatom cells, are transferred into light-harvesting pigments during LL phases following the HL illumination. Thus, the Ddx/Dtx-containing MGDG phase may act as a reservoir providing the substrates for the enzymes of the biosynthesis pathway leading to the main light-harvesting pigment Fx.

Function of the Protein-Bound Ddx Cycle Pigments within PSI

According to our results, Ddx bound to the Lhcr proteins connected with the PSI core complex serves as an accessory pigment. Once Ddx has been replaced by Dtx in its protein binding, Dtx might exert an important photoprotective function in PSI other than NPQ. This is based on the observation that a damaged PSI core, in contrast to PSII, must be completely rebuilt, which might take several days in vivo (Zhang and Scheller, 2004). Effective protection of PSI against damage is provided by the LHCI in vascular plants (Alboresi et al., 2009). Carotenoid molecules located within the LHCI scavenge ^{3}Chl *a*, which is preferentially generated by the red Chls. Based on these results, we propose a similar mechanism for the PSI of diatoms, where Dtx bound to the Lhcr proteins may serve to scavenge ^{3}Chl *a* and $^1O_2$ before oxidative damage to the PSI core takes place. Note that for the detoxification of ^{3}Chl *a*, Dtx and Chl *a* must be in close proximity, which cannot be realized by the lipid-dissolved Dtx molecules. It is also possible that the Ddx cycle pigments in PSI act as scavengers for superoxide, which may be produced by pseudocyclic electron flow during HL illumination, albeit at a lower rate than in vascular plants (Wilhelm et al., 2006). A comparable function for xanthophylls has been described for neoxanthin in the LHCII of vascular plants by Dall'Osto et al. (2007).

Function of the Protein-Bound Ddx Cycle Pigments within the Peripheral Antenna

The protein-bound Ddx cycle pigments of the peripheral antenna may participate in the process of NPQ. These special pigments are enriched in the FCPa complex compared with FCPb. The existence of different pools of protein-bound Dtx is in line with the observation that NPQ in diatoms can be heterogeneous and may be localized in different antenna complexes (i.e. a peripheral antenna complex and an FCP in closer proximity to the PSII core complexes; Grouneva et al., 2008; Miloslavina et al., 2009). It is also interesting that the FCPa complex, which is enriched in Ddx cycle pigments, was shown to undergo strong fluorescence quenching in the presence of Dtx (Gundermann and Büchel, 2008).

Although the linear correlation between the amount of Dtx and NPQ is lost in continuous HL-cultivated diatom cultures (Schumann et al., 2007), an increased Ddx cycle pool generated during an intermittent light regime correlates perfectly with an increased NPQ (Lavaud et al., 2002). There, the additionally synthesized Ddx exhibits an absorption maximum at 495 nm (Lavaud et al., 2003), in contrast to the absorption maximum of 488 nm obtained in this study for HL-synthesized Ddx. This indicates that intermittent illumination leads to the formation of Ddx, which is presumably protein bound to an L2-like site. Therefore, when assessing the quenching capability of diatoms, it is necessary to determine both the total amount of Ddx cycle pigments and their distribution and localization within the different pigment protein complexes. The localization of Ddx cycle pigments must be compared with changes in the protein composition of the peripheral antenna, with a special focus on changes in the amount of the Lhcx (also called Lhcsr or Li818) and probably also Lhcr proteins. The

Lhcx proteins in particular, which were shown to be involved in NPQ in green algae (Peers et al., 2009), seem to be of similar importance for the process of NPQ in diatoms (Zhu and Green, 2010).

CONCLUSION

In this study, three different Ddx cycle pigment pools were identified. Two are bound to special antenna proteins within the PSI and the FCP, respectively, while the largest pool of HL-synthesized Ddx cycle pigments is most likely localized within an MGDG shield surrounding the FCP. This spatial separation indicates a functional heterogeneity of the respective Ddx cycle pigments. It is assumed that protein-bound Ddx cycle pigments within the peripheral antenna participate in the mechanism of NPQ. Protein-bound Ddx cycle pigments in PSI might function as light-harvesting pigments and, in addition, might play a role in the photoprotection of PSI via detoxification of ^{3}Chl *a* and $^1O_2$. For the lipid-dissolved Ddx cycle pigments, an antioxidant function is hypothesized, where Dtx scavenges $^1O_2$ and peroxy-lipids. Furthermore, the existence of different Ddx cycle pigment pools has implications for the architecture of the thylakoid membrane. While Ddx interacts with MGDG, the large amounts of SQDG, which are present in the membrane, are separated from the Ddx cycle pigments. This means that the thylakoid membrane is differentiated into parts that are enriched in SQDG and other areas where MGDG, FCPs, and Ddx cycle pigments are densely packed. Accordingly, specific thylakoid membrane domains might exist in diatoms, whose existence has to be investigated in future studies.

MATERIALS AND METHODS

Cultivation of Algae

Cyclotella meneghiniana (1020-1a; Sammlung von Algenkulturen Göttingen) was grown in F/2 medium according to Guillard (1975) with the following modifications: the salt content of the medium was reduced to 50%, and the amount of silica was doubled. *Phaeodactylum tricornutum* (1090-1a; Sammlung von Algenkulturen Göttingen) was grown in modified ASP2 medium according to Lohr and Wilhelm (2001). Cells were cultivated as sterile airlift cultures (*C. meneghiniana*) or batch cultures (*P. tricornutum*) in a light/dark regime of 14/10 h at a temperature of 20°C. The intensity of the light was set to 10 to 15 μmol m^{-2} s^{-1} PAR for LL conditions or to 160 to 180 μmol m^{-2} s^{-1} for HL conditions.

Preparation of Thylakoids and Pigment Protein Complexes

Isolation of thylakoids was performed as described by Lepetit et al. (2007). In our experiments, the cells for thylakoid isolation were taken directly from the light conditions used for cultivation (see above). In one experiment, HL-cultivated cells were LL adapted for 45 min to allow the complete epoxidation of Dtx to Ddx before the preparation of the thylakoid membranes.

The isolation of pigment protein complexes followed the same protocol as applied by Lepetit et al. (2007). In brief, equal amounts of thylakoids were solubilized with DM (Roth) for 20 min on ice using a ratio of DM per Chl of 10, 40, or 80, corresponding to 0.3%, 1.2%, and 2.4% (w/v) DM, respectively. After centrifugation for 20 min at 40,000*g* and 4°C, 200 to 250 μL of the supernatant was immediately loaded onto a linear Suc gradient (0.1–0.7 M Suc, in thylakoid isolation medium B; Lepetit et al., 2007). Ultracentrifugation was carried out for 18 h at 110,000*g* and 4°C. Fractions containing the different isolated pigment protein complexes were collected with a syringe and immediately used for the measurements.

Absorption Spectroscopy

Chl determination was carried out according to Jeffrey and Humphrey (1975). Absorption spectra of cells, thylakoids, and pigment protein complexes were recorded with a Specord M 500 spectrophotometer (Zeiss) in a wavelength range from 350 to 750 nm. The bandwidth was set to 1 nm.

For the determination of the xanthophyll absorption spectra in MGDG and DM, Ddx and Dtx were purified according to Goss et al. (2005). Purified Ddx (with a final concentration of 0.4 μM) was then added to different amounts of DM (for further details, see "Results") and injected in thylakoid isolation medium B supplemented with 0.2 M Suc. Addition of Suc to the medium was used to simulate the Suc concentration of the Suc density gradient, where the FCP fractions were located. In the case of the Ddx or Ddx/Dtx absorption in MGDG, 0.4 μM pigment was added to 2.9 μM MGDG, mixed, and injected into thylakoid isolation medium B.

77 K Fluorescence Spectroscopy

Low-temperature fluorescence excitation spectra of the different samples dissolved in 60% glycerin (v/v) were recorded with an F-3000 fluorescence spectrophotometer (Hitachi) at 77 K using a Chl concentration of 1 μg mL^{-1}. The emission wavelength was set to the Chl *a* fluorescence emission maximum, which was determined before the measurements of the excitation spectra. Excitation spectra were recorded in the wavelength range from 380 to 570 nm with settings of the excitation and emission bandwidths of 3 and 10 nm, respectively. The excitation spectra were corrected using a rhodamine B spectrum as a reference.

Pigment Analysis by HPLC

Pigments of thylakoids and pigment protein complexes were extracted and analyzed by HPLC as described by Lepetit et al. (2007). For the pigment extraction of intact cells, 5 mL of the algal culture was harvested on glass fiber filters (GF 6, 55 mm diameter; Schleicher & Schüll) and extracted with HPLC extraction medium composed of 90% methanol:0.2 M ammonium acetate (9:1, v/v) and 10% ethyl acetate by mechanical disruption in a glass bead homogenizer (MSK; Braun) for 20 s. The pigment extracts were centrifuged for 2 min at 20,000*g* and immediately injected into the HPLC column.

Lipid Analysis

Lipid extraction, separation, and quantification were performed essentially as described by Goss et al. (2009). Isolated lipids from cells, thylakoids, and purified pigment protein complexes, corresponding to a Chl *a* concentration of 2 μg, were subjected to two HPTLC Silica 60 plates (Merck). The first plate was developed in an eluent system according to Mock and Kroon (2002), which was composed of methylacetate:isopropanol:$CHCl_3$:methanol:KCl (0.25%, w/v) in the ratio 25:25:25:10:4 (v/v). The eluent system for the second plate was chosen according to Ventrella et al. (2007) and consisted of $CHCl_3$:methanol:acetic acid:water in the ratio 75:13:9:3 (v/v). The use of two different eluent systems was necessary in order to separate all lipid classes of cells, thylakoid membranes, and isolated pigment protein complexes. After visualization of the lipids with primuline (direct yellow 59; Sigma-Aldrich), the lipid spots were quantitatively assessed using a Biometra digital imaging system in combination with the program Biodoc Analyze (Biometra). The pixel intensity of the lipid spots was compared with the pixel intensity of marker lipids with a defined concentration in order to calculate the lipid concentration per Chl *a* of the different samples. The marker lipids MGDG, DGDG, SQDG, PG, and phosphatidylethanolamine were purchased from Lipid Products, while PC was obtained from Sigma-Aldrich.

Protein Analysis

The different fractions of the gradient centrifugation were concentrated using Amicon ultrafiltration devices (10-kD molecular mass cutoff; Millipore).

Proteins were separated by SDS-PAGE as described by Lepetit et al. (2007). Bands of interest were excised and in-gel digested with trypsin (grade mass spectrometry approved; Serva Electrophoresis). The in-gel digests were separated on a nanoAcquity UPLC device (Waters) coupled online to a LTQ Orbitrap XL ETD mass spectrometer equipped with a nanoESI source (Thermo Fisher Scientific). The temperature of the transfer capillary was set to 200°C, and the tube lens voltage was 120 V. The ion spray voltage (1.5 kV) was applied to a PicoTip online nanoESI emitter (standard coating, o.d. 360/20 μm, and tip internal diameter 10 μm; New Objective). The tryptic peptides were dissolved in eluent A (20 μL) and injected by the autosampler at a flow rate of 10 μL min^{-1} onto a trap column (nanoAcquity UPLC Symmetry C18, 180 μm $\times$ 20 mm, particle diameter 1.7 μm) and then separated on a nanoAcquity UPLC BEH130 C18 (100 μm $\times$ 100 mm, particle diameter 5 μm) using two linear gradients from 3% to 50% acetonitrile over 30 min and then up to 85% over 3 min. Eluent A was aqueous 0.1% formic acid, and eluent B was acetonitrile with 0.1% formic acid. Tandem mass spectra were acquired in data-dependent acquisition mode using collision-induced dissociation activation in the linear ion trap (isolation width 2, normalized collision energy 35%, activation Q 0.25, activation time 30 ms). The ion trap scan was repeated for the top six peaks with charge states of 2 or higher. Data were acquired with Xcalibur (version 2.0.7). The acquired tandem mass spectra were analyzed automatically with MASCOT or the Sequest (Proteome Discover 1.0; Thermo Fisher Scientific) software package in UniProt or the *P. tricornutum* database (Joint Genome Institute *P. tricornutum* version 2.0; http://genome.jgi-psf.org/Phatr2/Phatr2.home.html), allowing up to three missed cleavage sites, a mass tolerance of 15 ppm for precursor ion scans, and a mass tolerance of 0.8 D for the product ion scans.

TL

Freshly isolated thylakoids of LL-adapted HL-grown *P. tricornutum* cells were incubated in the dark for 15 min in reaction medium, pH 5.2 (350 mM sorbitol, 10 mM NaCl, 15 mM $MgCl_2$, and 40 mM MES), in the presence of 30 mM ascorbate. These conditions were shown to trigger the deepoxidation of Vx to Zx without inducing reversible NPQ in vascular plants (Goss et al., 2008). In control thylakoids, ascorbate was omitted to avoid Ddx deepoxidation. After the incubation, the thylakoids were centrifuged at 40,000*g* for 15 min and resuspended in reaction medium, pH 7.6. Resuspended thylakoids with a Chl concentration of 20 μg mL^{-1} were illuminated for 10 min with 5,000 μmol m^{-2} s^{-1} PAR, and fluorescence kinetics were recorded with a PAM 101 fluorometer (Walz; Gilbert et al., 2004). The thylakoids were dark adapted for 5 min at 20°C, cooled to 2°C, and illuminated with one single turnover flash to induce charge separation in PSII. Afterward, the samples were heated from 2°C to 180°C at a rate of 20°C min^{-1}, and light emission was detected by means of channel photomultipliers.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. 77 K fluorescence excitation spectra of LL and HL FCPa and FCPb of *C. meneghiniana*.

Supplemental Table S1. Identification of antenna proteins within the FCP and the PSI complex of *P. tricornutum* by peptide mass fingerprint and tandem mass spectrometry.

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