

Dynamic response of the transcriptome of a psychrophilic diatom, *Chaetoceros neogracile*, to high irradiance

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Abstract Large-scale RNA profiling revealed that high irradiance differentially regulated 577 out of 1,439 non-redundant genes of the Antarctic marine diatom *Chaetoceros neogracile*, represented on a custom cDNA chip, during 6 h of treatment. Among genes that were up- or down-regulated more than twofold within 30 min of treatment (310/1,439), about half displayed an acclimatory response during 6 h under high light. Expression of the remaining non-acclimatory genes also rapidly returned to initial levels within 30 min following a shift to low irradiance. High light altered expression of most of the photosynthesis genes (48/70), in contrast to genes in other functional categories. In addition, opposite response patterns were provoked in genes encoding fucoxanthin chlorophyll *a/c* binding protein (FCP), the main component of the diatom light-harvesting complex; high irradiance caused a decrease in expression of most *FCP* genes, but drove the rapid and specific up-regulation of ten others. *C. neogracile* responded very promptly to a change in light intensity by rapidly adjusting the tran-

script levels of *FCP* genes up-regulated by high light, and these dynamic adjustments coincided well with diatoxanthin (Dtx) levels formed by the xanthophyll cycle under the same conditions. The observation that the non-photochemical quenching (NPQ) capacity of this polar diatom was highly dependent on Dtx, which could bind to FCP and trigger NPQ, suggests that the up-regulated *FCP* gene products may participate in a photoprotective process as Dtx-binding proteins.

Keywords Transcriptome · Antarctic diatom · High irradiance · Xanthophyll cycle · Fucoxanthin chlorophyll *a/c* binding protein · NPQ (non-photochemical quenching)

Abbreviations

DTT	Dithiothreitol
NPQ	Non-photochemical quenching
Ddx	Diadinoxanthin
Dtx	Diatoxanthin
DDE	Diadinoxanthin de-epoxidase
FCP	Fucoxanthin chlorophyll <i>a/c</i> binding protein
LHC	Light-harvesting complex

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Introduction

The ocean is the largest ecosystem on Earth, most of it comprising very cold environments (Morgan-Kiss et al. 2006). Despite its low average primary productivity, the ocean makes the largest contribution to Earth's total net primary production because of its huge size.

Chromophytes are a very prevalent group of oxygenic phototrophs found in low-temperature environments. Diatoms, in particular, are the dominant chromophytes in

marine and sea ice habitats where the amount of light available to unicellular photosynthetic organisms is extremely variable (Lavaud et al. 2002a). For example, light can be a limiting factor when photosynthetic cells are located deep in the water column, causing them to invest energy in maximizing light absorption and photosynthetic efficiency. However, when residing close to the surface, cells can be exposed to irradiance that is 10- to 20-fold higher than that required for photosynthesis (van de Poll et al. 2005).

High irradiance negatively affects the viability, growth, and productivity of photosynthetic organisms. The photoprotective and photoacclimatory responses of plants and algae to high light have been studied at the molecular, cellular and whole-organism level. The acclimation processes essential for functional photosynthesis include enhanced repair, reduction in the number of light-harvesting antennae molecules and changes in PSI/PSII stoichiometry (Allen and Pfannschmidt 2000; Jin et al. 2003). Protection from high irradiance can be achieved by non-photochemical dissipation of absorbed light energy as heat and by alternative electron flow around PSI or to molecular oxygen (Müller et al. 2001; Ledford and Niyogi 2005). A variety of carotenoids are also known to play important roles in photoprotection. For example, xanthophylls are involved in the process of non-photochemical energy dissipation (Demmig-Adams et al. 1990; Havaux and Niyogi 1999; Niyogi 1999; Horton et al. 2005).

As a photosynthetic psychrophilic alga, *C. neogracile* is an important contributor to biomass production in the extreme environment of the Southern Ocean (Choi et al. 2008). Previously, we constructed the first expressed sequence tag (EST) library to analyze the transcriptome of this Antarctic diatom (Jung et al. 2007). A cDNA chip was customized based on the EST data and was used to study broad-scale changes in the genomic profile of *C. neogracile* in response to thermal stress. This analysis revealed that the transcriptome showed a coordinated response to thermal changes and that molecular homeostasis was rapidly established (Hwang et al. 2008).

Several researchers have investigated high irradiance- and oxidative stress-induced changes in the transcriptomes of mesophiles (Hihara et al. 2001; Rossel et al. 2002; Seki et al. 2002; Park et al. 2006). However, only a few studies have been reported thus far regarding the response of the transcriptomes of photosynthetic psychrophilic organisms to environmental stimuli (Mock and Valentin 2004; Mock et al. 2006; Hwang et al. 2008; Krell et al. 2008).

Previously, the *C. neogracile* transcriptome displayed an acclimatory response to thermal stress over time (Hwang et al. 2008). Therefore, we examined the expression profiles of those genes that were rapidly up- or down-regulated following exposure to high irradiance to determine whether this organism also exhibits molecular acclimation to light

stress. In the present study, we performed large-scale comparative and quantitative analyses of the *C. neogracile* transcriptome under different light regimes using the aforementioned cDNA chip. We also investigated the *FCP* genes whose products may play a photoprotective role in Antarctic diatoms in general under high irradiance. The concerted induction of *Dtx*, the xanthophyll cycle pigment, and NPQ under high light were also examined.

Materials and methods

Algal strain and growth conditions

The Antarctic marine diatom *Chaetoceros neogracile* (KOPRI AnM0002) was kindly provided by the Korea Ocean Polar Research Institute (KOPRI; Incheon, Korea) and cultured as previously described (Jung et al. 2007). For analysis of the growth rate, 400 ml of cells in a modified f/2 medium were axenically cultured in growth chambers under low, moderate, and high irradiance (20, 200 and 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively) at approximately 4°C. Cell densities were determined by microscopic counting using hemacytometer (Neubauer, Marienfeld, Germany) at indicated day and are presented as the means \pm SD. The photon flux density (PFD) was measured using a quantum meter (Li-Cor, Lincoln, NE). The specific growth rate (μ) was determined using the following equation $\mu = \text{Ln}(N2/N1)/(t1 - t2)$, where $N1$ and $N2$ are cell numbers at time $t1$ and $t2$, respectively.

For microarray experiments, 5-day-old cells were transferred from a light intensity of 20–600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 30 min, 60 min or 6 h. High light treated cells were transferred back to an initial growth chamber of low irradiance at time zero to induce recovery for 30 min, 60 min or 3 h. Dithiothreitol (DTT), an inhibitor of diadinoxanthin de-epoxidase was used at concentrations of 0.5 mM to analyze the pigment and chlorophyll fluorescence.

Chlorophyll fluorescence measurements and NPQ analysis

Chlorophyll (Chl) fluorescence was measured using an FMS2 pulse-amplitude-modulation fluorometer (Hansatech Instruments Ltd., UK). *C. neogracile* cells were dark-adapted for 15 min at 4°C prior to measurement, then subjected to a 0.7-s flash of saturated white light ($3,000 \mu\text{mol m}^{-2} \text{s}^{-1}$) in order to measure F_m . Algal cultures were then illuminated with white actinic light (approximately $200 \mu\text{mol m}^{-2} \text{s}^{-1}$) for 10 min followed by a 10-min dark recovery period, during which the cells were subjected once per minute to a flash of saturated white light of the same intensity used to measure F_m in order to determine F_m' .

NPQ was determined by the formula $(F_m - F_m')/F_m$, where F_m' is the maximum PSII fluorescence in the light-adapted state and F_m is the maximum PSII fluorescence in the dark-adapted state.

Pigment analysis

Two milliliters of algal suspension were centrifuged in an Eppendorf centrifuge at 14,000 rpm for 2 min. Pigments were extracted from cells by the addition of 200 μ L filtered 90% acetone to the pellet followed by vortexing at maximum speed for 1 min. The extract was centrifuged at 14,000 rpm and the resulting supernatant was filtered through a 0.2 μ m nylon filter. The filtrate was subjected to analysis on a Shimadzu Prominence HPLC model LC-20AD equipped with a Waters Spherisorb S5 ODS2 4.6 \times 250-mm cartridge column. Solvent concentrations were: 90% acetonitrile, 10% water and 0.01% triethylamine from 0 to 1 min; 86% acetonitrile, 9.6% water, 0.01% triethylamine and 5% ethyl acetate from 2 to 14 min; and 100% ethyl acetate from 15 to 21 min. A post-run was performed for 9 min using the initial solvent mixture. The flow rate was maintained at 1.0 mL per minute throughout the run. Pigments were detected at a wavelength of 445 nm using a reference wavelength of 550 nm. Concentrations of individual pigments were determined from HPLC profiles of chlorophyll and carotenoid calibration standards. Pigments were identified by retention time and absorption spectra with reference to pigment standards (DHI 14C Centralen; Denmark).

cDNA microarray construction and experiment

We previously prepared custom cDNA chips by spotting polymerase chain reaction (PCR) amplicons of 1,744 ESTs representing 1,439 different isolated genes (Hwang et al. 2008). These chips were used to investigate changes in the *C. neogracile* transcriptome in response to high irradiance. Total RNA was extracted from the samples described above using TRI reagent (Molecular Research Center, Inc., USA) and purified with RNeasy Mini Columns (Qiagen, Valencia, CA, USA). For microarray experiments, cDNA was labeled with the fluorescent dye Cy-3 (low light samples) or Cy-5 (high light samples in time course experiments). All of the experiments were carried out as previously described by Hwang et al. (2008).

Data analysis

Graphing and statistical analyses of microarray data, as well as scatter plot analysis, were performed using Microsoft Excel. A *k*-means clustering analysis was carried out using EPCLUST software from the European Bioinformat-

ics Institute (<http://www.bioinf.ebc.ee/EP/EP/EPCLUST/>). For distance measure, Euclidean distances were used to cluster the data and the number of clusters employed for analysis was 6 ($k = 6$). The deduced amino acids of *LHCs* from different diatoms including green algal LI818 protein were aligned with the ClustalW program and a phylogenetic tree was constructed using the distance method as implemented in MEGA 4.0 (<http://www.megasoftware.net>) and Neighbor-Joining clustering algorithm. The sequences (accession number given in parentheses) of *CnFCPs* (EL622214, EL621966, EL621146, EL621467, EL621834, EL622068, EL622410, *C. neogracile*), *FCP2* and *FCP6* (CAA04178.1, CAA04402.1, *Cyclotella cryptica*), *TpLhcs* (XP_002295258.1, EED87488.1 XP_002290755.1, XP_002287075.1, XP_002295183.1, XP_002294608.1, XP_002294608.1, *Thalassiosira pseudonana*), *PtLhcs* (EEC 51450.1, XP_002182760.1, XP_002183709.1, XP_002178860.1, *Phaeodactylum tricorutum*), *LI818* (XP_001696064.1, *Chlamydomonas reinhardtii*) were used.

Quantitative real-time RT-PCR

PCR was performed using a QuantiTech SYBR Green RT-PCR Kit (Qiagen) for 45 cycles and the accumulation of fluorescent products was monitored using a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). All procedures were performed according to the manufacturers' instructions. Supplementary Data 1 summarizes the primer sequences used in this study.

Results and discussion

High light retards the growth of *C. neogracile*

Chaetoceros neogracile is a psychrophilic diatom carrying out oxygenic photosynthesis in the southern polar region where variation in environmental factors such as light and temperature is usually extreme (Thomas and Dieckmann 2002). We were interested in the molecular responses of this organism to high irradiance. Therefore, we cultured diatoms in growth chambers under low, moderate, and high irradiance (20, 200 and 600 μ mol photons $m^{-2} s^{-1}$) and examined growth (Fig. 1). Control cells grown under low light at 4°C showed sigmoidal growth, a typical growth pattern, with a fivefold increase in cell number in less than 7 days. While light of moderate intensity did not significantly affect growth as compared to the control, high irradiance caused a noticeable retardation of growth in 1-day-old cultures and limited the growth to 65–80% of that of the control over the period of growing time. The specific growth rate of the cells under 600 μ mol photons $m^{-2} s^{-1}$

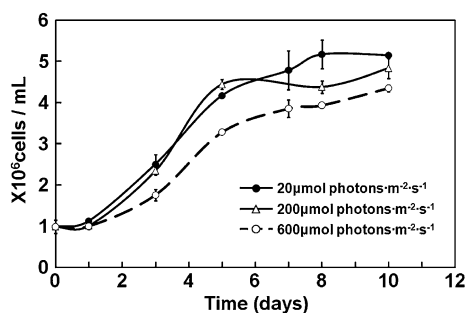


Fig. 1 Growth curves for *C. neogracile* under different irradiances. Cells were cultured in growth chambers at three different irradiances (20, 200 or 600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 4°C for 10 days and the number of cells was determined on the indicated day by counting under a microscope. The data presented are the mean \pm SD of three independent experiments

was significantly lower at the initial stage of growing period than those of cells under 20 and 200 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, but it was recovered during the late log phase of growth and then declined after (Supplementary Data 4). Due to its negative effect on the physiology of *C. neogracile*, the highest light intensity (600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) was selected for study of the transcriptional response of this diatom to light stress.

The *C. neogracile* transcriptome responds dynamically to high irradiance

We used a previously prepared cDNA chip containing 1,744 ESTs representing 1,439 different genes (Hwang et al. 2008) to investigate alterations in the *C. neogracile* transcriptome in response to high light. Inferred from the number of the predicted genes in *P. tricornutum* and *T. pseudonana*, where the whole genome has been fully sequenced, our chip is likely to represent about 14% of total genes of *C. neogracile*. The microarray experiment was performed with total RNA isolated from cells cultured at 4°C under low (20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) or high light (600 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$).

A twofold difference in relative transcript levels is a threshold commonly used in the analysis of microarray data to define differentially expressed genes. Therefore, we collected data for those genes that displayed more than a twofold difference in expression when compared to the level obtained under low light at any point during 6 h of culturing. As a result, we found that high irradiance differentially regulated more than 40% of the *C. neogracile* genes represented on the chip (577/1,439 non-redundant sequences). Upon further analysis, it was found that differentially expressed genes comprised slightly more of those that were up-regulated (325/577, 56%) than those that were down-regulated (252/577, 44%). We sorted these genes according

to the putative functional categories assigned to them in an earlier experiment (Jung et al. 2007). Among those genes represented on the chip, about 30–40% in most of the categories were differentially affected by the shift to high irradiance. Almost 70% (48/70) of the genes belonging to the photosynthetic group displayed differentially regulated expression (Fig. 2a). Overall, the total numbers of such genes within each functional group were similar. However, up-regulation was prominent in those genes involved in genetic information processing (53/66, 80%), followed by structural (4/6, 67%) and defense-related genes (10/16, 63%). High light-induced suppression was more evident in transport-related genes (16/24, 67%) and photosynthesis genes (29/48, 60%) (Fig. 2a).

We selected some of the genes displaying differential expression for real-time RT-PCR to confirm the microarray data. As shown in Fig. 2b, the expression patterns of several genes displayed in the microarray experiment were highly consistent with the results obtained by PCR. The correlation factor between the two sets of data was about 0.937, indicating the high reliability of the microarray data (Fig. 2c).

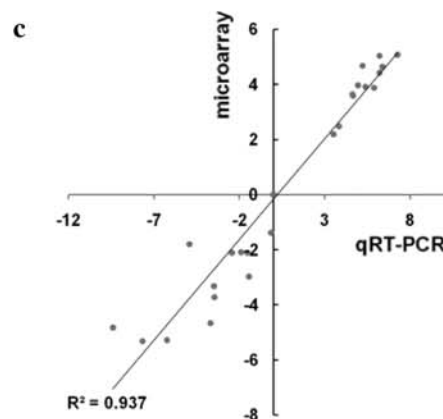
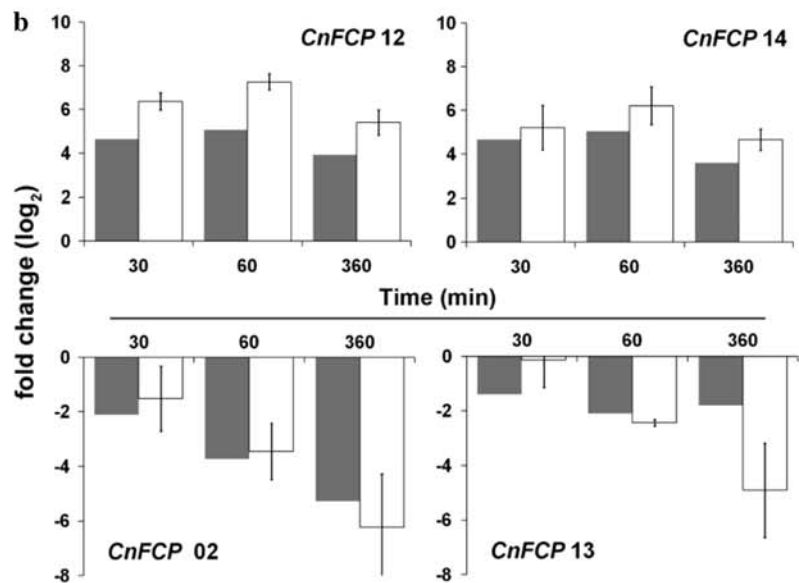
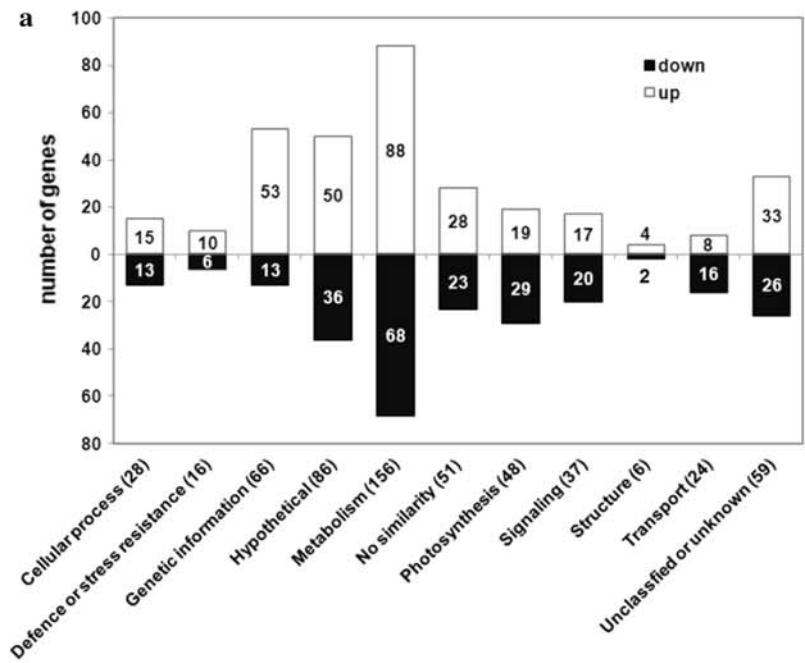
Some groups of *C. neogracile* genes showed a rapid response to the change in light intensity

Figure 3 shows the alterations in expression profiles induced by the upshift and subsequent downshift in light intensity over time for all of the genes represented on the chip. All of the genes were first arrayed on a continuum in rank order of descending fold change after 30 min under high light, then changes in expression were tracked in conjunction with treatment time, which was 6 h for high light and up to 3 h for recovery under low light. Many genes responded rapidly to high light, showing up to a 30-fold increase in expression level within 30 min, while many more genes responded gradually. Within 30 min of the downshift in light intensity, the transcript levels of most of the highly up-regulated genes were rapidly reduced, while down-regulated genes and slowly up-regulated genes did not appear to be significantly affected. These results indicate that the expression of genes that are rapidly up-regulated in response to high irradiance is in a dynamic balance that depends on light conditions.

Self-organizing map analysis of the response of the *C. neogracile* transcriptome to high irradiance

To further investigate the light stress response, a *k*-means clustering analysis was performed in order to group *C. neogracile* genes according to their similarity in expression profiles. The 1,439 genes represented on the chip were clustered into six different groups depending on the pattern

Fig. 2 Effects of high irradiance on the *C. neograticle* transcriptome. **a** Number of up- and down-regulated genes among differentially regulated genes in each functional category under high irradiance. The numbers of up-regulated and down-regulated genes are indicated by *white* and *black bars*, respectively. Total number of probes on the chip belonging to each functional category was presented in the bracket. **b** Validation of microarray data by real-time RT-PCR. Real-time RT-PCR was performed on those *CnFCP* genes that displayed a contrasting expression pattern in microarray experiments. Cells cultured in growth chambers under low irradiance ($20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) were transferred to a growth chamber under high irradiance ($600 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) at time zero and cultured for an additional 6 h. The relative expression ratio was calculated for each gene by comparing the expression value at time zero to that at the indicated time during high irradiance. The fold change in the amount of gene expression induced by high irradiance is expressed on the \log_2 scale. *Gray bar* and *white bar* represent the data from microarray and real-time RT-PCR, respectively. **c** Correlation between fold change in expression based on the cDNA microarray and real-time RT-PCR (qRT-PCR) data shown in Fig. 2c



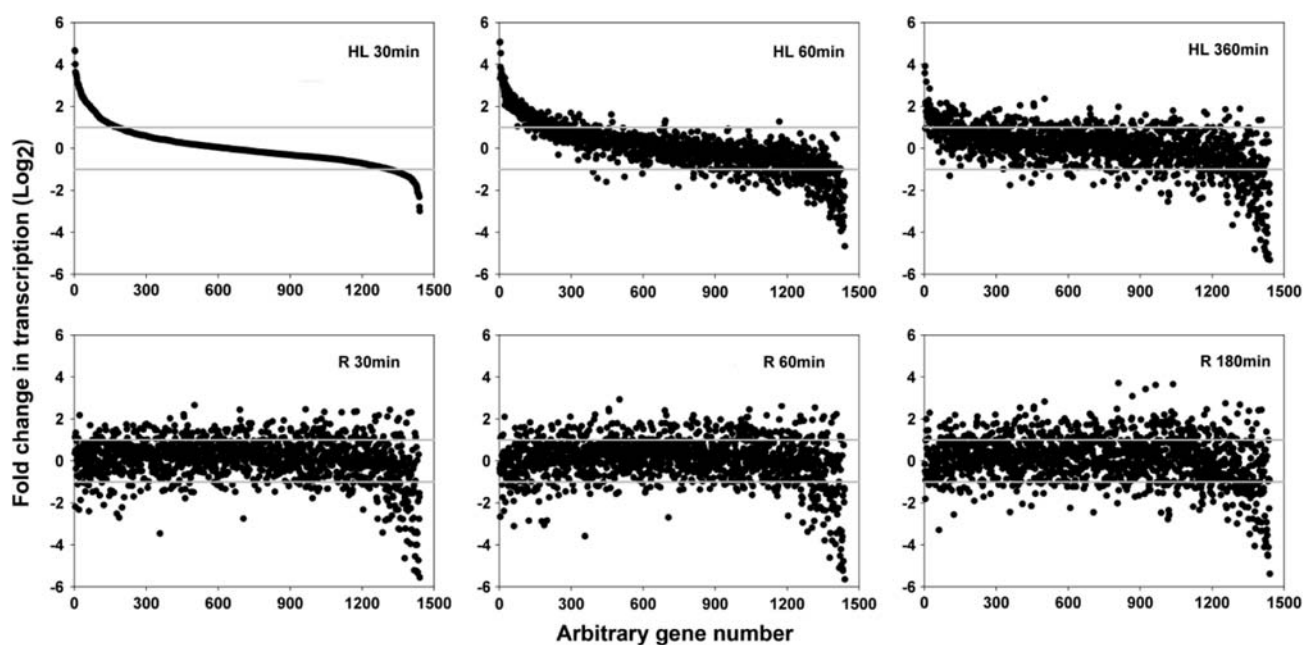


Fig. 3 Time-dependent changes in the *C. neogracile* RNA profile in response to two different light regimes. Cells cultured in a growth chamber under low irradiance were transferred to a growth chamber under high irradiance at time zero, then cultured for 6 h and returned to the first chamber to induce recovery. The relative expression ratio was calculated for each gene by comparing the expression value at time zero to that at the indicated time. The fold change in the amount

of gene expression induced by high irradiance is expressed on the \log_2 scale. All of the genes represented on the chip were first arrayed in rank descending order of fold change, then changes in expression level were tracked relative to time under high irradiance. *Gray lines* represent a twofold change in the ratio between expression level under high light (*HL*) and low light. *R* indicates recovery after the shift from high light to low light

in which their expression changed in response to the upshift in light intensity. Each cluster was visualized as a specific pattern of fold changes plotted against incubation time (Fig. 4). This self-organizing map analysis revealed that certain groups of genes rapidly and significantly responded to the change in light intensity. Highly and moderately up-regulated genes were grouped in two Clusters, A and B. Cluster C represents genes that were significantly down-regulated under high light while the group of genes in Cluster D were moderately down-regulated. However, a majority of the genes on the chip (900 out of 1,439) showed very slow or little light-dependent expression (Clusters E and F), emphasizing the relative importance of highly responsive genes.

The most highly responsive genes (Cluster A) included several genes for light-harvesting complex proteins, as well as genes for cell division proteins (FstHs), electron transport (succinate:ubiquinone oxidoreductase subunit 2, NADH dehydrogenase subunit 4), among others (see Supplementary Data 2 for complete results). Interestingly, some genes for transcription factors (HACA, Myb, etc.) and possible signaling components (GCN5-related *N*-acetyltransferase, histone deacetylase and ring finger protein 25) were included in this cluster. The group of genes in Cluster B, which were noticeably up-regulated but to a lesser degree, also contained possible signaling components such as zinc finger proteins, protein kinases and phosphatases,

and ubiquitin ligase, etc. Metabolism-related genes in this group included some genes of carbohydrate metabolism (phosphoglycerate kinase, pyruvate kinase, PEP carboxykinase, aldo-keto reductase, phosphoglucomutase, etc). The genes of glutathione metabolism (glutathione reductase, glutathione peroxidase, glutamate-cysteine ligase) and ribosome genes were found in this cluster as well. Seventy-five percent of the most rapidly down-regulated genes (Cluster C) were photosynthesis-related genes, including those for light-harvesting complex proteins, photosystem II extrinsic protein, triose-phosphate isomerase, etc. Another group of down-regulated genes (Cluster D) also included many genes for photosynthesis, such as light-harvesting complex proteins and FCPs. Several transporter genes (ABC transporter family protein, PEP/pyruvate translocator and formate transporter) and radical scavenging proteins (glutathione *S*-transferases and superoxide dismutases) were also present in group D. Cluster E represented a large number of genes that were slowly up-regulated to a lesser extent at 6 h of exposure to high light. This group contained most of the probe sets for ribosomal subunit genes on the chip. Along with several genes for translation initiation factors and heat-shock proteins, translation-related genes constituted more than 11% of the genes in Cluster E. These results indicate that a gradual increase in protein synthesis would be expected under high light.

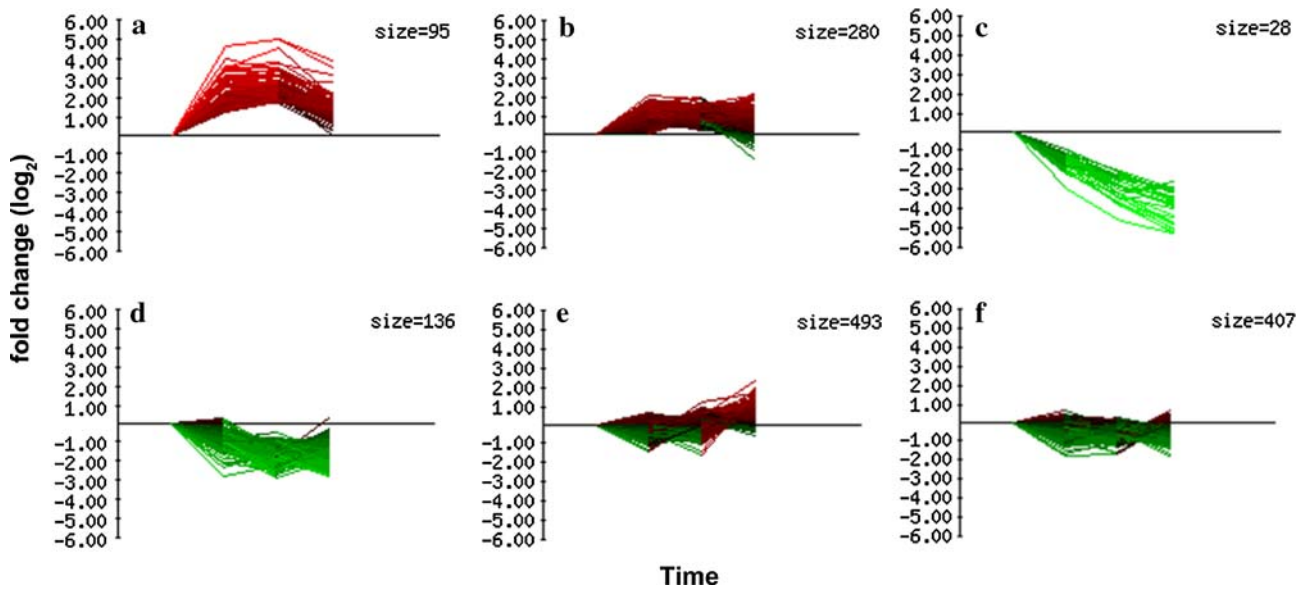


Fig. 4 Self-organizing map analysis of the *C. neogracile* RNA profile in response to high irradiance. A *k*-means clustering analysis was performed on the expression patterns of a total of 1,439 ESTs on the chip

under high irradiance. *Red* and *green* indicates an increase or a decrease in transcript abundance, respectively. The number of genes in each *k*-means cluster is indicated as its size

KEGG pathway mapping (<http://www.genome.jp/kegg/pathway.html>) was performed for the genes of each cluster (Supplementary Data 6) to predict high light-mediated metabolic changes. Around 11–39% of the genes of each cluster could not be analyzed for their roles since they were annotated to hypothetical and unknown proteins (the amount of hypothetical or unknown protein genes of each cluster is indicated as a percentage in Supplementary Data 2). Genes of most metabolic pathways including lipid, nucleotide turnover, energy production, etc., did not show any noticeable abundance in clusters of up- or down-regulated genes. Clusters of moderately and slowly up-regulated genes (Clusters B and E, respectively) contained more carbohydrate metabolism genes than clusters of down-regulated genes (Clusters C and D), with a comparable amount of genes still not affected by high irradiance (Cluster F). As mentioned above, prominent down-regulation in photosynthetic genes and up-regulation in translation-related genes were observed in cluster C and E, respectively. More of genes in amino acid metabolism, and protein folding, sorting and degradation processes were included in clusters of moderately and slowly up-regulated genes (Clusters B and E). However, a limited amount of genes analyzed makes it difficult to expect a reliable prediction for the metabolic change induced by high irradiance.

Acclimatory and light regime-dependent responses of high light-early responsive genes in *C. neogracile*

Since the *C. neogracile* transcriptome previously, displayed an acclimatory response to thermal stress over time (Hwang

et al. 2008), its molecular acclimation to light stress was also investigated. Figure 5 shows the alterations in expression profiles induced by the upshift and subsequent downshift in light intensity over time for rapidly up- or down-regulated genes represented on the chip within 30 min. About 45% (86/193) of the rapidly up-regulated genes and 41% (54/131) of the rapidly down-regulated genes showed an acclimatory response during 6 h under high light. When the culture was transferred back to low light after 6 h, the expression of 70% (75/107) of the up-regulated, non-acclimated genes was dynamically readjusted within 30 min, while only 16% (12/77) of the down-regulated, non-acclimated genes were affected (Fig. 5).

A possible photoprotective role for psychrophilic *FCP* genes and the Ddx–Dtx cycle against high irradiance

Various patterns of responses were displayed in such photosynthesis-related genes of *C. neogracile* represented on the chip as the genes of light-harvesting complex proteins and chloroplast ferredoxin NADP oxidoreductase, etc (Supplementary Data 3). For example, expression of many genes for light-harvesting complex proteins and photosystem II 12 kDa extrinsic protein were significantly reduced under high light stress but chloroplast ferredoxin NADP oxidoreductase genes and the subunit of chloroplast ATP synthase genes were not significantly changed under high irradiance.

In our analysis of the *C. neogracile* *FCP* genes represented on the chip, we found that a group of genes encoding *FCPs* responded to high light in opposite ways; some of them were up-regulated while others were down-regulated

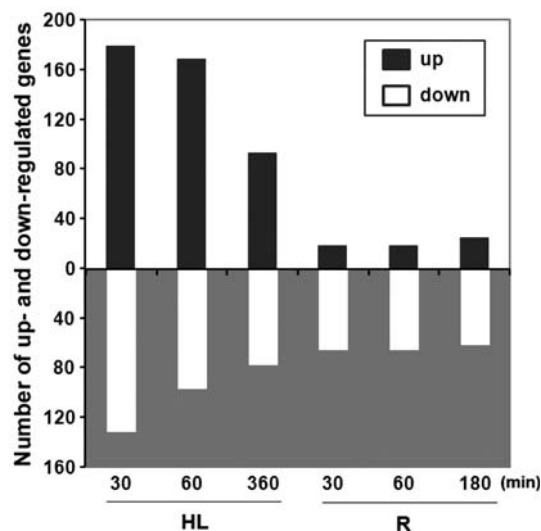


Fig. 5 Changes in expression of high light-early responsive genes to high irradiance. The total number of high light-early responsive genes showing more than twofold expression change under high light (HL) or during recovery (R) after the shift from high to low light was counted at the indicated time. Up- and down-regulated genes are represented by black and white bars, respectively

by the same environmental cue (Fig. 6a). FCPs are the main components of the diatom light-harvesting complex (LHC), and as such are important features of the photosynthetic apparatus. Their pigment composition is therefore significantly different from that of CAB, an LHC of green plants, in its enrichment with fucoxanthin and chlorophyll *a/c* as the main carotenoid and secondary chlorophyll, respectively (Wilhelm et al. 2006). Diatom LHCs collect light energy and funnel it to the photosystem reaction centers for photosynthesis in the same manner as LHCs in green plants (Pysznik and Gibbs 1992; Grossman et al. 1995). In addition to the function of FCPs in powering the light reactions of photosynthesis, another distinctive biological role is implicated by the presence of differentially expressed *FCP* multigenes. Previously, Southern blotting and denaturing gradient gel electrophoresis (DGGE) analysis indicated that the *FCP* complex in *Cyclotella cryptica*, a mesophilic diatom, contains at least 21 genes (Eppard and Rhiel 1998; Eppard et al. 2000). Similarly, six to approximately 30 *FCP* genes have been reported for *P. tricornutum*, *T. pseudonana* and *C. neogracile* (Bhaya and Grossman 1993; Eppard et al. 2000; Armbrust et al. 2004; Bowler et al. 2008; Hwang et al. 2008). In *C. cryptica*, steady-state levels of *FCP* multigene transcripts were differentially established depending on light intensity (Oeltjen et al. 2004). The specific accumulation of the corresponding FCP proteins was confirmed by immuno-electron microscopic quantification (Becker and Rhiel 2006). *C. neogracile* *FCP* gene expression showed a differential response to thermal stress as well (Hwang et al. 2008).

Figure 6a shows the two opposing patterns of response to light stress observed for *C. neogracile* *FCP* genes. Within 30 min of the shift to high irradiance, the transcript levels of *CnFCP* 12, 14 and 15 rapidly increased up to 30-fold, while others were down-regulated 10-fold. The simultaneous but contrasting responses induced by the high light stimulus in these unicellular organisms strongly suggests the possibility that FCPs play a role in the response to light stress.

FCP proteins are the major diatom antenna proteins, containing the xanthophylls diadinoxanthin (Ddx) and diatoxanthin (Dtx). Diadinoxanthin de-epoxidase (DDE) catalyzes the conversion of Ddx to Dtx in a process that is called the Ddx–Dtx cycle. Similar to the xanthophyll cycle in green plants, this cycle is likely to alleviate light stress by quenching chlorophyll fluorescence, a process known as non-photochemical quenching (NPQ).

We monitored the levels of Ddx and Dtx under different light regimes to investigate the possible photoprotective role of xanthophyll pigments in *C. neogracile*. Figure 6b shows that the relative amounts of Ddx and Dtx were adjusted immediately and to a significant degree in cultures illuminated at different intensities, coinciding well with the dynamic adjustments of high light-up-regulated *FCP* transcript levels under the same conditions. A rapid increase in Dtx and a concomitant decrease in Ddx were observed in cells within 30 min of exposure to high light; transferring the culture back to low irradiance reversed these changes within a comparable amount of time. Under a constant light intensity, the relative amounts of both pigments remained fairly invariable, indicating that such a rapid change in the relative amounts of both pigments is under dynamic control of the light fluence. Dithiothreitol (DTT), an inhibitor of DDE (Lohr and Wilhelm 1999; Goss et al. 2006, 2008), completely abolished the light response of *C. neogracile* with respect to Ddx and Dtx levels (Fig. 6c); Ddx and Dtx concentrations did not respond at all to changes in light fluence in the presence of DTT, suggesting that high irradiance efficiently induces the de-epoxidation of Ddx to Dtx by DDE in *C. neogracile*.

Since NPQ is known to be a photoprotective means of dissipating excess absorbed light energy, we examined changes in NPQ in *C. neogracile* under different light conditions. Induction of NPQ was clearly evident in diatoms grown under both low and high irradiance for 6 h. Diatoms grown under high light exhibited relatively higher NPQ than diatoms grown under low light (Fig. 6d), suggesting that NPQ plays a certain photoprotective role in this organism as well. Furthermore, the addition of DTT completely abolished the induction of NPQ in diatoms grown under both low and high light, a result that indicates a direct relationship between Dtx formation and NPQ induction. In other diatoms, the presence of Dtx was required for NPQ

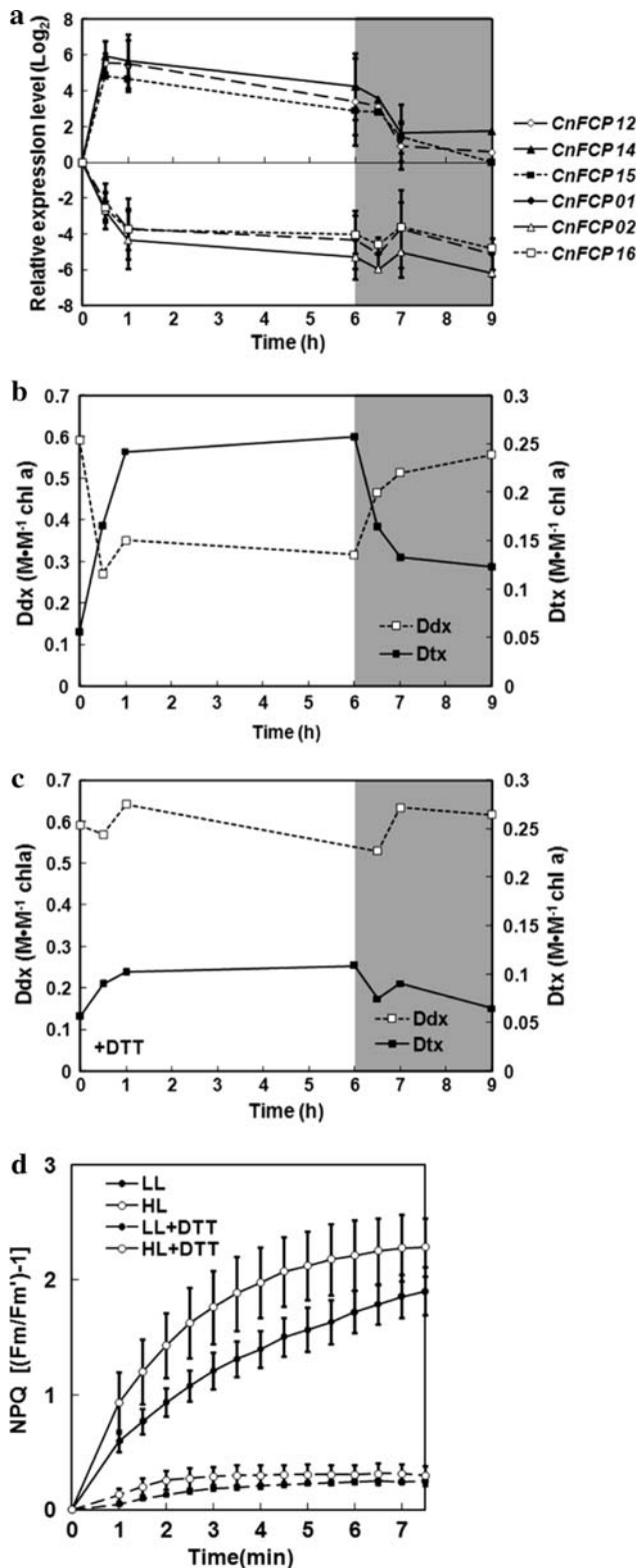


Fig. 6 Irradiance-specific responses of *C. neogracile* FCP genes and xanthophylls. Cells were cultured as described in Fig. 3 and total RNA and xanthophylls were isolated from samples at the indicated time. The effect of different light regimes was examined in respect to the expression of six different FCP genes by using real-time RT-PCR (a) and the levels of Dtx and Ddx in the absence (b) or presence (c) of 0.5 mM DTT. The values depicted in the figure are the mean of three measurements \pm SD. White and gray regions represent high irradiance for 6 h and low irradiance for 3 h, respectively. d Induction kinetics of NPQ. Cells were cultured under low (solid circle, LL) and high irradiance for 6 h (open circle, HL) and NPQ capacity was examined with (dotted line) or without (solid line) treatment with DTT. Values are the mean of three measurements \pm SD

associated with FCPs (Lavaud et al. 2003; Guglielmi et al. 2005; Gundermann and Büchel 2008). The FCP complexes also differ in the relative content of Ddx and Dtx pigments (Guglielmi et al. 2005; Gundermann and Büchel 2008). In *C. cryptica*, high light can differentially induce the production of Dtx in each FCP complex. For example, the Dtx content in FCPa is specifically increased by high illumination and is able to effectively quench chlorophyll fluorescence. Interestingly, there seems to be a strong correlation between the Dtx content and the number of FCP6 gene products in the trimeric FCPa (Beer et al. 2006). In addition, high irradiance can induce a differential change in Dtx/Ddx composition in FCP complexes, resulting in a differential efficiency of chlorophyll fluorescence quenching (Gundermann and Büchel 2008). Thus, antenna systems such as FCP complexes are likely to participate in mediating the NPQ response. In the present study, the expression of high light-inducible FCP genes was highly responsive to changes in light intensity, and the pattern of this expression was well matched with the pattern of the Ddx/Dtx conversion cycle under different light regimes. Therefore, high light-inducible FCP genes may encode FCP complexes involved in photoprotection as major antenna proteins containing xanthophylls, the cycling of which is known to alleviate light stress via NPQ.

The question remains of how and where Dtx-mediated NPQ occurs in a diatom. The fundamental NPQ mechanism appears to be built into the molecular structure of LHCs, which are able to switch rapidly and reversibly between two different conformations that are structurally very similar but functionally very different: a light-harvesting state and a dissipative quenching state (Horton et al. 2005). In the light-harvesting state, LHCs efficiently transfer absorbed light energy to the photosystems. However, LHCs in the dissipative quenching state are responsible for the thermal dissipation of light energy that is absorbed in excess. The conformational switch from the light-harvesting mode to the dissipative mode can be triggered by several cellular signals induced by high irradiance. For example, high light-driven acidification of the thylakoid

and NPQ capacity was strongly correlated with the buildup of Dtx (Lavaud et al. 2002a, b, c; Ruban et al. 2004; Goss et al. 2006).

In diatoms, Dtx has been found to occur not only in thylakoid membranes, unbound to any protein, but also mainly

lumen protonates specific sites on LHC proteins, causing conformational changes in antennae that lead to the dissipation of excess energy (Kramer et al. 2003).

In higher plants, zeaxanthin produced by the de-epoxidation of violaxanthin via the xanthophyll cycle serves as a signal to trigger NPQ by binding to LHC proteins (Pascal et al. 2005; Ahn et al. 2008) and this xanthophyll must bind to PsbS protein, a minor component of the LHC, in order to develop NPQ (Li et al. 2000; Holt et al. 2004). PsbS has been proposed to be a “sensor” for the increase in the thylakoid lumen pH induced by excessive light absorption, and PsbS may function as an antenna “organizer” and likely drives LHCI antennae into the quenched state through conformational change under excess light (Li et al. 2004; Kiss et al. 2008; Horton et al. 2008). However, no match for a PsbS ortholog has been found thus far in either the Diatom EST Database or in the fully sequenced *T. pseudonana* and *P. tricornutum* genome (Armbrust et al. 2004; Maheswari et al. 2005; Bowler et al. 2008). Instead, LI818-like proteins in diatoms have been suggested to play a similar role of PsbS in responding to high light stress (Zhu and Green 2008). LI818 is unique family member in the LHC originally discovered in the green algae, with no putative homologues in vascular plants and the expression of *LI818* genes are highly up-regulated under high light stress (Gagne and Guertin 1992; Savard et al. 1996; Yamano et al. 2008). After completing genome sequence of *T. pseudonana* and *P. tricornutum* (Armbrust et al. 2004; Bowler et al. 2008), several *LI818* homologous genes have been found through

phylogenetic analysis and designated as *Lhcx* proteins. So far, *LI818*-like genes are known to be present in *T. pseudonana*, *P. tricornutum* and *C. cryptica*. We performed a phylogenetic analysis for some of *C. neogracile* *FCP* genes which were differentially responsive to high irradiance to examine their similarity to the *Lhcx* genes. Based on the protein sequences of *LHC* homologues from *T. pseudonana*, *P. tricornutum* and *FCP2* and *FCP6* from *C. cryptica* as well as *LI818* from green algae, the deduced amino acid sequences of 21 *LHC*s were aligned (see alignment result in Supplementary Data 5) and neighbor-joining cladogram is depicted in Fig. 7. Two distinct clades were notable from this tree. The first group is the *LI818*-like proteins designated as *Lhcx* proteins. The second group represents the major light-harvesting proteins named *Lhcf* proteins (Zhu and Green 2008). Phylogenetic analysis clearly showed that high light-up-regulated *CnFCP12*, 14 and *CnFCP15* proteins are in the same clade with *Lhcx* proteins, whose functions are implicated in photoprotection (Oeltjen et al. 2002; Becker and Rhiel 2006; Yamano et al. 2008; Zhu and Green 2008). These results further supported the possible role of up-regulation of some *FCP* genes under high light in the thermal dissipation for photoprotection from high irradiance.

In the present study, we have demonstrated that the transcriptome of *C. neogracile*, a psychrophilic diatom, dynamically responds to light stress. In addition, we clearly showed that the induction of NPQ in this organism was caused by the formation of Dtx via the xanthophyll cycle,

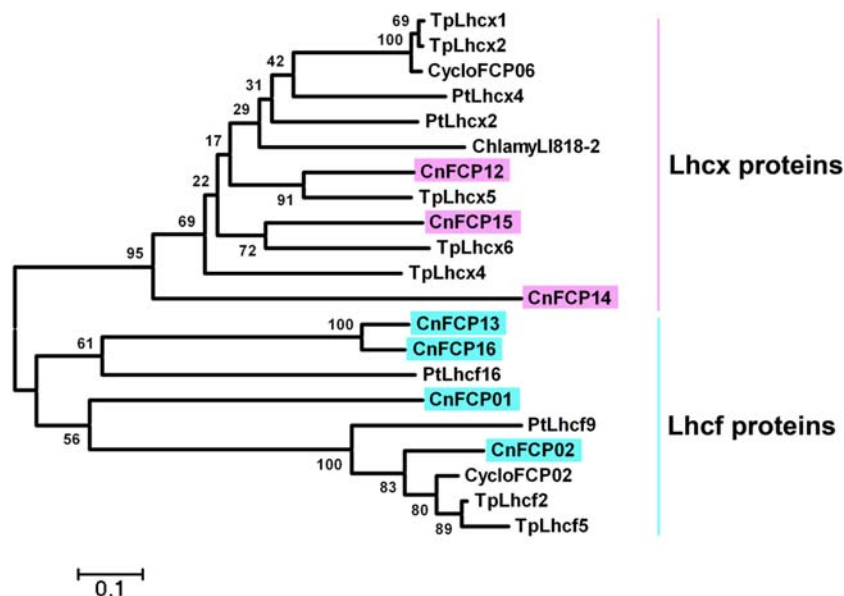


Fig. 7 Phylogenetic analysis for high light responsive CnFCPs and other LHCs from different diatoms. The neighbor-joining tree was constructed based on alignment of a data set of amino acids of LHCs from different diatoms presented in Supplementary Data 5. Numbers along branches denote bootstrap values which were calculated over 1,000

iterations. The colored areas represent the up-regulated (pink) and down-regulated (sky blue) CnFCPs as a result of high irradiance. *Tp*, *Thalassiosira pseudonana*; *Cyclo*, *Cyclotella cryptica*; *Pt*, *Phaeodactylum tricornutum*; *Chlamy*, *Chlamydomonas reinhardtii*; *Cn*, *Chaetoceros neogracile* used in this study

as has been observed in mesophilic diatoms (Olaizola and Yamamoto 2004; Goss et al. 1999, 2006). These results indicate that Dtx-dependent NPQ is also a photoprotective mechanism in *C. neogracile*. Recent studies, however, suggest that NPQ in diatoms is a multi-component system that includes both transient and steady-state quenching (Grouneva et al. 2008). It has also been proposed that the observed variable Dtx quenching efficiency is due to the presence of multiple Dtx reservoirs in the diatom antenna system (Grouneva et al. 2008). Therefore, the possibility of multi-component NPQ in *C. neogracile* and the potential contribution of FCP complexes containing Dtx to NPQ will be studied further.

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