Journal of Experimental Botany, Vol. 64, No. 2, pp. 483–493, 2013
doi:10.1093/jxb/ers340 Advance Access publication 25 November, 2012
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Journal of Experimental Botany
www.jxb.oxfordjournals.org

RESEARCH PAPER

Blue light is essential for high light acclimation and photoprotection in the diatom *Phaeodactylum tricornutum*

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Received 29 August 2012; Revised 24 October 2012; Accepted 30 October 2012

Abstract

The objective of the present study was to test the hypothesis that the acclimation to different light intensities in the diatom *Phaeodactylum tricornutum* is controlled by light quality perception mechanisms. Therefore, semi-continuous cultures of *P. tricornutum* were illuminated with equal amounts of photosynthetically absorbed radiation of blue (BL), white (WL), and red light (RL) and in combination of two intensities of irradiance, low (LL) and medium light (ML). Under LL conditions, growth rates and photosynthesis rates were similar for all cultures. However, BL cultures were found to be in an acclimation state with an increased photoprotective potential. This was deduced from an increased capacity of non-photochemical quenching, a larger pool of xanthophyll cycle pigments, and a higher de-epoxidation state of xanthophyll cycle pigments compared to WL and RL cultures. Furthermore, in the chloroplast membrane proteome of BL cells, an upregulation of proteins involved in photoprotection, e.g. the Lhcx1 protein and zeaxanthin epoxidase, was evident. ML conditions induced increased photosynthesis rates and a further enhanced photoprotective potential for algae grown under BL and WL. In contrast, RL cultures exhibited no signs of acclimation towards increased irradiance. The data implicate that in diatoms the photoacclimation to high light intensities requires the perception of blue light.

Key words: Blue light, diatoms, non-photochemical quenching, photoacclimation, photoprotection, photoreceptors.

Introduction

Diatoms are major contributors to the marine primary production (Geider *et al.*, 2001). They have evolved by secondary endosymbiosis which led to a different genetic regulation of the nucleus–plastid interaction compared to organisms with plastids derived from primary endosymbiosis (Wilhelm *et al.*, 2006). The evolutionary success of diatoms in the ocean as well as in freshwater environments is supposed to be closely linked to their ability to adapt to dynamic light conditions (Depauw *et al.*, 2012). Diatoms are exposed not only to oscillations of light intensity (Lavaud *et al.*, 2004), but also to changes of light quality. In the open ocean, the ratio of blue to red light increases with the depth, because red light as well as far-red light is strongly attenuated within the upper layer of the water column, whereas blue light penetrates deeper (Kirk, 1994). Beyond a certain depth, neither far-red light nor solar red light are present. Accordingly, a vertical transfer of algae in the water column does not only change the ambient light intensity but also the ambient light quality.

In higher plants and green algae, it was shown that the acclimation of the photosynthetic apparatus towards the ambient light conditions is mainly triggered by both, by the reduction states of the components of the redox switch (the plastoquinone pool and the thioredoxin system) (Durnford and Falkowski, 1997; Pfannschmidt *et al.*, 1999; Bräutigam

et al., 2010) and by photoreceptors. In these organisms, both redox pools are typically oxidized in darkness and become more reduced under illumination. This reduction triggers short-term reaction mechanisms like state transitions, but also induces a signal cascade which alters the nuclear gene expression via retrograde signalling (Pesaresi *et al.*, 2009). In addition to the measurement of the light intensity, green chloroplasts are able to perceive the light quality via the reduction state of the plastoquinone pool. In higher plants and green algae, the antennae of photosystem II (PSII) consist mainly of LhcII proteins with a high relative content of chlorophyll *b*, whereas the antennae of photosystem I (PSI) consist of light-harvesting proteins with only minor amounts of chlorophyll *b*. Accordingly, both photosystems possess different excitation spectra, and an illumination with a light quality absorbed preferentially by one of the two photosystems influences the reduction state of the plastoquinone pool and triggers the respective acclimation mechanisms.

In addition to the reduction state of the thioredoxin system and the plastoquinone pool, the reduction state of other stromal compounds, the proton gradient across the thylakoid membrane, and the occurrence and concentration of reactive oxygen species influence the photoacclimation of green algae and higher plants (Li *et al.*, 2009). Interestingly, the main function of photoreceptors was thought to be restricted to the control of photomorphogenesis (Strasser *et al.*, 2009), chlorophyll biosynthesis (Stephenson and Terry, 2008), and light-induced movements like stoma opening (Kinoshita *et al.*, 2001), phototropism (Foster *et al.*, 1984) and chloroplast high-light avoidance movements (Kasahara *et al.*, 2004). Accordingly, it was stated that photoreceptors do not play an important role in the photoacclimation of green chloroplasts (Walters *et al.*, 1999; Bräutigam *et al.*, 2010). However, in *Arabidopsis thaliana*, 77 of 992 high light-induced genes were recently shown to be misregulated in cryptochrome deficient mutants (Kleine *et al.*, 2007), which indicates an involvement of photoreceptors in the regulation of photoacclimation and illustrates the complexity of the regulatory network.

Interestingly, several specific traits of diatoms indicate that there are probably remarkable differences between the regulation of photoacclimation in diatoms and organisms with green chloroplasts. First, there is no evidence for major differences in the pigmentation of the antennae of PSI and PSII in diatoms (Lepetit *et al.*, 2011). Therefore, a preferential excitation of either PSI or PSII by illumination with specific wavelength bands has not been observed in diatoms so far. Second, the reduction state of the plastoquinone pool is not only determined by the relative activity of PSI and PSII, but also by electron donation by stromal components, e.g. via chlororespiration (Dijkman and Kroon, 2002; Grouneva *et al.*, 2009). It has been shown that in diatoms the chlororespiratory electron flow in darkness is sufficient to establish a proton gradient across the thylakoid membrane, thereby leading to an activated xanthophyll cycle (XC) after prolonged periods of darkness (Jakob *et al.*, 1999). Therefore, in darkness the plastoquinone pool becomes reduced in diatoms whereas it becomes oxidized in green chloroplasts. Third, the light-induced redox switch of the enzymes of the Calvin cycle and the oxidative pentose phosphate cycle seems to be restricted to a comparatively small number of proteins (Michels *et al.*, 2005; Kroth *et al.*, 2008; Kikutani *et al.*, 2012). This indicates an altered functionality of the thioredoxin system in diatoms compared to higher plants and green algae, which could also apply to the involvement of this system in photoacclimation. Taken together, little is known about the regulatory processes which control photoacclimation in diatoms and how they act together. However, it is probable that there are major differences to the regulatory system of organisms with green chloroplasts. For example, it seems possible that the response to light quality might depend to a larger extent on the excitation of photoreceptors.

Diatoms exhibit several classes of photoreceptors (Depauw *et al.*, 2012), including the red light photoreceptor family of phytochromes as well as the blue light photoreceptors families of cryptochromes and aureochromes (AUREOs), which were found in the genome sequences of both the centric diatom *Thalassiosira pseudonana* and the pennate diatom *Phaeodactylum tricornutum* (Armbrust *et al.*, 2004; Montsant *et al.*, 2005; Bowler *et al.*, 2008). In contrast to phytochromes and cryptochromes, which can be found in nearly all eukaryotic organisms, AUREOs are restricted to stramenophiles (Ishikawa *et al.*, 2009). They were first described in the xanthophyte *Vaucheria frigida* (Takahashi *et al.*, 2007). AUREOs contain a LOV (light-oxygen-voltage) domain as well as a basic zipper (bZIP) domain and thus can be considered as blue light (BL)-mediated transcription factors. However, the specific function of AUREO photoreceptors in unicellular algae is unclear until now.

The objective of the present study was to analyse the physiological acclimation of the diatom *P. tricornutum* to different light qualities. Cultures of *P. tricornutum* were grown in chemostats under illumination with white light (WL), monochromatic blue light (BL), or monochromatic red light (RL). Cultures were adjusted to equal amounts of absorbed photons under low light (LL) and medium light (ML) conditions, respectively. After internal conversion, the absorption of blue photons results in the same excited state of chlorophylls as the absorption of red photons. Therefore, with the prerequisite of the same amount of absorbed quanta, different light qualities should result in comparable photosynthetic electron transport rates. Furthermore, due to the equal pigmentation of PSI and PSII in diatoms, the light quality should not directly influence the reduction state of the plastoquinone pool. Therefore, it is assumed that changes of cellular physiology are induced by a differential activation of light quality perception mechanisms, like photoreceptors.

Materials and methods

Cultivation of algae

P. tricornutum UTEX 646 was grown semi-continuously at 20 °C in an air-lifted rectangular bioreactor with a depth of 3 cm. Algae were cultivated in modified artificial seawater medium f/2 (according to Guillard and Lorenzen, 1972) without silica and with 50% of the original salt content. Flora LED-panels (CLF Plant Climatics, Wertingen, Germany) were used for illumination with monochromatic BL and RL

at wavelengths of 469 ± 10 nm and 659 ± 11 nm, respectively. White fluorescence tubes (18W/865, Osram, Munich, Germany) provided illumination with WL. The spectral composition of the light sources was recorded with a spectroradiometer (Tristan, Hamburg, Germany). Cultures were illuminated with a 14/10 light/dark regime. For LL conditions, algae were illuminated with either 24 µmol photons m^{-2} s^{-1} of BL, 40 µmol photons m^{-2} s^{-1} of WL, or 41 µmol photons m^{-2} s^{-1} of RL. In this way, it was assured that irrespectively of light quality, the same amount (10 µmol photons m^{-2} s^{-1}) was absorbed by the algal cultures. The amount of photosynthetically absorbed radiation (Q_{Phar}) was calculated according to Gilbert *et al.* (2000). For ML conditions, algae were illuminated with 72 µmol photons m^{-2} s^{-1} of BL, 120 µmol photons m^{-2} s^{-1} of WL, or 123 µmol photons m^{-2} s^{-1} of RL, resulting in an absorbed radiation of about 30 µmol photons m^{-2} s^{-1}. These light conditions are known to significantly change the light adaptation status of diatom cells (Schumann *et al.*, 2007; Lepetit *et al.*, 2010). To achieve full adaptation to the applied light conditions, algae were grown for at least 1 week under BL, WL, and RL in combination with LL and ML conditions. The chlorophyll *a* concentration was adjusted to 1.6 µg chlorophyll *a* ml^{-1} for LL cultures and 1.2 µg chlorophyll *a* ml^{-1} for ML cultures every day in the afternoon and increased to about 2 µg chlorophyll *a* ml^{-1} in the morning of the next day.

Cellular parameters

The concentrations of chlorophyll *a* and chlorophyll *c* were determined spectrophotometrically after pigment extraction with 90% acetone according to Wagner *et al.* (2006) using the equations of Jeffrey and Humphrey (1975). Growth rates were calculated from the daily increase of chlorophyll *a* content under steady-state conditions. From the same sample, cell numbers were counted with a Z2 particle counter (Beckman Coulter, Krefeld, Germany) and the chlorophyll *a* content per cell was calculated. The morphotype of the cells was fusiform. *In vivo* absorption spectra from 400 to 750 nm were recorded with a spectrophotometer (Specord M500, Zeiss, Oberkochen, Germany) adjusted to a bandwidth of 1 nm. The determination of dry weight was done according to Su *et al.* (2012).

Estimation of photosynthesis rates and quantum requirement

Oxygen-based photosynthesis rates (P_O) and fluorescence parameters were measured simultaneously under illumination with WL according to Wagner *et al.* (2006). In preceding experiments, photosynthesis/irradiance curves were recorded for the different cultures under illumination with blue, white, and red actinic light. Thereby, no difference of the photosynthesis rates plotted against Q_{Phar} were detected for the different actinic light sources (data not shown). Non-photochemical quenching (NPQ) of chlorophyll *a* fluorescence was calculated according to Bilger and Björkman (1990).

The carbon-related biomass production rate (B_C) and the quantum requirement of carbon-based biomass production ($1/F_C$) were calculated according to Su *et al.* (2012).

Pigment isolation

An aliquot (10 ml) of a dark-adapted culture were harvested on a glass fibre filter and freeze dried overnight (Labconco FreeZone, ILMVAC, Ilmenau, Germany). Pigment extraction and HPLC separation was done according to Su *et al.* (2012). For the measurements of de-epoxidation state (DES) of the XC pigments diadinoxanthin (Ddx) and diatoxanthin (Dtx), cultures were illuminated with 1,000 µmol photons m^{-2} s^{-1} WL for 10 min prior to harvesting. The DES was calculated as the ratio of Dtx to (Ddx + Dtx).

Isolation of chloroplast membranes

Isolation of chloroplast membranes was performed according to Lepetit *et al.* (2007). Chloroplast membrane isolation and subsequent mass spectrometry were performed with BL and RL cultures grown under LL conditions. All isolation steps were carried out at 4 °C under dim light. A culture volume of 600 ml was harvested by centrifugation (3400 *g*, 6 min). Cells were resuspended in 20 ml isolation medium A (10 mM MES pH 6.5, 2 mM KCl, 5 mM EDTA, 1 M sorbitol) and disrupted by using a pre-cooled French press (Thermo Spectronic, Rochester, USA) at 86.18 MPa. After centrifugation (1000 *g*, 10 min) the pellet contained mostly unbroken cells and was resuspended in 20 ml isolation medium A and passed through the French press a second time. The sample was centrifuged (1000 *g*, 10 min). The supernatants of both preparation steps were merged together and centrifuged again (40,000 *g*, 20 min). The pellet was resuspended in 1 ml isolation medium B (10 mM MES pH 6.5, 2 mM KCl, 5 mM EDTA).

Protein sampling, liquid chromatography–electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), and data analysis

Protein (60 µg) from the chloroplast membrane fraction of *P. tricornutum* cultures grown either under BL or RL conditions were separated by a 10% SDS-PAGE containing 0.3% of piperazin diacrylamide as cross-linker (Wagner *et al.*, 2004). One technical replicate of the RL and BL samples, respectively, as well as a further biological replicate were included in the following procedure. Gels were stained using NOVEX Colloidal Blue Staining Kit (Invitrogen, Darmstadt, Germany). Complete lanes were dissected into pieces of approx. 2.5 mm in height and prepared for in-gel tryptic digest over night and nLC-ESI-MS/MS according to Schmidt *et al.* (2006).

Data analysis was carried out via the Thermo Electron Corp Proteome Discoverer software (version 1.0) including the Sequest algorithm (Link *et al.*, 1999) as described in Veith *et al.* (2009) using a combined protein database of JGI PhaTr2 (http://genome.jgi-psf.org/Phatr2/Phatr2.download.ftp.html) and the 132 plastid-encoded proteins listed in NCBI (http://www.ncbi.nlm.nih.gov/nuccore/118410962). The false discovery ratewas set to be equal or smaller than 1% and the minimum Xcorr for the three charge states was set to 2.0 for +1, 2.5 for +2, and 3.0 for +3. All found peptides from any slice belonging to a given protein were grouped together and further analysed by an in-house-developed bioinformatic analysis tool. Proteins identified with at least two unique peptides in one of the samples were further classified.

Proteins fulfilling the search criteria were classified as chloroplast membrane specific or as probable contaminants, including proteins with transmembrane domains (TMDs) or yet others. Therefore, proteins were tested for chloroplast localization. All proteins encoded by the plastid genome were assigned to be localized in the chloroplast. For nuclear-encoded proteins, the occurrence of chloroplast-targeting signal peptides was tested using the program HECTAR (http://www.sb-roscoff.fr/hectar, Gschloessl *et al.*, 2008). All proteins with the prediction of a plastid signal peptide were assigned to the proteome of the chloroplast. The ASAFAP signal peptide is characteristic for the import of proteins into the chloroplast of diatoms (Kilian and Kroth, 2005). Several proteins known to be chloroplast localized from literature were misleadingly classified as proteins with non-plastidic signal motive by HECTAR. These proteins and others known from literature to be situated in the thylakoid membrane or to be associated with plastidic membrane protein complexes (e.g. Lhcf proteins or subunits of PSI and PSII) were assigned to be chloroplast membrane specific regardless of the results of the prediction program. Accordingly, proteins known to be located in other cellular compartments than chloroplast membranes (e.g. RbcL) were assigned to be probable contaminants regardless of the results of the prediction programs.

Proteins were tested for membrane localization using the prediction programs TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM), DAS membrane prediction (http://www.sbc.su.se/~miklos/DAS/maindas.html) according to Cserzo *et al.* (1997), and OCTOPUS membrane prediction (http://octopus.cbr.su.se) according to Viklund and Elofsson (2008). Proteins were assigned to be membrane specific if at least two of three membrane prediction programs predicted membrane localization.

To compare the abundance of identified proteins between the RL and BL samples, the normalized spectral abundance factor (NSAF) was calculated according to Zybailov *et al.* (2006). Accordingly, the total

number of identified peptides per protein in a sample represented as spectral counts (SpC) was divided by its number of amino acids (L). For the estimation of the relative abundance the ratio SpC/L was divided by the sum of SpC/L for all identified proteins, including probable contaminants. To avoid dividing by zero, a correlation factor of 0.16 was added to every individual spectral count as practised before (Zybailov *et al.*, 2006). Afterwards, the average of the technical replicate was calculated and averaged thereafter with the NSAF of the biological replicate. To determine the increase or decrease factor of protein abundance, the averaged NSAF of the BL sample was divided by the NSAF of the RL sample. Proteins were classified as upregulated under BL if the ratio of the average NSAF of BL samples to the average NSAF of RL samples was above or equal to 1.33 and classified as downregulated under BL if the ratio was below or equal to 0.75.

Promoter analyses

The genes of all detected nuclear-encoded chloroplast membrane proteins (64 out of 85) were tested for the occurrence of the *V. frigida* AUREO1 DNA-binding motive TGACGT and the reverse complement ACGTCA (Takahashi *et al.*, 2007) 50–500 bp upstream of the predicted transcription start. The gene models of seven proteins were incomplete and did not include a 5'-untranslated region prior to the start codon. Accordingly, the promoter regions of these genes were unknown and they were excluded from the analysis.

Statistics

The statistical analysis of the physiological data was carried out by one-way analysis of variance (ANOVA) followed by Tukey's test for pairwise multiple-comparison using the program Sigma Plot 11.0 and a P-value <0.05 for the rejection of the null hypothesis. For MS data evaluation, mean NSAF values of thylakoid membrane proteins isolated from RL and BL cultures were compared using Student's t-test along with P-values <0.05 and <0.1, respectively, for the rejection of the null hypothesis. It has to be mentioned that no correction of the required minimum P-values of the t-test was made in order to counteract the problem of multiple comparisons. This statistical procedure was not applicable due to the small number of replicates of this large-scale proteome analysis. Therefore, the obtained list of regulated proteins depending on the light quality will include false positives. Hence, proteins that were not exclusively found under BL and RL, respectively, but are up- or downregulated under BL should be regarded as potential blue-light regulated candidates.

Results

Cellular parameters

Under LL conditions, growth rates were similar for all cultures of *P. tricornutum* irrespective of the quality of the incident light (Table 1). The same holds true for the chlorophyll *a*-specific absorption (a^*_{Phy}) and the quantum requirement of carbon-based biomass production ($1/\Phi_C$; Table 1). Under BL, the cellular content of chlorophyll *a* as well as the dry weight was increased compared to RL conditions. At higher irradiance, growth rates were found to be higher for all light qualities compared to LL conditions. However, under RL, this increase was not as pronounced as under BL and WL conditions. Accordingly, $1/\Phi_C$ significantly increased in RL cultures in comparison to LL conditions (Table 1). In BL and WL cultures, the quantum requirement of biomass production did not change in comparison to LL conditions. This observation is in line with the photosynthesis–irradiance curves (see next section) and confirms that the intensity of irradiance during the cultivation of algae under ML conditions was still in the range of the light-limited part of photosynthesis. The chlorophyll *a* content per cell under ML in comparison to LL decreased under all conditions of different light qualities while stronger decreases were observed in WL and BL cultures compared to RL cultures. As already observed in the

Table 1. Cellular parameters of *Phaeodactylum tricornutum* cultures grown under illumination with blue, white, and red light under low and medium light conditions For excess light treatment, samples were illuminated with 1000 µmol photons m^{-2} s^{-1} for 10 min. Values are mean ± standard deviation (n = 3 for $1/F_C$; n = 9 for other parameters). a^*_{Phy}, Chlorophyll *a*-specific absorption; $1/\Phi_C$, quantum requirement of carbon-based biomass production; Chl, chlorophyll; Ddx, diadinoxanthin; Dtx, diatoxanthin; DES, de-epoxidation state of the Ddx cycle pigment pool [Dtx/(Ddx + Dtx)]. Values marked with same letters are not significantly different (Tukey's pairwise multicomparison test; $P < 0.05$).

Parameter	Low light			Medium light		
	Blue	White	Red	Blue	White	Red
Chl a (pg $cell^{-1}$)	7.2 ± 1.2^{a}	5.2 ± 0.4^{bc}	5.6 ± 0.7^{b}	4.3 ± 0.3^{d}	3.4 ± 0.3^{e}	4.6 ± 0.5^{cd}
a^*_{Phy} [m^2 (g Chl a)$^{-1}$]	9.8 ± 0.7^{a}	10.0 ± 0.4^{a}	9.9 ± 0.5^{a}	9.8 ± 0.2^{a}	9.9 ± 0.4^{a}	9.9 ± 0.3^{a}
Growth rate (µ d^{-1})	0.43 ± 0.04^{a}	0.43 ± 0.11^{a}	0.44 ± 0.12^{a}	1.01 ± 0.13^{b}	1.08 ± 0.22^{b}	0.78 ± 0.15^{c}
Dry weight (pg $cell^{-1}$)	22.1 ± 2.2^{a}	19.6 ± 1.5^{b}	15.9 ± 0.9^{c}	18.9 ± 0.9^{b}	14.6 ± 0.8^{c}	18.5 ± 1.7^{b}
$1/\Phi_C$ [mol photons (mol C)$^{-1}$]	14.3 ± 2.4^{a}	13.9 ± 0.4^{a}	13.8 ± 0.7^{a}	13.4 ± 0.1^{a}	14.6 ± 1.8^{a}	20.1 ± 0.5^{b}
Pigments [mmol (mol Chl a)$^{-1}$]						
Chl *c*	118 ± 4	119 ± 2	109 ± 4	120 ± 2	114 ± 2	105 ± 4
Fucoxanthin	757 ± 11	759 ± 12	726 ± 16	751 ± 8	753 ± 12	704 ± 11
Ddx	109 ± 5^{a}	109 ± 3^{a}	86 ± 8^{b}	130 ± 20^{c}	124 ± 6^{c}	84 ± 3^{b}
β-Carotene	58 ± 3	59 ± 2	63 ± 3	64 ± 4	63 ± 1	62 ± 1
Violaxanthin	9 ± 5	17 ± 2	11 ± 3	14 ± 4	14 ± 3	18 ± 5
Excess light						
Ddx	70 ± 6.6	68 ± 3.2	69 ± 5.0	72 ± 2.4	69 ± 2	65 ± 5.1
Dtx	40 ± 5.7	45 ± 1.6	24 ± 2.4	59 ± 2.1	59 ± 5.7	27 ± 2.2
DES	0.38 ± 0.01^{a}	0.40 ± 0.01^{a}	0.26 ± 0.01^{b}	0.45 ± 0.01^{c}	0.44 ± 0.04^{c}	0.28 ± 0.02^{b}

comparison of the different light quality under LL conditions, a^*_{Phy} did not change under ML conditions.

Photosynthesis rates

Gross oxygen-based photosynthesis rates were derived from measurements of photosynthesis–irradiance curves. In line with the comparable values of a^*_{Phy}, the α-slopes (light-limited part of the curves) were found to be similar under all light conditions (Fig. 1A–C). In contrast, the maximum photosynthesis rates (P_{max}) changed depending on the culture conditions. Whereas under LL conditions comparable values for P_{max} [about 190 µmol O_2 (mg chlorophyll a^{-1}) h^{-1}] were observed for BL, WL, and RL cultures, under ML conditions P_{max} was clearly increased in cultures grown under BL and WL [about 240 µmol O_2 (mg chlorophyll a^{-1}) h^{-1}]. Interestingly, P_{max} of RL cultures grown under ML did not increased significantly in comparison to that of LL cultures under RL conditions.

Non-photochemical quenching and pigmentation

Measurements of the capacity of NPQ were performed in parallel to the measurements of light saturation curves and revealed clear differences between the different cultures conditions (Fig. 1D–F). Most surprisingly, the intensity of the irradiance at growth influenced the maximum quenching capacity under WL but exhibited no influence under BL and RL. Instead, light quality exerted the dominating influence on the light adaptation status of the cells. Thus, RL cultures were characterized by low values of the maximum quenching capacity (about 0.45) for both growth light intensities. The highest NPQ values observed in BL cultures were found to be more than twice as high as under RL. NPQ values of cells grown under WL were in the middle of the range of NPQ values observed in cells of BL and RL cultures.

The changes of the XC pool size and the DES (Table 1) are in good agreement with the observed differences in the maximum NPQ values depending on light quality and quantity. Cells grown under RL possessed the smallest pool size of XZ pigments and the lowest DES after 10 min of illumination with excess light in comparison to cells grown under BL and WL. Whereas under RL no changes in neither the XC pool size nor the DES were observed depending on the intensity of the growth irradiance, in cells grown under BL and WL an increase of XC pool size and DES was found under ML conditions in comparison to LL conditions. Interestingly, the XC pool size and DES were comparable for BL and WL cells grown under LL and ML, respectively which stands in contrast to the differences observed for the maximum NPQ.

Proteomic analysis

Chloroplast membranes including outer chloroplast membranes were enriched from cells grown under BL or RL. Proteins from both samples were separated by SDS-PAGE (Supplementary Fig. S1, available at *JXB* online). The lanes were dissected into slices. After in-gel tryptic digestion, resulting peptides were analysed by LC-ESI-MS/MS. In total, 319 proteins with at least two unique peptides in one sample (BL

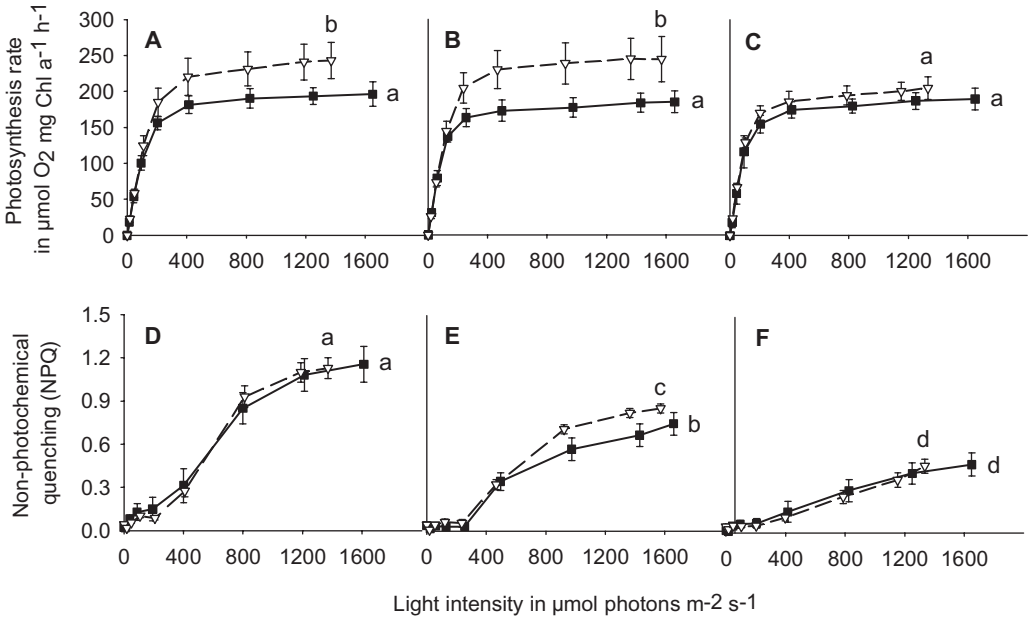

Fig. 1. Photosynthesis rates (A, B, C) and non-photochemical quenching (D, E, F) of *Phaeodactylum tricornutum* cultures grown under illumination with blue (A, D), white (B, E), or red (C, F) light depending on the light intensity in the measuring cuvette. For each light quality, algae were cultivated under low light (filled squares and solid lines) and medium light (open triangles and dashed lines). Values are mean ± standard deviation (n = 9–12). Maximum values of photosynthesis rates and non-photochemical quenching were tested for significant differences using Tukey's pairwise multi-comparison test. Values marked with same letters are not significantly different ($P < 0.05$).

or RL) were detected, and 85 of these proteins were assigned to be chloroplast membrane specific according to the criteria described. Detailed information about all identified peptides of each protein identified either under BL or RL as well as the protein function are given in Supplementary Table S1. The identified proteins included 27 different light-harvesting proteins which matched comparatively well with the antenna proteins found by Grouneva *et al.* (2011) in *P. tricornutum* cultures grown under white light. Of the antenna proteins detected by Grouneva *et al.* (2011), FCP 48798, Lhcr2, Lhcf12, and Lhcx2 were not detected in the present study, whereby the protein sequence coverage of Lhcr2 and Lhcf12 was very low in Grouneva *et al.* (2011). On the other hand, Lhcf6/7, Lhcf13, Lhcf14, and Lhcf15 were only found in the present study. In addition to the antenna proteins, seven different subunits of the ATPase, six proteins from PSI, and nine from PSII as well as three of the Cyt b_6/f complex were identified. Further 21 proteins belong to other functional groups and 12 are of unknown function.

In the BL sample, 84 of the proteins were identified (Table 2). Three of them were exclusively detected in BL and not in RL samples and one additional protein was only found in RL samples (Table 3). Quantitative comparison of proteins from BL and RL samples were performed based on the NSAF (Zybailov *et al.*, 2006); 10 proteins were found to be upregulated by a factor ≥1.33 under BL compared to RL (Table 3; Supplementary Table S1). With respect to the physiological adaptation in response to BL and RL the upregulation of the photoprotective protein Lhcx1, the zeaxanthin epoxidase, the Rieske protein, and a triosephosphate transporter should be highlighted. Furthermore, four proteins were found to be downregulated under BL in comparison to RL by a factor of ≤0.75. These include the light-harvesting proteins Lhcf15 (where the strongest downregulation was observed) and Lhcf2 as well as the PSI protein PsaF.

A relatively large number of proteins were considered as probable contaminants mostly due to contamination of the purified fraction with membrane remnants of other compartments (Supplementary Table S2A) or yet others (Supplementary Table S2B). In *P. tricornutum*, the chloroplast, the mitochondrium, and the endoplasmatic reticulum are in close conjunction. In combination with the robust cell wall, which requires comparatively harsh conditions to break the cells and to isolate the chloroplast membranes, this leads to the high number of probable contaminants in the present study. However, the summed NSAF of chloroplast membrane-specific proteins was clearly higher than the summed NSAF of probable contaminants. Probable contaminants accounted for 25.2 ± 1.8% of the summed NSAFs in BL cultures and 26.9 ± 2.1% of the summed NSAFs in RL cultures, indicating the successful enrichment of chloroplast membranes.

Promoter analysis

The genome sequence of the nuclear-encoded chloroplast membrane proteins was analysed with respect to the occurrence of AUREO1a-binding motives in the respective promoter region (50–500 bp prior to transcription start, Fig. 2A). The mean number of AUREO1a-binding motives in the genome sequence of proteins upregulated under BL was about 50% higher compared to nonregulated proteins. Noticeably, no AUREO1a-binding sites were found in the promoter regions of proteins upregulated under RL (Fig. 2B). However, this should only be regarded as a trend due to the low number of proteins upregulated under RL, which could be tested.

Discussion

In the present study, the impact of light quality on photosynthesis, photoprotective potential, and growth of *P. tricornutum* was studied after acclimation to BL, WL, and RL conditions. The different light qualities were combined with two different intensities of irradiance to change the light acclimation status of the cells. The amount of incident irradiance was carefully adjusted to assure the same amount of absorbed photons under the respective conditions of different light qualities. This is different to previous studies and avoids any superposing effects of light quality and light intensity, which might otherwise occur due to differences of the specific absorptivity of diatom cells in the blue and red spectral regions.

Interestingly, under LL conditions the light quality had no effect on basic cellular parameters of *P. tricornutum*, such as absorption properties of the cells, gross oxygen-based photosynthesis rates, growth rates, and the quantum requirement of biomass production. This picture changed under ML conditions where the cultivation under BL and WL resulted in clearly increased maximum photosynthesis rates and growth rates compared to LL conditions. This was not observed in RL cultures. Since growth conditions under different light qualities were adjusted to the same amount of Q_{Phar}, the lower growth rates of cells under RL were inevitably correlated with a higher quantum requirement of biomass production under RL compared to BL and WL. Thus, the same amount of light was absorbed but the usage of light energy for biomass production was less efficient in RL-grown cells. A possible explanation could be an increased thermal dissipation of absorbed quanta by, for example, a NPQ mechanism. However, under growth light conditions, the NPQ values for all cultures were close to zero. Another explanation could be suspected in a dissipation of cellular energy by increased mitochondrial respiration in RL cultures in comparison to BL and WL cultures. Thus, respiration rates were recorded prior to the measurement of light response curves with no significant differences between the cultures (data not shown). However, these measurements were always performed 2–5 hours after the onset of the illumination. Hence, it is possible that ML RL cultures exhibit increased respiration rates during the dark period which might be responsible for the increased quantum requirement.

The proteomic analysis revealed a reorganization of the thylakoid proteome which is evidenced by the fact that the expression level of 18 out of 85 identified proteins were actively regulated by light quality (Table 3). These proteins

Table 2. Functional categorization and characterization of chloroplast membrane-specific proteins identified in samples isolated from *Phaeodactylum tricornutum* cultures grown under low intensities of blue light. Protein IDs are according to JGI version 2.0 or NCBI reference sequence. Names are according to JGI version 2.0. Proteins are sorted in alphabetical order of their names within each functional category. Proteins of unknown function are sorted by their accession numbers. Only the number of different peptides identified in the blue light samples is shown. In contrast, Supplemental Table S1 lists all peptides identified in blue and red light samples with detailed information. If any peptide has the same amino acid sequence, but differs only in the oxidation status of any Met, it has been counted only once. Transmembrane domain (TMD) predictions were done with TMHMM2, Octopus, and DAS: +, TMDs predicted by all three programs; (+), TMDs predicted by two programs; –, TMDs predicted by only one or no program.

Protein ID	Name or homology of depicted proteins	No. of different peptides	TMDs
Light-harvesting proteins			
jgi|Phatr2|17531	FCP 17531	5	+
jgi|Phatr2|24119	FCP 24119	4	(+)
jgi|Phatr2|13877	FCP 47485	3	+
jgi|Phatr2|6062	FCP 6062	3	+
jgi|Phatr2|18049	Lhcf 1	5	+
jgi|Phatr2|25172	Lhcf 2	6	+
jgi|Phatr2|25168	Lhcf 3/4	8	(+)
jgi|Phatr2|30648	Lhcf 5	5	+
jgi|Phatr2|29266	Lhcf 6/7	5	+
jgi|Phatr2|22395	Lhcf 8	7	(+)
jgi|Phatr2|30031	Lhcf 9	7	(+)
jgi|Phatr2|22006	Lhcf 10	6	(+)
jgi|Phatr2|51230	Lhcf 11	6	+
jgi|Phatr2|22680	Lhcf 13	3	(+)
jgi|Phatr2|25893	Lhcf 14	7	+
jgi|Phatr2|48882	Lhcf 15	2	+
jgi|Phatr2|34536	Lhcf 16	2	(+)
jgi|Phatr2|16322	Lhcf 17	3	(+)
jgi|Phatr2|11006	Lhcr 1	3	+
jgi|Phatr2|9799	Lhcr 3	3	+
jgi|Phatr2|17766	Lhcr 4	6	(+)
jgi|Phatr2|23257	Lhcr 11	2	+
jgi|Phatr2|54027	Lhcr 12	3	+
jgi|Phatr2|14442	Lhcr 13	3	+
jgi|Phatr2|14386	Lhcr 14	5	+
jgi|Phatr2|27278	Lhcx 1	4	+
jgi|Phatr2|17326	LHL 1	5	+
ATPase			
YP_874424.1	ATPase subunit F0 B	5	+
YP_874423.1	ATPase subunit F0 B'	4	+
jgi|Phatr2|49053	ATPase subunit F0 D	5	(+)
YP_874426.1	ATPase subunit F1 α	12	–
YP_874407.1	ATPase subunit F1 β	17	–
jgi|Phatr2|20657	ATPase subunit F1 γ	11	–
YP_874425.1	ATPase subunit F1 δ	6	–
PSI			
YP_874359.1	PsaA	11	+
YP_874358.1	PsaB	8	+
YP_874394.1	PsaD	3	–
YP_874428.1	PsaE	2	–
YP_874361.1	PsaF	3	+
YP_874366.1	PsaL	3	+
PSII			
YP_874444.1	PsbA (D1)	6	+
YP_874387.1	PsbB (CP 47)	13	+
YP_874376.2	PsbC (CP 43)	9	+
YP_874377.1	PsbD (D2)	7	+
jgi|Phatr2|20331	PsbO	9	(+)
jgi|Phatr2|54499	PsbQ	6	–
jgi|Phatr2|26293	PsbU	4	–
YP_874401.1	PsbV	4	(+)
jgi|Phatr2|9078	PsbZ	2	–
Cyt b6/f			
YP_874404.1	PetA	7	+
YP_874393.1	PetB	3	+
jgi|Phatr2|13358	Rieske protein	3	+
Others			
jgi|Phatr2|43037	Aminoacyl-tRNA synthetase	10	(+)
jgi|Phatr2|45335	Calmodulin	5	+
YP_874484.1	CcsaA (cytochrome c biogenesis)	2	+
jgi|Phatr2|46336	Chloride channel	2	+
jgi|Phatr2|26635	Chloride channel	2	+
jgi|Phatr2|26422	Cytochrom P450	4	+
YP_874427.1	FtsH-like protein (PSII repair cycle)	12	+
jgi|Phatr2|17504	FtsH-like protein (PSII repair cycle)	9	(+)
jgi|Phatr2|42361[a]	FtsZ (chloroplast division)	1	(+)
jgi|Phatr2|44908[a]	Ketoacyl acyl carrier protein reductase	1	+
jgi|Phatr2|33017	Mg-chelatase	6	(+)
jgi|Phatr2|45515[a]	Nucleoside diphosphate epimerase	1	+
jgi|Phatr2|19030	Phosphate translocator	2	+
jgi|Phatr2|29157	Phosphoglycerate kinase	4	(+)
Others			
jgi|Phatr2|43657	Protein transport protein	4	+
jgi|Phatr2|30690	Protochlorophyllide a reductase	3	(+)
jgi|Phatr2|12155[a]	Protochlorophyllide a reductase	1	(+)
jgi|Phatr2|50540	Tic110 (protein transport)	14	(+)
jgi|Phatr2|41856	Transketolase	16	(+)
jgi|Phatr2|24610	Triosephosphate translocator	2	+
jgi|Phatr2|45845	Zeaxanthin epoxidase	2	+
Proteins with unknown function			
jgi|Phatr2|35625	Unknown function	2	+
jgi|Phatr2|42543	Unknown function	5	+
jgi|Phatr2|42612	Unknown function	10	(+)
jgi|Phatr2|43233	Unknown function	3	(+)
jgi|Phatr2|46529	Unknown function	6	(+)
jgi|Phatr2|47006	Unknown function	4	(+)
jgi|Phatr2|47612	Unknown function	13	(+)
jgi|Phatr2|48524	Unknown function	3	+
jgi|Phatr2|49618	Unknown function	8	+
jgi|Phatr2|49850	Unknown function	2	(+)
jgi|Phatr2|54465	Unknown function	3	+

[a]Protein was present with two or more unique peptides under red light, but only one under blue light.

Table 3. Chloroplast membrane-specific proteins of *Phaeodactylum tricornutum* only identified under blue and red light, respectively, or presumably regulated by light quality during growth. Protein IDs are according to JGI version 2.0 or NCBI reference sequence. Names are according to JGI version 2.0. Differences between blue and red light are significant (t-test, $P < 0.05$).

Protein ID	Name or homology of depicted proteins
Proteins identified only under blue light	
jgi|Phatr2|26635	Chloride channel
jgi|Phatr2|26422	Cytochrome P450
jgi|Phatr2|33017	Mg-chelatase
Proteins upregulated by a factor of ≥1.3 under blue in comparison to red light	
jgi|Phatr2|51230	Lhcf 11
jgi|Phatr2|11006	*Lhcr 1*
jgi|Phatr2|27278[a,b]	Lhcx 1
jgi|Phatr2|13358[c]	Rieske protein
jgi|Phatr2|41856[a]	Transketolase
jgi|Phatr2|45335	Calmodulin
jgi|Phatr2|24610[c]	Triosephosphate translocator
jgi|Phatr2|45845[a,b]	Zeaxanthin epoxidase
YP_874427.1[c]	FtsH-like protein (PSII repair cycle)
jgi|Phatr2|48524	Unknown function
Protein identified only under red light	
jgi|Phatr2|33768	Unknown function
Proteins downregulated by a factor of ≤0.75 under blue in comparison to red light	
jgi|Phatr2|48882	Lhcf 15
jgi|Phatr2|25172	Lhcf 2
YP_874361.1	PsaF
jgi|Phatr2|12155	Protochlorophyllide a reductase

[a] $P < 0.1$.
[b] Proteins involved in photoprotection.
[c] Proteins which are typically associated with an acclimation to high light conditions.

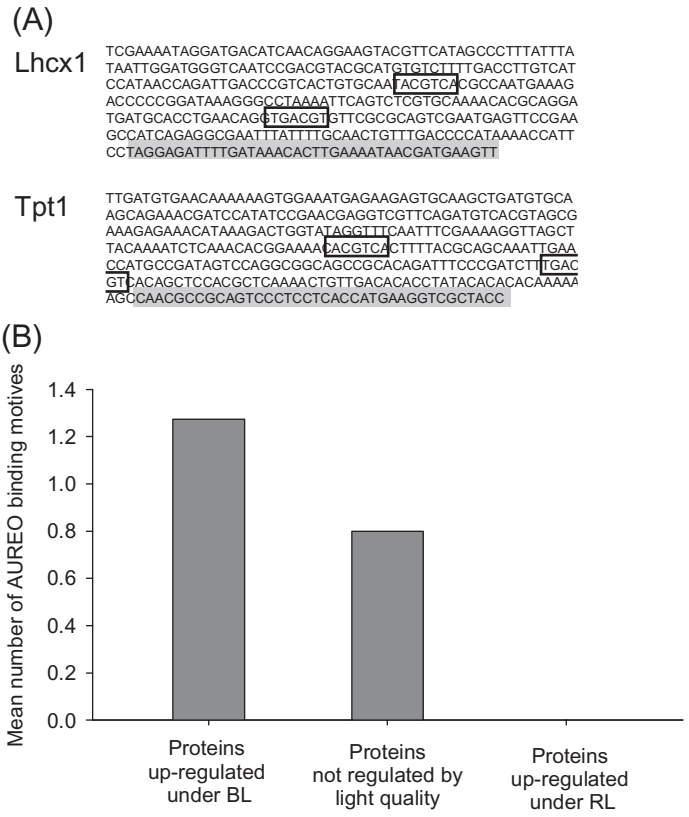

Fig. 2. (A) Promoter regions of the genes of the Lhcx1 protein (protein ID 27278) and the triosephosphate translocator protein (Tpt1, protein ID 24610). Grey background depicts the start of the transcript. Black frames indicate putative AUREO1a-binding sites. (B) Mean number of AUREO-binding motives in promoter regions 50–500 bp prior to the transcription start of nuclear-encoded proteins upregulated under blue light (BL; $n = 11$), not regulated by light quality ($n = 42$), and upregulated under red light (RL; $n = 4$).

can be separated into three classes. First, proteins with unknown function or whose role in light response appear to be not correlated to physiological data based on current knowledge. A second group covers proteins which are typically associated with an acclimation to high light conditions, e.g. proteins involved in the PSII repair cycle, the cytochrome c biogenesis, and the phosphate translocator (Table 3 note c). Although there is no direct verification by physiological data, the upregulation of these proteins is in line with the observed general high light syndrome of BL cells compared to RL cells. The changes in the expression level of LHC genes encoding for proteins which are specific for PSI (Lhcr1) could be interpreted as a remodelling of the antenna organization, but the functional consequence, for example an alteration in the absorption cross-section of both photosystems, can not be deduced from these data. Interestingly, Lhcf15, which was found neither by Grouneva *et al.* (2011) nor by Lepetit *et al.* (2011), is strongly upregulated in RL and downregulated in BL, which might indicate a RL-specific expression. The third class of proteins is upregulated under BL and is specifically involved in photoprotection (Table 3 note b), which has been studied also on the physiological level.

Diatoms are known for their large capacity to dissipate excessively absorbed light energy safely as heat by the NPQ mechanism (for a recent review, see Goss and Jakob, 2010). In addition, it is known that the extent of NPQ is triggered by the amount of Dtx to a large extent (Schumann *et al.*, 2007). Thus, to assess the photoprotective state of the cells the XC pigment pool size, the DES after 10 min of illumination under excess light conditions and the maximal NPQ during the measurement of light saturation curves were compared.

Under LL conditions, the pool size of XC pigments and the maximal NPQ values of cells grown under BL and WL were comparable to previous studies with similar irradiance (Lavaud *et al.*, 2002; Grouneva *et al.*, 2009). However, under RL conditions, cells of *P. tricornutum* possessed a significantly reduced photoprotective potential with a smaller pool size of XC pigments, a lower DES, and extraordinarily low values of the maximal NPQ. Moreover, the increase of growth irradiance under ML did not change the photoprotective potential of RL-grown cells whereas cells under BL and WL increased the

XC pigment pool size and possessed higher DES. Obviously, in *P. tricornutum* the acclimation to higher light intensities depends on the presence of BL and is inactive in monochromatic RL. Although proteomic analysis was performed on cells grown under LL conditions, the expression level of chloroplast membranes proteins of *P. tricornutum* showed an increase of several proteins typically related to HL acclimation in BL cultures compared to RL cultures. Therefore both, physiological and proteomic data point to the requirement of BL in the process of acclimation to higher light intensities.

The several zeaxanthin/Dtx epoxidases were suggested to be involved not only in the backward reaction of the XC but also in the carotenoid biosynthesis (Wilhelm *et al.*, 2006; Coesel *et al.*, 2008). Therefore, the larger pool size of XC pigments in BL cells in comparison to cells from RL cultures could be seen in line with the increased concentration of the zeaxanthin epoxidase observed in the plastidic proteome of BL cultures. Furthermore, the increased amounts of zeaxanthin epoxidase could be a consequence of the higher DES under BL and WL. A high concentration of Dtx under excess light conditions requires a fast epoxidation reaction back to Ddx under low light conditions to efficiently switch from a light protecting into a light-harvesting state (Goss *et al.*, 2006). Another interesting result is the upregulation of the Lhcx1 protein in BL cultures compared to RL cultures. Proteins of the Lhcx family and the homologous LHCSR family were recently shown to be essential for the formation and extent of NPQ in diatoms and green algae (Peers *et al.*, 2009; Bailleul *et al.*, 2010). It could be concluded that the observed differences in the maximum quenching capacity in BL cultures compared to RL cultures are related to a light quality-dependent regulation of the Lhcx1 protein level. A blue light-dependent enhancement of Lhcx1 expression might also explain the differences between the maximum NPQ of WL and BL cultures despite of their similar Ddx concentrations and DES after illumination.

Taken together, these results show that, even under the light-limited growth conditions of the present study, significant differences in the photoprotective status of BL-, WL-, and RL-grown cells are observed. Interestingly, the differences in parameters related to photoprotection of BL and RL cultures resemble the differences typically observed between high and low light-adapted WL cultures. Therefore, it is concluded that photoprotection in diatoms is regulated rather by light quality (particularly BL) than by the overall light intensity. This is clearly different to the situation in green algae and higher plants (Bräutigam *et al.*, 2010). Furthermore, the increase of maximum photosynthesis rates and XC pigment pool size in BL and WL cells grown under ML compared to LL indicates that the total amount of irradiance amplifies the light quality-induced effects of photoacclimation. This is in line with the results of previous studies examining effects of light intensity on the photophysiology of *P. tricornutum* (Lavaud *et al.*, 2002; Grouneva *et al.*, 2009).

Several perception mechanisms might be involved in the integration of the light intensity signal into the photoacclimatory signal transduction. First of all, under ML conditions the fraction of activated photoreceptors and/or their activity might be increased compared to LL conditions. It is also possible that some photoreceptors require high photon flux densities and are only activated under ML conditions. Furthermore, an increase of incident irradiance might additionally influence the reduction state of the plastoquinone pool, the thioredoxin system, the luminal pH, the NADPH to $NADP^+$ ratio, the ATP to ADP ratio, or the generation of reactive oxygen species. These processes were shown to be involved in photoacclimation in higher plants and green algae (Walters, 2005; Li *et al.*, 2009). However, the lack of a high light response of RL cells grown under ML compared to LL conditions indicates that light quality perception mechanisms dominate the photoacclimation in diatoms. In this context, it should be emphasized that high intensities of RL are highly artificial. In the natural environment of diatoms, high overall light intensities are usually combined with large amounts of BL (Kirk, 1994). Therefore, a BL-mediated photoacclimation seems to be the most appropriate strategy for these organisms.

For an assignment of the observed physiological changes during photoacclimation to specific regulatory mechanisms, experiments with photoreceptor deficient mutants will be required. However, the *in silico* analysis for the occurrence of AUREO1a-binding sites revealed that the promoter regions of genes encoding thylakoid membrane proteins upregulated under BL exhibited a mean value of 1.27 AUREO1a-binding sites, whereas no AUREO1a-binding sites were found in the promoter regions of genes encoding for thylakoid membrane proteins upregulated under RL (Fig. 2B). Interestingly, the promoter region of the Lhcx1 gene possesses even two AUREO1a-binding sites, as well as the promoter region of the triosephosphate translocator gene (*Tpt1*) (Fig. 2A). This might indicate that under BL conditions the binding of AUREO1a proteins induces an enhanced transcription of Lhcx1 and Tpt1 which could have triggered the observed changes of the protein concentrations. Interestingly, Coesel *et al.* (2009) showed that the overexpression of the crytochrome *Pt*CPF1 in *P. tricornutum* resulted in a downregulation of Lhcx1, Lhcx2, and Lhcx3 transcription rates after BL illumination, indicating that *Pt*CPF1 influences Lhcx transcription. Hence, blue light photoreceptors do probably play an important role in the regulation of photoacclimation of diatoms. Therefore, an important issue of future studies will be the generation of photoreceptor-deficient mutants. Based on the hypothesis that aureochromes participate in the regulation of the acclimation to light quantity, it could be expected that AUREO-deficient mutants will show a reduced photoprotective potential and no light intensity-dependent changes in photosynthesis rates. Finally, this should result in a reduced quantum efficiency of biomass production at medium levels of incident light as observed in RL-acclimated cells.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Comparison of the protein pattern of two different isolated thylakoid fractions from *P. tricornutum* grown under blue or red light.

Supplementary Table S1. Identified peptides of chloroplast membrane-specific proteins identified in samples isolated from *P. tricornutum* cultures grown under blue or red light.

Supplementary Table S2. Classification of probable contaminants of a chloroplast membrane fraction from *P. tricornutum*.

Acknowledgements

The authors thank Sandra Künzel for her help with the LC-ESI-MS/MS data evaluation. They are grateful to Cordula Herzog, Marcus Mann, and Claudia Nestler for their technical assistance. This work was supported by the DFG (grants Mi373/11-1 and 764/19, part of the DFG research group 1261).

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