Action Spectra of Chlorophyll *a* Biosynthesis in Cyanobacteria: Dark-Operative Protochlorophyllide Oxidoreductase-Deficient Mutants

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Z. Naturforsch. **64c**, 117–124 (2009); received April 28/July 4, 2008

Both light-dependent and light-independent (dark) protochlorophyllide (Pchlide) reductase account for catalyzing the reduction of Pchlide to chlorophyllide during the biosynthesis of Mg-tetrapyrrole pigments in cyanobacteria. To gain more insight into the interaction between the wavelength of the light and these two chlorophyll synthetic pathways in *Synechocystis* sp. PCC 6803, the spectral effectiveness of the formation of chlorophyll *a* was investigated during the regreening process in *chlL*$^-$ and *chlN*$^-$ mutants, which could not synthesize chlorophyll during growth in the dark. The action spectra showed obvious maxima around 450 nm and 650 nm, similar to those of higher plants except that the intensities of two peaks are reversed. The mRNA levels of *chlL* and *chlN* and chlorophyll *a* content under different wavelengths of light in the wild-type strain were also measured. The RT-PCR analysis revealed that the transcripts of *chlL* and *chlN* were up-regulated in red light but simultaneously down-regulated in green light which resulted in corresponding changes of the chlorophyll content. This fact indicates that the regulation of dark-operative protochlorophyllide oxidoreductase (DPOR) in the transcriptional level is essential for cyanobacteria to synthesize appropriate chlorophyll for acclimating in various light colour environments.

Key words: Dark-Operative Pchlide Oxidoreductase (DPOR), Light-Dependent Pchlide Oxidoreductase (LPOR), *Synechocystis* sp. PCC 6803

Introduction

Chlorophyll and bacteriochlorophyll, the Mg-tetrapyrrole pigments, serve as a class of key light-sensing molecules in a wide range of phototrophic organisms from photosynthetic bacteria to higher plants. The metabolic pathway for the *de novo* synthesis of Mg-tetrapyrrole represents a complex bioprocess. Taking chlorophyll for example, it is synthesized via fifteen sequential enzymatic steps, initiated from L-glutamate. Among them, the reaction which is responsible for stereospecific double-bond reduction of ring D of protochlorophyllide (Pchlide) to form chlorophyllide *a* (Chlide) is considered to be the rate-limiting step (von Wettstein *et al*., 1995; Fujita, 1996; Schoefs, 2001). This step is catalyzed by two enzymes: the dark-operative pchlide oxidoreductase (DPOR), a nitrogenase-like protein, and the light-dependent NADPH-Pchlide oxidoreductase (LPOR) (Rüdiger, 2003; Masuda and Takamiya, 2004), which is stringently regulated by light radiation (Fujita and Bauer, 2003). In photosynthetic organisms, the distribution of LPOR and DPOR is species-specific. Non-oxygen-evolving photosynthetic bacteria contain only DPOR (Xiong *et al*., 1998). In contrast, DPOR and LPOR coexist in cyanobacteria, green algae and gymnosperms (nonflowering plants), whereas angiosperms only contain LPOR (Fujita, 1996; Armstrong, 1998).

Genetic investigation of the purple nonsulfur bacterium *Rhodobacter capsulatus* suggested that at least three genes, *bchL*, *bchN*, and *bchB*, are involved in the dark form of Pchlide reduction during the generation of bacteriochlorophyll (Yang and Bauer, 1990; Burke *et al*., 1993; Armstrong, 1998). Other studies with the cyanobacterium have also confirmed that homologous genes, named as *chlL*, *chlN*, and *chlB*, are utilized for the light-independent Pchlide reduction during chlorophyll synthesis. These conclusions were further supported by the experimental evidence obtained by Wu and Vermaas (1995). They demonstrated that the deletion of either *chlL* or *chlN* in *Synechocystis* sp. PCC 6803 led to the block of chlorophyll formation in the dark.

As another key catalyst for the reduction of Pchlide, LPOR in photosynthetic organisms has also been characterized at the physiological, bio-

0939–5075/2009/0100–0117 $ 06.00

chemical and molecular levels. From a wide variety of photosynthetic organisms, including cyanobacteria (Suzuki and Bauer, 1995; Fujita and Bauer, 2000), green algae (Li and Timko, 1996), bryothytes (Takio *et al.*, 1998; Nishiyama *et al.*, 2003) and seed plants (Forreiter and Apel, 1993; Fujita, 1996; Reinbothe *et al.*, 1996; Sato-Nara *et al.*, 2004), the *por* gene which encodes the LPOR enzyme has been identified. Blast analysis of deduced amino acids of *por* surprisingly showed significant sequence similarity even among species which were considered to be evolutionarily very divergent (Dahlin *et al.*, 1999).

Although the genetic basis of the light-dependent Pchlide reduction has been clarified, there still remains a major problem: how do the specific amounts and wavelengths of photosynthetically active radiation affect the activity of LPOR? Studies on the synthesis efficiency of chlorophyll in light of different wavelengths during the greening process of higher plants from the dark to continuous light radiation provided a part of answers to this question (Ogawa *et al.*, 1973). As mentioned above, since DPOR functions in the dark independently, illustration of the action spectra of chlorophyll synthesis in cyanobacteria, green algae and gymnosperms was not an easy task. Meanwhile, the comparative contributions of the LPOR and DPOR path to chlorophyll formation responding to different light colours were also difficult to evaluate.

Fortunately, the construction of "targeting" mutants in which DPOR is blocked brings us an efficient tool to exactly solve these problems. In the present study, the action spectra of chlorophyll in cyanobacteria were successfully analyzed by using *chlL*$^-$ and *chlN*$^-$ mutants of *Synechocystis* sp. PCC 6803, which can not synthesize chlorophyll in the dark. Furthermore, in order to provide insight on how light quality regulates LPOR and DPOR, the transcriptional level of *chlL* and *chlN* in the wild-type strain under different wavelengths of light was also investigated. According to our knowledge, this is the first report on an action spectrum of chlorophyll biosynthesis in prokaryotes.

Materials and Methods

Growth conditions and light treatment

Synechocystis sp. PCC 6803 wild-type and deletion mutants (*chlL, chlN*) were reserved in our laboratory and the construction of *chlL, chlN* mutants was undertaken as previously described (Wu and Vermaas, 1995). These three strains were propagated in glass flasks with air bubbling at 28 °C. BG11 medium (Rippka *et al.*, 1979) which was supplemented with 1.5 μg/ml glucose was employed. After 3 d of growth under white light radiation, they were transferred into fresh liquid BG11 media and the initial cell densities were adjusted to 2 μg/ml. The transparent glass flasks containing new broths were, respectively, grown under different monochromatic light environments, including blue (450 nm), green (530 nm), red (660 nm) and infrared (more than 700 nm) light radiation. Specifically, irradiation with monochromatic light was provided by Philips 20 W fluorescent lamps coupled with Toshiba clear filters (No. 75, 53, 20, 781, Toshiba, Tokyo, Japan). The half band width of every monochromatic light beam was around 20 nm and the light intensities were normalized to 20 μmol m^{-2} s^{-1}. Cultivation under white light was also performed as a positive control. The growth of *Synechocystis* sp. PCC 6803 was monitored daily by measuring the absorbance at 730 nm (OD_{730}).

RNA isolation and RT-PCR

For total RNA extraction, cells grown in BG11 medium were harvested by centrifugation and washed twice with 10 mM tris(hydroxymethyl)aminomethane-HCl, 0.1 mM EDTA (pH 7.5). Approx. 300 μl of resuspended cells were mixed with the double volume of TRIzol reagent and 200 μl of glass bead, which was then broken by using a minibeadbeater (Biospec, Bartlesville, OK, USA) . The mixture was cooled on ice and then centrifuged at 20000 × *g* after adding 200 μl phenol/chloroform (1:1, v/v). The supernatant was transferred into another Eppendorf tube. After addition of 2.5 volumes of ethanol, RNA as precipitated at −70 °C, centrifuged and the precipitate washed once with 75% ethanol. The resulting pellet was dissolved in 20 μl diethyl pyrocarbonate (DEPC)-treated water and treated with RNase-free DNase I (Takara, Dalian, China) to eliminate contaminating DNA. The amount and purity of the RNA was estimated by measuring the optical density (OD) at 260 nm and 280 nm, where 1 OD at 260 nm is equivalent to approx. 40 mg/ml RNA. An OD_{260}/OD_{280} ratio of 1.7–1.8 is indicative of acceptable purity.

The expression levels of both *chlN* and *chlL* genes in *Synechocystis* sp. PCC 6803 under light of different wavelengths were relatively quantified

by the reverse transcriptase (RT)-mediated PCR technique. A reverse transcription reaction was performed in a 30 μl reaction volume using AMV-RT (New England Biolabs, Ipswich, MA, USA) and 0.6 mg of total RNA. The reaction mixtures were subsequently amplified by PCR. Specific primers were utilized for amplification of *chlN* (forward, 5′-GTCGTAAAGCTGACCAGGA-3′, and reverse, 5′-CATGCCACAGCGAATGAG-3′), *chlL* (forward, 5′-ACCCTGACTGGCTTTCTC-3′, and reverse, 5′-AGTCCCGTTTGGAAGTGC-3′), and *16S rRNA* (positive control) (forward, 5′-CAACTGGGACTGAGACACG-3′, and reverse, 5′-CCACGCCTAGTATCCATCGT-3′). PCR was carried out in a PTC-150 MiniCycler (MJ Research, Watertown, MA, USA) under the following conditions: initial denaturation at 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and then a final extension step at 72 °C for 10 min. To detect possible DNA contamination, control reactions were also performed without RT but with Taq polymerase. The resultant products were routinely measured on an 1.5% agarose gel and analyzed with a GEL-DOC2000 densitometer and a computer-aided image processing system (Bio-Rad, Hercules, CA, USA). The identities of all RT-PCR products were confirmed by DNA sequencing.

Quantitative analysis of photosynthetic pigments

To determine the chlorophyll content, *Synechocystis* sp. PCC 6803 cells were immersed in 3 ml methanol under dim light conditions after centrifugation at 12000 × *g* for 5 min. Then the cell suspension was concentrated again under the same conditions. The liquid residue of cells above was collected to determine the optical density at 666 nm by an ultraviolet spectrometer. The concentration of chlorophyll was calculated according to the following equation (MacKinney, 1941):

$$\text{concentration of chlorophyll } (\mu\text{g/ml}) = 13.9 \cdot A_{666},$$

where A_{666} represents the OD value of chlorophyll at 666 nm.

Phycocyanin (PC) and allophycocyanin (APC) were extracted according to the method developed by Su *et al.* (1992). The concentration of PC and APC was calculated according to the following equations:

$$\text{PC (mg/ml)} = (OD_{615} - 0.474 \cdot OD_{652})/5.34,$$

$$\text{APC (mg/ml)} = (OD_{652} - 0.208 \cdot OD_{615})/5.09.$$

Determination of action spectra of chlorophyll biosynthesis of cyanobacteria

ChlL and *chlN* deletion mutants were grown in darkness with air bubbling at 28 °C until the chlorophyll completely disappeared. Then these cell suspensions were equally distributed over 4 flasks and transferred into different irradiation environments (450 nm, 530 nm, 660 nm, white light) for growth. During a 3 h incubation, the cells were harvested for quantification of the chlorophyll content and to further calculate the relative efficiency of chlorophyll biosynthesis.

Results

Analysis of action spectra of chlorophyll formation in DPOR-deficient mutants

Fig. 1A shows the action spectra of chlorophyll synthesis in the blenched *Synechocystis* sp. PCC

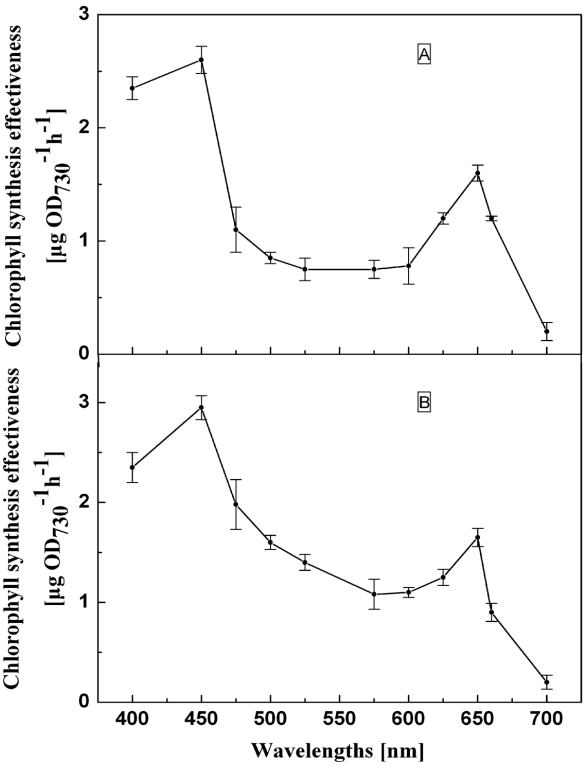

Fig 1. Action spectra of the formation of chlorophyll in (A) *Synechocystis* sp. PCC 6803 *chlL*-deletion mutant and (B) *chlN*-deletion mutant during 3 h of illumination with light of different wavelengths.

6803 *chlL*-deletion mutant during 3 h of illumination with light of different wavelengths, while Fig. 3B demonstrates the result obtained by a similar experiment with the *chlN*-deletion mutant. The profiles of the curves are almost identical, presenting a main peak at blue light (450 nm) and a smaller peak at around red light (650 nm). The obvious concaves of the curves in the range of 500–600 nm indicate comparative lower efficiency of chlorophyll biosynthesis in the green light region. These results indicate that a greater effect of chlorophyll synthesis occurs in the range of blue and red light, respectively, which is exactly consistent with absorbance spectra of Pchlide. Previous studies on angiosperms suggested that during the process of reduction of Pchlide to Chlide, Pchlide, LPOR and NADPH combine to form a tri-complex in which Pchlide accepts light energy and acts as the primary light receptor. As a consequence, the Pchlide absorbance spectrum accounts for the fluctuation of efficiency for light-mediated chlorophyll biosynthesis. Our observation strongly supports the notion that light active in the reduction of Pchlide is absorbed by Pchlide itself. Interestingly, we also found that the efficiency of chlorophyll synthesis in the blue light region is higher than in the red light region (shown in Fig. 3). These results are entirely opposite to those obtained with higher plants (Ogawa *et al.*, 1973).

Absorption spectra of Synechocystis sp. PCC 6803

Attempts to further illustrate the relative contribution of LPOR and DPOR in *Synechocystis* sp. PCC 6803 under light of different wavelengths drove us to perform a series of biochemical experiments. At first, the integrative absorption spectra of *Synechocystis* sp. PCC 6803 wild-type cells grown under various wavelengths were measured using a ultraviolet-visible spectrometer; the profile is shown in Fig. 2. The common feature of these curves is the presence of three absorption peaks at 436 nm, 625 nm and 680 nm, respectively. The peaks located at 436 nm and 680 nm represent the chlorophyll combined to the photosynthetic systems (Scheer, 1991), and the peak located at 625 nm indicates the special absorption of phycocyanin (PC), the main pigment for phycobilisomes.

Comparison of curves measured under different wavelengths of light showed that the absorption at 436 and 680 nm of cells grown in green light (530 nm) and infrared light (>700 nm) is lower than of those grown in white, blue (450 nm) and red light (660 nm). Particularly, the peaks of infrared light-grown cells almost disappeared. These observations suggest that in green light (GL), the ability of *Synechocystis* sp. PCC 6803 to synthesize chlorophyll is lower than in other light wavelengths. Under infrared light, *Synechocystis* sp.

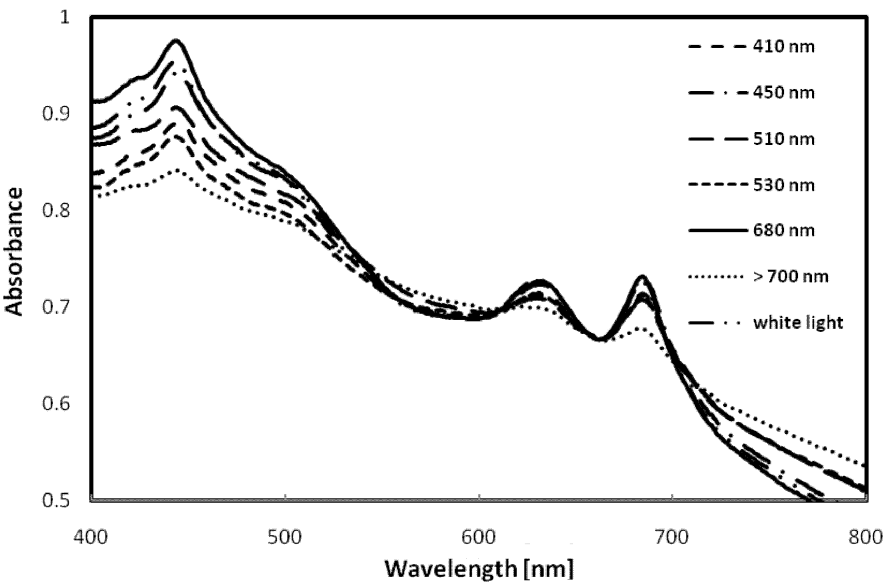

Fig 2. Absorption spectra of *Synechocystis* sp. PCC 6803 wild-type cells growing at different wavelengths.

PCC 6803 could hardly synthesize chlorophyll. In addition, compared with the peaks at 625 nm, we also found that the PC content obviously decreased in GL and infrared light environments.

Changes in the chlorophyll content under different light qualities

To further study the chlorophyll levels under different wavelengths, the relative chlorophyll content in the *Synechocystis* sp. PCC 6803 wild strain and DPOR deletion mutants was analyzed after algal cells were cultured under the conditions mentioned in Materials and Methods. As shown in Fig. 3, among the three strains grown in white,

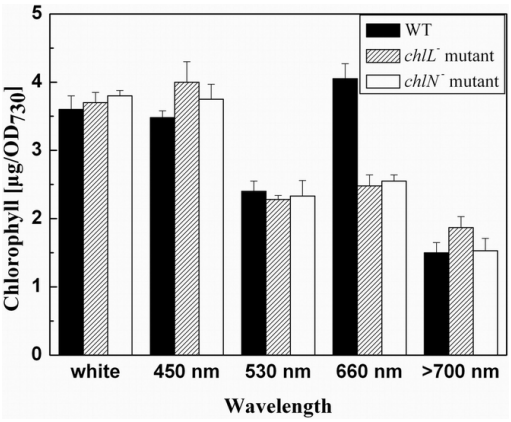

Fig 3. Chlorophyll content of *Synechocystis* sp. PCC 6803 wild-type (WT), *chlL* and *chlN* mutants at different wavelengths.

blue, green and infrared light, the content of chlorophyll has no significant difference, while under red light (RL) radiation the content of chlorophyll in *Synechocystis* sp. PCC 6803 wild-type cells is much higher than in *chlL*$^-$ and *chlN*$^-$ mutants, indicating that the deletion of the DPOR subunit, either *chlL* or *chlN*, led to a remarkable decrease of the chlorophyll content in these two mutants under RL. In addition, considering each strain alone, the chlorophyll content of wild-type *Synechocystis* sp. PCC 6803 in RL radiation is highest, whereas under blue light (BL) it is at the second-highst level. In contrast, blue illumination in *chlL* and *chlN* strains leads to a higher chlorophyll level than red light does, suggesting that the inactivation of DPOR, which exerts activity independently in the darkness, might be responsible for these changes.

Transcriptional analysis of DPOR subunits

To test whether the expression of DPOR subunits is affected by the light quality, we examined their transcriptional activities under light of various wavelenghts by RT-PCR. The 16S rRNA encoded gene which was expressed in different light qualities at approx. the same level was used as controls. No PCR product was detected using

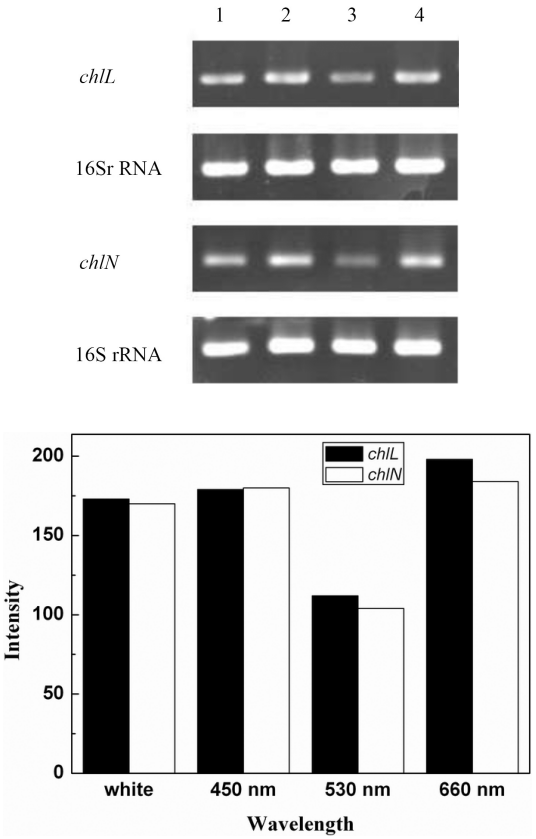

Fig 4. (A) RT-PCR analysis of subunits of DPOR in *Synechocystis* sp. PCC 6803 wild-type grown at various wavelengths. Lane 1, white light; lane 2, blue light (450 nm); lane 3, green light (530 nm); lane 4, red light (660 nm). (B) Quantification of the DPOR subunits by densitometry.

RNA as the template. As shown in Fig. 4B, the transcription levels of *chlL* and *chlN* present no significant difference in the same light, indicating that they are cotranscribed in the genome. However, among different light colours, different amounts of mRNA for DPOR appeared. *ChlL* and *chlN* were transcribed at the lowest level under GL conditions, while the expression pattern reached its maximum in RL.

By comprehensive analysis of the results mentioned above, we can assume that the obvious increase of the chlorophyll content under RL in wild-type *Synechocystis* is due to the compensational effect of DPOR, which was up-regulated in the mRNA level. Under GL radiation it was found opposite. Because both LPOR and DPOR maintained a comparative lower level, the total efficiency of chlorophyll biosynthesis was decreased, leading to cells with less chlorophyll.

Discussion

As for photosynthetic organisms, a change in the chlorophyll content is a common response triggered by different light signals (*i.e.* light intensities and wavelengths). LPOR which is responsible for light-mediated Pchlide reduction has been widely accepted as a key enzyme involved in this adaptive mechanism. However, the light-independent approach for chlorophyll increases the complexity of chlorophyll regulation in cyanobacteria. In the present study, the action spectra of chlorophyll synthesis were successfully measured in DPOR-inactivated mutants of *Synechocystis* sp. PCC 6803. We found that the chlorophyll synthesis efficiency is higher in blue light (BL) than in RL when the DPOR pathway was blocked in *chlL*$^-$ and *chlN*$^-$ mutants. This result indicates a remarkable difference to higher plants. Ogawa *et al.* (1973) reported that in higher plants the action spectra of chlorophyll synthesis are similar to those of DPOR deletion mutants in *Synechocystis* sp. PCC 6803, except that comparative intensities of two peaks are reversed: the main peak locates at the red range and the smaller peak lies in the blue region. According to Virgin (1986), it was regarded to be the strong absorption of beta-carotenoid in the blue region that decreases the absorbance of Pchlide in blue light which results in the reduction of the chlorophyll synthesis efficiency in the blue region. As a consequence, in angiosperms the highest chlorophyll synthesis efficiency occurs in the RL region.

As we reasoned, two major factors might account for the opposite phenomenon observed in *Synechocystis*. First, cyanobacteria, unlike higher plants, possess phycobilisomes as antennas making the light-harvesting function of carotenoids dispensable. Although their are also contained in *Synechocystis* as photosynthesis protective pigments their relative amounts are lower than in higher plants. According to our unpublished data and relative report (Schagerl and Muller, 2006), the ratio of carotenoids to chlorophyll in cyanobacteria is at around 0.19, which is not as high as that in leaves of higher plants. Second and more important, the absorbance maximum of phycocyanin (PC), the main light-harvesting pigment in phycobilisomes of *Synechocystis* sp. PCC 6803, is located at 620 nm. PC competes with Pchlide in absorbing RL, which leads to a decrease of the corresponding peak in that area of the spectra. Our results support the notion that the spectral effectiveness in formation of chlorophyll relies not only upon absorbance spectra of Pchlide but also upon those of other light-sensitive pigments presented. It can be also concluded that the action spectra for chlorophyll biosynthesis are almost consistent among evolutionarily divergent photosynthetic organisms from prokaryotes to higher plants. Thus, in further investigations, more attention needs to be paid to integrated mechanism including both light regulatory molecules and signal transduction channels.

Another interesting observation from the present work is that the expression of *chlL* and *chlN*, the core subunits of DPOR, is down-regulated in GL. This effect coupled with the lowest synthetic efficiency of LPOR under GL radiation results in a decreased chlorophyll content. In striking contrast to these results, Stowe-Evans *et al.* (2004); who did research about the spectral effect on transcriptionomics of the cyanobacterium *Fremyella diplosiphon*, found that the mRNA level of *chlL* and *chlN* enhances when cells were grown in GL. The opposite consequence occurred in two species of cyanobacteria which may relate to the so-called "complementary chromatic adaptation" (CCA), namely the ability of cyanobacteria to change the phycobilisome composition to adapt to various light conditions (Grossman *et al.*, 1994). When *F. diplosiphon* was grown in GL, phycoerythrin (PE, absorbance maximum at around 560 nm) accumulates to promote the efficiency of light harvesting. As a result, the increase in absorbance of photons might require greater amounts of chlorophyll a than can be synthesized by the light-dependent form of Pchlide reductase alone. Thus, DPOR up-regulates as the compensation for chlorophyll production. However, it should be noted that PE is not present in *Synechocystis* sp. PCC 6803. So, the efficiency of light absorbance in GL is correspondingly repressed, re-

sulting in the slowdown of photosynthesis as well as the growth rate. In this context, DPOR is not essential to up-regulate the production of chlorophyll *a*. In fact, as reported in numerous reports, the biosynthesis of redundant chlorophyll harms the growth of photosynthetic organisms. Therefore, this type of transcriptional regulation is essential for cyanobacteria to acclimate to changing light conditions. Recently, the evidence that expression of DPOR is altered by UV light, iron deficiency, and changes in the redox state of the photosynthetic electron transport chain has been reported in other studies (Hihara *et al*., 2001, 2003; Huang *et al*., 2002; Singh *et al*., 2003). Our data further indicate that the light colour is also an important trigger to regulate DPOR, although visible light is not required for its activity.

In summary, the relative contribution of the DPOR pathway for chlorophyll synthesis under light of different wavelengths in *Synechocystis* sp. PCC 6803 could be assessed to some extent. In RL, DPOR functioned as a compensational path to produce sufficient chlorophyll *a* for the rapid growth in this light environment. Due to the decreased efficiency in light harvesting caused by the absence of PE, under GL radiation, DPOR maintained at the basic level and the cooperative effort for chlorophyll synthesis did not occur. To date, although the corresponding biochemical process has not been described in detail, it is still worth for further investigation. In our laboratory, studies including the identification of new light colour-responsive proteins in *Synechocystis* sp. PCC 6803 by utilizing 2-DE based proteomic techniques are in progress.

Acknowledgement

This work was supported by the NSFC projects 30670476 and u0633009.

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