A Novel “Oxygen-induced” Greening Process in a Cyanobacterial Mutant Lacking the Transcriptional Activator ChlR Involved in Low-oxygen Adaptation of Tetrpyrrole Biosynthesis*

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Rina Aoki1, Yuto Hiraide, Hisanori Yamakawa, and Yuichi Fujita2
From the Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464-8601, Japan

**Background:** ChlR activates the transcription of genes encoding low-oxygen-type enzymes in response to hypoxia in cyanobacteria.

**Results:** The chlR-lacking mutant showed a novel “oxygen-induced” greening process upon exposure to air.

**Conclusion:** The contents of photosystems were correlated well with the chlorophyll contents in the greening process.

**Significance:** Oxygen-induced greening provides a promising alternative system to investigate the biogenesis of photosystems.

ChlR activates the transcription of the chlAII-ho2-hemN operon in response to low-oxygen conditions in the cyanobacterium Synechocystis sp. PCC 6803. Three genes in the operon encode low-oxygen-type enzymes to bypass three oxygen-dependent reactions in tetrpyrrole biosynthesis. A chlR-lacking mutant, ΔchlR, shows poor photoautotrophic growth due to low chlorophyll (Chl) content under low-oxygen conditions, which is caused by no induction of the operon. Here, we characterized the processes of etiolation of ΔchlR cells in low-oxygen conditions and the subsequent regreening of the etiolated cells upon exposure to oxygen, by HPLC, Western blotting, and low-temperature fluorescence spectra. The Chl content of the etiolated ΔchlR cells incubated under low-oxygen conditions for 7 days was only 10% of that of the wild-type with accumulation of almost all intermediates of the magnesium branch of Chl biosynthesis. Both photosystem I (PSI) and photosystem II (PSII) were significantly decreased, accompanied by a preferential decrease of antenna Chl in PSI. Upon exposure to oxygen, the etiolated ΔchlR cells resumed to produce Chl after a short lag (～2 h), and the level at 72 h was 80% of that of the wild-type. During this novel “oxygen-induced” greening process, the PSI and PSII contents were largely increased in parallel with the increase in Chl contents. After 72 h, the PSI content reached ~50% of the wild-type level in contrast to the full recovery of PSII. ΔchlR provides a promising alternative system to investigate the biogenesis of PSI and PSII.

Chlorophyll (Chl)3a plays an essential role in harvesting light and the initial process of photosynthetic electron transfer to convert light energy to chemical energy. Chl a molecules bind noncovalently to protein complexes of photosystem I (PSI) and photosystem II (PSII). In addition, peripheral antennas such as LHC (light-harvesting Chl a/b-binding proteins), which bind both Chl a and Chl b, are used for harvesting light in higher plants (1). In cyanobacteria and red algae, a unique light-harvesting complex, phycobilisome, which binds bilin pigments such as phycocyanobilin, is used as a peripheral antenna.

The two main photosynthetic pigments of cyanobacteria, Chl a and phycocyanobilin, are synthesized in the tetrpyrrole biosynthetic pathway (2, 3). In addition to the two pigments, heme, a prosthetic group of heme proteins, is also synthesized in the same pathway. Tetrpyrrole biosynthesis contributes not only to photosynthesis but also to a variety of biological functions, including respiration. Tetrpyrrole biosynthesis for Chl a and phycocyanobilin consists of at least 18 reactions (Fig. 1). The first nine reactions starting from glutamate to protoporphyrin IX constitute the common core pathway (4). Subsequent biosynthetic pathway is divided into two branches, magnesium branch and iron branch, by the insertion of metal ions, Mg2+ and Fe3+, into protoporphyrin IX, respectively. The magnesium branch is specific for Chl, and the iron branch is specific for heme and phycocyanobilin biosynthesis. One of the important regulatory steps in the magnesium branch is protochlorophyllide (Pchlide) reduction, the penultimate step in Chl a biosynthesis (5–8). In angiosperms, a unique enzyme light-dependent Pchlide oxidoreductase, which requires light for the catalysis, converts Pchlide to chlorophyllide a by the C17=C18 double bond reduction. As a result, seedlings of angiosperm grown in the dark become pale yellow because the Chl biosynthesis is arrested at the Pchlide reduction. The etiolated seedlings have the ability to become green upon exposure to light followed by the biogenesis of photosystems and LHC (9). In contrast, some gymnosperms, green algae and cyanobacteria have an alternative Pchlide reductase, dark-operative Pchlide oxidoreductase (DPOR), which consists of three subunits, protoporphyrin IX monomethylester; PSI, photosystem I; PSII, photosystem II; Pchlide, chlorophyllide.
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ChlL, ChlN, and ChlB, together with light-dependent Pchlide oxidoreductase. DPOR catalyzes Pchlide reduction even in the dark (8). Therefore, Pchlide reduction is independent of light in these organisms (6).

In the greening of etiolated seedlings of angiosperms, PSI and PSII start to assemble *de novo* coincident with the onset of Chl production. Thus, the light-induced greening process is suitable for analysis of biogenesis of photosystems (10). Although the green alga *Chlamydomonas reinhardtii* has the ability to green in the dark, the *y*-mutant, which lacks the DPOR activity by a nuclear genome mutation (11), is etiolated when grown heterotrophically in the dark. The etiolated *y*-cells have the ability to become green upon exposure to light due to the activity of light-dependent Pchlide oxidoreductase such as angiosperm (12). The *y*-mutant has been widely used for analysis of chloroplast biogenesis (13–15). In cyanobacteria, which are photosynthetic prokaryotes, Chl biosynthesis is also independent of light due to the presence of DPOR (16). DPOR-lacking mutants of cyanobacteria have also been used as an alternative suitable tool for greening analysis (17–19) because (i) gene manipulation is easy, (ii) all Chl molecules bind to PSI and PSII due to the lack of LHC, and (iii) genes related to photosynthesis are encoded by the single chromosome rather than two nuclear and chloroplast genomes in photosynthetic eukaryotes. We have also isolated a *chll*-lacking mutant of the cyanobacterium *Leptolyngbya boryana* (formerly *Plectonema boryanum*) and successfully prepared “etiolated” cells with a Chl content of only 0.5% of that of the WT by a semi-continuous heterotrophic cultivation in the dark. The etiolated cells quickly became green upon illumination, similar to the etiolated seedlings of angiosperms (18).

In tetrapyrrole biosynthesis of the cyanobacterium *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803), there are three oxygen-dependent reactions, coproporphyrinogen III (CPgen) oxidation, Mg-protoporphyrin IX monomethyl ester (MPE) cyclization, and heme cleavage, which are catalyzed by two types of enzymes (Fig. 1). These two types of enzymes are functionally differentiated to “aerobic-type enzymes”, which operate under aerobic conditions, and “low-oxygen-type enzymes”, which are specifically used under low-oxygen conditions to bypass the aerobic-type enzymes. In CPgen oxidation, HemF is an aerobic-type enzyme, and HemN is a low-oxygen type enzyme (20). HemF and HemN are structurally unrelated enzymes with different evolutionary origins (analogous enzymes) (21). MPE cyclization and heme oxygenation are catalyzed by two isoform pairs, ChlAⅢ/ChlAⅡ and HO1/HO2, respectively. Low-oxygen-type isoforms, ChlAⅡ and HO2, might have some special biochemical properties enabling to operate under low-oxygen conditions (22, 23). The three genes, *hemN*, *chlAⅢ*, and *ho2*, encoding the low-oxygen-type enzymes form a small operon, *chlAⅢ-ho2-hemN*, in *Synechocystis* 6803, and are transcribed only under low-oxygen conditions, whereas the other three genes, *hemF*, *chlAⅡ*, and *ho1*, encoding aerobic-type enzymes are expressed constitutively (20, 22, 23).

![FIGURE 1. The tetrapyrrole biosynthetic pathway in cyanobacteria. Only major intermediates are shown in the core pathway from glutamate to protoporphyrin IX. One arrow corresponds to one enzyme involved in the respective reaction. Enzymes operating under aerobic and low-oxygen conditions in the three reactions are shown by blue and red arrows, respectively. Intermediates detected in ΔchlR cells are shown in green.](image)

Previously, we identified a novel transcriptional regulator ChlR involved in the induction of the *chlAⅢ-ho2-hemN* operon in response to low-oxygen conditions (24). Here, we report the initial characterization of “etiolated” ΔchlR cells in low-oxygen conditions and a greening process of the etiolated ΔchlR cells after a shift to aerobic conditions. The etiolated cells contained only small amounts of PSI and PSII. Upon exposure to air, PSI and PSII contents were largely increased in parallel with the increase in Chl content. This novel oxygen-induced greening of ΔchlR would be regarded as a promising alternative system to investigate the biogenesis of photosystems.

**EXPERIMENTAL PROCEDURES**

Cyanobacterial Strains and Growth Conditions—*Synechocystis* sp. PCC 6803 and its derivative strains used in this study were cultivated in BG-11 medium as described (23). To prepare cells grown under aerobic and low-oxygen conditions, we incubated agar plates in air and in an anaerobic jar (BBL GasPak Anaerobic Systems, Becton Dickinson and Company, Franklin Lakes, NJ; Gas Generating Kit Anaerobic System, Oxoid, Basingstoke, Hants, UK), respectively. Agar plates were
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follows (Fig. 2 and Table 1). The DNA fragment containing the recombinant plasmid carrying DNA fragment was cloned into EcoRI site of pUC118. The 6803 with a pair of primers (Table 1) by a standard

illumination of Synechocystis 6803 (FLR40SW, Hitachi, Tokyo, Japan) under both conditions.

Construction of Plasmids for Gene Disruption and Transformation of Synechocystis 6803—A plasmid for the chlA_{II}, ho2-hemN operon (sll1874-sll1875-sll1876) operon disruption was constructed by overlap extension PCR (Fig. 2 and Table 1) (22). DNA fragments of the upstream (f1 and r1 primers) and downstream (f2 and r2 primers) regions of the target operon were amplified by PCR from genomic DNA of Synechocystis 6803 with a standard thermal cycle (KOD-plus DNA polymerase; Toyobo, Osaka, Japan). A kanamycin-resistant cartridge (Km) with a standard thermal cycle (KOD-plus DNA polymerase; Toyobo) and reverse primers for RT-PCR (Table 2) according to the manufacturer's instructions. Thus obtained cDNA preparation was used as the template for PCR amplification with the specific primers and cycle numbers (Table 2).

SDS-PAGE and Western Blotting—Cells were collected by centrifugation, and the cell pellets were resuspended in HEPES buffer (50 mM HEPES-KOH, pH 7.5, and 10 mM MgCl2). The harvested cells (500 µl) were disrupted by vigorous shaking (“Bug Crasher” GM-01, TAITEC, Saitama, Japan) with glass beads (100 mg, 150–212 microns, Sigma) for 60 min at 4 °C. After adding 2 mM phenylmethanesulfonyl fluoride, the resulting suspension was centrifuged at 1,360 × g for 5 min at 4 °C to discard unbroken cells. Proteins of the supernatants were separated by SDS-PAGE and Western blot analysis. The proteins separated by SDS-PAGE were transferred onto PVDF membranes (Immobilon P, Merck Millipore) and reacted with antiseras against D1, CP47, 33 kDa, PsaA/B, and PsaC (18). The specific protein bands were visualized by a chemiluminescent substrate (ECL Western blotting analysis system, GE Healthcare) with a lumino-image analyzer (LAS-3000mini, Fujifilm, Tokyo, Japan).

Preparation of Membrane Fractions—Collected cells were resuspended in thylakoid buffer (50 mM HEPES-KOH, pH 7.5, 10 mM MgCl2, 5 mM CaCl2, and 20% (w/v) glycerol) and disrupted as described above. The membrane fractions were prepared by ultracentrifugation at 100,000 × g for 60 min at 4 °C (S55A2 rotor, CS100GXII, Hitachi). The pellet was washed once, and the resulting pellet was resuspended in thylakoid buffer.
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TABLE 1

Oligonucleotides used in this study

| Primer name       | Sequence                                      | Use                                      |
|-------------------|-----------------------------------------------|------------------------------------------|
| pEcoI874E3        | 5'-CCKAAATCCGACTACATGAAAGGACCCAGACCAC-3'      | fl primer for Δoperon (EcoRI)            |
| pEcoI874E3        | 5'-GGATCCGGATCCAGAGTGCAACACATCGCTCTTACCCCGGAGACGTTTA-3' | fl primer for Δoperon (EcoRI)            |
| pEcoI874E3        | 5'-CCCGTGGGCTCAGCTGAGGAATTCATTATAGAAACAC-3' | fl primer for Δoperon (EcoRI)            |
| pEcoI874E3        | 5'-GTAACGGCCTTCTCCATCGGGAAATC-3'             | fl primer for Δoperon (EcoRI)            |
| psbAI-r           | 5'-AGAATTCCTGTGCGAGCACGC-3'                  | fl primer for ΔpsbA1 (EcoRI)            |
| psbAI-r           | 5'-GCGATGTTGTAGGTTTCTTCC-3'                  | Reverse primer for ΔpsbA1 (EcoRI)       |
| 1214specfcl       | 5'-CGAGGCTGATTCTCTATTTACTCCTGGTCGGAGGCTC-3' | Forward primer for spectinomycin cartridge |
| 1214speccl        | 5'-GTAACGGCCTTCTCCATCGGGAAATC-3'             | Reverse primer for spectinomycin cartridge |
| psfRT1874Hf       | 5'-AAACCACTTGGGCGCCGAGTATTTTTACTTCCTGGTCGGAGGCTC-3' | Reverse primer for segregation check of Δoperon |
| psfKhemnN1r1      | 5'-GCGATTGTGCTTCATCTTACATTTAATTCTGCGAGAAAAC-3' | Reverse primer for segregation check of ΔpsbA1 |
| PpsbAI-f2         | 5'-CTTGCATGCGCTTCCATGCGGAAATC-3'             | Reverse primer for segregation check of ΔpsbA1 |
| RT-sl1181-r       | 5'-CAGTTGTGTTAGTTCTCCCTTCCCT-3'              | Reverse primer for segregation check of ΔpsbA1 |

Low-temperature Fluorescence Emission Spectra—Fluorescence emission spectra of membrane fractions at 77 K were recorded with excitation and emission bandwidths of 20 and 3 nm, respectively, using a fluorescence spectrophotometer (model FP777w with a low-temperature unit PMA-281, JASCO). The excitation wavelength was 430 nm. The membrane fractions were adjusted to a protein concentration of 6 μg ml⁻¹.

RESULTS

Pigment Analysis of ΔchlR Grown under Low-oxygen Conditions—The growth of the ΔchlR-deficient mutant ΔchlR was severely retarded, and the Chl content was markedly decreased under low-oxygen conditions. Previously, we also showed that the chlAᵢ₋, ho₂-hemN operon and psbA1 are not expressed in ΔchlR even though under low-oxygen conditions (24). To determine the gene responsible for the severe phenotype, we isolated a chlAᵢ₋-operon-lacking (Δoperon), a psbA1-lacking (ΔpsbA1), and a chlAᵢ₋-operon and psbA1-lacking (Δoperon/ΔpsbA1) mutants (Fig. 3) and then compared their phototrophic growth and Chl contents with those of ΔchlR. Only the two mutants, Δoperon and Δoperon/ΔpsbA1, showed severe growth retardation with a marked decrease of Chl contents similar to ΔchlR (Fig. 3). These results indicated that the phenotype of ΔchlR is caused by the defect of the chlAᵢ₋-ho₂-hemN operon rather than that of psbA1.

To determine which step in Chl biosynthesis is interrupted in ΔchlR under low-oxygen conditions, we extracted the pigments from the ΔchlR cells with methanol and analyzed them by HPLC. As shown in Fig. 4, the ΔchlR cells accumulated a variety of Chl precursors (trace b), whereas only Chl was detected in the WT (trace a, peak 8). The precursors in ΔchlR were coproporphyrin III (peak 1), Mg-protoporphyrin IX (peak 2), divinyl Pchlide (peak 3), monovinyl Pchlide (peak 4), MPE (peak 5), protoporphyrin IX (peak 6), and demetallated MPE (peak 7). In ΔchlR, three genes, hemN, chlAᵢ₋, and ho₂, encoding oxygen-independent CPgen oxidase (HemN), low-oxygen-type MPE cyclase (ChlAᵢ₋), and heme oxygenase 2 (HO2), respectively, are not expressed under low-oxygen conditions because of the loss of chlR (24). We previously isolated three mutants, ΔhemN, ΔchlAᵢ₋, and Δho₂, in which hemN, chlAᵢ₋, and ho₂ were disrupted individually (20, 22, 23). To identify which gene is responsible for the pigment accumulation in ΔchlR, we compared the pigment profiles of these mutants with that of ΔchlR (Fig. 4, traces c–e). Only Chl was detected in Δho₂, similar to the WT (trace e), which was consistent with the normal growth phenotype as shown previously (23). In ΔhemN, only coproporphyrin III, which is the auto-oxidation product of CPgen during the extraction step, was accumulated as shown previously (Fig. 4, trace c) (20). The ΔchlAᵢ₋ mutant showed a pigment accumulation profile similar to that of ΔchlR except for coproporphyrin III (Fig. 4, trace d). These results suggested that the anomalous pigment accumulation of ΔchlR is due to the additive effect of the deficiency of the two genes, hemN and chlAᵢ₋. In addition, the growth defect with the accumulation of Chl precursors in ΔchlR is considered to be caused by the loss of induction of hemN and chlAᵢ₋ under low-oxygen conditions.

To examine whether the expression of genes for Chl biosynthesis other than hemN and chlAᵢ₋ is affected in ΔchlR, we performed RT-PCR analysis on 23 genes involved in Chl biosynthesis in the WT and ΔchlR (Fig. 5). The effect of the loss of chlR was limited to the two genes hemN and chlAᵢ₋, and the mRNA levels for all other 21 genes were substantially the same as the levels in the WT (Fig. 5B).

Oxygen-induced Greening of ΔchlR—Despite the poor growth of ΔchlR and the pale blue color due to Chl deficiency under low-oxygen conditions, the loss of chlR appeared not to be lethal. We examined whether the ΔchlR cells restored the ability to grow photoautotrophically upon exposure to air. The ΔchlR cells were incubated under low-oxygen conditions for 7 days and then transferred to the aerobic conditions (Fig. 6). During the first 12 h after the transfer, no apparent change was observed in the pale blue ΔchlR colonies on the plates. However, the cells began to grow from 24 h, and the cells returned to the normal green shading after 72 h (Fig. 6A). We measured the Chl contents during this process by HPLC (Fig. 6B). The initial Chl content of the ΔchlR cells was only ~10% of the WT. Such Chl-deficient cells could be regarded as etiolated cells. Upon exposure to air, the Chl content started to increase with a 2-h lag and reached ~80% of the WT level after 72 h. In parallel, the MPE contents were also determined by HPLC (Fig. 6C). The initial MPE content of the etiolated ΔchlR cells was ~12 times higher than that of the WT. Upon exposure to air, the MPE content decayed rapidly with a half-life of ~2 h and reached a level equivalent to the WT substantially after 48 h.
The recovery of Chl contents with a sharp decrease in MPE could be regarded as a novel greening process, which is induced by the exposure to oxygen. In contrast to the so-called greening of etiolated seedlings initiated by light, we propose to call this an oxygen-induced greening process.

**Western Blot Analysis of PSII and PSI Subunits**—To investigate the effect of Chl deficiency on the contents of PSI and PSII, we harvested the cells during the oxygen-induced greening process and subjected the total crude extracts to Western blot analysis with specific antisera against D1, CP47, 33 kDa, and PSII proteins. In the etiolated ΔchlR cells before greening, the contents of all subunits of PSI and PSII were drastically decreased to ~5% of the WT contents except for 33 kDa and CP47 (~50 and 25% of the WT contents, respectively; Fig. 7, panels c and b). Upon exposure to air, the contents of all subunits started to increase toward the WT levels. However, the recovery profiles were not the same. For the Chl-binding proteins D1 and CP47 of PSII, the levels became similar to those of the WT after 72 h (Fig. 7, panels a and b). In contrast, the contents of PsaA/B and PsaC reached ~50% of those of WT after 72 h (Fig. 7, panels d and e). The profiles indicated that the recovery of the PSI proteins is significantly faster than that of PSII proteins during the oxygen-induced greening.

There are three psbA copies psbA1 (slr1811), psbA2 (slr1311), and psbA3 (slr1867) in the genome of *Synechocystis* 6803. The psbA2 and psbA3 genes encode the normal type of D1 protein with identical amino acid sequences. The psbA1 gene encoding D1’ protein, whose amino acid sequence shows 85%
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Identity to the D1 protein, is expressed only under low-oxygen conditions (25), and the expression of psbA1 is activated by chlR (24). Therefore, the defect of psbA1 in ΔchlR may affect the amounts of D1 under low-oxygen conditions. We examined the levels of D1 protein in ΔchlR and the WT in comparison with those in other mutants (Fig. 8). The D1 level in ΔpsbA1 detected by the antisera against D1 was the same as that in the WT, although the two mutants, Δoperon and Δoperon/ΔpsbA1, showed a significant decrease of D1 protein under low-oxygen conditions similar to ΔchlR. The result confirmed that the decrease of D1 protein in the etiolated ΔchlR cells is caused by the Chl deficiency rather than the defect of psbA1.

To examine whether the Chl deficiency caused by the loss of chlR affects the transcript levels of genes encoding some subunits of photosystems, we carried out RT-PCR analysis of these genes (Fig. 9). The transcript levels of all five genes in the cDNA preparations from the WT were similar to the levels in the ΔchlR. This result indicated that Chl deficiency does not affect the transcript levels of genes encoding PSII and PSI subunits and that D1, PsaA/B, and PsaC proteins are decreased at post-transcriptional levels in the etiolated ΔchlR cells.

Low-temperature Fluorescence Emission Spectra of Membrane Fractions—To address how Chl molecules are associated with PSII and PSI in the cells during the etiolation and the greening processes, we measured low-temperature (77 K) fluorescence emission spectra of the membrane fractions in the WT and ΔchlR (Fig. 10). In the WT membrane fractions, the ratios of two emission peaks at 688–691 and 722–724 nm derived from PSII and PSI, respectively, were almost constant over time during the aerobic incubation (Fig. 10A, traces a–f).

In contrast, the 687-nm peak (slightly blue-shifted) was much higher than the 718-nm peak (slightly blue-shifted) in the ΔchlR membrane fraction (Fig. 10B, trace g), suggesting the marked decrease of peripheral Chl molecules localized in PSI antenna and the preferential accumulation of Chl molecules in PSII. The shape of spectrum did not significantly change 2 h after the aerobic incubation (trace h), which is consistent with the 2-h lag in Chl accumulation (Fig. 6B). The 718-nm peak started to increase after 12 h (trace i) and reached almost the same level (as a 723-nm peak) as that in the WT after 72 h (traces j–l).

Taking together the Chl and protein contents (Figs. 6B and 7), it was suggested that the biogenesis of PSII proceeds significantly faster than that of PSI and the full restoration of PSI would take a longer time (>72 h).

DISCUSSION

Oxygen-induced Greening of ΔchlR—In this study, we developed a novel etiolation/greening system in the ΔchlR mutant of Synechocystis 6803. ChlR is a transcriptional regulator involved in the activation of transcription of the chlA14-ho2-hemN operon in response to low-oxygen conditions (24). The loss of chlR causes a severe decrease of Chl under low-oxygen conditions because of the defect of the two bypass reactions, CPgen.
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oxidation catalyzed by HemN and MPE cyclization by ChlA
(Fig. 4). Upon exposure to oxygen, the etiolated ΔchlR cells resumed the Chl biosynthesis and the level of Chl recovered eventually to that of the WT. In the oxygen-induced greening process, the factor leading to the Chl deficiency (oxygen) and the responsible reactions in Chl biosynthesis (CPgen oxidation and MPE cyclization) are completely different from the conventional light-induced greening in angiosperms (light, Pchlide bication) are completely different from the con-
ventionalexperimental conditions for 2 days. Low-oxygen samples (lanes 1 and 2) were prepared from cells grown under low-oxygen conditions for 5 days and then transferred to air to be cultivated under aerobic conditions for 2 days. Low-oxygen samples (lanes 2 and 4) were prepared from cells grown under low-oxygen conditions for 7 days. The housekeeping gene rm16Sα was used as a control.

FIGURE 5. The Chl biosynthetic pathway (A) and transcript levels of genes involved in Chl biosynthesis in the WT and ΔchlR cells (B). Total RNAs were isolated from the WT (lanes 1 and 2) and ΔchlR (lanes 3 and 4) cells. Aerobic samples (lanes 1 and 3) were prepared from cells grown under low-oxygen conditions for 5 days and then transferred to air to be cultivated under aerobic conditions for 2 days. Low-oxygen samples (lanes 2 and 4) were prepared from cells grown under low-oxygen conditions for 7 days. The housekeeping gene rm16Sα was used as a control.

Etiolation of ΔchlR Cells under Low-oxygen Conditions—The Chl content of ΔchlR grown under low-oxygen conditions was only 10% of that in the WT. The decrease of Chl is probably caused by dilution of the pre-existing Chl in the parental ΔchlR cells rather than degradation of Chl. We previously reported the decrease of Chl due to dilution by cell division during the etiolation process of a DPOR-lacking mutant (18). While the Chl biosynthesis in the parental ΔchlR cells is arrested under low-oxygen conditions, cell division of ΔchlR could proceed because the cells still have the ability to carry out photosynthesis using the pre-existing Chl at an early stage after the inoculation. The Chl content is decreased by dilution while cell division progresses. When the Chl content per cell is reduced to some threshold level, the cells are not able to continue growth. The threshold level may be a point where the energy production by photosynthesis is comparable with the energy consumption to drive cell division and to maintain cellular activities. We observed that the ΔchlR cells continued to grow slightly but significantly during the first few days after the inoculation to low-oxygen conditions. Considering that the Chl content of the etiolated ΔchlR cells was 10% of that of the WT, it is presumed that the ΔchlR cells divided three or four times during the incubation to produce etiolated cells.

PSII and PSI in Oxygen-induced Greening—In etiolated ΔchlR cells, the amounts of PsaA/B and PsaC of PSI were decreased significantly as well as those of D1 and CP47 of PSII. A comparable decrease in the proteins of PSI and PSII was observed in etiolated states of Δpor (a light-dependent Pchlide oxidoreductase-lacking) and ΔchlL (a DPOR-lacking) mutants of Synechocystis 6803 (19). In another cyanobacterium, L. boryana, we showed a preferential decrease of PSI than PSII during the etioliation process in the DPOR-lacking mutant (18). However, not only the protein amounts of PSI but also those of D1 and CP47 decreased significantly in the
etiolated cells (18). The constant levels of mRNAs for the subunits of PSI and PSII in both cyanobacterial species confirmed that the levels of Chl-binding protein of photosystems are regulated at post-transcriptional levels. Taken together, wherever Chl biosynthesis is arrested, Chl deficiency causes the decrease in the Chl binding proteins of PSI and PSII at post-transcriptional levels.

The low-temperature fluorescence emission spectrum of the etiolated membrane (Fig. 10, trace g) suggested that the Chl molecules in PSI were decreased preferentially over PSII. The fluorescence emission at 722–724 nm is derived from peripheral antenna Chl molecules of PSI (Fig. 10, traces a–f). The
fluctuation results of Photosystems in detail. In addition to
light-induced greening is a fast reaction (completed
during the oxygen-induced greening process suggest that this
feature of fluorescence emission spectrum of a CP47 sub-
membrane (688–690 nm). This blue shift is consistent with the
10
two-h lag of Chl supply, 2) the
PSII subunits (D1, CP47, and 33 kDa) were almost restored to
the WT levels after 72 h, and 3) the PSI subunits (PsaA/B and
PsaC) reached ~50% of the WT levels after 72 h. This faster
recovery of PSI than PSI might be supported by the novel
mechanism to maintain the PSII biogenesis in the etiolated
cells. Or this may simply reflect that the PSI content is much
higher than that of PSII in cyanobacterial cells (30).

CP47 showed interesting behavior slightly different from
that of the other Chl-binding proteins (Fig. 7, panel b).
Although the contents of D1 and PsaA/B were reduced to ~5% of
those of the WT, the content of CP47 stayed ~25% in the
etiolated cells. This level was kept until 12 h and subsequently
increased reaching the WT level after 72 h. This suggests that
CP47 is significantly more tolerant to Chl deficiency than D1
and PsaA/B. A modular assembly model has been proposed for
the PSII biogenesis, in which four subcomplexes D1, D2, CP47,
and CP43 are consecutively assembled to form a monomeric
core complex of PSII (31). In the etiolated ΔchlR cells, the D1
subcomplex is degraded by Chl deficiency and the CP47 sub-
complex that is more tolerant to Chl deficiency than D1 may be
accumulated not to form RC47 complex (31). This assumption is supported by low fluorescence spectra (Fig.
10b). The emission peak of PSII in the etiolated membrane is
687 nm, which is 1–3 nm shorter than that in the WT mem-
brane (688–690 nm). This blue shift is consistent with the
feature of fluorescence emission spectrum of a CP47 sub-
complex isolated from a D1–FtsH2 mutant of Synechocystis
sp. PCC 6803 (32).

Overall profiles of Chl-dependent biogenesis of PSI and PSII
during the oxygen-induced greening process suggest that this
novel greening process is similar to that of the conventional
light-induced greening. However, time scale is quite differ-
ent. The light-induced greening is a fast reaction (completed
within 20–24 h) in angiosperm seedlings and the cyanobac-
terial DPOR-lacking mutants. In contrast, the oxygen-
induced greening of ΔchlR is a relatively slow reactions that
takes more than 72 h to complete. Thus, ΔchlR provides a
promising alternative system for analysis of the biogenesis pro-
cesses of photosystems in detail. In addition to ΔchlR, mutants
lacking chlA11, such as ΔchlA11 and Δoperon may also serve as the
model system.

Oxygen, an Important Factor for Chl Production and Assem-
bly of Photosystems—The biosynthesis of Chl needs to be finely
coordinated with Chl-binding proteins such as subunits of pho-
tosystems because upon illumination, free Chl and its inter-
mediates generate reactive oxygen species, which severely damage
the cells. In angiosperms such as Arabidopsis thaliana, most
genes that encode enzymes in Chl biosynthesis depend on
light. In contrast, in cyanobacteria Chl biosynthesis is inde-
pendent of light due to the presence of DPOR. Thus, light is
less important for the fine-tuning of Chl supply and photo-
system biogenesis in cyanobacteria than in angiosperms.
The most important environmental factor for cyanobacteria
to grow would be oxygen, which is the major limiting factor
in Chl biosynthesis because cyanobacteria thrive under var-
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ious oxygen levels. In cyanobacteria, ChlR and its regulons constitute an important piece of the elaborate mechanisms to ensure photosynthetic growth under oxygen-limited conditions.

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