Formation of prolamellar-body-like ultrastructures in etiolated cyanobacterial cells overexpressing light-dependent protochlorophyllide oxidoreductase in *Leptolyngbya boryana* (Received September 27, 2019; Accepted January 20, 2020; J-STAGE Advance publication date: April 2, 2020)

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Protochlorophyllide (Pchlide) reduction is the penultimate step of chlorophyll (Chl) biosynthesis, and is catalyzed by two evolutionarily unrelated enzymes: dark-operative Pchlide oxidoreductase (DPOR) and light-dependent Pchlide oxidoreductase (LPOR). Because LPOR is the sole Pchlide reductase in angiosperms, dark-grown seedlings of angiosperms become etiolated. LPOR exists as a ternary complex of Pchlide-NADPH-LPOR to form paracrystalline prolamellar bodies (PLBs) in etioplasts. Because LPOR is distributed ubiquitously across oxygenic phototrophs including cyanobacteria, it would be important to determine whether cyanobacterial LPOR has the ability to form PLBs. We isolated a DPOR-less transformant \(\Deltachl/LPORox\), carrying a plasmid to overexpress cyanobacterial LPOR in the cyanobacterium *Leptolyngbya boryana*. The transformant did not produce Chl in the dark and became etiolated with an accumulation of Pchlide and LPOR. Novel PLB-like ultrastructures were observed in etiolated cells, which disappeared during the early stage of the light-dependent greening process. However, the rate of Chl production in the greening process of \(\Deltachl/LPORox\) was almost the same as that observed in the control cells, which carried an empty vector. An *in vitro* LPOR assay of extracts of dark-grown \(\Deltachl/LPORox\) cells suggested that the PLB-like structures are deficient in NADPH. Low-temperature fluorescence emission spectra of membrane fractions of the etiolated cells indicated the absence of the photoactive form of Pchlide, which was consistent with the inefficiency of the greening process. Cyanobacterial LPOR exhibited an intrinsic ability to form PLB-like ultrastructures in the presence of the co-accumulation of Pchlide; however, the PLB-like structure differed from the authentic PLB regarding NADPH deficiency.

**Key Words:** cyanobacteria; etiolation; greening; light-dependent protochlorophyllide oxidoreductase; prolamellar body; protochlorophyllide; transmission electron microscopy

**Abbreviations:** Chl, chlorophyll \(a\); Chlide, chlorophyllide \(a\); DPOR, dark-operative protochlorophyllide oxidoreductase; LPOR, light-dependent protochlorophyllide oxidoreductase; Pchlide, protochlorophyllide; PLB, prolamellar body

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**Introduction**

Chlorophyll \(a\) (Chl), which is synthesized from glutamate through at least 15 reactions, is a tetrapyrrole pigment that is essential for photosynthesis, and is the most abundant natural pigment on Earth (Chen, 2014; Fujita and Yamakawa, 2017; Kobayashi and Masuda, 2016; Masuda and Fujita, 2008; Tanaka and Tanaka, 2007). Reduction of protochlorophyllide (Pchlide) is one of the most important regulatory steps in the Chl biosynthetic pathway. In angiosperms, this reduction is catalyzed by the light-dependent Pchlide oxidoreductase (LPOR), which requires light for catalysis (Zhang et al., 2019); therefore,
seedlings of angiosperms grown in the dark accumulate a large amount of Pchlide in colorless plastids, which are called etioplasts (Gabruk and Mysliwa-Kurdziel, 2015; Masuda et al., 2009). LPOR accumulates as a ternary complex with its substrates Pchlide and NADPH, to form paracrystalline aggregates called prolamellar bodies (PLBs) in etioplasts. Once the etiolated seedlings are exposed to light, Pchlide molecules in the ternary complex are immediately converted to chlorophyllide a (Chlide), followed by Chl production; subsequently, the PLBs are degraded to form thylakoid membranes in parallel with massive de novo Chl biosynthesis. Analyses of PLBs have revealed that physiological functions, morphological dynamics in the greening phase, and protein and lipid composition, as well as the roles of galactolipids in this system (Blomqvist et al., 2008; Fujii et al., 2017, 2018, 2019; Gabruk et al., 2017; Grzyb et al., 2013; Solymosi and Schoefs, 2010).

In contrast to angiosperms, many phototrophs have an alternative Pchlide reductase that works in a light-independent manner, and is called dark-operative Pchlide oxidoreductase (DPOR); thus, the photosynthetic organisms that carry DPOR can synthesize Chl even in the dark (Fujita and Bauer, 2000, 2003; Masuda et al., 2009; Moser et al., 2013; Muraki et al., 2010; Reinbothe et al., 2010; Vedalankar and Tripathy, 2019). DPOR consists of three subunits ChlL, ChlN, and ChlB, the amino acid sequences of which show significant similarity to those of the nitrogenase subunits NiIF, NiD, and NiK, respectively (Fujita and Bauer, 2003). Most oxygenic phototrophs, with the exception of angiosperms, employ both LPOR and DPOR in their Chl biosynthetic pathways, and have the ability to produce Chl even in the dark (Fujita et al., 1992; Stolárik et al., 2017, 2018; Yamamoto et al., 2011, 2017). These organisms maintain Pchlide levels at a minimum even in the dark, and no PLB is formed, with some exceptions. In seedlings of larch (Larix decidua) grown in the dark, the expression of chloroplastic DPOR genes (chlL, chlN, and chlB) is suppressed, resulting in the accumulation of Pchlide and the formation of small PLBs in etioplasts, which are intermediate plastids between chloroplasts and etioplasts (Stolárik et al., 2018). This indicates that the LPOR of angiosperms has the ability to form the PLB structure.

LPOR is believed to have emerged in ancestral cyanobacteria from a gene family encoding for the short-chain dehydrogenases/reductases, followed by the transfer to eukaryotic photosynthetic organisms via endosymbiosis. Currently, LPOR is distributed ubiquitously across oxygenic photosynthetic organisms. Cyanobacteria harboring both LPOR and DPOR have the ability to produce Chl in the dark (Fujita et al., 1992), and the expression level of the por gene, which encodes LPOR, is kept low even in the dark (Plohnke et al., 2015). Some morphological changes were observed in the thylakoid membranes of dark-grown cyanobacteria, but no PLB structure was detected (Barthel et al., 2013; Peschek and Sleytr, 1983; Plohnke et al., 2015). Thus, it would be important to determine whether cyanobacterial LPOR can form PLB structures under Pchlide accumulation conditions.

In this study, we used a chlL-knockout mutant, YFC2 (ΔchlL), of the cyanobacterium Leptolyngbya boryana dg5 (Kada et al., 2003). A plasmid for the overexpression of LPOR of L. boryana was introduced into the ΔchlL cells and the transformant ΔchlL/LPORox was isolated. Using transmission electron microscopy, novel PLB-like ultrastructures were observed in the transformant cells grown in the dark. Moreover, morphological changes in the membrane structures upon light exposure were detected as a light-dependent greening process. In addition, LPOR activity was assayed in extracts of ΔchlL/LPORox cells containing the PLB-like structures. Based on these results, the intrinsic and hidden characteristics of cyanobacterial LPOR and the intracellular environments in cyanobacterial cells that are necessary for the formation of PLB-like structures were discussed.

Materials and Methods

Cyanobacterial strains and growth conditions. Cyanobacterial strains were cultivated in BG-11 medium supplemented with 10 µg ml⁻¹ chloramphenicol for plasmid maintenance, as described previously (Yamamoto et al., 2009). The chlL-disrupted mutant ΔchlL (YFC2, Kada et al., 2003), derived from the cyanobacterium Leptolyngbya boryana IAM-M101 strain dg5 (formerly Plectonema boryanum) (Fujita et al., 1996; Hiraide et al., 2015) was used as the host strain to overexpress LPOR. For heterotrophic growth in the dark, 30 mM glucose was added to BG-11 medium and cultures were kept in the dark using black cloths. After the etiolating step, the cultures were inoculated into BG-11 (without glucose) and illuminated at a light intensity of 50 µmol photons m⁻² s⁻¹, to start the greening process. The overexpression of LPOR was induced by the addition of 1 mM IPTG.

Construction of the overexpression plasmid. The coding region of the por gene (LBDD_07590) of L. boryana was amplified by PCR using a primer pair (por 1,5′-GTTTCCATGGCACAGGATCAAAAAACCC-3′; and por 3,5′-TTAGGATCTTAAAGCGAGTCCAACGAGCTT-3′), the NcoI and BamHI recognition sequences are underlined and double underlined, respectively) and genomic DNA from L. boryana as the template. The amplified fragment was cloned into the NcoI-BamHI site of pQE60 (Qiagen, Chatsworth, CA, USA), to yield pPORII (H. Takagi, Y. Fujita and T. Hase, unpublished). To overexpress LPOR in L. boryana, we constructed the shuttle plasmid pYF102, which contained the Xhol-PnuII fragment consisting of the T₂ promoter, the por gene, and the terminator from pPORII in the Xhol and EcoRV sites of the shuttle vector pPBH201 (Walton et al., 1993). A lacF gene was introduced into pYF102 to regulate the expression of LPOR in cyanobacterial cells. The KpnI-SalI fragment carrying a lacF gene fragment from pTrC99A (Amann et al., 1988) was inserted into the KpnI-Xhol site of pYF102, to yield pYFP202 (Y. Fujita, unpublished). pYFP202 was introduced into cyanobacterial cells by electroporation (Fujita et al., 1992) and the transformant ΔchlL/LPORox was isolated. For E. coli heterologous expression, phKPPpor, which expresses LPOR as a Strep-tag fusion protein, was constructed. The por fragment was amplified by PCR with
the primer pair Pbporf1 (5′-ATCTAAGGTCTCTGGCCGCCAGAGATCAAAAAACCAG-3′, the BsaI site is underlined.) and Pbporr1 (5′-GATAGCGGCTCATACTTAAAGCGAGTCACAGGCTTC-3′, the BsaI site is underlined.) using the L. boryana genomic DNA as a template and was introduced into the BsaI sites of pASK-IBA5plus (IBA, Göttingen, Germany).

**Determination of Chl and Pchlide content.** Pigments in the cyanobacterial cells were extracted in 90% (v/v) methanol as described previously (Yamazaki et al., 2006). The content of Chl and Pchlide in the methanol extracts was determined by spectroscopic (Kada et al., 2003) and HPLC methods. For HPLC analysis, aliquots (20 µl) of the methanol extracts were applied to a 4.6 × 150 mm Symmetry C8 3.5 µm column (Waters), and pigments were separated as described previously (Goto et al., 2010; Zapata et al., 2000).

**Western blot analysis.** Western blot analysis was carried out essentially as described previously (Yamazaki et al., 2006). Proteins were transferred from SDS-PAGE gels onto PVDF membranes, which were incubated with the antiserum (anti-LPOR-6xHis; Fujita et al., 1998). The LPOR protein signals were visualized using a chemiluminescent substrate (Yamamoto et al., 2009). Protein concentrations were determined using the Protein Assay (Bio-Rad) and bovine serum albumin as a standard.

**Transmission electron microscopy analysis.** Cyanobacterial cells grown on BG-11 plates with or without 30 mM glucose in the dark (heterotrophic) or light (photoautotrophic) conditions were collected and suspended in BG-11 containing 1% (v/v) glutaraldehyde. Specimens were prepared for electron microscope observation as described previously (Okhi and Fujita, 1996). Thin sections were stained with uranyl acetate and lead citrate and ultrastructures were observed with an electron microscope (JEM1210, JIEL, Tokyo).

**Immunocytochemical detection.** To visualize the localization of LPOR in the cyanobacterial cells, immunoocytochemical detection was performed as described previously (Taniuchi et al., 2008). The cyanobacterial cells were resuspended in PBS containing 3% (w/v) paraformaldehyde (PFA, Nakarai, Kyoto) and kept at 4°C for 6 h. The cells were collected, resuspended in chilled ethanol and stored at −30°C until use. For permeabilization and blocking, the cells incubated with 5% DMSO and 15% neutral sheep serum (Cosmo bio, Japan) in PBS at 4°C for overnight. The cells were washed with PBS twice and incubated with the anti-POR-6xHis antiserum (1:2000 dilution in PBS) at 4°C for 4 h. After the incubation, the cells were washed twice with PBS containing 0.2% Triton X-100 and incubated with the secondary antibody (fluoro-chrome-conjugated goat anti-rabbit IgG (Alexa Fluor 488, Molecular Probes)) in PBS at room temperature for 1 h. The immunoreactions were detected under an epifluorescence microscope (BX51, Olympus) equipped with a CCD camera (CP70, Olympus). The filter set used for the observation of autofluorescence from phycobiliproteins and Alexa Fluor 488 fluorescence were U-MWIG3 (DM570/BPB530-550/BA575IF/DM570) and U-MINIB3 (DM505/BP470-495/BA510IF/DM505), respectively.

**In vitro LPOR assay.** LPOR activity in the dark-grown ∆chlL/LPORox cells was detected using total extracts. ∆chlL/LPORox cells were heterotrophically grown for 14 days in the dark. All subsequent procedures were performed in the dark or under dim green light at 4°C. The cells were suspended in lysis buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 20% (w/v) glycerol, and 0.1% (v/v) Triton X-100) and homogenized using a Bug Crashar (TAITEC, Koshigaya) using glass beads (Sigma, USA) for 60 min. Unbroken cells and glass beads were separated from the extracts by pelleting at 1,300 × g for 3 min. Aliquots of the crude extracts were illuminated with fluorescent lamps (50 µmol photons m⁻² s⁻¹) for 70 min at 30°C. NADPH (1 mM) or purified LPOR were added to the extracts, as required. The reactions were stopped by...
the addition of methanol (final concentration, 90%). Pigments in the methanol extracts were separated by HPLC to determine the formation of Chlide. The kinetic parameters of LPOR from *L. boryana* were also determined using *E. coli* heterologous expression. LPOR was expressed in *E. coli* with a Strep-tag, as described previously (Yamamoto et al., 2008). Harvested cells were suspended in the lysis buffer and disrupted by sonication. The soluble fraction was collected by ultracentrifugation at 110,000 × g for 30 min (Rotor, RP-50; Hitachi). Assays of LPOR were performed in a 100 µl volume containing 50 mM Tris-HCl (pH 7.5), 200 µM NADPH, 0.1% (v/v) Triton X-100, 0.1% (v/v) β-mercaptoethanol, 2–20 µM Pchlide, and an *E. coli* crude extract expressing LPOR. All assays were carried out at 30°C with illumination provided by fluorescent lamps (50 µmol photons m⁻² s⁻¹). The LPOR protein was purified from the *E. coli* crude extract using a Strep-Tactin sepharose column as described previously (Yamamoto et al., 2008).

**Results**

**Overexpression of LPOR in *L. boryana* YFC2**

To create special cellular environments in which large amounts of Pchlide and LPOR would accumulate concomitantly, we isolated the transformant ΔchlL/LPORox, which overexpresses LPOR in the DPOR-deficient mutant YFC2 (ΔchlL) of *L. boryana* dg5. We isolated three transformants as control strains: WT/CV (the wild-type strain carrying the control vector), WT/LPORox (the wild-type strain carrying LPOR-overexpression plasmid), and ΔchlL/CV (ΔchlL carrying the control vector). Overexpression of LPOR in ΔchlL/LPORox was confirmed by Western blot analysis using the anti-LPOR-6xHis antiserum (Fig. 1A). The content of the LPOR protein in ΔchlL/LPORox cells was much higher than that detected in ΔchlL/CV and WT/CV cells. The LPOR content in ΔchlL/LPORox in the presence of IPTG was about 15 times higher than that of the control strain WT/CV. Subsequently, the content of Chl and Pchlide in these transformants was compared (Fig. 1B). The Chl content of ΔchlL/CV, ΔchlL/LPORox, WT/CV, ΔchlL/CV and ΔchlL/LPORox were grown in light (A) or dark (B) conditions in the presence of 1 mM IPTG and 30 mM glucose. Representative images of transverse (upper panels) and longitudinal (lower panels) sections are shown for each transformant.
CV, and WT/LPORox was almost identical, with undetectable levels of Pchlide in the light conditions (data not shown). However, when grown in the dark for 20 days, the Chl content of ΔchlL/CV and ΔchlL/LPORox was reduced to very low levels, 0.41 and 0.57 µM OD₇₃₀⁻¹, respectively, because of the loss of DPOR; this was in contrast with the normal Chl contents, 4.0 and 5.5 µM OD₇₃₀⁻¹, observed in dark-grown WT/CV and WT/LPORox, respectively (Fig. 1B). Large amounts of Pchlide were accumulated in ΔchlL/CV and ΔchlL/LPORox (2.3 and 2.7 µM OD₇₃₀⁻¹, respectively), which were about 80 times higher than those of WT/CV and WT/LPORox (both 0.03 µM OD₇₃₀⁻¹) (Fig. 1B). The Chl and Pchlide content of ΔchlL/LPORox was almost identical to that of ΔchlL/CV (Fig. 1B), suggesting that the overexpression of LPOR has little impact on the metabolic flow of Chl biosynthesis. These results confirmed that the cultivation of ΔchlL/LPORox in the dark caused the co-accumulation of LPOR and Pchlide in cyanobacterial cells.

Detection of ultrastructures in the etiolated cyanobacterial cells

The intracellular structures in these cyanobacterial transformants grown in the light or dark were compared by transmission electron microscopy (Figs. 2 and 3). All transformants grown in the light conditions developed normal thylakoid membranes, which are typical multilayer structures located just inside the cytoplasmic membranes (Fig. 2A). Dark-grown WT/CV cells also exhibited the normal thylakoid membranes, which is consistent with the normal Chl content detected, even in the dark (Fig. 2B). In contrast, dark-grown ΔchlL/CV, which contains only a small amount of Chl (Fig. 1B), showed an aberrant membrane pattern, with indistinct fragmented membranes dispersed in the cytoplasm (Fig. 2B). In some cells, the fragmented membranes formed a few stacked layers. These fragmented membrane structures were probably caused by Chl deficiency. Interestingly, in dark-grown ΔchlL/LPORox cells, aberrant ultrastructures were observed (Fig. 2B, ΔchlL/LPORox). These ultrastructures were classified into two types: cross-crystal-like lattice structures (Fig. 3, arrows 1 with solid lines) and shapeless cloudy structures (Fig. 3, arrows 2 with broken lines). The lattice structures were found only in ΔchlL/LPORox, whereas the cloudy structures were also found in some ΔchlL/CV cells. Of note, these unique structures were not detected in the light-grown ΔchlL/LPORox cells or in the dark-grown WT/LPORox cells (data not shown), both of which accumulated LPOR in large amounts, with normal levels of Chl. These observations suggest that the lattice structures are specific to the cells that co-accumulate LPOR and Pchlide.

Fig. 3. Transmission electron micrographs of ΔchlL/LPORox cells grown in the dark in the presence of 1 mM IPTG and 30 mM glucose.

To visualize the unique ultrastructures in dark-grown ΔchlL/LPORox cells, four additional images (A–D) are shown. The lattice structures are indicated by solid arrows (1) and the cloudy structures are indicated by broken arrows (2).

Fig. 4. Immunocytochemical detection of LPOR in the transformants. ΔchlL/LPORox were grown in the light (A) and dark (B). WT/LPORox and WT/CV were grown in the dark (C) and light (D), respectively. All cells were fixed in 1% glutaraldehyde, followed by incubation with the anti-LPOR-6xHis antiserum, and specific signals were detected by Alexa Fluor 488 (right panels). Autofluorescence (red fluorescence) derived from phycobiliproteins in the cyanobacterial cells is shown in the left panels on the left. Bars, 10 µm.
The localization of the overexpressed LPOR protein in the cyanobacterial cells was examined using the immunocytochemical detection method (Taniuchi et al., 2008). After the permeabilization step, cyanobacterial cells were incubated with the anti-LPOR-6xHis antiserum and the localization of LPOR was visualized using a secondary antibody conjugated to Alexa Fluor 488 and an epifluorescence microscope. The autofluorescence of cyanobacterial cells (Fig. 4, left panels) and the fluorescence from Alexa Fluor 488 (Fig. 4, right panels) were observed. While green signals from Alexa Fluor 488 were not detected in WT/CV cells because of a low content of LPOR (Fig. 4D), the two transformants ΔchlL/LPORox and WT/LPORox showed significant signals from Alexa Fluor 488, indicating that the fluorescence resulted from the overexpression of LPOR. In the light-grown ΔchlL/LPORox and the dark-grown WT/LPORox cells, the green fluorescence signals from Alexa Fluor 488 were distributed uniformly in the cells. In contrast, the green fluorescence signals were heterogeneous, with some dark-grown ΔchlL/LPORox cells showing strong spot fluorescence signals (Fig. 4B, right panels). These results suggest that the overexpressed LPOR aggregates and is distributed heterogeneously only in dark-grown cells with Pchlide accumulation (Fig. 4B), while in the light-grown cells the overexpressed LPOR is dispersed throughout the cytoplasm not to form such aggregations and normal thylakoid membranes develop in the presence of a normal level of Chl (Fig. 4A, C; right panels).

**Greening process of etiolated cyanobacterial cells**

Dark-grown ΔchlL cells containing a small amount of Chl and a large amount of Pchlide start to produce Chl upon illumination, i.e., the light-dependent greening process of etiolated cyanobacterial cells (Kada et al., 2003). To examine whether the large accumulation of LPOR accelerates the greening process, the rates of Chl synthesis and Pchlide reduction were compared between ΔchlL/LPORox and ΔchlL/CV (Figs. 5A and B). Before light exposure, ΔchlL/LPORox and ΔchlL/CV grew heterothropically in the dark over 1 month. The dark-grown ΔchlL/LPORox and ΔchlL/CV cells contained very low amounts of Chl (0.26 μM OD730⁻¹ and 0.31 μM OD730⁻¹, respectively). After light exposure, the Chl levels of both the contents of Chl (A) and Pchlide (B) in the dark-grown ΔchlL/LPORox cells (filled circles; solid line) and the dark-grown ΔchlL/CV cells (open circles; dotted line) were determined during the greening process. As a wild-type control, the Chl content of WT/CV is also shown (open squares; dashed line). The greening process of ΔchlL/LPORox cells (C, D, and E) and ΔchlL/CV cells (F, G, and H) was observed using transmission electron microscopy at 0 h (C and F), 6 h (D and G), and 23 h (E and H) after light irradiation.
strains started to increase, with a short lag (about 5 h), to 4.1 μM OD_{730}^{-1} and 3.7 μM OD_{730}^{-1}, respectively, in 24 h. Seventy-three hours after light exposure, the Chl levels reached 6.3 μM OD_{730}^{-1} and 6.2 μM OD_{730}^{-1}, respectively. The Pchlide levels of ∆chlL/LPORox and ∆chlLI/CV cells (1.3 μM OD_{730}^{-1} and 2.1 μM OD_{730}^{-1} at 0 h, respectively) dropped sharply after light exposure, followed by a gradual decrease to undetectable levels within 20 h (Fig. 5B). The rates of the Chl increase and the decay of Pchlide levels in ∆chlL/LPORox cells were similar to those observed in ∆chlLI/CV.

During the greening process, the development of thylakoid membrane in these cyanobacterial cells was observed by transmission electron microscopy (Figs. 5C–H). In ∆chlL/LPORox, the dark-grown initial cells (0 h) showed undeveloped thylakoid membranes and the unique ultrastructures as shown in Fig. 3 (Fig. 5C). Six hours later, the unique ultrastructures had disappeared and new thylakoid membranes were formed, but remained fragmentary, which would develop to form stacked typical thylakoids (Fig. 5D). Furthermore, oval-shaped granules (major axis, 140–180 nm; minor axis, 90–100 nm) were observed near the developing thylakoid membranes at 23 h (major axis, 140–180 nm; minor axis, 90–100 nm) were thylakoids (Fig. 5D). Furthermore, oval-shaped granules would develop to form stacked typical thylakoids as shown previously (Fig. 5F). Six hours later, developing thylakoid membranes were detected (Fig. 5G) and the oval-shaped granules were also observed in the 23-h cells, as reported for ∆chlL/LPORox (Fig. 5H).

These results suggest that LPOR overexpression and the unique ultrastructures do not have any significant effects on the greening process that is triggered by light irradiation in the etiolated cyanobacterial cells.

**Kinetic parameters of LPOR and in vitro LPOR assay of etiolated cells**

To confirm the biochemical properties of the overexpressed cyanobacterial LPOR, we determined the kinetic parameters of LPOR (Fig. 6). The apparent $K_m$ value for Pchlide was $8.5 \pm 0.7 \mu M$ in the presence of 200 mM NADPH. Similarly, the $K_m$ value for NADPH was $7.8 \pm 0.5 \mu M$ in the presence of 4 μM Pchlide. These kinetic parameters of LPOR were comparable to those of LPOR from the cyanobacterium *Synechocystis* sp. PCC 6803 (Table 1, Masuda et al., 2009).

To determine whether the LPOR that accumulated in the dark-grown ∆chlL/LPORox cells is competent for the catalysis of light-dependent Pchlide reduction, an *in vitro* LPOR assay of the crude extracts from dark-grown ∆chlL/LPORox cells was performed. Upon light exposure, the crude extract itself did not show any Chlide formation activity, even after 70 min of incubation in the light (Fig. 7, trace 2). No Chlide formation was observed even when the purified LPOR protein was added to the crude extract (Fig. 7, trace 4). However, the addition of 1 mM NADPH to the crude extract led to significant formation of Chlide (Fig. 7, trace 3). These results suggest that the overexpression of LPOR in dark-grown ∆chlL/LPORox cells does not lead to photoconversion of Pchlide because of a deficiency of NADPH, which would be consistent with the observation that the co-accumulation of LPOR and Pchlide did not accelerate the light-dependent greening process.

**Low-temperature fluorescence emission spectra of the membrane fractions of the etiolated cells**

Low-temperature fluorescence emission spectra of the membrane fractions of the etiolated cells were obtained to examine whether the photoactive Pchlide was contained in these cells (Fig. 8). In angiosperm etioplastic membrane fractions, Pchlide molecules exist in the photoactive and nonphotoactive forms, which exhibit fluorescence emission peaks at 655 nm and 633 nm in low-temperature fluorescence emission spectra, respectively. Only the photoactive Pchlide (F655) undergoes photoconversion to form Chlide upon the application of a light flash, whereas the nonphotoactive Pchlide (F633) persists even after the application of the flash and does not contribute to photoconversion.

We prepared membrane fractions from dark-grown ∆chlL/LPORox and ∆chlLI/CV cells and recorded low-temperature fluorescence emission spectra in the presence of NADPH (Fig. 8). Both membrane fractions yielded fluorescence emission spectra with a peak at 632 nm, which were similar to those of ∆chlL reported previously by us.
Upon irradiation with a flash of light, there was no substantial change in the two membrane fractions was observed. However, after 10 min of incubation in the light, a new peak appeared at 660 nm (corresponding to Chlide) in $\Delta$chlL/\text{LPORox}, but not in $\Delta$chlL/\text{CV}.

The appearance of Chlide after 10 min of incubation, rather than just after the flash irradiation, in $\Delta$chlL/\text{LPORox} and the absence of Chlide formation in $\Delta$chlL/\text{CV} are consistent with the content of LPOR in these transformants.

Discussion

In this study, we isolated a transformant ($\Delta$chlL/\text{LPORox}) that overexpressed LPOR and accumulated Pchlide in the dark. In addition, we observed unique PLB-like ultrastructures in the dark-grown $\Delta$chlL/\text{LPORox} cells and a light-dependent greening process in the etiolated cyanobacterial cells. These results suggest that cyanobacterial LPOR contributes to the formation of the PLB-like structures in the presence of Pchlide. However, analyses of the greening process and in vitro LPOR activity, and low-temperature fluorescence spectra, suggested that the overexpressed LPOR exists as a binary complex, LPOR-Pchlide, not including NADPH.

**PLB-like ultrastructures in etiolated cyanobacteria**

The cloudy structures observed in the dark-grown $\Delta$chlL/\text{LPORox} cells (Fig. 3) were also found in dark-grown $\Delta$chlL/\text{CV} cells, which did not overexpress LPOR, suggesting that the cloudy structure is caused by the Chl deficiency or the accumulation of Pchlide. Similar cloudy structures were detected in the dark-grown $\gamma$-I mutant of the green alga *Chlamydomonas reinhardtii* (Friedberg et al., 1971), which accumulated Pchlide because of the loss of DPOR activity in the dark. Given that a similar structure was also observed in dark-grown *Synechocystis* sp. PCC 6803 (wild-type) (Barthel et al., 2013), the cloudy structure may be caused by Chl deficiency, rather than by Pchlide accumulation.

The lattice structure detected in dark-grown $\Delta$chlL/\text{LPORox} cells is regarded as a specific structure that resulted from the co-accumulation of LPOR and Pchlide. In the lattice structures, short-flat membranes are stacked, form many layers, and are extended in a disordered fashion. These features appear to be different from those of the authentic etioplast PLBs, which form a regular and
ordered lattice structure (Kowalewska et al., 2016). Electron microscopy showed that some cells were filled with the lattice structure, whereas other cells had few of them (Fig. 3). This heterogeneous distribution of the lattice structures appears to be consistent with the heterogeneous LPOR distribution found in the dark-grown ∆chlL/LPORox cells, as visualized by immunocytochemical detection (Fig. 4B). It would be reasonable to assume that LPOR is localized in the lattice structure in ∆chlL/LPORox cells, although we cannot rule out the possibility that this non-uniform distribution of LPOR may be caused by unknown physiological differences in individual cells.

**Light-dependent greening of the etiolated cyanobacterial cells**

Accumulation of Pchlide in the cell causes severe photooxidative damage upon illumination. Therefore, plant cells keep the Pchlide level in the etioplast at a low level, so as not to lead to photooxidative damage, through the controlled supply of Pchlide (Meskauskiene et al., 2001) and the formation of the photoactive ternary complex with LPOR and NADPH (Paddock et al., 2012). In the light-dependent greening process of ∆chlL/LPORox, a short lag time (ca. 5 h) was observed before the onset of increase of the Chl content upon illumination. It is unlikely that this short lag time represents the photooxidative damage for the following reasons: 1) A short lag time before the Chl increase was also observed in light-dependent greening process of plants (Ohashi et al., 1988; Yoshida et al., 1995). 2) In the previous experiment of light-dependent greening of ∆chlL using semi-continuous cultures, no such lag time was observed (Kada et al., 2003), in which glucose containing BG-11 was used commonly for both cultivations before and after the light exposure. In the greening experiment of ∆chlL/LPORox (Fig. 5), we cultivated ∆chlL/LPORox in glucose-containing BG-11 in the dark and observed the light-dependent greening in BG-11 without glucose. The cellular metabolism of ∆chlL/LPORox should switch the growth mode from chemoheterotrophic to photoautotrophic modes. The short lag time would represent this switching period. 3) In dark-grown ∆chlL cells, a significant amount of Pchlide is excreted into the culture medium (Fujita et al., 1992). This active efflux of Pchlide would contribute to the maintenance of the intracellular Pchlide concentration at a low level, so as not to lead to severe photooxidative damage in the cyanobacterial cells.

The Chl production rate of ∆chlL/LPORox during the greening process was almost the same as that of the control ∆chlL/CV, indicating that the PLB-like structures do not play any active roles in the greening process. This is in clear contrast with the authentic PLB of plant etioplasts, in which the photoactive Pchlide-NADPH-LPOR ternary complex contributes to the production of Chlde immediately upon light illumination. The in vitro LPOR assay indicated that the rapid photoreduction of Pchlide was arrested by NADPH deficiency (Fig. 7), suggesting that the overexpressed LPOR does not form a photoactive Pchlide-NADPH-LPOR ternary complex in cyanobacterial cells. This hypothesis was supported by the low-temperature fluorescence spectra of the membrane fractions of dark-grown ∆chlL/LPORox, which showed that Pchlide existed in the nonphotoactive form (F632), rather than the photoactive one (Fig. 8).

As mentioned before, in the immunocytochemical images (Fig. 4), the fluorescence intensity of the dark-grown ∆chlL/LPORox cells was heterogenous. A small portion of cells showed a much higher intensity of fluorescence intensity than those of other cells (Fig. 4B), suggesting that these specific cells contain larger amounts of LPOR than those of other cells. We cannot rule out the possibility that the rates of Pchlide reduction and Chl production of such specific cells are significantly faster than other cells during the greening process, because such faster greening by only a small number of cells could be masked by a large number of cells showing the normal greening rate in this batch experiment. In the future microscopic spectroscopy of single-cells (Nozue et al., 2017; Sugiuira and Itoh, 2012) should be applied to investigate whether a higher accumulation of LPOR promotes the rate of the greening process.

The enzymatic properties of LPOR of *L. boryana* and the cellular level of NADPH in the *L. boryana* transformant may be important for understanding why the photoactive Pchilde-NADPH-LPOR ternary complex was not formed in the cyanobacterial cells. As shown in Table 1, the $K_m$ values for Pchilde and NADPH of LPOR from *Synechocystis* sp. PCC 6803 and *Gloeobacter violaceus* PCC 7421 restored the PLB size in the mutant (Masuda et al., 2009). This heterologous expression of cyanobacterial LPORs in *A. thaliana* clearly showed the contribution of the cyanobacterial LPORs to the formation of the PLB structure in etioplasts. However, the level of photoactive Pchlide was not restored by the overexpression of the cyanobacterial LPORs in the PORA knockout mutant, suggesting that the cyanobacterial LPORs do not contribute to the formation of the photoactive ternary complex in the etioplasts of *A. thaliana*. This observation is consistent with the results of our experiments.

The level of NADPH in cyanobacterial cells should also be considered. The cellular NADPH pool is 30–50% reduced in the dark in *Synechocystis* sp. PCC 6803 (Kauny and Setif, 2014). Although we did not determine the NADPH pool in cells grown in the continuous dark conditions in this study, the NADPH/NADP+ ratio may have been too low to allow the formation of the photoactive ternary complex in ∆chlL/LPORox cells. A detailed *in vitro* kinetic analysis of the LPOR from *Thermosynechococcus elongatus* suggested a sequence for the formation of the photoactive ternary complex: the first step is the binding of NADPH to LPOR, followed by the binding of Pchlide to the binary complex (NADPH-LPOR), to form the photoactive ternary complex (Heyes et al., 2008). A low NADPH level may interfere with the efficient formation of the binary NADPH-LPOR complex, which would preclude the formation of the photoactive ternary complex. However, as ∆chlL/LPORox exhibited a good heterotrophic growth in the dark, it is unlikely that the NADPH
level was very low. Further studies are needed to determine why the photoactive ternary complex is not formed in cyanobacterial cells.

Other factors that participate in the formation of PLB: carotenoids and phycobilisome

The PLBs of plant etioplasts contain carotenoids, mainly lutein. Inhibition of lutein biosynthesis results in the complete loss of PLB formation in etioplasts (Cuttris et al., 2007; Park et al., 2002). Lutein is distributed only in Chlorophylla, land plants, and some Rhodophyta species, but not in cyanobacteria (Takaichi, 2011). This difference in carotenoid composition might also explain the inability of the PLB-like structure in cyanobacteria.

Cyanobacteria possess phycobilisomes, which are a large complex of antenna located on the thylakoid membranes, rather than the light-harvesting Chl complexes that are present in higher plants. A PSI-less mutant of Synechocystis sp. PCC 6803 showed organized regular arrays of phycobilisomes on tubular and sheet-like thylakoid membranes (van de Meene et al., 2012). Plastids in the red alga Cyanidioschyzon merolae grown heterotrophically in the dark produced unique pentagonal tubular structures instead of thylakoid membranes, with phycobilisomes lined up on the surface of the tubular structure (Moriyama et al., 2019). Because the dark-grown ΔchlL/ΔPOR cells retained a significant amount of phycobilisomes, the presence of arrays of phycobilisome complexes on the rudimentary thylakoid membranes may have interfered with the formation of an ordered paracrystalline PLB ultrastructure in cyanobacterial cells.

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