Functional Evaluation of a Nitrogenase-Like Protochlorophyllide Reductase Encoded by the Chloroplast DNA of Physcomitrella patens in the Cyanobacterium Leptolyngbya boryana

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Abstract

Dark-operative protochlorophyllide (Pchlide) oxidoreductase (DPOR) is a nitrogenase-like enzyme consisting of the two components, L-protein (a ChlL dimer) and NB-protein (a ChlN–ChlB heterotetramer), to catalyze Pchlide reduction in Chl biosynthesis. While nitrogenase is distributed only among certain prokaryotes, the probable structural genes for DPOR are encoded by chloroplast DNA in lower plants. Here we show functional evaluation of DPOR encoded by chloroplast DNA in a moss Physcomitrella patens by the complementation analysis of the cyanobacterium Leptolyngbya boryana and the heterologous reconstitution of the moss L-protein and the cyanobacterial NB-protein. Two shuttle vectors to overexpress chlN-chlB from P. patens were introduced into the cyanobacterial chlL- and chlB-lacking mutants, respectively. Both transformants restored the ability to perform Chl biosynthesis in the dark, indicating that the chloroplast-encoded DPOR components form an active complex with the cyanobacterial components. The L-protein of P. patens was purified from the cyanobacterial transformant, and DPOR activity was reconstituted in a heterologous combination with the cyanobacterial NB-protein. The specific activity of the L-protein from P. patens was determined to be 118 nmol min⁻¹ mg⁻¹, which is even higher than that of the cyanobacterial L-protein (76 nmol min⁻¹ mg⁻¹). Upon exposure to air, the activity of the L-protein from P. patens decayed with a half-life of 30 s, which was eight times faster than that of the cyanobacterial L-protein (240 s). These results suggested that the chloroplast-encoded L-protein functions as efficiently as the cyanobacterial L-protein but is more oxygen labile than the cyanobacterial L-protein.

Introduction

Chl a is synthesized from glutamate through a complex pathway consisting of at least 15 reactions (e.g. Vavilin and Vermaas 2002, Tanaka and Tanaka 2007, Masuda and Fujita 2008). Protochlorophyllide (Pchlide) reduction is the penultimate step of Chl a biosynthesis, catalyzed by two different enzymes (Fujita 1996, Armstrong 1998, Fujita and Bauer 2003, Reinhothe et al. 2010). One is the dark-operative (light-independent) Pchlide oxidoreductase (DPOR) and the other is the light-dependent Pchlide oxidoreductase (LPOR). Although these two enzymes catalyze the same stereospecific reaction of the Pchlide D-ring to form chlorophyllide (Chlide), there is no significant sequence similarity between them. DPOR is distributed widely not only among anoxygenic photosynthetic bacteria but also among oxygenic phototrophs such as cyanobacteria, green algae and gymnosperms (Fujita and Bauer 2003). LPOR is employed by all oxygenic photosynthetic organisms including angiosperms. Anoxygenic photosynthetic bacteria and angiosperms use DPOR and LPOR as their sole Pchlide reductases, respectively. Most oxygenic photosynthetic organisms including cyanobacteria utilize both enzymes. It has been elusive as to how the two enzymes contribute in Pchlide reduction, though light and oxygen are key factors in their differentiation in cyanobacteria (Fujita et al. 1998, Yamazaki et al. 2006).

DPOR from the photosynthetic bacterium Rhodobacter capsulatus has been studied most extensively (Fujita and Bauer 2000, Nomata et al. 2005, Nomata et al. 2006, Nomata et al. 2008, Muraki et al. 2010, Kondo et al. 2011). The amino acid sequence of DPOR has been determined from R. capsulatus and 13 other anoxygenic photosynthetic bacteria as well as eukaryotic green algae and gymnosperms. DPORs from anoxygenic photosynthetic bacteria have 35%–50% identity with DPORs from oxygenic phototrophs. DPORs from anoxygenic photosynthetic bacteria are composed of two components: L-protein (a ChlL dimer) and NB-protein (a ChlN–ChlB heterotetramer), like DPOR in oxygenic phototrophs. The L-protein and NB-protein of oxygenic phototrophs exist only in the dark, whereas the L-protein and NB-protein of anoxygenic phototrophs exist both in the dark and light. While the light-dependent NPR has been well studied, the dark-operative DPOR has not. The identification of the structural genes for DPOR would contribute to understanding the mechanism of Chl a biosynthesis.

The structural genes for DPOR are encoded by chloroplast DNA in lower plants. The amino acid sequence of the L-protein has been determined from tomato (Solanum lycopersicum) and tobacco (Nicotiana tabacum) (Nomura et al. 1992, 1996). Furthermore, the NB-protein genes of the tomato have been cloned and sequenced (Nomura et al. 1998). The amino acid sequence of the NB-protein has been determined from Arabidopsis thaliana, tomato, and tobacco (Nomura et al. 1996, 1998). DPOR is distributed not only among anoxygenic photosynthetic bacteria but also among oxygenic phototrophs such as cyanobacteria, green algae and gymnosperms. Although the amino acid sequence of the L-protein and NB-protein has been determined from several plant and bacterial species, the structural genes for DPOR have not been identified so far. The identification of the structural genes for DPOR would contribute to understanding the mechanism of Chl a biosynthesis.

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acid sequences of the three structural proteins of DPOR, Bchl, BchN and BchB, show significant similarity to those of nitrogenase subunits, NiIFh, NiID and NiIFr, respectively. The nitrogenase-like features have been confirmed by reconstitution with the two purified components, L-protein (a Bchl dimer) and NB-protein (a BchN–BchB heterotetramer) (Fujita and Bauer 2000). L-protein is an ATP-dependent reductase component specific for NB-protein. A [4Fe–4S] cluster is chelated by the two protomers of L-protein (Nomata et al. 2006). The crystal structures of L-protein and NB-protein suggested a common structural framework in DPOR and nitrogenase (Sarma et al. 2008, Muraki et al. 2010, Bröcker et al. 2010).

All DPORs that have been biochemically studied so far are of prokaryotic organisms such as photosynthetic bacteria and cyanobacteria. DPOR from cyanobacteria, which are oxygenic phototrophs, has properties common to those of DPORs from photosynthetic bacteria (Yamazaki et al. 2006, Bröcker et al. 2008a, Bröcker et al. 2008b, Yamamoto et al. 2009, Bröcker et al. 2010). Probable DPOR genes, chlL, chlN and chlB, which are the orthologs of bchL, bchN and bchB, respectively, are found in chloroplast DNA from many algae and lower plants. Some gymnosperms and green algae can produce Chl even in the dark where LPOR does not catalyze the reaction (Mariani et al. 1990, Mukai et al. 1991, Kusumi et al. 2006), indicating that the chloroplast-encoded DPOR does operate in the chloroplasts. In the green alga Chlamydomonas reinhardtii, the three knockout mutants of chlL, chlN and chlB in chloroplast DNA showed a common phenotype, a defect in Chl synthesis in the dark, namely yellow-in-the-dark, which is indirect evidence that these chloroplast genes encode functional DPOR subunits (Choquet et al. 1992, Suzuki and Bauer 1992, Li et al. 1993, Liu et al. 1993). As is the case for C. reinhardtii, the increase in Chl content of cells during heterotrophic growth in the dark provides a simple indication for the operation of DPOR in the organism. However, since many eukaryotic algae lack heterotrophic growth ability in the dark, such a simple evaluation is not applicable for them (Fujita and Bauer 2003).

In addition, there are many photosynthetic eukaryotes lacking greening ability in the dark even though all DPOR genes are seemingly present in chloroplast DNA. For example, Ginkgo biloba and Larix decidua do not have the ability to produce Chl in the dark even though they have a full set of DPOR genes (Kusumi et al. 2006). Therefore, the presence of probable DPOR genes in chloroplast DNA does not necessarily mean the operation of DPOR in the organism. In some gymnosperms, RNA editing causing amino acid substitution might play a role in the regulation of DPOR activity (Demko et al. 2008). Thus, quantitative evaluation of the individual components of DPOR is required for understanding how DPOR contributes to Chl synthesis in these organisms.

From an agricultural point of view, nitrogenase is a critical enzyme for leguminous crop productivity. However, nitrogenase is distributed only among limited members of prokaryotes, and only leguminous crops have the ability to fix nitrogen by a symbiotic relationship with rhizobia. In spite of considerable efforts, the creation of nitrogen-fixing crops by transfer of nif genes from diazotrophic prokaryotes has been left unfulfilled. The chloroplast DNA-encoded nitrogenase-like enzyme DPOR presents a promising lead (Beatty and Good 2011). Thus, characterization of chloroplast DPOR would provide a valuable contribution toward this long-standing interest.

The moss Physcomitrella patens is a model plant, and its genome sequence has recently become available (Rensing et al. 2008). Though all three DPOR genes are found in the chloroplast DNA (Sugiura et al. 2003), there has been no indication of the operation of DPOR since P. patens lacks heterotrophic ability. Previously, we established a DPOR overexpression system in the cyanobacterium Leptolyngbya bor-yana to evaluate whether the expressed proteins are active as DPOR components in vivo (Yamamoto et al. 2009). Here we report the functional expression of the DPOR components encoded by chloroplast DNA from P. patens using the cyanobacterial expression system and the reconstitution of DPOR activity with the purified moss L-protein and the cyanobacterial NB-protein in vitro. The specific activity of the moss L-protein was even higher than that of the cyanobacterial L-protein, suggesting that the moss L-protein potentially operates as the DPOR component in chloroplasts of P. patens as efficiently as the cyanobacterial L-protein. We also examined the oxygen lability of the moss L-protein upon exposure to air, and found that the moss L-protein is more labile than the cyanobacterial L-protein. This cyanobacterial DPOR expression system could be applied for characterization of DPOR from many other photosynthetic eukaryotes lacking heterotrophic growth ability.

**Results**

**Overexpression of chloroplast-encoded ChlL and ChlN–ChlB in L. boryana for in vivo complementation**

To clarify the origin of DPOR, we designate the DPOR components with a subscript of the organism, for example Lp-protein (from P. patens) and Lb-protein (from L. boryana), as reported previously (Yamamoto et al. 2009). Previous studies on DPORs from prokaryotes (Fujita and Bauer 2000, Nomata et al. 2005, Nomata et al. 2006, Nomata et al. 2008, Yamamoto et al. 2009, Bröcker et al. 2010, Muraki et al. 2010) suggested that the chloroplast DPOR also consists of two components: L-protein, a ChlL dimer; and NB-protein, a ChlN–ChlB heterotetramer. We employed the in-vivo DPOR complementation system to evaluate indirectly whether Lp-protein and NBp-protein are active as the DPOR components. We constructed two plasmids, pHBLc3 and pHBNB3, which were designed to overexpress ChlL and ChlN as C-terminal and N-terminal fusion proteins with the Strep tag (WSHQPEFK) in L. boryana, respectively (Fig. 1). These plasmids were introduced into the cells of YFC2 (a chlL-disrupted mutant; Fujita et al. 1992, Kada et al. 2003) and YFB14 (a chlB-disrupted mutant; Fujita et al. 1996).
of *L. boryana* by electroporation, giving rise to the two transformants, YFC2/Lc3 (YFC2 expressing Lp-protein) and YFB14/NB3 (YFB14 expressing Nb-protein), respectively.

In both mutants YFC2 and YFB14, Chl synthesis is arrested at the Pchlide reduction step in the dark (Fujita et al. 1992, Fujita et al. 1996, Kada et al. 2003). If the introduced Lp-protein and Nb-protein formed active heterologous complexes with the endogenous cyanobacterial components, the ability to perform Chl synthesis in the dark would be restored in the transformants. The Chl contents of the negative controls (YFC2 and YFB14 harboring an empty vector pPBH202) were kept at the level of the inoculation. The Chl contents of YFC2/Lc3 and YFB14/NB3 cells grown in the dark were 1.2 \( \mu \text{g} \text{ml}^{-1} \) OD\( _{730} \) and 1.5 \( \mu \text{g} \text{ml}^{-1} \) OD\( _{730} \) (Fig. 1), which are significantly higher than those of the negative controls. This result indicated that both Lp-protein and Nb-protein expressed in *L. boryana* are functional as DPOR components and form active complexes with the cyanobacterial Nblb-protein and Llb-protein, respectively.

**Reconstitution of DPOR activity with purified Lp-protein in heterologous combination**

The L-protein is the target component of oxygen inactivation in *L. boryana* (Yamamoto et al. 2009). It would be of interest to determine whether the chloroplast L-protein is labile to oxygen as well. Thus, we focused on the initial quantitative evaluation on the L-protein. The Lp-protein was purified from *L. boryana* YFC2/Lc3 cells grown under mixotrophic conditions, although there was slight contamination by an unknown 20 kDa protein (Fig. 3C, lane 2). We then tried to reconstitute DPOR activity with purified Lp-protein and the cyanobacterial Nblb-protein in a heterologous combination. The Nblb-protein was provided as a crude extract of *Escherichia coli* overexpressing Strep–ChlN and ChlB proteins (Yamamoto et al. 2009). Chlide formation was detected when the Lp-protein was mixed with the crude extract containing the Nblb-protein, and the rate of Chlide formation was dependent on the amount of the Lp-protein (Fig. 2A). This clearly indicated that the chloroplast-encoded Lp-protein forms an active DPOR heterologous complex with the Nblb-protein. A fixed amount of Nblb-protein in the *E. coli* crude extracts was titrated with the purified Lp-protein (Fig. 2A inset, filled circles). As the positive control, the purified Llb-protein was used for the titration (open circles).

In this titration, the Chlide formation rate increased as the amount of Lp-protein increased. The ratio of the Chlide formation rate to the amount of the Lp-protein was much lower than that of the Llb-protein (Fig. 2A inset). The relationship between the Chlide formation rate and the L-protein concentration followed Michaelis–Menten kinetics (Fig. 2A inset, **B**). In the Lineweaver–Burk plot, the two sets of plots from the Lp-protein and Llb-protein form straight lines intersecting at one point on the y-axis (Fig. 2B).
apparent $V_{\text{max}}$ values of the NB$_{Lb}$-proteins were very similar to each other (62.5 pmol min$^{-1}$ using the L$_{PP}$-protein and 65.4 pmol min$^{-1}$ using the L$_{LB}$-protein). The kinetic characteristics suggested that the L$_{PP}$-protein binds to the NB$_{Lb}$-protein at the same site as the L$_{LB}$-protein in the homologous complex.

The kinetic parameters of the L$_{PP}$-protein were determined by titration of the NB$_{Lb}$-protein (Fig. 3). In this case, NB-protein is regarded as the substrate for L-protein. A fixed amount of the purified L$_{PP}$-protein was titrated with the NB$_{Lb}$-protein purified from E. coli (Fig. 3C). As the control, a fixed amount of purified L$_{LB}$-protein was also titrated with NB$_{Lb}$-protein (Fig. 3A). The relationship between the Chlide formation rate and the NB-protein concentration also followed Michaelis–Menten kinetics (Fig. 3A) giving two straight lines in the Lineweaver–Burk plot (Fig. 3B). Their $V_{\text{max}}$ values were calculated to be 42.5 pmol min$^{-1}$ for the L$_{PP}$-protein and 129 pmol min$^{-1}$ for the L$_{LB}$-protein. When the $V_{\text{max}}$ values were normalized by the amount of L-protein added to the assay mixture, the specific activity (the normalized $V_{\text{max}}$ values) of the L$_{PP}$-protein

Fig. 2 (A) Reconstitution of DPOR activity in a heterologous combination of L-protein and NB-protein. The DPOR assay was carried out as described (Yamazaki et al. 2006). Various amounts of the purified L$_{PP}$-protein (0 µg, black; 0.55 µg, blue; 1.1 µg, violet; and 1.65 µg, red) were added to the crude extract (140 µg protein) containing NB$_{Lb}$-protein that was prepared from the transformant of E. coli. The volume of the reaction mixture was 250 µl. Inset: Michaelis–Menten plot of this assay with the L$_{PP}$-protein as the substrate for the NB$_{Lb}$-protein. The rate of Chlide formation in the assay (A) was estimated and plotted (filled circles). As a control assay, various amounts of the cyanobacterial L$_{PP}$-protein were added to the crude extract and the Chlide formation rates were determined (open circles). (B) Lineweaver–Burk plot to determine the $K_m$ and $V_{\text{max}}$ values of NB$_{Lb}$-protein for L$_{PP}$-protein (filled circles) and L$_{LB}$-protein (open circles).

Fig. 3 (A) Michaelis–Menten plot of the DPOR assay with the NB$_{Lb}$-protein as the substrate for fixed amounts of purified L$_{PP}$-protein (5.5 nM; filled circles) and L$_{LB}$-protein (25 nM; open circles). The volume of the reaction mixture was 250 µl. (B) Lineweaver–Burk plot to determine the $K_m$ and $V_{\text{max}}$ of the L$_{PP}$-protein (filled circles) and L$_{LB}$-protein (open circles) for NB$_{Lb}$-protein. (C) An SDS profile of L$_{PP}$-protein (lane 1), L$_{PP}$-protein (lane 2) and NB$_{Lb}$-protein (lane 3) used in this experiment. L$_{LB}$-protein and L$_{PP}$-protein were purified from the cyanobacterial transformants YFC2/Lc2 and YFC2/Lc3, respectively. NB-protein was purified from E. coli harboring pHANB2 (Yamamoto et al. 2009).
In this study we confirmed the potential operation of a nitrogenase-like enzyme encoded by chloroplast DNA for the first time using the cyanobacterial in-vivo complementation system. Further, we purified the LPP-protein from a cyanobacterial transformant overexpressing ChlL and determined the specific activity of the LPP-protein by heterologous combination with the cyanobacterial NB-L-protein.

**Discussion**

In this study we confirmed the potential operation of a nitrogenase-like enzyme encoded by chloroplast DNA for the first time using the cyanobacterial in-vivo complementation system. Further, we purified the LPP-protein from a cyanobacterial transformant overexpressing ChlL and determined the specific activity of the LPP-protein by heterologous combination with the cyanobacterial NB-L-protein.

(118 nmol min⁻¹ mg⁻¹ protein⁻¹) was even higher than that of the LLL-protein (76 nmol min⁻¹ mg⁻¹ protein⁻¹). This result suggested that chloroplast LPP-protein potentially operates even more efficiently than LLL-protein in the presence of the homologous NB-protein in the chloroplasts.

**Oxygen sensitivity of the chloroplast-encoded L-protein**

Using this heterologous DPOR assay system, we examined the oxygen lability of the LPP-protein. The purified LPP-protein and LLL-protein were exposed to air for various times, and then the activity with the NB-L-protein was measured. LPP-protein activity diminished with a half-life of about 30 s (Fig. 4), while LLL-protein activity decreased with a half-life of about 240 s. This indicated that the chloroplast-encoded L-protein is much more vulnerable to oxygen than the cyanobacterial L-protein.

**In-vivo heterologous combination of DPOR components**

The Chl contents of the cyanobacterial transformants grown in the dark suggest that the plasmid-bone proteins are active as DPOR components (Fig. 1). Previously we demonstrated that the overexpression of the endogenous DPOR components successfully restored Chl production in the dark in this complementation system (Yamamoto et al. 2009). In this study, we confirmed that this complementation system is applicable for the evaluation of DPOR components encoded by chloroplast DNA from *P. patens*. However, in the case of complementation by the moss NB-protein, the Chl content of YFB14/NB3 was only half that of the positive control (Fig. 1). The reason for this partial complementation could be lower DPOR activity in the heterologous combination than in the homologous combination. The other reason is that the contents of Strep–ChlN and ChlB were too low to complement DPOR activity fully in YFB14/NB3. Because the Strep–Chl protein was not detected by a Strep–Tactin–peroxidase conjugate in Western blot analysis (data not shown), the amount of Strep–ChlN expressed in YFB14/NB3 could be estimated to be <0.1 µg mg⁻¹ protein⁻¹, the sensitivity limit of the Strep–Tactin–peroxidase conjugate. Although the complementation is partial, this in-vivo system is useful for evaluation of the DPOR activity of the individual components from various oxygenic photosynthetic organisms.

It should be noted that the confirmation of protein expression in the chloroplasts in *P. patens* is necessary to reach any conclusions on the contribution of DPOR in Chl production in *P. patens*. In Bryophytes, the Chl protein has been immunologically identified in the soluble fraction of chloroplasts in *Marchantia polymorpha* (Fujita et al. 1989).

**The moss L-protein activity in a heterologous combination in vitro**

We demonstrated that the moss LPP-protein is active as the DPOR component by the in vitro reconstitution of DPOR activity in a heterologous combination. Wätzlich et al. (2009) reported DPOR activity of various heterologous combinations between L-proteins and NB-proteins from a green bacterium *Chlorobium tepidum*, a thermophilic cyanobacterium *Thermosynechococcus elongates*, and a marine cyanobacterium *Prochlorococcus marinus*. However, they determined the relative activity of the heterologous complexes at an L-protein to NB-protein ratio of <3, which is too low to exert the maximal activity of the NB-proteins. We carried out a preliminary experiment to examine how much excess LLL-protein is needed for the maximal activity of NB-L-protein in the homologous combination. The maximal activity of NB-L-protein was obtained at the L-protein/NB-protein ratio of >17 (Supplementary Fig. S1), which is much higher than that of *R. capsulatus* (about 3; Nomata et al. 2008) but is comparable with the Fe-protein/MoFe-protein ratio of nitrogenase from *Klebsiella pneumoniae* (>10; Thornery and Lowe 1984). In this study, taking this observation into consideration,
we evaluated the L<sub>Pp</sub>-protein in the heterologous combination by Michaelis–Menten kinetics.

In one titration, the NB<sub>Lb</sub>-protein in the E. coli extract was regarded as the enzyme (Fig. 2). The L<sub>Pp</sub>-protein behaved as a substrate similar to the L<sub>Lb</sub>-protein with the same V<sub>max</sub> value and K<sub>m</sub> value that is about six times higher than that of the L<sub>Lb</sub>-protein (Fig. 2C). This behavior of the L<sub>Pp</sub>-protein suggested that it binds to a docking site on the NB<sub>Lb</sub>-protein where the L<sub>Lb</sub>-protein binds. On the other hand, in the other titration, L-protein was regarded as an enzyme to reduce a substrate NB-protein. The specific activity of L<sub>Pp</sub>-protein was estimated to be 118 nmol min<sup>−1</sup> mg<sup>−1</sup>, which is even higher than that of the cyanobacterial L<sub>Lb</sub>-protein (Table 1). This estimation suggested that the L<sub>Pp</sub>-protein exerts DPOR activity even more efficiently than cyanobacterial L<sub>Lb</sub>-protein when an excess amount of the homologous NB-protein is present and the environment is kept anaerobic. During photoautotrophic growth of P. patens, the chloroplast DPOR probably plays an important role in the supply of Chl. We would like to emphasize that the specific activity of the L<sub>Pp</sub>-protein was determined by the heterologous combination with the NB<sub>Lb</sub>-protein even though the homologous partner NB<sub>Pp</sub>-protein was not available. This hypothesis should be confirmed by the determination of the kinetic parameters with the homologous combination when the NB<sub>Pp</sub>-protein becomes available.

**Interaction between L-protein and NB-protein**

Similar heterologous combinations between Fe-protein and MoFe-protein have been reported in nitrogenase (Detroy et al. 1968, Emerich and Burris 1978). Most heterologous Fe-protein and MoFe-protein complexes are active except for Fe-protein from Clostridium pasteurianum (Jacobson et al. 1990). The sequence similarities among these compatible Fe-proteins (NifH proteins) are more than about 70% identity. Thus, as a rough estimation, >70% identity between a heterologous combination with L-protein and a homologous L-protein to a given NB-protein would be required for the formation of catalytically active DPOR complex. Another heterologous combination of the NB<sub>Lb</sub>-protein with the L<sub>Rc</sub>-protein from the photosynthetic bacterium *Rhodobacter capsulatus* supported this similarity threshold. In this heterologous combination, no DPOR activity was detected (data not shown). Bchl from *R. capsulatus* is much more diverged (50% identity) from the cyanobacterial Chl than chloroplast Chl. Chloroplast Chl normally shows >80% identity to the cyanobacterial Chl (Fujita 1996). Thus, a given L-protein from any eukaryotic organisms could be used to determine the specific activity with the cyanobacterial NB<sub>Lb</sub>-protein.

A set of amino acid residues of Fe-protein is involved in formation of the nitrogenase complex with MoFe-protein (Peters et al. 1994). Based on the structural comparison between Fe-protein and L-protein from *Rhodobacter sphaeroides* (L<sub>Rc</sub>-protein), six amino acid residues, Cys126, Tyr129, Gln133, Gly161, Gln168 and His169, in Bchl were proposed to interact with the NB<sub>Rc</sub>-protein to form the DPOR complex (Sarma et al. 2008) (Fig. 5A). These six residues are spatially arranged to surround the [4Fe–4S] cluster (Fig. 5B). Tyr127 in Chl from *P. marinus* (corresponding to Tyr129) has been reported to be involved in the interaction with the NB-protein by site-directed mutagenesis (Wätzlch et al. 2009). Three residues, Gln133, Gln168 and His169, of the six are commonly altered to glutamate, asparagine and tyrosine in Chl proteins from *L. boryana* and *P. patens*, respectively. Thus, the conservation of these residues might be critical to form a productive heterologous complex with the cyanobacterial NB<sub>Lb</sub>-protein.

An amino acid sequence alignment among five ChlL/Bchl proteins from *P. patens*, *L. boryana*, *P. marinus*, *R. sphaeroides* and *R. capsulatus* is shown in Fig. 5A. Focusing on the sequence comparison between *P. patens* and *L. boryana*, there are 25 residue differences within the overlapping 271 residues except for the different C-terminal extensions (beyond the 272nd residues). The 25 residues are distributed throughout the structure, except for the probable interaction surface centered around the [4Fe–4S] cluster. These differences in the ChlL sequence appear to cause the low affinity in the heterologous combination. Changes in the five residues, Glu60 to leucine, Ala70 to proline, Asp114 to tyrosine, Val195 to cysteine and Asp226 to glutamate, seem to be important, because they were altered to amino acid residues with different chemical properties in *P. patens*. The three changes (Glu60Leu, Asp114Tyr and Asp226Cys) affect charge distributions on the L-protein surface based on the crystal structure of L<sub>Rc</sub>-protein (Fig. 5B; Sarma et al. 2008). In addition, the C-terminal extension of *P. patens* is nine amino acid residues longer than that of *L. boryana*. While the C-terminal region of *L. boryana* is rich in negatively charged residues, the numbers of positively and negatively charged residues are the same in *P. patens* ChlL. This difference in charge distribution might affect the affinity between L-protein and NB-protein in the heterologous DPOR complex. The structure of a homologous complex between L-protein and NB-protein needs to be determined for a detailed discussion about the interaction between the components, as is the case for nitrogenase Fe-protein–MoFe protein complexes (Schindelin et al. 1997, Tezcan et al. 2005).

**Table 1** Comparison of the kinetic parameters of DPOR components from several photosynthetic organisms

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<tr>
<th>Organisms</th>
<th>L-protein</th>
<th>NB-protein</th>
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<tr>
<td></td>
<td>Specific activity&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Half-life&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Specific activity&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Physcomitrella patens</td>
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<td>ND</td>
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<tr>
<td>Leptolyngbya boryana</td>
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<td>240</td>
<td>12.0</td>
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<tr>
<td>Rhodobacter capsulatus</td>
<td>96&lt;sup&gt;e&lt;/sup&gt;</td>
<td>20</td>
<td>26.2&lt;sup&gt;e&lt;/sup&gt;</td>
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<sup>a</sup> ChlL formation activity (nmol min<sup>−1</sup> mg<sup>−1</sup>).
<sup>b</sup> Half-life in the oxygen inactivation (s).
<sup>c</sup> Determined by the heterologous combination with NB<sub>Lb</sub>-protein.
<sup>d</sup> Nomata et al. (2006).
<sup>e</sup> Nomata et al. (2008).
Fig. 5 (A) Amino acid sequence alignment of ChlL (BchlL) from *P. patens* (Pp), *L. boryana* (Lb), *P. marinus* (Pm), *R. capsulatus* (Rc) and *R. sphaeroides* (Rs). Two conserved cysteine residues involved in holding the [4Fe–4S] cluster are shown by red ♦. Based on structural comparison with nitrogenase Fe-protein, six amino acid residues proposed to be involved in the interaction of the NB Rs-protein in the DPOR complex (Sarma et al. 2008) are shown in red. Twenty amino acid residues out of the 25 residues which differed between *P. patens* and *L. boryana* are shown in cyan, and five amino acid residues out of 25, E60, A70, D114, V195 and D226, in *L. boryana* with different chemical properties from those in *P. patens* are shown in blue, violet, yellow, green and orange, respectively. (B) Stereo-views of crystal structure of L Rs-protein from *R. sphaeroides* (3END; Sarma et al. 2008). The [4Fe–4S] cluster and the ADP molecules are shown in a stick model. The 20 amino acid residues different between *P. patens* and *L. boryana* are shown in cyan, and the five amino acid residues mentioned are shown with side chains in a stick model with the same color as in the above alignment (A).
Oxygen sensitivity

Our comparative analysis of L-protein clearly demonstrated that the lability of L-protein to oxygen is variable in the organisms (Table 1). The cyanobacterial L_p-protein is about 10 times more tolerant to oxygen exposure than the other two L-proteins. This suggests that L-protein has the potential to evolve resistance to oxygen. It would be of interest to determine which amino acid residue(s) in the 25 different residues from those of P. patens are important for the oxygen tolerance in further study.

There has been a long-standing interest in creating nitrogen-fixing crops. One of the probable approaches is introduction of nitrogenase genes into plant cells for expression in chloroplasts. A transgenic tobacco and a green alga C. reinhardtii expressing the nifH gene have been reported (Dixon et al. 1997, Cheng et al. 2005). In C. reinhardtii, the yellow-in-the-dark phenotype caused by the lack of chlL was complemented by the nifH gene (Cheng et al. 2005). To confer oxygen tolerance to Fe-protein is a very important issue for functional expression of nitrogenase in chloroplasts where ATP and reducing power are rich but there is a powerful oxygen-generator PSII. Given the structural similarity between Fe-protein and L-protein (Sarma et al. 2008), the structural basis of oxygen tolerance of L-protein would provide an invaluable clue to create an oxygen-tolerant Fe-protein.

Oxygen tolerance of L-protein might have developed during the evolution of the cyanobacterial lineage, because the concentration of oxygen in cyanobacterial cells would be much higher than that in R. capsulatus cells due to oxygenic photosynthesis. However, the chloroplast L_p-protein of P. patens shows oxygen sensitivity comparable with that of L-protein from R. capsulatus in spite of the oxygenic photosynthetic organism. Photosynthetic eukaryotes might have evolved to have very efficient scavenging systems such as the water–water cycle to remove oxygen and reactive oxygen species in the chloroplast (Asada 1999). That could be why there has been no need to develop oxygen tolerance for the chloroplast DPOR. Further study is required to clarify how the oxygen-labile DPOR operates in chloroplasts.

Materials and Methods

Cyanobacterial strains, mutants and cultivation conditions

The mutants YFC2 (lacking chll; Kada et al. 2003) and YFB14 (lacking chlb; Fujita et al. 1996) derived from L. boryana IAM-M101 strain dgs (formerly Plectonema boryanum) were used as host strains to overexpress the chloroplast DPOR components, L-protein and NB-protein, respectively. These mutants were cultivated in BG-11 medium with 15 µg ml⁻¹ kanamycin under medium intensity light conditions (40 µmol photon m⁻² s⁻¹) as described (Yamazaki et al. 2006). The transformants harboring the overexpression plasmids were cultivated as above in medium supplemented with 10 µg ml⁻¹ chloramphenicol for plasmid maintenance. The medium was supplemented with glucose (30 mM) for heterotrophic growth in the dark.

Construction of chloroplast protein expression plasmids

To express L-protein and NB-protein from the chloroplast DNA of P. patens as Strept-tag fusion proteins (ChlL–Strep and Strept–ChlN) in E. coli, we constructed the plasmids using pASK-IBA3plus (IBA, Göttingen, Germany) for chlL and pASK-IBA5plus (IBA) for chln-chlb as described (Yamamoto et al. 2009). The coding region of chil was amplified by PCR using the primers PpLf2 and PpLr2 (Supplementary Table S1) with amplified DNA fragment LA4 (Sugiura et al. 2003) from the P. patens chloroplast genome as the template. PCR was carried out with KOD DNA polymerase (KOD-plus; Toyobo) under standard thermal cycling conditions. The PCR-amplified fragment was cloned into Bsal sites of pASK-IBA3plus to yield pHCL3. The Strept-tag and a linker sequence (SAWSHPQFEK) are connected to the original C-terminus of ChlL. The coding regions of the chloroplast chln and chlb genes were amplified using the primer pairs Ppchlnf2 and Ppchlnr2; and Ppchlb2 and Ppcplbr2 (Supplementary Table S1), and amplified DNA fragments LA4 and LA6 (Sugiura et al. 2003) as the template, respectively. These two amplified fragments were connected by the overlapping sequence between them in the second PCR with the primers Ppchlnf2 and Ppchlnr2 (Sambrook and Russell 2001). The amplified chln-chlb fragment was cloned into Bsal sites of pASK-IBA5plus to yield pHANB3. In pHANB3, chln and chlb were artificially connected to form a small operon for co-expression as described (Yamamoto et al. 2009). A Strept-tag with a linker sequence (MSAWSHPQFEKGA) is connected to the N-terminus of Chln.

To overexpress the chloroplast DPOR components in the cyanobacterium L. boryana, we constructed two shuttle vectors as follows. The coding regions of PpChIL and PpChLN–ChlB were amplified by PCR using the primers PBHclL2 (Supplementary Table S1) and PBH-AS3kr1 (Yamamoto et al. 2009), and PBHl18-f1 (Yamamoto et al. 2009) and Ppchlb2 (Supplementary Table S1), with pHCL3 and pHANB3 as the templates, respectively. These PCR fragments were introduced into the SphI–BamHI sites of pPBHL118 (Yamamoto et al. 2009) to yield pPHLC2 and pHBNB3, respectively (Fig. 1). As positive controls, we used pHBLc2 and pHBNB2, which carry the cyanobacterial chil and chln-chlb, respectively (Yamamoto et al. 2009). These plasmids were introduced into the cyanobacterial cells by electroporation (Fujita et al. 1992), and the resulting transformants were selected on BG-11 plates containing 30 mM glucose and 10 µg ml⁻¹ chloramphenicol.

Sequence information of DPOR subunits

During the confirmation of the nucleotide sequences of the constructed plasmids, we found two nucleotide mismatches
causing amino acid substitution in the chlB genes; the Gly330 codon (GGT) and the Thr356 codon (ACT) in the database (accession No. AP005672.1) should be Ser330 (AGT) and Ile356 (ATT), respectively. Both amino acid residues, serine and isoleucine, are the same as those in the liverwort Marchantia polymorpha and black pine Pinus thunbergii (Fujita and Bauer 2003). We confirmed both codons by sequencing the PCR products amplified from the total DNA of P. patens.

**Determination of Chl**

Cyanobacterial cell suspensions (in 100 μl of water) were mixed with 900 μl of methanol to extract pigments as described previously (Yamamoto et al. 2009). The Chl concentration in the methanol extracts was determined as described (Yamazaki et al. 2006).

**Assay of DPOR activity**

YFC2/Lc3 (YFC2 carrying pHBLc3) was grown in BG-11 containing 30 mM glucose under low light conditions as described (Yamamoto et al. 2009). The preparation of the DPOR components was carried out in an anaerobic chamber as described previously (Yamamoto et al. 2006). We used an E. coli expression system to purify Strep-tagged NB Lb-protein. Crude extracts of gate (Bio-Rad) for the anti-ChlB-6 Tactin–peroxidase conjugate (Bio-Rad), followed by incubation with the antiserum against ChlB from L. boryana (Fujita et al. 1996) or Strep–Tactin column from crude extracts was performed as described previously (Yamamoto et al. 2006). Western blot analysis was carried out as described (Yamazaki et al. 2006). A polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore) was incubated with the antiserum containing 30 mM glucose under low light conditions as described (Yamamoto et al. 2008). Affinity purification with a Strep–Tactic column from crude extracts was performed as described (Nomata et al. 2008). DPOR activity was assayed and oxygen sensitivity was analyzed as described previously (Nomata et al. 2006).

**Western blot analysis**

Western blot analysis was carried out as described (Yamazaki et al. 2006). A polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore) was incubated with the antiserum against ChlB from L. boryana (Fujita et al. 1996) or Strep–Tactic–peroxidase conjugate (Bio-Rad), followed by incubation with goat anti-rabbit IgG–horseradish peroxidase conjugate (Bio-Rad) for the anti-ChlB-6 × His. The specific protein bands were visualized with a chemiluminescent substrate (ECL Western Blotting Analysis System; GE Healthcare).

**Supplementary data**

Supplementary data are available at PCP online.

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**References**


